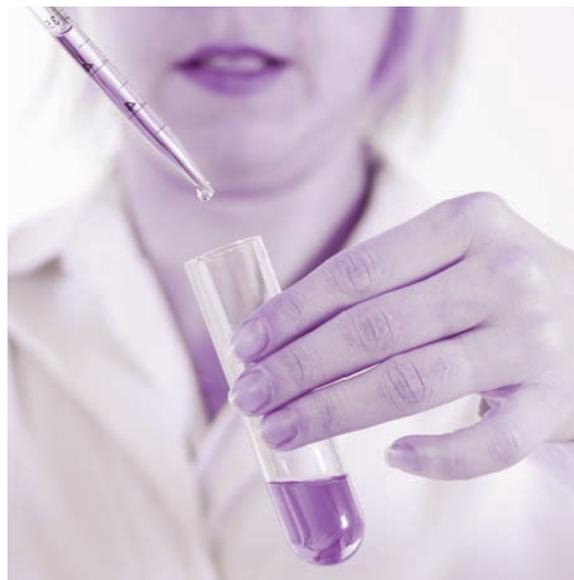


Skin Care and Aging



Skin Care and Aging

Zoe Diana Draelos, MD, Editor

Alluredbooks

Skin Care and Aging

ISBN: 978-1-932633-83-2

Copyright 2011, by Allured Business Media. All Rights Reserved.

Editorial

Book Editor: Angela C. Kozlowski
Indexer: Julie Grady
Cover Design: Bryan Crowe
Page Layout: Bryan Crowe, Anita Singh

Administration

Publisher: Marian Raney
Book Sales Executive: Marie Kuta
Book Coordinator and Web Support: Anita Singh

Neither this book nor any part may be reproduced or transmitted in any form by any means, electronic or mechanical, including photocopying, microfilming and recording, or by any information storage retrieval system, without permission in writing from the publisher.

NOTICE

To the best of our knowledge the information in this book is accurate. However, in a time of rapid change, it is difficult to ensure that all information provided is entirely accurate and up-to-date. Therefore, the author and the publisher accept no responsibility for any inaccuracies or omissions and specifically disclaim any liability, loss, or risk, personal or otherwise, which is incurred as a consequence, directly or indirectly, of the use and/or application of any of the contents of this book. Mention of trade names or commercial products does not constitute endorsement or recommendation for use by the Publisher.

Alluredbooks

A division of Allured Business Media
336 Gundersen Drive, Suite A, Carol Stream, IL 60188 USA
Tel: 630-653-2155 • Fax: 630-653-2192

www.AlluredBooks.com
E-mail: books@allured.com

Table of Contents

Part I: Skin Pigmentation

1. **A Direct Connection to Melanocytes**
Katie Schaefer with David E. Fisher, PhD 1
2. **Antiaging in a Different Light: Assessing How Chromophores Color Perception**
Philippe Mondon, Nada André, Emmanuel Doridot, Olga Gracioso and Karl Lintner, PhD 5
3. **Grapefruit Extract Cream: Effects on Melanin and Skin**
Naveed Akhtar, Mahmood Ahmed, Nazar M. Ranjha, Ahmad Mahmood 9
4. **Lightening, Boosting and Protecting with Colorless Carotenoids**
Liki von Oppen-Bezalel 39

Part II: Looking for Youth in Skin Aging

Aging Skin

5. **Innovations in Dermatology: Rethinking the Aging Face**
Zoe Diana Draelos, MD 51
6. **Slowing Intrinsic and Extrinsic Aging: A Dual Approach**
Liki von Oppen-Bezalel 57
7. **Sirtuins: A Breakthrough in Antiaging Research**
Isabelle Imbert, PhD; Claude Dal Farra, PhD; Nouha Domloge 65
8. **Pep Talk: Slowing Down Aging**
Rachel Chapman with Donald R. Owen 73
9. **Drawing the Line on Wrinkles**
Diane Bilodeau, PhD and Isabelle Lacasse 77
10. **Modeling UVD-induced Formation of Photoproducts in Human Keratinocytes**
C. Lenaers, D. Boudier, V. Barruche, B. Closs 91

Genomics and Aging Skin

11. **Mutations in Mitochondrial DNA as Principal Aging Factor**
Daniel Schmid and Fred Züllli 103
12. **Mitochondrial Nourishment and Protection for Antiaging Effects**
KG Sabarinathan, PhD 111

13. DNA Repair and Photoaging	
<i>Alan Walfield; Daniel Yarosh, PhD; Mindy Goldstein, PhD</i>	123
14. Understanding Extracellular Proteolytic Enzymes	
<i>Sanford Simon, PhD</i>	133
15. RNA Interference and Therapeutic Applications	
<i>Mindy Goldstein, PhD</i>	141
16. Stimulating Stem Cells in Younger Skin	
<i>Katie Schaefer with Louis Rinaldi</i>	147
The Behavior of Aging Skin	
17. Cutaneous Blood Flow in Aging Skin	
<i>Jeanette Waller and H.I. Maibach, MD</i>	151
18. Cutaneous Biochemistry in Aging Skin	
<i>Jeanette Waller and H.I. Maibach, MD</i>	155
19. Thickness of Aging Skin	
<i>Jeanette Waller and H.I. Maibach, MD</i>	163
20. Stimulation of Dermal and Epidermal Metabolism: An Approach to Antiaging	
<i>Maud Jouandeaud-Le Guillou, Sylvie Bordes, Catherine Soulie, Brigitte Closs and Patrice Andre</i>	171
21. In vitro Approaches to Antiaging Testing	
<i>Robert Holtz</i>	181
Part III: Novel Cosmeceutical Ingredients	
22. Tetrapeptide Targets Epidermal Cohesion	
<i>G. Pauly, MD; P. Moussou, PhD; J.-L. Contet-Audonneau, MD; C. Jeanmaire, PhD; O. Freis, PhD; M. Sabadotto; L. Danoux; V. Bardey, PhD; I. Benoit; and A. Rathjens, PhD</i>	187
23. Chitin Nanofibrils: A Natural Compound for Innovative Cosmeceuticals	
<i>Pierfrancisco Morganti, Gianluca Morganti, Riccardo Muzzarelli and Corrado Muzzarelli</i>	203
24. Biomimetic Tripeptides for Improved Dermal Transport	
<i>Hugo Ziegler and Marc Heidl</i>	215
25. Strategies of Antiaging Actives in Sunscreen Products	
<i>Claire Mas-Chamberlin, Philippe Mondon, Francois Lamy, Olivier Peschard and Karl Litner</i>	227

26. Antiaging Effects of a Skin Repair Active Principle	
<i>L. Rigano, C. Andalfatto and F. Rastrelli</i>	245
27. Wrinkle Reduction by Stimulation of the Skin's Mechanical Resistance	
<i>Catherine Lenaers, David Boudier, Christa Chauprade, Delphine Rondeau and Brigitte Closs</i>	257
28. Peptides in the Pipeline for Antiaging	
<i>Bud Brewster</i>	271
29. An Herbal Blend for Antiaging Effects: TCM in Personal Care	
<i>Daniel Schmid, Esther Belser and Fred Züllli</i>	279
30. An ECM-derived Tetrapeptide to Counterbalance ECM Degeneration	
<i>Mike Farwick, Ursula Maczkiewitz, Peter Lersch, Tim Falla, Susanne Grether-Bech and Jean Krutmann</i>	289

Part IV: Antioxidants and Skin Care

31. Understanding Reactive Oxygen Species	
<i>Paolo Giacomoni, PhD</i>	297
32. Measuring Reactive Oxygen Species in Skin with Fluorescence Microscopy	
<i>Nancy Kinkade, PhD</i>	305
33. Using Photochemiluminescence to Quantify the Antioxidative Capacity of Topicals	
<i>Hongbo Zhai, MD and Howard Maibach, MD</i>	315
34. Protecting the Genome of Skin Cells from Oxidative Stress and Photoaging	
<i>Louis Danoux, Christine Jeanmaire, Vincent Bardey, Gilles Perie, Marie-Danielle Vazquez-Duchene, Veronique Gillon, Florence Henry, Philippe Moser and Gilles Pauly</i>	321
35. From the Sea: Algal Extracts for Skin Homeostasis	
<i>Diane Bilodeau and Isabelle Lacasse</i>	335
36. Measuring the Antioxidant Potential of an Acai Extract	
<i>Karina Coyado Bispo</i>	353
37. Protecting Skin from UV Oxidative Stress with a New (Cys-Gly)₂ Dimer Peptide	
<i>A. Plantivaux, E. Bauza, T. Marchand, C. Dal Farra and N. Domloge</i>	359

Part V: Skin Moisturization

- 38. An Aquaporin-inspired Lipid Concentrate for Mature Skin**
Mike Farwick, Betty Santonnat, Peter Lersch, Kees Korevaar, Anthony V. Rawlings, Susanne Grether-Beck, Kathrin Medve-Koenigs and Jean Krutmann 367
- 39. Watering Holes in the Stratum Corneum**
Bud Brewster 377
- 40. Treating Wrinkles with Dimethylaminoethanol, Retinol and Mineral Salts**
C. Bertin, C. Robert, M. Jouselin, N. Issachar and E. Camel 387
- 41. Watermelon Survival Strategies for Skin DNA Protection**
Cornelia Huber and Thomas Schreier 399
- 42. γ -Poly Glutamic Acid: A Novel Peptide for Skin Care**
Natalie Ben-Zur and Daniel Goldman 409

Part VI: Nutricosmetics and Skin Health

- 43. Nutricosmetics: Feeding the Skin**
Bud Brewster 421
- 44. Macroalgae in Nutricosmetics**
J.H. Fitton, PhD and M. Irhimeh, PhD 431
- 45. Recent Advances in Slimming Treatments**
Karen Costa-Stachan, PhD 443
- 46. Macroalgal Fucoidan Extracts: A New Opportunity for Marine Cosmetics**
J. Helen Fitton, PhD, Mohammad Irhimeh and Nick Falk 451
- 47. Balancing Skin's Microflora with Probiotics**
Katie Schaefer with Aea Marc 467
- INDEX** 471

Introduction

This text is dedicated to everyone who is looking to improve the appearance of our everyday “wash ‘n wear clothing,” known as skin. Skin is the body’s largest organ and has amazing versatility. It keeps the world “out” and the rest of us “in.” Skin regulates the water balance in the body, represents the first line of defense against infection, maintains an even 98.6° Fahrenheit temperature, provides our sensory contact with the outside world, and represents our body image. It tells our gender and, unfortunately, our age. Through the centuries, man has looked for the secret to young skin. This book tries to scientifically examine aging by highlighting the intricacies of skin aging, followed by an analysis of genomics and how aged skin reflects the changes of aging DNA. It looks at the behavior of aging skin and how this can be modified with cosmeceuticals, antioxidants, moisturizers and nutritional supplements. Yet, to truly understand the value of this text, it is important to examine the “antiaging” goal we are trying to achieve.

What exactly is antiaging? At first glance, the answer seems obvious. Antiaging is simply not getting old, but the aging process is inevitable, thus this answer is somewhat ridiculous. No, antiaging must mean something more. The whole process of life is that of maturation. We are born with plump cherubic round faces punctuated by eyes too large and noses too small for our facial proportions. With childhood, the surface area of the face increases, the nose enlarges, and the eyes appear relatively smaller. As adolescence approaches, the jaw line begins to assume prominence and the round face becomes elongated with an increase in the size of the forehead. Young adulthood is characterized by carefully sculpted cheekbones, growth of the ears, and development of a mature hairline. And, finally, adulthood sees drooping of the nasal tip, lines of expression around the eyes and on the forehead, and loss of the creamy texture of the skin. Given these observations, what exactly is antiaging?

I think each stage in life brings certain joys, accompanied by sorrows. Infancy is the epitome of flawless skin, but having to live

in diapers is rather restrictive. Youth has the tremendous advantage of physical health, but the disadvantage of flawed skin due to acne. Most people would not aspire to have the countenance of a 13-year-old teenager ever again, but might wish to have the physical stamina. It is wonderful to have the insight of 40 years, but the onset of glabellar frown lines is not very desirable. Seeing your child reach adulthood is magnificent, but the onset of high blood pressure at age 50 is not so great. How do these observations figure into antiaging?

Looking and feeling wonderful at a mature age is antiaging. This is the broad goal that we should work towards in our skin care products and in our research. This text scientifically examines antiaging from a functional, genomic, and physiologic standpoint while presenting cosmeceutical, antioxidant, and moisturizational topical agents and nutricosmetic oral agents to achieve the one goal desired by all ... to be perceived as beautiful!

Looking for Youth in Aging Skin

The single most important factor in looking young and healthy is great skin, a fact that cannot be ignored. It is simply not possible to be humanly beautiful without great skin. It is unfortunate that with aging comes unattractive skin, unless steps are taken to prevent unnecessary skin damage. This chapter examines the mechanisms of aging and the modulation of substances, such as sirtuins, that may slow skin aging by prevention of genetic damage. Much of skin aging has been linked to DNA damage that does not allow the replication of quality skin. To better understand how this aging can be manipulated, it is important to examine the behavior of aging skin to initiate changes consistent with youthful skin performance. This chapter presents some new insights in skin aging.

Skin Pigmentation

Skin discoloration in persons of all degrees of skin pigmentation is a challenge that has defied resolution in either the over-the-counter or prescription realm. Yet, the need to achieve even pigmentation is a common consumer desire. The melanocyte holds the key to skin pigmentation, but among the 30 or so defined varieties of skin color,

only one is the proper match for any individual. Too much pigmentation is associated with photodamage and inflammation, while too little pigmentation is associated with skin disease and aging. This chapter examines the melanocyte and skin color perception, while presenting some new ingredients and their role in skin color normalization.

Skin Moisturization

The quickest way to improve the appearance of skin is through adequate hydration, more commonly known as moisturization. Moisturizers decrease trans-epidermal water loss and substances such as glycerin and urea may even modulate the osmotic balance in the skin through aquaporins. Water allows the skin to function as a plastic covering over the body, providing for unlimited movement and facial expression. This chapter examines the newly discovered aquaporins and new ingredients with moisturizing properties.

Antioxidants and Skin Care

The biggest insult the skin sustains is oxidative damage. It is amazing to realize that the same oxygen that breathes life into the body also slowly destroys it with minute repetitive injuries. Yet, the body is well equipped to deal with this damage through endogenous and exogenous antioxidants. This chapter examines the nature of reactive oxygen species and elucidates measurement techniques while presenting some new ingredients for oxidative protection.

Novel Cosmeceutical Ingredients

Cosmeceuticals are a category of topical agents designed to improve the appearance of the skin, which can be found in the over-the-counter arena yet may have profound skin effects. Cosmeceuticals must be distinguished from colored cosmetics, designed only to adorn the body using pigments. Cosmeceuticals are intended to produce an improvement in skin functioning by modifying texture, roughness, pigmentation, and redness. Many novel ingredients have been brought forth to accomplish these ends, some of which are discussed in this chapter.

Nutricosmetics and Skin Health

The old adage that “you are what you eat” has new meaning with a better understanding of nutricosmetics and skin health. The consumption of certain substances has been found to be beneficial to the skin, as the blood stream rapidly brings a variety of nutrients to the dermis. Enhancing skin appearance from the inside must complement the use of cosmeceuticals to enhance the skin from the outside. It may be necessary to feed your skin to look great throughout life. This chapter examines the concept of nutricosmetics and the role that some ingredients may play in skin health.

A Direct Connection to Melanocytes

Katie Schaefer

Cosmetics & Toiletries magazine

KEY WORDS: *melanocytes, UV, pigmentation, skin, sun*

ABSTRACT: *Research and evaluation of a compound has begun that is believed to darken skin pigmentation without the use of self-tanners or sun exposure.*

An academic study of the pathway of UV-induced pigmentation may have created a safe tan for sunseekers. Scientists at the Dana-Farber Cancer Institute, Harvard Medical School, have begun research and evaluation of the compound forskolin, which they believe darkens skin pigmentation successfully without the use of self-tanners and without sun exposure. David Fisher, MD, PhD, director of the melanoma program in the department of medical oncology at Dana-Farber Cancer Institute, Harvard Medical School, led the research in Boston to discover what he hopes to be a safe alternative to UV exposure.

The Discovery

The major scientific breakthrough according to Fisher was in that the target of UV rays was not found to be melanocytes but rather keratinocytes, which are superficial cells that lie next to the melanocytes.

“After you go out in the sun, UV exposure triggers keratinocytes to synthesize and secrete melanocyte stimulating hormone (MSH). The MSH then activates a receptor protein on the surface of adjacent

melanocytes, which in turn activates an enzyme within melanocytes to trigger the melanin synthesis cascade,” said Fisher. People with red hair usually have a variant form of the receptor protein on the melanocyte surface, and their variant form often is unable to respond to MSH, which explains why red-headed individuals rarely tan.

The researchers discovered that they could bypass the variant receptor with a drug that penetrated melanocytes and directly activated the key enzyme, thereby creating pigmentation in red-haired mice.

“Forskolin is simply the first drug we chose to test. This portion of the pathway has many drugs that are capable of activating it. The first one is rarely the best one,” added Fisher. Researchers reportedly are in the process of systematically testing and optimizing different drugs. According to Fisher, they have not yet concluded which drugs are both effective and safe for human use.

Application

Red-haired mice were chosen for the experiment. “We chose to use red-haired mice because they have a block on the surface protein of the melanocyte that hinders their ability to tan,” said Fisher. The researchers hypothesized that the skin of the red-haired mice would become tan with repeated application.

Within a couple of days, the mice’s pigment darkened. “We examined the pigment in the skin of the red-haired mice and it was indistinguishable from the pigment in naturally darker-haired mice,” added Fisher, which demonstrated to the research team that they had found a way to increase pigment without UV exposure.

Testing

The researchers have been testing the compound in a variety of situations. They also are testing the compound to determine its safety. Part of this process includes testing to determine whether the compound helps to protect against sunburn, formation of DNA mutations or skin cancer.

“The red-haired mice were exposed to UV light and they exhibited the same protection as mice that were born black-haired,” added Fisher. The mice exposed to UV light that had been treated

with the compound also had a decreased incidence of skin cancer.

If this approach is determined to be effective and safe for human use, protection for fair-skinned individuals may be more easily achieved with the application of this topical treatment.

The tan induced by the application of the compound, however, fades in time. The half-life of the compound in mice is two weeks, which according to Fisher, is similar to the rate of a tan's fading in most humans.

In determining whether the compound will be safe for human use, an important challenge is the delivery of the drug.

"The biology appears to operate in a similar way to human skin, although additional work is ongoing. So far, we believe that a major difference between the tan in mice and in humans is structural: human skin is thicker, and it is essential to deliver the drug to the appropriate cells," said Fisher.

Testing on human skin samples is underway. Fisher believes that the compound's chemical structure could remain more stable in human skin and therefore, the tan could last longer.

A Look Ahead

Once human safety is determined, Fisher believes the process will help numerous individuals. "Any condition of photosensitivity is likely to be better tolerated with this kind of strategy, though careful studies will be necessary to examine the degree of safety and benefit," said Fisher. He also added that the incidence of skin cancer could be diminished, if the compound is proven safe for human use and substituted in place of UV light exposure. Fisher hopes that, if the compound is determined safe, the sunless induction of pigmentation could impact public health in two ways:

- 1) direct protection of the skin; and
- 2) diminished sunseeking behavior.

Antiaging in a Different Light: Assessing How Chromophores Color Perception

Philippe Mondon, Nada André, Emmanuel Doridot, Olga Gracioso and Karl Lintner, PhD

Sederma SAS, Perray-en-Yvelines, France

KEY WORDS: *chromophores, collagen, heterogeneity, darutoside, oridonin, spectrophotometric intracutaneous analysis*

ABSTRACT: *Aging influences cutaneous parameters that give rise to progressive changes in three skin chromophores, altering the visual homogeneity of skin. To address these changes, the authors developed and examined the effects of a complex based on Siegesbeckia orientalis and Rabdosia rubescens using a novel skin imaging technique.*

In general, beautiful skin is perceived as being blemish-free, a characteristic often associated with youthful skin. During aging, a progressive decline in functions in the skin gives rise to, among other things, the emergence of visible heterogeneity of the skin including redness, blemishes, blotches, wrinkles and rough spots that are more readily noticeable. Pro-inflammatory messengers induce local redness and greater sensitivity of the blood vessels to environmental or behavioral stress. Similarly, oxidative stress and pro-inflammatory messengers stimulate pigmentation. Also, the dendricity of melanocytes increases while the phagocyte activity of keratinocytes on melanosomes is augmented. This results in the localized appearance of lentigines and hyperpigmentation.¹

Chromophores

Chromophores are molecules able to absorb light at a certain wavelength. The two most common cutaneous types are brown and red chromophores, i.e. melanin produced by melanocytes and stored in neighboring keratinocytes and hemoglobin contained in the blood, respectively.² In young skin, the distribution of these two types is very homogeneous. Thus, young healthy Caucasian type skin has a fresh pink complexion with no apparent blood vessels. The vascular structure consists of a dense network of superficial, practically invisible microcapillaries³ that supply the skin with nutrients and oxygen. Regarding pigmentation, young skin exhibits little melanin chromophore visual asperity, and stimulation of melanin genesis by exposure to the sun results in the homogeneous pigmentation of skin.

With age and repeated daily minor stresses, however, the situation tends to change. The skin loses its bloom due to the impairment of the chromophore balance.² Locally, certain melanocytes become more productive and lentigines of variable size and shape appear on the skin. In parallel, in certain places—frequently those most exposed to the sun—vascularization becomes visible either in the form of vessels or diffuse red areas of variable intensity (see **Figure 1**).⁴

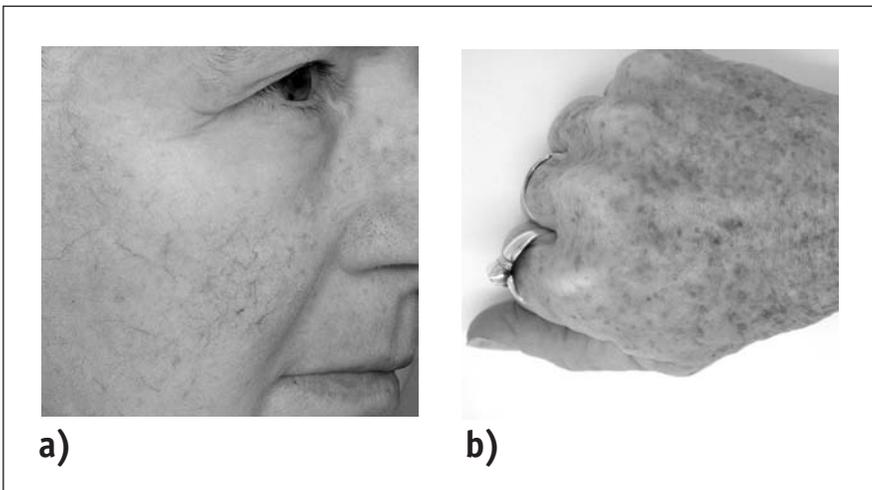


Figure 1. Elderly skin with lentigines and redness on the a) face and b) hand

In addition to chromophores that are visible to the naked eye and readily quantified, dermal collagen also has the ability to absorb

certain types of photons and thus constitutes a particular chromophore. It is well-known that, with age and stress, fewer proteins are produced in the extracellular matrix and those that are present exhibit greater degradation. This weakens the support network of the skin, once again heterogeneously. The degradation, particularly of the most abundant collagen molecules I and III, renders the skin thinner; thus, underlying structures and defects become visible.

Oxidative Stress and Visible Implications

The human organism possesses various lines of defense against external aggressions. One such cell protection scheme is based on the nuclear factor erythroid-2 related factor-2 (Nrf2) enzymatic system. This system stimulates the synthesis of various proteins including key enzymes necessary for the formation and efficacy of reduced glutathione,⁵ one of the major endogenous protective compounds produced by the cell.

Glutathione is a tripeptide that is involved in the management of oxygen free radicals, peroxides and nitric oxides.⁶ Nrf2 stimulation typically occurs in response to inflammatory or oxidative stress, but also thanks to dietary plant-derived molecules such as curcumin and allicin. The effect of these molecules to strongly stimulate Nrf2 expression explains, to a great extent, their beneficial effects with respect to certain diseases and forms of cancer.⁷ Reduced glutathione is the substrate of glutathione peroxidases and transferases, which not only neutralize hydrogen peroxide, but also other toxic organic peroxides formed by the oxidation of fatty acids and cholesterol.⁶ Glutathione is thus central to antioxidant defense.

VEGF, Redness and Wrinkles

As previously noted, skin aging sometimes gives rise to a localized increase in facial or décolleté skin redness. Exposure to the sun activates and intensifies this phenomenon by increasing the erythema, vasodilatation and fragility of micro-capillaries. Under normal conditions, Vascular Endothelial Growth Factor (VEGF) is little expressed; however, it is synthesized by keratinocytes and fibroblasts in response to inflammatory phenomena such as UV radiation, wound healing, irritation, etc. in order to induce a temporary

increase in micro-vascularization.⁸ The constitutional over-expression of VEGF induces a marked increase in the microvascular network. In addition, the vessels are more fragile, hyper-permeable and surrounded by inflammatory cells.⁸ This makes the skin more receptive to oxidative UV stress.

Interestingly, it also has been shown that over-expression of VEGF has other unexpected effects such as an increase in the formation of UV radiation-induced wrinkles. The over-expression of VEGF in skin leads to a pro-inflammatory state that triggers the formation of inflammatory stimuli⁹ such as PGE2 and interleukins 6 and 8.¹⁰

Reducing Heterogeneity as an Antiaging Approach

As described, chronological or photo-induced aging will influence the various cutaneous parameters that give rise to progressive changes in three skin chromophores—i.e., brown, red and collagen. This change in the homogeneity of skin is responsible for the perception by others as skin appearing aged. To address these changes in the appearance of skin homogeneity, the authors developed and examined the effects of a phytocomplex^a based on the combination of two plants: *Siegesbeckia orientalis*, which is rich in darutoside, and *Rabdosia rubescens*, which contains oridonin.¹¹ In the present article, this will be referred to as the *SR complex*.

Darutoside, extracted and purified from *Siegesbeckia orientalis*, a plant of Madagascar, is reputed to possess soothing and healing effects. It is a diterpene whose structure is shown in **Figure 2**. *Rabdosia rubescens* has been used in traditional Chinese medicine to treat sore throats, tonsillitis, insect stings and snake bites. The herb is endowed with antibacterial and anti-inflammatory activity.¹² From this plant, the authors isolated oridonin in a pure form (**Figure 3**), which was found to reduce UVB-induced pro-inflammatory mediators such as PGE2 by 55% ($p < 0.01$), IL 6 by 57% ($p < 0.01$) and IL 8 by 47% ($p < 0.01$) (data not shown). In addition, initial molecular biology studies showed the value of the two molecules and their combination for combating the effects of aging and maintaining the balance of cutaneous chromophores. Further investigation for cosmetic skin care purposes was thus warranted.

^a Chromocare (INCI: Butylene Glycol (and) Water (aqua) (and) Siegesbeckia Orientalis Extract (and) Rabdosia Rubescens Extract) is a product of Sederma.

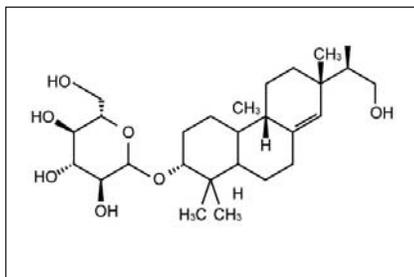


Figure 2. Darutoside structure

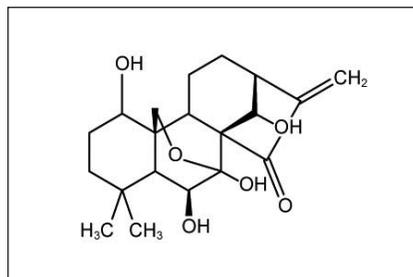


Figure 3. Oridonin structure

Materials and Methods

Twelve different *in vitro* studies were carried out using human dermal fibroblasts (HDF), human keratinocytes (HK) and human melanocytes under classical conditions of culture. Incubation times with the SR complex varied, depending on the assayed end point, i.e. 24, 45, 120 or 450 hr. Extracted mRNA allowed for a quantitative analysis of gene expression, assessed by text and data mining software.¹³

Collagen I synthesis, assessed in HDF monolayer and collagen I and III in full thickness skin^b were evaluated by immune labeling and image quantification. MMP-1 was measured in HDF by ELISA; human melanocytes incubated with the combination of both molecules were lysed after five days and their melanin content was assayed; and the phagocytosis of melanosomes was followed in HK by intracellular fluorescence. VEGF after UVB irradiation of HK was determined by ELISA, and anti-inflammatory activity was studied using a modified HET-CAM protocol: the SR complex was applied to the surface of the chorioallantoic membrane to which, after incubation, a surfactant was added as an irritant. The inflammation index was then scored by an experienced operator.

Assessing Heterogeneity

The cutaneous heterogeneity of chromophores can be monitored using systems such as spectrophotometric intracutaneous analysis (SIA), designed to see chromophores through the skin.^{2, 14, 15} The SIA portable scanner^c combines a camera and spectroscope that takes a photograph upon contact with the skin and analyzes, to a depth of 2 mm beneath the skin surface, the manner in which light

^b Full Thickness Skin is a product of Phenion.

from 400 nm to 1000 nm is absorbed or re-emitted. It thereby makes it possible to quantify the various chromophores and analyze the heterogeneity of the distribution of the collagen chromophore.

In addition, a photographic chamber for the face^d enables imaging under visible and cross-polarized light. Standardization of the images is ensured by a computer-aided repositioning system and by complete control of the photography conditions. From the pre- and post-images, it is possible to obtain the redness or vascularization index and the melanin lentiginosities index.

Clinical studies on 26 panelists from 45–71, with a mean age of 58, investigated changes in melanin chromophores using the photographic chamber^d, hemo-globin using the SIA portable scanner^c and photographic chamber^d, and collagen using the SIA portable scanner^c and echography on both the face and décolleté. Hormonal consistency in the three months preceding and during the study was required. In addition, UV radiation, sun exposure sessions and tanning product application were prohibited in the month preceding and during the study. Subjects were required to use only the cosmetics supplied throughout the duration of the study (see **Formula 1**). The study was conducted using noninvasive methods versus a control site, with subjects acting as their own control. The 3% SR complex cream and control cream were applied to opposite sides of the face and décolleté using slight massaging twice daily for two months.

Results: *In vitro*

Collagen: With respect to connective tissue matrix repair, the authors observed increased collagen I synthesis in monolayer fibroblasts (immune labeling; +170% vs. control, $p < 0.01$) and a significant increase in collagen I and collagen III production in the full thickness skin model after artificial aging (betamethasone protocol) (see **Figure 4**). The application of the SR complex was found to enable repair and improve the maintenance of damaged skin.

^c The Spectrophotometric Intracutaneous Analysis (SIAscope) device is manufactured by AstronClinica.

^d The VISIA System photographic chamber is manufactured by Canfield.

Formula 1. Test formulas for *in vivo* evaluations

	Placebo	Active formula
Water (<i>aqua</i>)	qs to 100.00% w/w	qs 100.00% w/w
Carbomer	0.25	0.25
Butylene Glycol	2.00	2.00
Phenoxyethanol	qs	qs
Steareth-2 (Volpo S2, Croda)	0.40	0.40
Steareth-10 (Volpo S10, Croda)	1.20	1.20
Cetearyl Alcohol (and) Dicityl Phosphate (and) Ceteth 10 Phosphate (Crodafos CES, Croda)	4.00	4.00
Cetearyl Alcohol (Crodacol CS90, Croda)	0.50	0.50
Laurocapram (Azone)	2.50	2.50
Cyclopentasiloxane (and) Cyclohexasiloxane (DC 345, Dow Corning)	2.00	2.00
Diethylhexyl Succinate (Crodamol OSU, Croda)	7.00	7.00
Potassium Sorbate	0.10	0.10
Water (<i>aqua</i>)	3.00	3.00
NaOH, 30%	0.40	0.40
Excipient	3.00	—
SR Complex (Chromocare, Sederma)	—	3.00

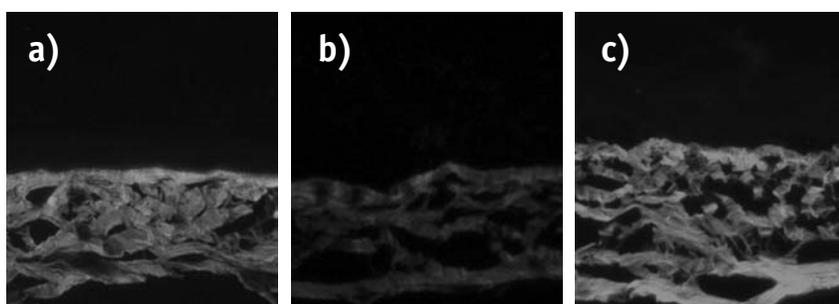


Figure 4. Collagen I immunolabeling of reconstructed skin specimens; a) control, b) aged skin and c) aged skin + 3% SR complex

These results were further corroborated by the fact that, as predicted by the DNA array results, MMP-1 formation was reduced by 43% ($p < 0.01$) in HDF cultures. Thus, the authors confirmed the ability of these substances to reorder and protect collagenous tissue, which has a visible and measurable effect on the skin's chromophores and appearance.

Melanin: With age, melanocytes stimulated by UV radiation or pro-inflammatory messengers locally increase melanin production. The melanosomes are transferred to keratinocytes by a network of extensions known as dendrocytes. Neighboring keratinocytes absorb the melanosomes by phagocytosis.

Table 1 shows that human melanocytes produced less melanin when incubated with the SR complex. Concomitant with this result, the substances contained in the SR complex also reduce the ability of keratinocytes to phagocyte melanosomes. **Figure 5** illustrates this phenomenon. The nuclei were Hoechst-counterstained; the melanosome models appear as green dots. By reducing the melanin content of melanocytes and the ability of keratinocytes to phagocyte melanosomes, the SR complex reduces the chromophore melanin.

Table 1. Change in melanin content of human melanocytes after five days of incubation with SR complex (n = 4)

		Melanin content ($\mu\text{g}/\text{mL}/106$ cells)	Change (%)
Control	—	44.7 ± 1.1	Reference
SR complex	1%	33.1 ± 1.0	-26%; $p < 0.01$
	2%	24.7 ± 1.1	-45%; $p < 0.01$
Arbutin	0.03%	20.9 ± 0.8	-54%; $p < 0.01$

Hemoglobin: To address the question of the third chromophore in the skin, i.e. hemoglobin, the authors examined the effects of oridonin and darutoside on the synthesis of VEGF. As noted, VEGF is stimulated by inflammation, leading to increased redness of the skin. Incubation of UVB-stressed human keratinocytes with the SR complex depressed VEGF expression by 44% ($p < 0.01$). In addition,

the anti-inflammatory and anti-vascularization activity of these ingredients could be demonstrated visibly by using the HET-CAM protocol described above.

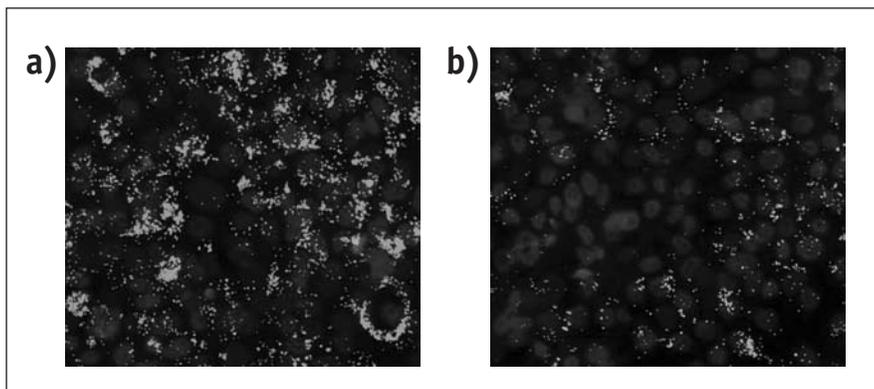


Figure 5. Inflammation index of a) the control; green = model melanosomes; blue = cell nucleus; and b) treatment with 3% SR complex

A moderately irritating product induces instantaneous changes in the capillary density of the chorioallantoic membrane. Previously unseen blood vessels become clearly visible under the effect of vasodilatation and this property was exploited to evaluate the effects of the product. The SR complex diluted in physiological water (NaCl 0.9%) was applied to the surface of the chorioallantoic membrane and, after incubation, a surfactant was applied as an irritating agent to the surface of the membrane.

A score was determined by an experienced operator. A 60% ($p < 0.01$) reduction (versus the untreated, irritated control) of the inflammation index was observed for the membrane protected with the SR complex, which compares favorably with an 80% reduction by aspirin, the positive control (see **Figure 6**). The SR complex thus clearly and significantly reduced the contribution of the chromophore hemoglobin.

Results: Clinical Studies

Adult women with lentigines or hyperpigmented, red areas on the face or décolleté, were selected; subjects with two criteria in the same area were preferred. An initial joint panel of 26 subjects was used. Each subject was subsequently classified as a function of chro-

mophores. Since it was sometimes difficult to obtain three pertinent chromophores at the same site, an optimal panel of between 22 and 25 subjects meeting the screening criteria for each chromophore and site was used. The products were well-tolerated by all test subjects.

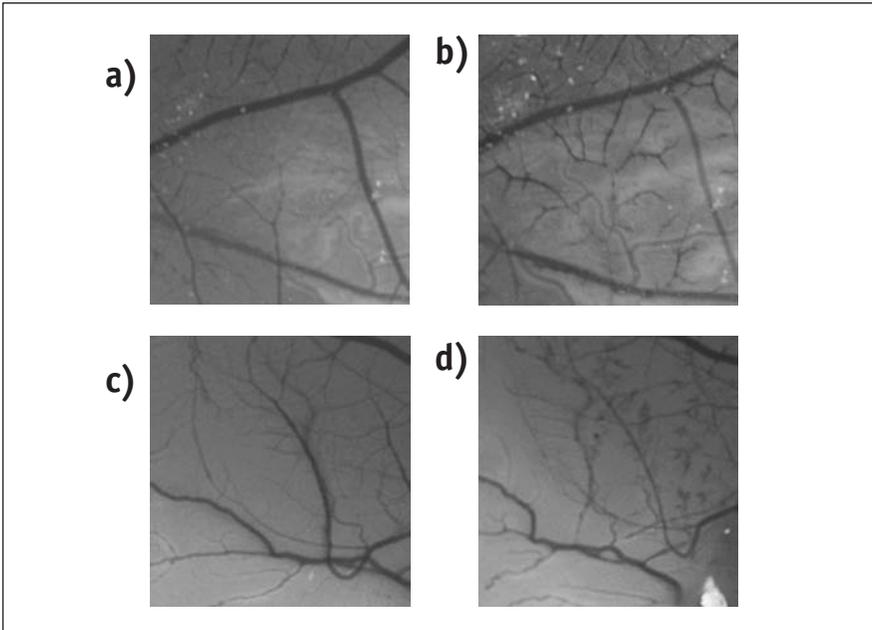


Figure 6. Results of the HET-CAM test; a) irritant control at T0; and b) T 4.5 min; and c) treatment with SR complex at T0; and d) T 4.5 min

Evaluation of collagen chromophore: The density of the dermis and thus that of its principal component, collagen, can be determined by echography. The intensity of the sound waves or echoes reflected by the corresponding tissues is a function of tissue density. Younger skin is highly echogenic since it is denser than more mature skin, which has areas of heterogeneity, i.e. variable numbers of irregular non-echogenic fields. **Figure 7**, recorded using an echography system^e and a probe operating at 20 MHz, illustrates this. **Figure 8** demonstrates that treatment of the skin with the SR complex leads to highly significant increase in skin density, reflecting an increase in dermal collagen fibers.

The results obtained with the SIA scanner were correlated with published results,¹⁵ in order to appreciate the pre- versus post-change

^e The Dermalcan C echography system is manufactured by Cortex.

in the heterogeneity of collagen distribution, and expressed as a variation of age. Although absolute values of change appear to be small, they are, in fact, significant, including when they are compared with the vehicle-only treated sites: The heterogeneity values dropped from 2.51 to 2.46 ($p < 0.05$), whereas they were unchanged to the third decimal point, 2.475, on placebo sites (see **Figures 9** and **10**).

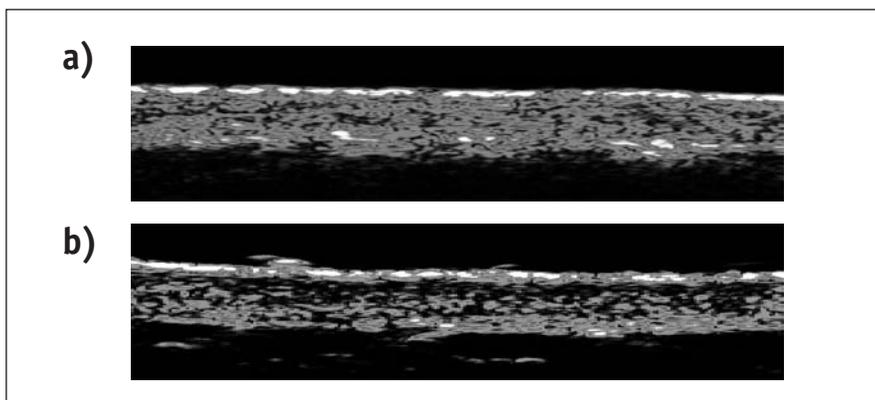


Figure 7. Echographic scan of a) the dermis of a subject aged 20 years, and b) the dermis of a subject aged 68 years

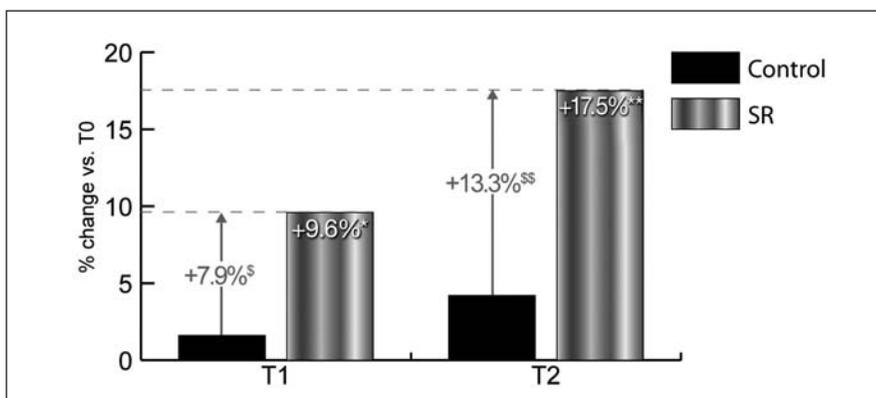


Figure 8. Change in dermal collagen (echography + image analysis; $n = 25$); * = $p < 0.05$ versus T0; ** = $p < 0.01$ versus T0; \$ = $p < 0.05$ versus control; and \$\$ = $p < 0.01$ versus control

Melanin chromophore: Following application of the test products for one and two months, the authors measured a decrease in melanin lentigines by 9.3% as soon as the first month, which again is significant ($p < 0.05$ /control) when compared with the control (see **Figure 11**).

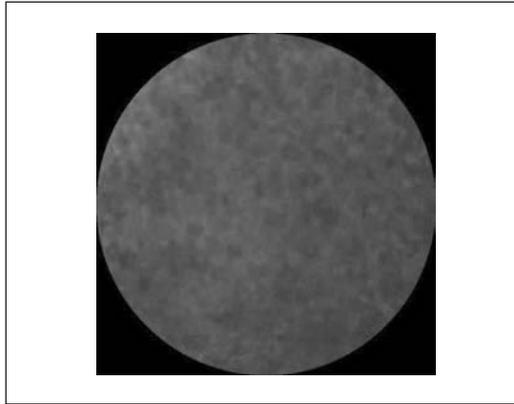


Figure 9. Collagen distribution visualized by SIA scanner

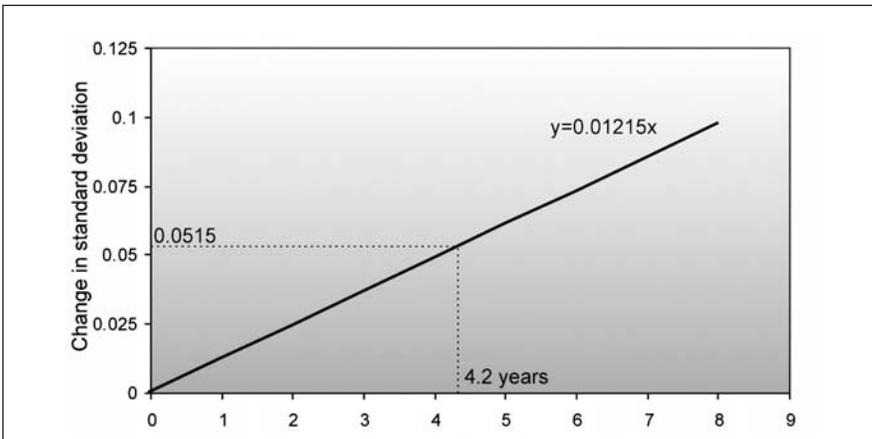


Figure 10. Correlation between the change in standard deviation and change in theoretical age for collagen homogeneity

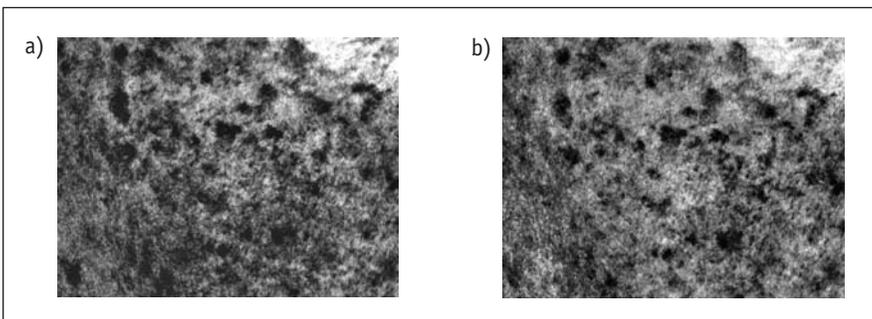


Figure 11. Melanin chromophores a) at T0, and b) after one month of treatment

Hemoglobin: To assess redness, the photographic chamber^d with a red/brown subsurface analysis was used.² After two months of treatment with the SR complex, a significant improvement ($p < 0.05$) in the vascularization index was observed: -8.2% after the first month, and -10.8% after the second month; $n = 24$ with 3 measurements (see **Figure 12**).

The portability of the SIA scanner made it possible to take measurements of the hemoglobin chromophores on the décolleté. However, not all panelists had vascularization and visible redness on the décolleté; thus, albeit for pertinent results, only 17 volunteers participated in this evaluation. An average of > 12% reduction in the redness index was observed after two months, when compared with the before and placebo treatments, which is significant ($p < 0.01$). **Figure 13** illustrates the reduction in vascularization (> 12% reduction in the SIA scanner vascularization index; $p < 0.01$).

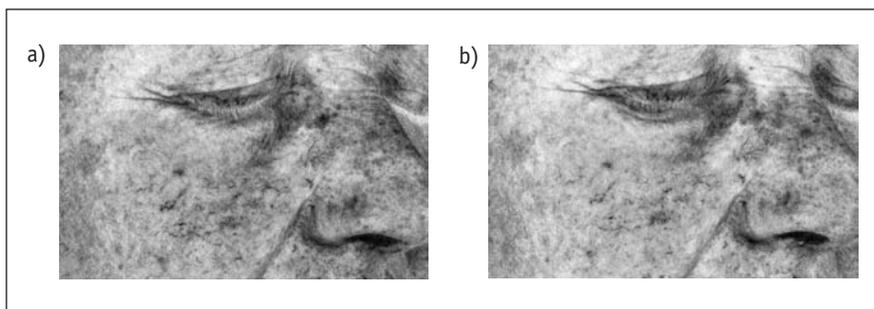


Figure 12. Hemoglobin chromophores a) at T0, and b) after one month of treatment

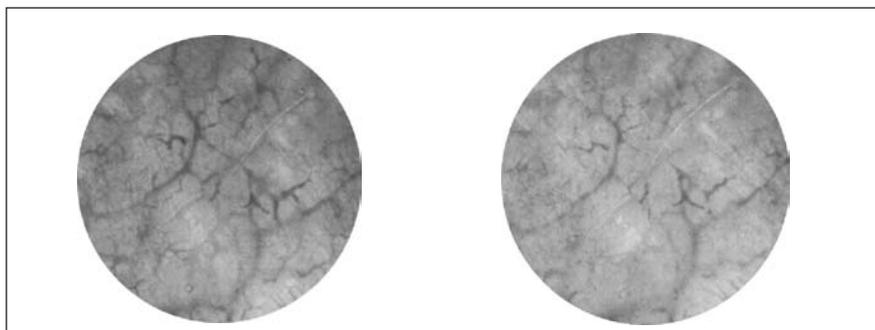


Figure 13. SIA scan of the décolleté a) at T0, and b) at T2 months, showing a reduction in vascularization

Conclusion

Aging is not just a matter of wrinkles; after all, they are just a symptom. The consequences of oxidative aging are as visible and effectively perceived by the consumer: brown spots, redness and dermal decline. An effective solution for the treatment of and protection against these visible effects of aging has thus far been lacking. The present concept is a holistic approach to evaluate and appreciate skin beauty based on the understanding of human perception of skin tone, homogeneity differences, and the chromophores influencing these parameters, well explained by Matts et al.¹⁶ The described SR complex, based on two plant extracts rich in oridonin and darutoside, addresses the ensemble of aspects in a targeted and coherent way.

Published August 2010 *Cosmetics & Toiletries* magazine

References

1. GE Costin and VJ Hearing, Human skin pigmentation: Melanocytes modulate skin color in response to stress, *The FASEB J* 21 976–994 (2007)
2. R Demirli et al, RBX Technology overview, company document, Canfield Imaging Systems (2007)
3. B Fink, K Grammer and R Thornhill, Human (*Homo sapiens*) facial attractiveness in relation to skin texture and color, *J Comparativ Psychol* 115 92–99 (2001)
4. M Yaar, Clinical and histological features of intrinsic versus extrinsic skin aging, in: *Skin Aging*, Gilchrest and Krutmann, Springer-Verlag Berlin Heidelberg (2006) p 9–21
5. YJ Suhr, Cancer chemoprevention with dietary phytochemicals, *Nature Reviews Cancer*, 3 768–780 (2003)
6. A Favier, Le Stress Oxydant, *Actualité Chimique* Nov–Dec 108–115 (2003)
7. RL Thangapazham, A Sharma and RK Maheshwari, Multiple molecular targets in cancer chemoprevention by curcumin, *The AAPS Journal* 8 E443–E449 (2006)
8. M Detmar, The role of VEGF and thrombospondins in skin angiogenesis, *J Dermatol Sci* 24 78–84 (2000)
9. S Hirakawa et al, Vascular endothelial growth factor promotes sensitivity to ultraviolet B-induced cutaneous photodamage, *Blood* 105 2392–2399 (2005)
10. I Strickland, LE Rhodes, BF Flanagan and PS Friedmann, Tnf-Alpha and Il-8 are upregulated in the epidermis of normal human skin after UVB exposure, *J Invest Dermatol* 108 763–768 (1997)
11. P Boiteau, *Plantes médicinales de Madagascar*, Ed. Karthala (1993)
12. Y Du et al, Oridonin confers protection against arsenic-induced toxicity through activation of the Nrf2-mediated defensive response, *Environ Health Perspective* 116 1154–1161 (2008)
13. P Benech, C Mas Chamberlin, P Mondon and K Lintner, PredictSearch: Understanding biological activity of cosmetic ingredients, *Personal Care Magazine* 61–65 (Sept 2007)
14. M Moncrieff, S Cotton, E Claridge and P Hall, Spectrophotometric intracutaneous analysis: A new technique for imaging pigmented skin lesions, *Br J Dermatol* 146 448–457 (2002)
15. PJ Matts, J Carey and SD Cotton, Chromophore mapping: A new technique to characterize aging human skin *in vivo*, presentation at the *Am Acad Dermatol* 63rd Annual Conference (2005)
16. PJ Matts, B Fink, K Grammer and M Burquest, Color homogeneity and visual perception of age, health and attractiveness of female facial skin, *J Am Acad Dermatol* 57 977–984 (2007)

Grapefruit Extract Cream: Effects on Melanin and Skin

Naveed Akhtar, Gulfishan and Mahmood Ahmed

The Islaima University of Bahawalpur, Bahawalpur, Pakistan

Nazar M. Ranjha

BZ University, Multan, Pakistan

Ahmad Mahmood

University of the Punjab, Lahore, Pakistan

KEY WORDS: *grapefruit extract, stability, emulsion, melanin, erythema*

ABSTRACT: *The current work aimed to formulate a stable w/o emulsion containing grapefruit extract by entrapping the extract in the inner aqueous phase. The final formula was found to have skin-whitening, moisturizing, cleansing and antiwrinkle effects, among others.*

Emulsions are thermodynamically unstable systems defined as microscopic dispersions of liquid droplets contained within another liquid, with a diameter ranging from 0.5 μm to 100 μm .¹ Emulsions usually consist of mixtures of an aqueous phase with various oils or waxes. The liquid that is broken up into droplets is termed the *internal* or disperse phase, whereas liquid surrounding the droplets is known as the *external* or continuous phase. Both phases are stabilized by a third component, the surfactant.²

The two most familiar types of emulsions are readily distinguished as o/w and w/o.³ The majority of skin care products and a significant percentage of toiletry products are emulsions.⁴ The basic components of these formulations are emulsifiers, emollients and consistency enhancers.⁵ Additional value can be given to these formulations by including active ingredients with specific cosmetic or dermatological

effects.⁴ Particularly advantageous cosmetic emulsion preparations are obtained when antioxidants are used as active ingredients.⁶

Based on a perceived safety benefit, the worldwide trend toward using natural additives has spurred interest in natural antioxidants found in plants.⁷ Extract from grapefruit is rich in natural antioxidants⁸ and provides some cosmetic benefits for the skin such as reduction in skin melanin, increase in skin moisture and anti-wrinkle effects.⁷ The best natural antioxidants present in grapefruit extract are ascorbic acid or vitamin C,⁹ flavonoids,¹⁰ beta carotene and lycopene.¹¹ Thus, the current work aimed to formulate a stable w/o emulsion containing grapefruit extract by entrapping the extract in the inner aqueous phase. Tests were performed on sample formulations to measure their effects on different physiological characteristics such as melanin, erythema, moisture, sebum, pH and TEWL.

Materials

Paraffin oil was used in the oily phase of a test formulation containing 1% grapefruit extract, as well as in the control (see **Formula 1**). Paraffin oil is a synthetic that is often preferred because of its benefits including being nontoxic and nonirritant to skin, as well as its ability to form an elegant white emulsion. It is a mixture of refined liquid saturated aliphatic (C₁₄–C₁₈) and cyclic hydrocarbons obtained from petroleum.¹²

In addition to the paraffin, cetyl PEG/PPG-10/1 dimethicone^a was chosen as the lipophilic emulsifier to form a w/o emulsion in both the test formulation and the control.¹³

Beeswax was incorporated in both formulas to increase the consistency of the cream and to stabilize the w/o emulsion.¹⁴

Grapefruit extract is water-soluble and was used as an aqueous extract in the test formula. During this study, 30 different test formulations were prepared but in the present work, data is presented comparing only one test formulation with the control.

^a Abil-EM 90 (INCI: Cetyl PEG/PPG-10/1 dimethicone) is a product of Evonik.

Preparing the Emulsion

The w/o test emulsion was prepared by adding the aqueous phase incorporating the grapefruit extract to the oily phase with continuous agitation (see **Formula 1**).¹⁵

Formula 1.

Control and test emulsions used in the described study

	<u>Control formula</u>	<u>Test formula</u>
<i>Oil Phase</i>		
Paraffin oil	20.00% w/w	20.00% w/w
Cetyl PEG/PPG-10/1 dimethicone (Abil-EM90, Evonik)	4.00	4.00
Beeswax	4.00	4.00
<i>Aqueous phase</i>		
Grapefruit extract	---	1.00 (concentrated)
Glycerin	1.00	1.00
Water (<i>aqua</i>)	qs to 100.00	qs to 100.00
Citric acid	qs to 100.00	qs to 100.00

The oily and aqueous phases were separately heated to $75^{\circ}\text{C} \pm 1^{\circ}\text{C}$, and one to two drops of citric acid were added to the aqueous phase to adjust the pH before heating. After heating, the aqueous phase was added to the oily phase, drop by drop.

Stirring was continued at 2000 rpm by the mechanical mixer for about 10 min until the complete aqueous phase was added. Two to three drops of lemon oil were added during this stirring time to give the emulsion a fragrance.

After complete addition of the aqueous phase, the speed of the mixer was reduced to 1000 rpm for homogenization for a period of 5 min, then the speed was further reduced to 500 rpm for another 5 min for complete homogenization, until the emulsion cooled to room temperature. The control formula, without grapefruit extract, was prepared in the same manner.

Emulsion Analysis

The test and control emulsions were visually analyzed organoleptically to test color, thickness, look and feel, and physically to measure creaming and phase separation.

Stability: In the cosmetics industry, product stability is one of the most important quality criteria.¹⁶ Final acceptance of an emulsion by the consumer depends on its stability and appearance,¹⁷ and one readily apparent requirement in a well-formulated emulsion is that the emulsion possesses adequate physical stability.¹⁸

In the present study, the base and test formulation each were divided into four samples separately and these samples were kept at different storage conditions—i.e., at 8°C in a refrigerator, at 25°C, 40°C, and at 40°C + 75% relative humidity (RH) in stability chambers. These samples were observed with respect to change in color, liquefaction and phase separation for a period of 28 days at definite time intervals.

Color: The freshly prepared base and test formulation appeared elegant white in color and no change in color was observed for either the control or test formula, up to an observation period of 28 days. This showed both emulsions were stable at the different storage conditions throughout the 28 days.

Liquefaction: The viscosity of an external oil phase is the key factor contributing to the formation of stable emulsions.¹⁹ According to Stoke's law, increased viscosity of the external phase is associated with an improved shelf-life of emulsions.²⁰ Cosmetic creams appear as stable, concentrated emulsions²¹ but as soon as an emulsion has been prepared, time- and temperature-dependent processes occur to affect its separation, leading to the decreased viscosity, which results in increased liquefaction.¹⁸

No liquefaction was observed with the naked eye in the control and test formulation samples stored at 8°C and 25°C during the 28 days but slight liquefaction occurred in the samples of the control kept at 40°C and 40°C + 75% RH from the 21st day of observation. No further increase in liquefaction was noted until the end of the study. On the other hand, a slight liquefaction was observed in test formulation samples stored at 40°C and 40°C + 75% RH at the 28th day of the observation period.

Stoke's law states that the rate of creaming is inversely proportional to the viscosity. As creaming increased, the viscosities of the base and the formulation gradually decreased at rising temperatures, resulting in liquefaction.²⁰

Phase separation: The instability of emulsions is explained by phase separation,¹⁶—i.e., any emulsion reverts back to the separate bulk phases. The separated phase can either cream or sediment.¹⁸ Destabilization is compounded mostly by coalescence and gives a first indication through extension of droplets.¹⁶ The two instability processes, coalescence and Ostwald ripening, result in droplet size growth with time.² According to Stoke's law, larger droplets cream much more rapidly than smaller particles.²⁰ The concentration of the disperse phase and the droplet size are key parameters in determining the type and time scale of the instability process.²

In the case of the control and test formulations, no phase separation was observed in any of the samples up to 28 days, indicating their stability.

Centrifugation: Centrifugation, if used judiciously, is an extremely useful tool for evaluating and predicting the shelf-lives of emulsions.¹⁸ The cream volume or the separation of phases at a given time is taken as a measure of the physical stability of emulsion.²¹ However, these are examples of situations that exist in any accelerated test—namely the tendency to “overkill” emulsions because the tests used introduce a new mechanism of instability by causing unreasonably high stress.¹⁸

In the described study, 5–10 g samples of the test and control formulas were rotated at 5000 rpm for 5 min. The test was performed for the formulations kept at the different storage conditions for up to 28 days at definite time intervals. No phase separation on centrifugation was noted in any of the samples thus indicating that both the test and control emulsions were stable for 28 days.

Electrical conductivity: Conductivity differences arise when an emulsion creams, the proportion of water increases in the lower part of the emulsion, and the proportion of oil increases in the separated upper portion of the emulsion. By measuring conductivity differences in the upper and lower parts as a function of storage time, instability can be determined.²¹ Conductivity tests

are also used to distinguish between emulsion types²²—o/w emulsions will conduct because water is the continuous phase, and since oils are poor conductors, w/o emulsions conduct poorly.²³

In the present study, a conductivity test was performed for all samples of the control and test formulation. No change in electrical conductivity was noted.

pH: pH is a significant parameter in so far as the effectiveness of the cream is concerned, and it can be used as an indicator of emulsion stability.¹⁶ For the formation of stable emulsions, the pH value of the aqueous phase is a key factor.¹⁹ The pH of skin ranges between 5 and 6; 5.5 is considered the average pH level. Therefore, formulations for application to skin should have pH close to this range.

In the present study, the pH of the freshly prepared control and test formulations was found to be 5.79 and 5.97, respectively (see **Table 1**).

Time	8 °C		25 °C		40 °C		40 °C+75% RH	
	Ctrl	Test	Ctrl	Test	Ctrl	Test	Ctrl	Test
0 hr	5.97	5.79	5.97	5.79	5.97	5.79	5.97	5.79
12 hr	5.94	5.81	5.92	5.82	5.74	5.58	5.68	5.83
24 hr	5.76	5.95	5.64	5.81	5.58	5.96	5.53	5.98
36 hr	5.57	5.86	5.41	5.63	5.01	5.67	5.05	5.82
48 hr	4.96	5.84	4.87	5.49	4.74	5.04	4.61	5.06
72 hr	4.86	6.62	4.78	5.93	4.6	5.47	4.65	5.84
7 days	4.69	6.62	4.58	5.84	4.51	5.75	4.47	5.93
14 days	4.62	6.01	4.56	5.5	4.43	5.33	4.39	5.69
21 days	4.56	6.07	4.47	5.85	4.41	5.26	4.36	5.84
28 days	4.45	6.09	4.39	5.86	4.35	5.35	4.27	5.85

The pH values decreased continuously from the first to the last day. On the 28th day, the pH levels of the control formulation samples were 4.45, 4.39, 4.35 and 4.27, respective to the different storage conditions. The pH of the control samples stored in different conditions showed slight variations with time, and slightly increased at the end of the study period. This could have been due to the production of metabolites; no such tendency is inherent in emulsions, but it can occur.

The pH values of the test formulation measured 6.09, 5.86, 5.35 and 5.85 on the 28th day, respective of their storage conditions—i.e., at 8°C in a refrigerator, at 25°C, 40°C, and at 40°C + 75% RH.

By using a two-way Analysis of Variance (ANOVA) technique at 5% level of significance, the change in pH of the samples of control formulation, at different levels of time and temperature, was found to be significant but there was an insignificant difference in changes of pH for samples of the test formulation.

A Least Significant Difference (LSD) test also was applied to determine the individual average effects of the pH levels of the control and test formulas at different temperatures with the passage of time. From this test it was concluded that at different storage conditions, a significant change occurred in the pH of the control formula but an insignificant change was observed in the test formulation samples.

Dermatological Tests

Patch test: Before application of the control and test formulations to a total of nine male and female human volunteers aged 20–25, patch tests for melanin and erythema were performed and the values measured are shown in **Table 2**.

After performing the patch test for 24 hr, the test and control formulations were found to produce no skin irritation; thus both creams were deemed safe for *in vivo* evaluation. This lack of irritation could have been attributed to the presence of a good emollient in both formulas—i.e., glycerin²⁴—or ascorbic acid, a natural antioxidant present in the formula from the grapefruit extract,^{25,26} which has been shown to reduce skin erythema.^{27,28}

Table 2. Percent change in melanin and erythema for control (Ctrl) and test (Test) formulas after a 24-hr patch test

Volunteer #	Melanin		Erythema	
	Ctrl	Test	Ctrl	Test
1	-22.35	-6.60	-4.64	0.54
2	-11.64	-10.32	13.76	-23.78
3	-11.38	-2.48	-3.78	-41.12
4	-0.94	-1.97	-11.62	-1.923
5	-1.69	-2.89	-1.89	-15.49
6	-2.48	-4.70	-6.76	3.51
7	-16.38	-12.83	-12.89	-2.33
8	3.83	-21.33	-3.62	-13.11
9	8.88	-6.76	-23.00	2.90
Mean ± SEM	-6.02 ± 3.36	-7.76 ± 2.09	-6.05 ± 3.31	-10.09 ± 4.98

Melanin

Melanocytes are present in the basal layer of the epidermis and they manufacture melanin, which is responsible for the color of skin. Melanin is formed through a series of oxidative reactions involving the amino acid tyrosine in the presence of the enzyme tyrosinase. Tyrosinase catalyzes three different reactions in the biosynthetic pathway of melanin in melanocytes.²⁹

Antioxidants have important physiological effects on skin.³⁰ Their main role is to scavenge free radicals such as peroxides²⁷ that contribute to tyrosinase activation and melanin formation.³¹ As previously mentioned, grapefruit extract is rich in natural antioxidants³² that capture free radicals, resulting in the inhibition of melanogenesis.³⁰

In the present study, the effect of the control and the test formulations on the production of skin melanin was examined. The amount of melanin was measured^b for four weeks at different time intervals in each test subject after application of the control and test formulation.

^b The Mexameter is a device of Courage-Khazaka, Germany.

It was found that the control formulation increased melanin content in the skin until the end of the 28-day test period, while the test formulation incorporating grapefruit extract decreased the melanin content throughout the study (see **Table 3**).

An ANOVA test found the control and test formulations to produce statistically significant effects on skin melanin content in volunteers; a paired sample t-test showed a significant difference in the melanin effects of the control versus the test formulation from the second week, lasting to the fourth week of the study, confirming the two creams had different effects on melanin.

Researchers concluded that the decreased melanin content after application of the test formulation could have been attributed to the antioxidant activity of the grapefruit extract—particularly its potent antioxidant vitamin C that causes inhibition of melanogenesis.^{9,30,31}

Table 3. Percent change in melanin content of the skin after application of the control (Ctrl) and test (Test) formulas

Volunteer #	First week		Second week		Third week		Fourth week	
	Ctrl	Test	Ctrl	Test	Ctrl	Test	Ctrl	Test
1	-5.11	-11.00	4.80	-12.62	17.42	-14.24	10.21	-20.71
2	5.34	-0.47	9.16	-3.50	13.99	-7.24	16.03	-9.35
3	-8.26	-0.32	-1.18	-8.06	5.31	-14.52	5.90	-19.03
4	0.96	-2.40	-2.30	-5.21	-1.54	-9.62	15.16	-10.62
5	-11.90	-1.04	-13.18	-5.19	-5.14	-7.27	15.11	-14.19
6	18.36	-2.03	12.46	-5.76	11.15	-13.90	5.57	-19.66
7	9.97	-12.67	13.90	-13.50	7.85	-10.47	16.62	-16.25
8	-6.80	-2.13	3.97	-3.66	-0.28	-7.93	-4.53	-9.15
9	-10.10	-11.01	1.63	-13.10	-0.98	-14.88	1.95	-27.98
Mean	-0.84	-4.79	3.25	-7.85	5.31	-11.12	9.11	-16.33
±SEM	± 3.42	± 1.72	± 2.79	± 1.38	± 2.61	± 1.09	± 2.47	± 2.08

Erythema

Skin irritation is caused by the direct toxicity of chemicals on cells or blood vessels in the skin and is different from contact allergy, which is caused by an immune response.²⁸

In the described *in vivo* study, irritation also was monitored weekly throughout the test period for both formulations. Erythema was measured^b and found to be slightly increased after application of the control formula for the first week but almost no change was observed during the second week, and a pronounced decrease was observed at the third and fourth week.

In comparison, after application of the test formulation, erythema was slightly increased after the first week; however, decreased at the second and fourth week.

An ANOVA test confirmed statistically that the control and test formulation produced insignificant effects on skin erythema. A paired sample t-test showed a significant variation in irritation with respect to the control and test formulation at the first and second week (see **Table 4**).

Table 4. Percent change in skin erythema content, or potential of a substance to produce irritation, after application of control (Ctrl) and test (Test) formulas

Volunteer #	First week		Second week		Third week		Fourth week	
	Ctrl	Test	Ctrl	Test	Ctrl	Test	Ctrl	Test
1	-4.87	-1.17	1.87	-2.92	-4.87	-9.75	-17.42	2.14
2	-10.60	-3.75	-12.90	-5.93	-15.82	-3.16	-30.41	-2.77
3	-7.92	15.64	-1.58	8.06	1.23	43.60	-5.46	9.72
4	-3.36	-2.39	-4.53	-3.75	-7.21	-4.78	-0.34	-12.12
5	2.47	5.80	-16.92	-2.46	11.79	10.71	-14.83	1.79
6	5.30	-0.19	2.65	-0.78	0.00	-1.75	-7.94	-7.60
7	29.00	-0.97	25.34	-1.93	25.34	1.16	23.52	8.12
8	19.45	6.13	7.00	-10.94	10.72	0.66	6.56	-9.41
9	-4.70	3.71	0.00	10.35	-22.82	8.20	-13.59	-7.42
Mean	2.75	2.53	0.10	-1.14	-0.18	4.99	-6.66	-1.95
±SEM	± 4.44	± 2.02	± 4.04	± 2.20	± 4.92	± 15.26	± 5.17	± 2.61

It was thus concluded that both the test and control formulations decreased erythema at the end of the study period and the overall effect of the test formulation on erythema was insignificant; thus it could be used safely without irritating skin. In addition, the active ingredient

in grapefruit extract is a good source of vitamin C,³³ which is used as an anti-inflammatory,²⁸ and topical vitamin C is claimed to inhibit UV-radiation-induced damage to porcine skin since it functions as a biological co-factor and an antioxidant.²⁹

Moisture Content

Moisturizing treatments involve factors such as repairing the skin barrier; retaining/increasing water content; reducing TEWL; restoring the lipid barrier's ability to attract, hold and redistribute water; and maintaining skin integrity and appearance.³⁴

A 100-g sample of grapefruit juice contains 36–40 mg of vitamin C,¹¹ which is known to increase the collagen fibers in the dermis.^{35–39} With an increase in collagen, conditions for hydration are improved.⁴⁰ In addition, vitamin C improves the barrier function of the stratum corneum (SC), in turn improving moisture in the skin.⁴¹

In both formulations tested, glycerin was incorporated in the internal aqueous phase to enhance the skin moisture level. Glycerin is a humectant with excellent sensorial properties that moisturizes the full thickness of the SC while creating an apparent “reservoir” of moisture in the skin. This makes the skin more resistant to dry conditions.²⁴

In the present study, a slight increase in moisture was found^c from the first to third week after application of the control, and a slight decrease was observed at the fourth week; however, after the application of the test formulation, the skin moisture content was more pronounced from the first to the fourth week.

ANOVA testing found that the control showed an insignificant change with respect to the basic values, at 0 hr before application of creams, whereas the test formulation showed a significant variation throughout the study period of 28 days.

By applying an LSD test, the change in moisture content was found to be statistically significant after application of the test formulation. A paired sample t-test confirmed a significant difference in the moisture values was produced at the fourth week when comparing the control to the test formulation (see **Table 5**).

^c The Corneometer is a device of Courage-Khazaka, Germany.

Table 5. Percent change in skin moisture content after application of control (Ctrl) and test (Test) formulas

Volunteer #	First week		Second week		Third week		Fourth week	
	Ctrl	Test	Ctrl	Test	Ctrl	Test	Ctrl	Test
1	27.83	34.62	16.10	41.00	-11.93	46.92	-13.52	59.00
2	56.04	17.38	39.61	22.80	-8.21	29.35	-41.30	33.41
3	14.11	42.29	3.60	28.08	20.87	39.86	-5.71	53.03
4	-1.09	4.63	-3.27	8.30	-26.73	17.76	-5.82	21.24
5	-3.20	6.26	-16.16	11.18	-13.39	14.31	-21.40	15.20
6	9.78	1.47	8.26	-4.26	8.94	0.29	26.81	11.91
7	-1.12	37.41	-3.54	35.37	23.84	41.50	19.37	54.20
8	10.59	26.90	-2.97	36.55	-2.97	42.98	-10.41	47.66
9	7.94	3.74	11.56	9.07	30.61	-6.05	45.58	7.12
Mean	13.43	19.41	5.91	20.90	2.34	25.21	-0.71	33.64
±SEM	± 6.21	± 5.40	± 5.30	± 5.19	± 6.54	± 6.52	± 8.89	± 6.78

Sebum Content

Sebaceous glands, located in each hair follicle, produce sebum to lubricate and protect the skin.³³ Sebum production is measured using a special opalescent plastic film^d that becomes transparent when it comes in contact with sebum lipids.⁴²

A probe is used to press a piece of the film onto the skin for a measured length of time and sebum is adsorbed onto the film, similarly to ink on blotting paper, thus turning the film transparent.

The probe is then placed into a device that radiates a light beam onto the film. A metal mirror behind the film reflects the beam back again through the film and into an instrument called a *photomultiplier*, which measures the amount of light in the beam. The more sebum on the skin, the more transparent the film and the greater the amount of light reflected.

In the present study, the effects of the control and test formulations on sebum content in human facial cheeks were investigated. Sebum was measured weekly in all test subjects using the control and test formulations; both samples were found to increase sebum content from the first to fourth week of the study period.

^d The Sebumeter is a device of Courage-Khazaka, Germany.

An ANOVA test confirmed a statistically significant effect of the control and test formulation on skin sebum throughout the study period. By applying an LSD test, it was evident that significant changes in sebum content were observed at different time intervals after application of both formulations (see **Table 6**).

Table 6. Percent change in sebum after application of control (Ctrl) and test (Test) formulas

Volunteer #	First week		Second week		Third week		Fourth week	
	Ctrl	Test	Ctrl	Test	Ctrl	Test	Ctrl	Test
1	34.43	180.33	139.34	17.24	183.61	25.00	180.33	96.55
2	47.19	205.62	84.27	49.52	115.73	56.19	205.62	147.62
3	47.19	205.62	84.27	49.52	115.73	56.19	205.62	147.62
4	58.06	464.52	103.23	192.31	170.97	276.92	464.52	976.92
5	271.43	771.43	309.52	81.25	771.43	140.00	771.43	196.25
6	10.42	206.25	64.58	82.35	108.33	105.88	206.25	402.94
7	-31.71	145.12	14.63	15.33	93.90	21.33	145.12	25.33
8	165.52	513.79	637.93	12.70	627.59	23.81	513.79	34.13
9	7.94	3.74	11.56	9.07	30.61	-6.05	45.58	7.12
Mean	61.86	294.99	169.96	104.84	273.85	151.22	294.99	314.50
±SEM	±32.90	±81.10	±66.09	±42.32	±86.22	±60.31	±81.10	±112.16

With a paired sample t-test it was found that the control and test formulations showed insignificant variations regarding the skin sebum content. Thus, researchers attributed the increase in sebum content to the oily nature of the w/o emulsions, since they contained paraffin oil.¹⁴

pH Values

The pH of skin ranges between 5 and 6; 5.5 is considered to be the average pH of skin. Therefore, formulations intended for application on skin should have a pH level close to this range.²⁸

pH values in the volunteers' skin were measured at different time intervals before and after the application of the control and test formulation for a period of four weeks (see **Table 7**).

Table 7. Percent change in skin pH values after application of control (Ctrl) and test (Test) formulas

Volunteer #	First week		Second week		Third week		Fourth week	
	Ctrl	Test	Ctrl	Test	Ctrl	Test	Ctrl	Test
1	-20.86	-8.60	-17.65	-33.84	-3.80	-24.20	-3.63	-27.33
2	3.57	-4.38	1.07	-11.17	4.63	-7.82	-1.52	1.46
3	90.59	10.89	122.32	7.46	98.91	7.46	-1.31	-7.46
4	45.86	0.60	73.83	3.41	112.75	-15.23	154.81	114.83
5	16.00	-2.04	15.34	-5.92	23.29	-1.63	39.51	156.33
6	-10.46	-36.10	-4.77	-27.80	-4.59	-15.95	125.87	-42.86
7	87.35	6.64	7.96	15.35	8.78	92.53	148.16	-7.26
8	94.02	17.67	-5.25	13.36	53.08	120.47	131.70	142.24
9	-7.43	-54.13	-49.17	-47.26	-8.21	-57.51	-21.11	-53.63
Mean	33.18	-7.72	15.96	-9.60	31.65	10.90	63.61	30.70
±SEM	± 15.70	± 7.70	± 17.16	± 7.42	± 15.38	± 19.19	± 24.92	± 27.62

After application of the control, pH values of the skin was increased throughout the study period, whereas application of the test formulation decreased pH values during the first and second week, and increased during the third and fourth week.

ANOVA testing found a significant effect on skin pH values produced by the control, whereas an insignificant effect was observed by the test formulation. A paired sample t-test concluded that the change in skin pH in volunteers by both the test and control formulation was significant in the first and fourth week.

The absence of change in the pH of skin from the test formulation could be attributed to the presence of potent natural antioxidants in the grapefruit extract,³² which can prevent the oxidative degradation of skin by scavenging free radicals, thus maintaining the natural integrity of human skin.

TEWL

Body loses water by constant evaporation through the skin, also known as trans-epidermal water loss (TEWL).⁴² TEWL changes are related to SC water-binding capacity.⁴² Water makes up 70–75% of the basal layer weight but only 10–15% of the SC. If the water

content of the SC falls below 10%, it becomes dry, less flexible and prone to damage, breakdown and infections.⁴³

In the present study, a decrease in TEWL values was noted after the application of the control and test formulation throughout the 28-day study (see **Table 8**).

Table 8. Percent change in TEWL values after application of control (Ctrl) and test (Test) formulas

Volunteer #	First week		Second week		Third week		Fourth week	
	Ctrl	Test	Ctrl	Test	Ctrl	Test	Ctrl	Test
1	21.36	-16.88	-10.91	-30.52	17.27	-21.10	-28.64	-48.05
2	-6.91	-4.50	-16.59	-8.00	-27.19	-19.50	-32.72	-24.50
3	16.23	13.44	1.05	6.45	5.76	3.76	-12.57	-2.69
4	-4.68	-17.76	-24.26	-23.03	-28.51	-32.57	-14.04	-37.50
5	-8.56	-13.10	-28.02	-13.54	-33.85	-11.35	-30.35	-32.31
6	-21.68	-9.84	-32.87	-12.99	-46.50	-40.55	-14.69	-28.74
7	-15.27	-21.85	-27.27	-23.33	-26.55	-28.15	-36.36	-36.67
8	-9.84	-39.11	-12.30	-14.92	-42.21	-37.50	-45.90	-34.27
9	5.16	-0.25	-13.10	-53.44	-27.78	-51.65	-19.05	-58.78
Mean	-2.69	-12.21	-18.25	-19.26	-23.28	-26.51	-26.03	-33.72
+SEM	± 4.75	± 4.91	± 3.57	± 5.53	± 7.04	± 5.54	± 3.86	± 5.18

ANOVA testing confirmed that changes in TEWL produced by both formulations were statistically significant during the four-week study. By applying an LSD test, the change in TEWL values became significant for both formulations after the first week of application. With a paired sample t-test, insignificant variation in TEWL was found with respect to the control and test formulation throughout the study.

Researchers concluded that both formulations prevented TEWL probably due to a number of factors including their glycerin and paraffin oil content. Glycerin moisturizes the full thickness of the SC²⁴ and paraffin oil, as mentioned previously, forms an occlusive covering on skin thus preventing TEWL. Therefore, due to moisture-retaining properties, both formulations enhanced the SC's ability to attract, hold and redistribute water, in turn reducing TEWL.^{24, 34, 44}

Panel Test

A questionnaire was given to each volunteer for sensory evaluation of each of the two creams. Average points were calculated for both the control and test formulations (see **Figure 1**).

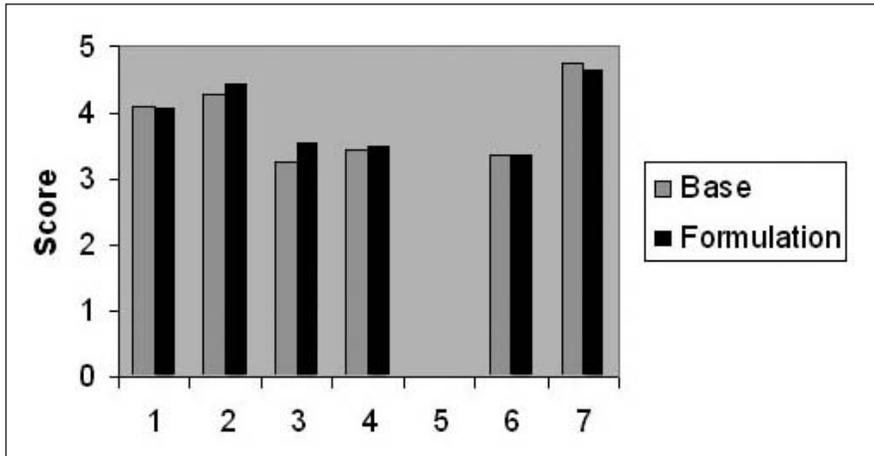


Figure 1. Average Values for Panel Test: 1= Ease of application; 2 = Spreadability; 3 = Sense just after application; 4 = Sense in long term (28 days); 5 = Irritation; 6 = Shine on skin; and 7= Sense of softness

The average points for ease of application were 4.12 and 4.08 for the control and test formulation, respectively, indicating ease of application on the skin. Points for spreadability rated the test formulation higher—4.29 for the control and 4.42 for the test formulation. Feel upon application was rated as 3.25 points for the control and 3.54 for the test formulation, an indication the test formulation felt better upon application to skin.

Average points for the 28-day application period were 3.43 and 3.5 for the control and test formulation, respectively. The numbers indicated the test formulation produced a more pleasant feeling; there also was no irritation on the skin in both formulations thus they were assigned 0.0 points for irritation by all volunteers.

Shine on skin was rated 3.35 for the control and 3.36 for the test formulation. This was expected since the two formulations contained the same quantity of paraffin oil. Similarly, the control produced a higher rating for softness of the skin; the average points was 4.74 for the base and 4.63 for the test formulation.

From a paired sample t-test, an insignificant difference was noted between the average points of sensitivity for both formulations. Thus, researchers concluded that little variation existed between the control and test formulation in regards to sensory evaluation. Both creams behaved similarly, from a sensory point of view.

Conclusion

Antioxidant-rich grapefruit extract, when used at a concentration of 1% in topical creams, was not found to realize its full skin benefit potential in a 28-period of application. It is therefore suggested, to maximize the cosmetic benefits, that the grapefruit extract be used in higher concentrations and for longer period of time.

Published January 2008 *Cosmetics & Toiletries* magazine.

References

1. JPF Macedo et al, Micro-emultocrit technique: A valuable tool for determination of critical HLB value of emulsions, *AAPS Pharm Sci Tech* 7(1) (2006)
2. K Welin-Berger, Formulations, release and skin penetration of topical anesthetics, Dissertation for the Degree of Doctor of Philosophy (Faculty of Pharmacy), Uppsala University (2001) pp 17–18
3. F Nielloud, G Marti-Mestres and MM Gilberte, *Pharmaceutical Emulsions and Suspensions*, New York: Marcel Dekker (2007)
4. G Kutz, P Biehl, M Waldmann-Laue and B Jackwerth, On the choice of oil-in-water emulsifiers for use in skin care products for sensitive skin, *SOFW J*, 123 145–150 (1997)
5. TO Ngai, SH Behrens and H Auweter, Novel emulsions stabilized by pH and temperature sensitive microgels, *The Roy Soci of Chem* (2004)
6. US Pat 7,138,128, Preparations of the W/O emulsion type with an increased water content, and comprising cationic polymers, B Andreas, K Rainer and Schneider Gu, assigned to Beiersdorf AG (Hamburg, Germany) (Nov 21, 2006)
7. NV Yanishlieva, E Marinova and J Pokorny, Natural antioxidants from herbs and spices, *Eu J of Lip Sci and Tech* 108 (9)776–793 (2006)
8. D Roberts, Antioxidant values in fruits and vegetables, Macular Degeneration Support Web site; www.mdsupport.org (Accessed Feb 27, 2007)
9. L Curtis, *Juice Up*, Northbrook, IL USA: Virgo Publishing (1997)
10. FP Mary et al, A furanocoumarin-free grapefruit juice establishes furanocoumarins as the mediators of the grapefruit juice–felodipine interaction, *Ameri J of Clinic Nutr*, 83(5) 1097–1105 (2006)
11. *Grapefruit*, Wikipedia, the free encyclopedia Web site; www.en.wikipedia.org/wiki/grapefruit#column-one; Wikimedia Foundation Inc. 15(59) (Accessed Mar 28, 2007)
12. *British Pharmacopoeia* 2 (2004) pp 1132–1134
13. Code of Federal Regulations, U.S. Food and Drug Administration, Sec.146.132, *Grapefruit juice*, Department of Health and Human Services, U.S. Government Printing Office 2 (2002) pp 439–440
14. RC Rowe, PJ Sheskey and PJ Weller, Dimethicone, mineral oil, wax white; wax yellow, *Handbook of Pharmaceutical Excipients*, 4th ed, Chicago: The PhP London, and Washington, D.C.: the APhA Washington (2003) pp 213–214, 395–396, 687–690

15. J Swarbrick, JT Rubino and OP Rubino, Coarse dispersions, in *Remington: The science and practice of pharmacy*, 21st edn, Philadelphia: Lippincot Williams and Wilkins (2006) pp 316–334
16. NM Mostefa, AH Sadok, N Sabri, A Hadji, Determination of optimal cream formulation from long-term stability investigation using a surface response modelling, *Int J of Cosmet Sci*, 28 (3) 211–218 (2006).
17. M Muehlbach, R Brummer and R Eggers, Study on the transferability of the time temperature superposition principle to emulsion, *Int J of Cosmet Sci*, 28(2) 109–116 (2006)
18. HA Lieberman, MM Rieger and GS Banker, Pharmaceutical Emulsions, in *Pharmaceutical Dosage Forms: Disperse Systems*, New York and Basel: Marcel Dekker 1 (1988) pp 199–240, 285–288
19. L Song, X Ge, M Wang and Z Zhang, Direct preparation of silica hollow spheres in a water in oil emulsion system: The effect of pH and viscosity, *J of Non-Cry Sol* 352(21–22) 2230–2235 (2006)
20. L Lachman, HA Lieberman and JL Kanig, in *Emulsions, The Theory and Practice of Industrial Pharmacy*, 3rd edn, Bombay: Varghese Publishing House (1990) p 502
21. A Abdelbary and SA Nour, Correlation between spermicidal activity and haemolytic index of certain plant saponins, *Pharmazie* 34(9) 560–561 (1979)
22. K Tauer, Emulsions, in *MPI Colloids and Interfaces*, D-14476 Golm, Germany: Am Mühlenberg (2006)
23. ST Mabrouk, The preparation and testing of a common emulsion and personal care product: Lotion, *J of Chem Edu* 81(1) 83–86 (2004)
24. TL Diepgen, Professional care for dry and sensitive skin, Medical and economic costs of skin disease, in *White Book of Dermatology*, Heidenberg, Germany: European Dermatology Forum, University of Heidenberg (2005)
25. Grapefruit, International Cyber Business Services Inc; www.holisticonline.com (2000)
26. TE Wallis, *Text book of Pharmacognosy*, 5th edn, New Delhi: CBS publishers, (2004) p 194
27. A Prakash, Antioxidant activity, What are antioxidants? *Medallion Laboratories* 19(2) (2001)
28. N Akhtar, Formulation and evaluation of a cosmetic multiple emulsion system containing macademia nut oil and two antiaging agents, Dissertation for the Degree of Doctor of Philosophy, the Department of Pharmaceutical Technology, Anadolu University (2001) pp 104,107
29. P Shoukat et al, Survey and mechanism of skin depigmenting and lightening agents, *Wiley Int Sci J* 20(11) 921–934 (2006)
30. DG Meyers and PA Maloley, Safety of antioxidant vitamins, *Arch of Int Med* 156(9) 925–935 (1996)
31. CD Villarama and HI Maibach, Glutathione as a depigmenting agent: An overview, *Int J of Cosmet Sci* 27(3) 147–153 (2005)
32. D Roberts, Antioxidant Values in Fruits and Vegetables, Macular Degeneration Support Web site; www.mdsupport.org (Accessed Feb 15, 2007)
33. G Mateljan, Grapefruit, The World's Healthiest Foods, Honolulu, Hawaii: McGraw Hill Professional, Science and Technology Encyclopedia, www.healthline.com (2007)
34. JN Kraft and CW Lynde, Moisturizers: What They Are and a Practical Approach to Product Selection, Skin therapy letter, indexed by the U.S. National Library of Medicine 10 (2005)
35. R Hata and H Senoo, L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation and formation of a three dimensional tissue like substance by skin fibroblasts, *J Cell Physiol*, 138(1) 8–16 (1989)
36. S Murad, D Grove, KA Lindberg, G Reynolds, A Sivaraja and SR Pinnel, Regulation of collagen synthesis by ascorbic acid, *Proc Natl Acad Sci USA* 78 2879–2882 (1981)
37. JC Geesin, D Darr, R Kaufmann, S Murad and SR Pinnel, Ascorbic acid especially increases type 1 and type III procollagen messenger RNA levels in human skin fibroblast, *J Invest Derma* 90(4) 420–424 (1988)

38. SR Pinnel, S Murad and D Darr, Induction of collagen synthesis by ascorbic acid: A possible mechanism, *Arch Derma* 123(12) 1684–1686 (1987)
39. K Scharffetter-Kochanek et al, Photoaging of the skin from phenotype mechanisms, *Exp Gerontol* 35(3) 307–316 (2000)
40. SJ Padayatty and M Levine, New insights into the physiology and morphology of vitamin C, *Canad Med Assoc J* 164(3) 353–355 (2001)
41. M Ponc et al, The formation of competent barrier lipids in reconstructed human epidermis requires the presence of vitamin C, *J Invest Derm* 109(3) 348–355 (1997)
42. J Gray, *The world of skin care*, ed 1, London: Macmillan Press (2000) p 7
43. D Mitsushiro, Influence of drug environment on epidermal functions, *J Derm Sci* 24(1) 22–28 (2000)
44. HK Biesalski and JUC Obermueller, UV light β -carotene and human skin beneficial and potentially harmful effect, *Arch Biochem Biophys* 389(1) 1–6 (2001)

Lightening, Boosting and Protecting with Colorless Carotenoids

Liki von Oppen-Bezalel

IBR Ltd., Ramat Gan, Israel

KEY WORDS: *phytoene, phytofluene, colorless carotenoids, antiaging, SPF booster, anti-inflammatory, brightening, whitening*

ABSTRACT : *Phytoene and phytofluene (P&P) are carotenoids and precursors in the biosynthetic pathway of other carotenoids. These occur naturally in microorganisms, algae and other plants and exhibit benefits for skin care including skin lightening, anti-inflammatory activity and protection against UV and oxidative damage.*

Biologically, carotenoids are a significant group of organic pigments with more than 700 members. They are found widely throughout nature but are synthesized only by plants, algae, fungi and bacteria, where they aid in the absorption of light and capture excessive energy, neutralizing tissue-damaging free radicals.¹ Chemically, carotenoids are isoprenoid, C-40 molecules that are either linear or cyclized at one or both ends of the molecule. The chemical structure determines their physico-chemical properties and biological activity.

Carotenoids, specifically β -carotene and lycopene, were used as a treatment against photosensitization as early as 1964.² Since then, a vast number of studies have shown that carotenoids act as antioxidants, anti-inflammatories and antimutagenic agents. These materials also are believed to potentially inhibit certain cardiovascular diseases and cancers. In addition, many carotenoids show beneficial immu-

nological effects. Thus, these molecules are of interest for protective applications against premature aging and age-related disorders resulting from oxidative damage and stress.

Unfortunately, most carotenoids are sensitive to light, a property that considerably limits their use and shortens the shelf life of products that contain them.³ In addition, most carotenoids have a distinctive visible color that is undesirable in most cosmetic and some food applications. In contrast to other carotenoids, however, phytoene and phytofluene (P&P) lack visible color and absorb light in the ultraviolet (UV) range. They are also precursors in the bio-synthetic pathway of other carotenoids (see **Figure 1**).

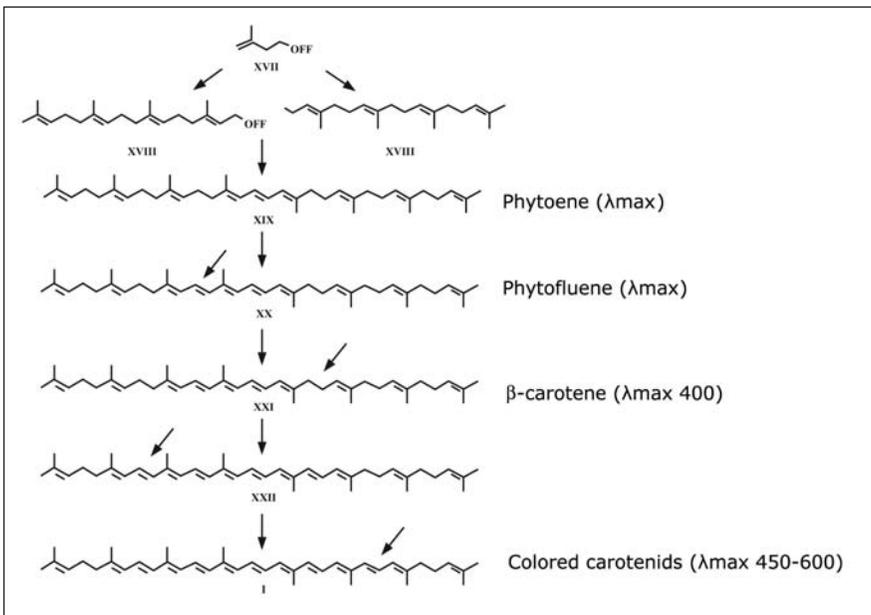


Figure 1. The biosynthetic pathway of carotenoids, where phytoene and phytofluene are the first precursors

Phytoene has been shown to act as an anticarcinogen in mouse skin cancer models⁴ and may play a role in cell-to-cell communication, as some studies with phytoene-producing transgenic mice have suggested.⁵ In addition, dietary P&P accumulate in human skin,⁶ offering protection by acting as UV absorbers, antioxidants and anti-inflammatory agents, suggesting they could play a protective role in beauty and skin applications.

Bioavailability and Tissue Distribution

Dietary carotenoids are found in most human tissues including the blood and skin.^{1,7-11} The distribution of carotenoids throughout the human body is not uniform; for example, higher concentrations are found in the skin, compared with the lungs, plasma or lymphocytes. In addition, P&P accumulate in greater amounts than other carotenoids.⁹ Interestingly, the carotenoid content found within the human body also is notably greater than the content found in foods containing the molecule.¹²

UV Protection

UV radiation generates reactive oxygen species (ROS) and free radicals, which lead to inflammation, lipid peroxidation, DNA damage, collagen degradation and more, resulting in premature skin aging and potential skin cancer. UV irradiation causes DNA damage in two primary ways:

1. UVB damages DNA directly. Energy from sunlight causes an alteration in the chemical composition of the nucleotide bases, altering the molecular structure and disrupting DNA transcription; and
2. UVA causes damage indirectly. Here, the sunlight itself does not change the physical structure of DNA but rather triggers an array of molecules in the cell to break down in turn producing free radicals and ROS. These mutagenic agents can cause alterations in the physical structure of DNA.

P&P^a carotenoids can be employed to protect skin from UVA and UVB damage since phytoene has been shown to absorb UVB, while phytofluene absorbs UVA (see **Figure 2**). Together, they reduce UV transmission through partial absorption by reflecting or emitting light (see **Figure 3**). Mathews-Roth has demonstrated that carotenoids and phytoene specifically have photo-protective capabilities and can prevent UV-induced skin cancer.^{4,13-15}

^a Phytoene and phytofluene are nutraceutical beauty supplements in the product Phytofloral TP and topical active ingredients in IBR-TCLC and IBR-CLC.

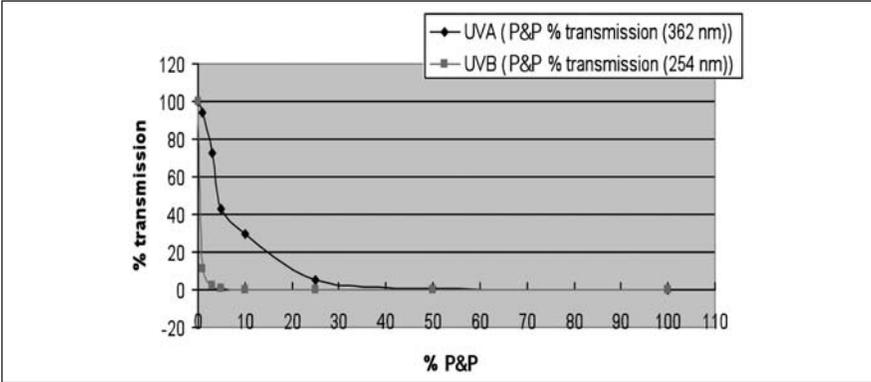


Figure 2. Transmission of UVB and UVA by P&P

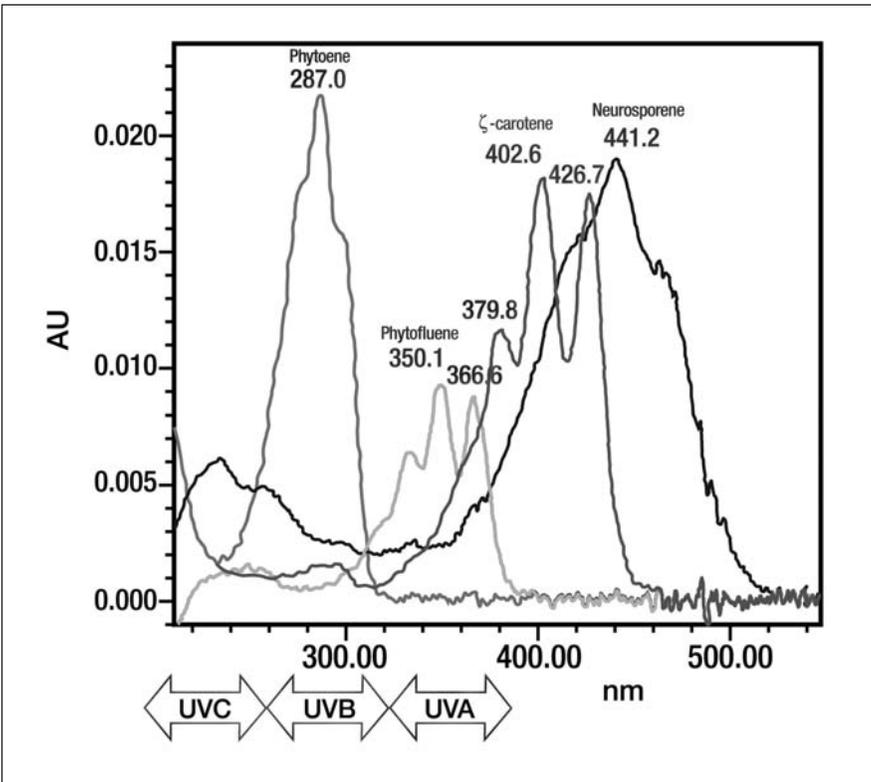


Figure 3. Typical absorption spectra for carotenoids

Hydroxyl Radical Quenching

Hydroxyl radicals play a significant role in UV damage and are several levels of magnitude more reactive toward cellular constituents

than superoxide radicals and hydrogen peroxide. This type of free radical can be formed from $\cdot\text{O}_2^-$ and H_2O_2 via the Harber Weiss reaction, which generates hydroxyl radicals from hydrogen peroxide and superoxide. The interaction of copper or iron with H_2O_2 also produces $\cdot\text{OH}$, as first observed by Fenton.

These reactions are significant, because the constituents ($\cdot\text{O}_2^-$, H_2O_2 and H_2O) are found within the body and on the skin, and can easily interact to generate damaging free radicals.¹⁶ Sunlight is a source for topical generation of hydroxyl radicals, as was demonstrated by Taira et al.¹⁷ This group suggested that skin exposed to sunlight may lead to hydroxyl radical generation and simultaneous lipid peroxidation.

P&P are some of the few antioxidants that show efficacy in quenching hydroxyl radicals ($\cdot\text{OH}$) by trapping and neutralizing them, as the DPPH assay in **Figure 4** shows; however, they are more than antioxidants, as their comparison with BHT shows in **Figure 5**.^{18–20}

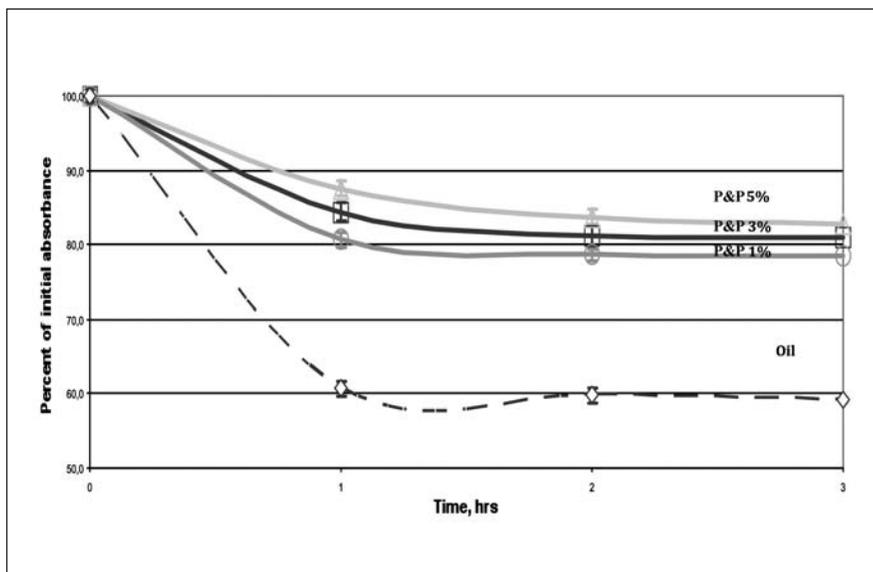


Figure 4. Hydroxyl radical scavenging activity of colorless carotenoids as shown by quenching of 1,1-diphenyl-2-picrylhydrazyl (DPPH)

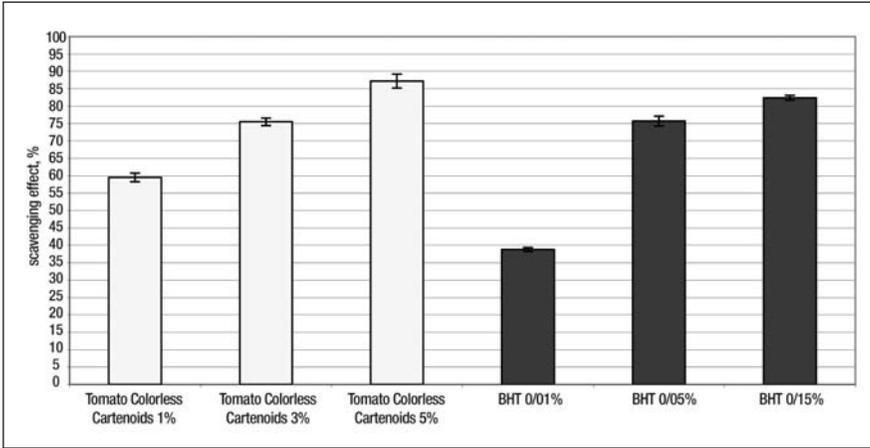


Figure 5. DPPH Scavenging activity comparing colorless carotenoids with BHT (courtesy of IBR Ltd.)

Anti-inflammation and DNA Damage

UVB light induces erythema formation. However, colorless carotenoids have been shown to significantly reduce the expression of inflammatory markers interleukin-6 and interleukin-12 in activated lymphocytes¹⁸ as well as reduce inflammation and potentially sun-damaged cells.^{18–22} Specifically, a 46% reduction in PGE-2 expression in interleukin-1-induced normal human fibroblasts has been observed.²¹ The anti-inflammatory activity of P&P carotenoids could thus reduce erythema formation.

Other studies have shown a reduction of UV-induced erythema with a dietary intake of a combination containing the colorless carotenoids P&P.^{7,23,24} In most of these studies, researchers noted that added protection was likely due to the ability of P&P to absorb UV light, providing additional protection against UV-generated free radicals.^{3,7,24–27}

Another detrimental effect of free radicals formed by UVA/UVB is collagen degradation, which leads to the premature skin aging expressed by wrinkles and loss of skin elasticity and shine. *In vitro* studies have shown that P&P are able to inhibit MMP-1 expression—which is involved in the breakdown of the extracellular matrix—at the low concentration of 15 $\mu\text{g}/\text{mL}$ by 14%, a mild but significant effect. Therefore, P&P carotenoids could reduce the downstream collagen degradation effect of UV light.²¹

P&P also have demonstrated protective effects against DNA damage caused by hydroxyl radicals formed *in vitro*. This was measured by a reduction in fluorescence derived from biotinylated base pairs introduced to a reaction system following free radical damage to plasmid DNA. The DNA was protected by P&P, as shown by the change in fluorescence level indicated in **Table 1**, as well as following repair (not shown).

Table 1. P&P protection against damage to plasmid DNA caused by free radicals ¹³						
Compound	Conc. µg/mL	% inhibition in the presence of hydroxyl radicals	% non-specific inhibition	% specific inhibition	Concentration giving 50% of activity	
Phytoene and phytofluene	140	86	24	62	11.2 µg/mL	
	14	74	19	55		
	1.4	6	7	1		
	0.14	1	1	0		
Positive control	1000	88	9	79	80 µg/mL	
	100	69	11	58		
	10	5	0	5		
	1	4	0	4		

These findings are in accord with an intervention study conducted by Porinni et al.⁹ with carotenoids from natural tomato extract containing mostly lycopene, phytoene, phytofluene and β -carotene. This study showed modification of plasma and lymphocyte levels and improved antioxidant protection against DNA damage to lymphocytes with increasing values of plasma and lymphocyte carotenoid levels, especially in the case of phytoene.

Skin Brightening with P&P

Human skin tone varies between fair to dark brown. Skin type, according to the Fitzpatrick scale, ranges from I to VI with the higher numerals indicating darker, ethnic skin tones. Although these darker types are more resistant to sun irradiation, they are also at risk for skin cancer.

Differences in skin color are primarily determined by the amount of melanin present in melanocytes. Melanin production is stimulated by UVA light, leading to a tanning effect in skin. Aging, exposure to sun, hormonal abnormalities and various skin disorders increase the deposition of melanin pigment in skin, resulting in dark spots and freckles. These pigmented spots are undesired in many cultures and also considered unhealthy.

To eliminate pigmentation on skin or achieve a lighter skin tone, whitening or bleaching compositions are useful. Typical lightening agents in cosmetic formulations include kojic acid, arbutin, licorice extract and vitamin C. These are effective tyrosinase inhibitors and antioxidants but tend to be unstable since they are easily oxidized and degraded. Moreover, these compounds carry a higher risk for potential skin irritation and inflammation.

A combination of P&P has demonstrated efficacy on skin pigmentation by reducing the melanin content in skin cells.²⁰ In addition, these natural carotenoids have not been shown to cause adverse effects when applied to skin or taken orally. An example of a skin-lightening formulation incorporating P&P carotenoids is shown in **Formula 1**.

P&P carotenoids in concentrations lower than known skin-lightening ingredients such as arbutin have in fact shown greater effects

on melanin synthesis than such ingredients. For instance, P&P carotenoids have shown a 22% reduction in melanin content in B16 murine melanocytes at a concentration of 7.5 μM with no effect on cell viability, whereas arbutin, a known skin-lightening agent, has shown an effect on melanin synthesis without an inhibitory effect on cell growth at maximum concentrations of 50 μM .²⁸ Although the lightening effect of P&P carotenoids has not been tested on ethnic skin, they have been formulated into ethnic products in Asia. These materials could in theory be used on ethnic skin to lighten, protect against UV damage and reduce inflammation.

Formula 1. Lightening o/w day cream

A. Potassium cetyl phosphate	3.00% w/w
Glyceryl stearate	0.50
Triethylhexanoin	6.00
Isopropyl isostearate	6.00
Cyclopentasiloxane	6.00
Phenyl trimethicone	2.00
B. Water (<i>aqua</i>)	qs to 100
Carbomer	0.40
C. Squalane (and) <i>Solanum lycopersicum</i> (tomato) fruit extract (IBR-TCLC, IBR)	0.50
Water (<i>aqua</i>) (and) <i>Narcissus tazetta</i> bulb extract (IBR Dormin, IBR)	1.50
D. Titanium dioxide (and) boron nitride (and) acrylates/ammonium methacrylate copolymer (WhiteCap2, Tagra)	1.00
E. Aluminum oxide	0.50
Glycerin	4.00
F. Propylene glycol (and) diazolidinyl urea (and) methylparaben (and) propylparaben (Sharomix DMP, Sharon Laboratories)	1.00

Procedure: Combine B with stirring. Separately combine A. Heat A and B at 80°C. Add A to B while stirring moderately (approx. 300 rpm). Homogenize AB for 90 sec. Prepare E and add to AB in the homogenizer. Homogenize again for 90 sec. Cool to 40-45°C and add C and F to batch, stirring slowly. Add D to batch without homogenization. Cool to RT stirring slowly. Adjust final pH if necessary to 6.5.

Boosting Sunscreen SPF

Frequently, when sunscreens are exposed to sunlight, some of the light energy goes toward generating harmful free radicals and ROS. However, an active molecule that could quench or prevent free radical generation may stabilize the formulation and increase its safety and efficacy.

P&P carotenoids were tested in this capacity. Two sunscreen creams were prepared with 3.5% w/w octyl methoxycinnamate and 1% butyl methoxydibenzoylmethane. One sunscreen cream contained 5% P&P carotenoids from algae while the control did not contain the compounds. The *in vitro* SPF of both sunscreens was determined using a testing substrate that mimics the surface properties of human skin^b. 2 mg/m² was applied to the substrate and irradiated. The reflected light was measured by transmittance analyzer^c. The Minimum Protection Factor (MPF) was obtained and SPF was calculated.

The P&P carotenoids were shown to provide stabilization and to reduce the generation of free radicals and damage derived from irradiation of TiO₂ (see **Figure 6**); in addition, they boosted the SPF of a test sunscreen (see **Table 2**).

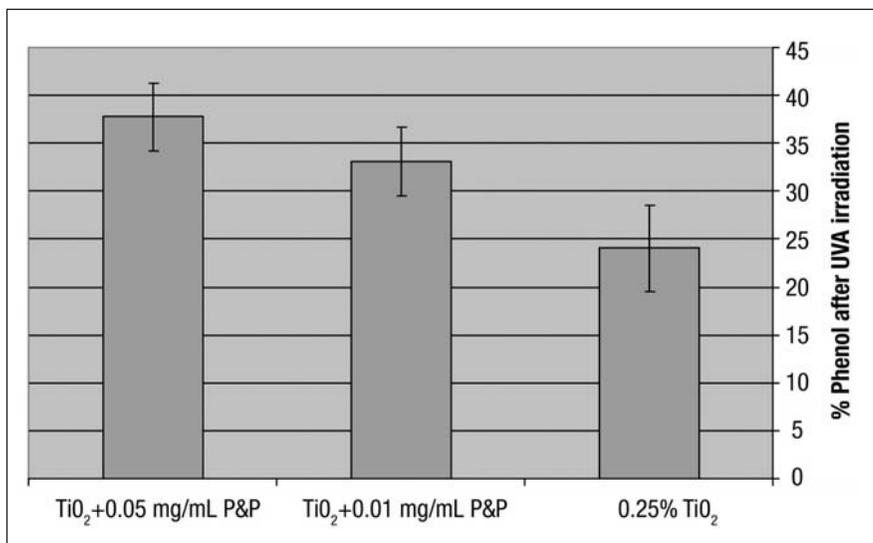


Figure 6. P&P reduce degradation of phenol in the presence of UV irradiated TiO₂

^bVitro-Skin is a product of IMS Inc.

^cUV-1000F Ultraviolet Transmittance Analyzer is a product of Labsphere.

Table 2. SPF of sunscreen creams with and without P&P

Sunscreen	SPF <i>in vitro</i> (382 nm)
Sunscreen cream	18.7 ± 0.4
Sunscreen cream + 5% P&P	32.5 ± 0.1

Conclusion

The colorless carotenoids P&P have been shown to protect the skin via oral and topical application against UV and oxidative damage that leads to premature aging and other disorders. This chapter summarizes the protective activities of colorless carotenoids against UV irradiation, free radicals and DNA damage as well as anti-inflammatory benefits, collagen production promotion and reduction of skin pigmentation. Finally, P&P carotenoids can efficiently stabilize sunscreens and boosts their sun protection capabilities.

Published March 2009 *Cosmetics & Toiletries* magazine.

References

1. A Bendich and JA Olson, Biological actions of carotenoids, *FASEB J* 3 1927–32 (1989)
2. MM Mathews, Protective effects of β -carotene lethal photosensitization by haematoporphyrin, *Nature* 203 1092 (1964)
3. NI Krinski, MM Mathews-Roth and RF Taylor (Eds), in: *Carotenoids, Chemistry and Biology*, New York, London: Platinum Press (1989)
4. MM Mathews-Roth, Antitumor activity of beta-carotene, canthaxanthin and phytoene, *Oncology* 39(1) 33–7 (1982)
5. Y Satomi, N Misawa, T Maoka and H Nishino, Production of phytoene, a carotenoid, and induction of connexin 26 in transgenic mice carrying the phytoene synthase gene crt, *B. Biochem Biophys Res Comm* 320 398–401 (2004)
6. F Khachik, L Carvalho, PS Bernstein, GJ Muire, DY Zhao and NB Katz. Chemistry, distribution, and metabolism of tomato carotenoids and their impact on human health, *Exp Biol Med* 227 845–851 (2002)
7. O Aust, W Stahl, H Sies, H Tronnier and U Heinrich, Supplementation with tomato-based products increases lycopene, phytofluene, and phytoene levels in human serum and protects against UV-light-induced erythema, *Int J Vitam Nutr Res* 75(1) 54–60 (2005)
8. IV Ermakov, MR Ermakova, W Gellermann and J Lademann, Noninvasive selective detection of lycopene and beta-carotene in human skin using Raman spectroscopy, *J Biomed Opt* 9(2) 332–8 (2004)

9. M Porrini, P Riso, A Brusamolino, C Berti, S Guarnieri and F Visioli, Daily intake of a formulated tomato drink affects carotenoid plasma and lymphocyte concentrations and improves cellular antioxidant protection, *Brit J of Nutrition*, 93(1) 93–99 (2005)
10. F Khachik, GR Beecher, MB Goli and WR Lusby, Separation, identification, and quantification of carotenoids in fruits, vegetables and human plasma by high performance liquid chromatography, *Pure App Chem*, 63(1) 71–80 (1991)
11. PD Fraser and PM Bramley, The biosynthesis and nutritional uses of carotenoids, *Progress in Lipid Research* 43 228–265 (2004)
12. TR Hata, TA Scholz, IV Ermakov, RW McClane, F Khachik, W Gellermann, LK Pershing, Non-invasive raman spectroscopic detection of carotenoids in human skin, *J Invest Dermatol*, 115(3) 441–8 (2000)
13. MM Mathews-Roth and NI Krinsky, Carotenoids affect development of UVB induced skin cancer, *Photochem Photobiol* 46(4) 507–9 (1987)
14. MM Mathews-Roth, Carotenoid functions in photoprotection and cancer prevention, *J Environ Pathol Toxicol Oncol*, 10(4–5) 181–92 (1990)
15. MM Mathews-Roth, Photoprotection by carotenoids, *Fed Proc* 46(5) 1890–3 (1987)
16. B Halliwell and JMC.Gutteridge, The chemistry of oxygen radicals and other oxygen-derived species. *Free Radicals in Biology and Medicine*, New York: Oxford University Press (1985) pp 20–64
17. J Taira, K Mimura, T Yoneya, A Hagi, A Murakami and K Makino, Hydroxyl radical formation by UV-irradiated epidermal cells, *J Biochem* 111(6) 693–5 (1992)
18. L Von Oppen-Bezalel, E Lerner, DG Kern, B Fuller, E Soudant and A Shaish, IBR-CLC, Colorless Carotenoids: Phytoene and Phytofluene from Unicellular Algae—Applications in Cosmetics, Wellness and Nutrition, *Fragrance J*, 34 48–53 (2006)
19. L Von Oppen-Bezalel, Colorless Carotenoids, phytoene and phytofluene for the skin: For prevention of aging/photo-aging from inside and out, *SÖFW*, 7 (2007)
20. L Von Oppen-Bezalel, UVA, A main concern in sun damage: Protection from the inside and outside with phytoene, phytofluene, the colorless carotenoids and more, *SÖFW*, 11 (2007)
21. BB Fuller, DR Smith, AJ Howerton, D Kern, Anti-inflammatory effects of CoQ10 and colorless carotenoids, *J Cos Derm* 5(1) 30–38 (2006)
22. MM Mathews-Roth and MA Pathak, Phytoene as a protective agent against sunburn (>280 nm) radiation in guinea pigs, *Photochem Photobiol*, 21(4) 261–26 (1975)
23. JP Cesarini, L Michel, JM Maurette, H Adhoue and M Bejot, Immediate effects of UV radiation on the skin: Modification by an antioxidant complex containing carotenoids, *Photodermatol Photoimmunol Photomed* 19(4) 182–9 (2003)
24. Y Sharoni, Lycopene, skin cancer and UV exposure, in *Conference notes: Examining the Health Benefits of Lycopene from Tomatoes*, Washington, DC, conference convened by the Center for Food and Nutrition Policy at Virginia Tech (Apr 1–2, 2003)
25. W Stahl, U Heinrich, S Wiseman, O Eichler, H Sies and H Tronnier, Dietary tomato paste protects against UV-induced erythema in humans, *J Nutr* 131 1449–1451 (2001)
26. J Lee, S Jiang, N Levine and RR Watson, Carotenoid supplementation reduces erythema in human skin after simulated solar radiation exposure, *Proc Soc Exp Biol Med* 223 170–174 (2000)
27. H Sies and W Stahl. Carotenoids and UV protection, *Photochem Photobiol Sci* 3(8) 749–52 (2004)
28. S Akiu, Y Suzuki, T Asahara, Y Fujinuma and M Fukuda, Inhibitory effect of arbutin on melanogenesis—Biochemical study using cultured B16 melanoma cells, *Nippon Hifuka Gakkai Zasshi*, 101(6) 609–13 (1999)

Innovations in Dermatology: Rethinking the Aging Face

Zoe Diana Draelos, M.D.

Department of Dermatology, Wake Forest University, North Carolina USA

KEY WORDS: *skin care, antiaging, skin thickness, humectancy, barrier function, anti-inflammatory, fillers*

ABSTRACT: *Dermatologic innovations are addressing the symptoms of aging skin by restoring skin thickness, enhancing humectancy, maintaining barrier function and reversing or preventing inflammation.*

Solutions to the age-old problem of the aging face require that we revise our understanding of the skin. Its thickness, barrier function, inflammation, and supporting structure all change with age. Now three new concepts discussed here are changing the way we think about the aging face.

The wrinkles of time eventually appear on every face. This is well accepted, but exactly what causes these wrinkles has been the subject of vigorous debate in dermatologic circles. For many years, it was thought that gravity ultimately produced the aging face by causing sagging resulting in drooping eyebrows, redundant upper eyelid skin, lower eyelid bags, a down-turned mouth and a double chin.

While this theory of gravity aging seems plausible, it has been replaced with what seems to be a more physiologically consistent explanation for the redundant skin on the face: decreased skin thickness and deterioration of the underlying facial structure. Rethinking fundamental ideas on the causes of aging is the first step toward finding effective solutions.

Restoring Skin Thickness

Fat redistribution: Researchers looking at mammalian patterns of aging have noticed the disappearance of subcutaneous fat on the face and beneath the skin of the body with a new deposition of fat around the organs inside the chest and abdomen. The exact cause of this fat redistribution is unknown, but is probably related to decreases in various hormones.

Why is this fat redistribution important to the cosmetic chemist? Because it provides insight regarding how products of the future should be formulated to optimize facial appearance.

The loss of facial fat means that the same amount of skin is now covering less material underneath. Folds and wrinkles of the face are not related to overabundant skin, but rather less substance to support the overlying skin. Beautiful smooth skin is simply a drape shaped and firmed by the fat, muscles and bones that it artistically covers. The skin is only as beautiful as the supporting framework. Thus, youthful skin requires a youthful frame.

Unfortunately, the bones of the face are not weight-bearing and thus are subject to the gradual loss that affects all bones of the body, especially in postmenopausal women. The muscles of the face are not skeletal muscles that can be exercised and enlarged. This leaves the skin and subcutaneous fat as possible targets of cosmetic therapy.

Surgery: In the past, the cosmetic surgeon focused on cutting away unwanted excess skin as a technique of minimizing the folds and wrinkles of the face. This pulling of the skin over a suboptimal frame left the face looking thin, bony and gaunt.

Newer concepts in cosmetic surgery have focused on methods of restoring fullness to the face by replacing subcutaneous fat and plumping thinning skin. This has led to fat transplant procedures whereby unwanted fat from the abdomen is harvested, frozen, and injected beneath the skin of the cheeks and around the mouth. It has led to the search for permanent fillers, such as artificial bone and medical-grade silicone to take up space and plump the thinning mature face.

Fillers: Currently, the hottest concept in dermatology is the use of fillers to restore the fullness of the skin. One of the most important substances lost from the skin with time is known as

ground substance composed of proteoglycans, such as hyaluronic acid. Hyaluronic acid is naturally found within the dermis of the skin and acts as a sponge holding water and thickening the skin. Injecting hyaluronic acid into the skin in the superficial dermis restores the water-holding capacity of the skin filling in folds and wrinkles. The hyaluronic acid remains in the skin for approximately 6 months and then must be replaced by reinjection.

Sites of thinning skin and subcutaneous fat loss that respond well to hyaluronic acid injection include the lips, the folds between the nose and the corner of the mouth (nasolabial folds), the folds between the corners of the mouth and the chin (melolabial folds), and the fine furrows of the upper lip.

Hyaluronic acid is nothing new to the cosmetic chemist. Hyaluronic acid has been used for years as an effective humectant to enhance hydration of the skin and prevent desiccation of the product as it sits on the shelf under low humidity conditions.

What can be learned from this new trend that brings increasing use of fillers in dermatology? One of the most important mechanisms of decreasing wrinkling of the skin is enhancing humectancy. Glycerin is one of the most important humectants owing to its ability to form a temporary reservoir in the stratum corneum. The search for methods of creating humectant reservoirs in the skin should be pursued because aging results in decreased humectancy. This could be accomplished via time-released humectants, such as those delivered via multilamellar vesicles, or novel humectants that are able to attract water without leaving the skin sticky.

Restoring Barrier Function

Disorganization of the barrier: In addition to thinning of the skin with age and the loss of the underlying framework, the skin also loses the nicely organized structure of the skin barrier. The skin barrier is constructed of protein-rich keratinocytes that are held together with intercellular lipids. With time, the intercellular lipids appear to be less well formed and the keratinocytes do not properly exfoliate. Whether this is attributable to failure of the skin cells to communicate proper functional messages or to a decrease in the biosynthetic abilities of the skin, the skin is not as smooth and even,

resulting in a phenomenon labeled by some as decreased radiance or luminosity.

Ceramides: One of the newer concepts in dermatology regarding barrier function is the incorporation of ceramides into moisturizers. Because ceramides are an important component of the intercellular lipids, theoretically ceramides might enhance the skin barrier resulting in increased skin surface smoothness and improved radiance. Moisturizers containing ceramide 3 are indeed recommended by many dermatologists as an adjuvant treatment to topical corticosteroids for the treatment of atopic dermatitis, a disease characterized by dry skin, hay fever, and asthma.

Reversing and Preventing Inflammation

Inflammation: Skin thickness and barrier function are important qualities to restore and maintain for the appearance of healthy skin. This article has focused on activities designed to restore skin functioning, but an equally important medical activity is the prevention of skin damage. Newer theories of skin aging place photodamage and carcinogenesis on the same spectrum. Thus, advanced photodamage is synonymous with skin cancer because both are caused by the inflammatory changes associated with exposure to ultraviolet A (UVA) (see **Inflammation and Skin Cancer**). Dermatology seeks to develop both oral and topical anti-inflammatory agents that someday may prevent skin cancer.

This interest in topical anti-inflammatories is also germane to the cosmetic chemist, because the ability to stop cutaneous inflammation or even shut down the inflammatory cascade could ultimately decrease or possibly prevent skin wrinkling. Topical anti-inflammatory cosmeceuticals are an important area of research because inflammation of the skin results in collagen and elastin breakdown through the activation of collagenases and elastases. In the female, collagen breakdown is further increased by the presence of estrogen that functions as a metalloprotease. It is for this reason, among others, that women age more quickly than men, especially after pregnancy. The ability to stop cutaneous inflammation or even shut down the inflammatory cascade could ultimately decrease or possibly prevent skin wrinkling.

A successful topical anti-inflammatory is any substance that interrupts any part of the inflammatory cascade, which offers diverse opportunities for efficacy. Currently available anti-inflammatory cosmeceuticals include niacinamide, green tea polyphenols, and salicylic acid.

Prevention: The flip side of reversing inflammation is prevention. Thus, any cosmetic that irritates the skin potentially enhances aging through activation of the inflammatory cascade. Theoretically, this was one of the problems with the stinging and burning induced by glycolic acid-containing moisturizers popular in the 1990s. While “no pain, no gain” may be the mantra of muscle building workout routines, no product applied to the skin should induce any noxious sensory stimuli. This is a sign that the inflammatory cascade has been activated.

Furthermore, UV photoprotection becomes an important mechanism to prevent inflammatory cascade activation. This means that the incorporation of broad-spectrum sun protectants into skin care products is a necessary requirement for the prevention of aging. Few excellent broad-spectrum sunscreen agents exist beyond zinc oxide and titanium dioxide. This points to the need for the U.S. Food and Drug Administration (FDA) to hasten the approval of novel agents as monographed sunscreen ingredients.

Inflammation and Skin Cancer

UVA damage actually begins in youth with the first ray of sunshine, but the cumulative effects are not seen usually until age 30. The exposure of skin to tanning booth light or residing closer to the equator hastens these changes. With continued UVA exposure, the inflammation accelerates and the skin loses the Langerhans cells that are responsible for detecting and eliminating cells with defective DNA.

Replication of this defective DNA results in cells that ultimately reproduce uncontrolled and unchecked, which results in the autonomous cells responsible for basal cell carcinoma, squamous cell carcinoma, and melanoma. These three forms of skin cancer are the end result of cumulative inflammatory skin insults.

Summary

Conceptual advances in understanding the aging process and developing new procedures to reverse the signs of aging are active topics

of dermatologic research. It is necessary to determine exactly what makes faces appear old.

Currently, an important part of aging is felt to be decreased skin thickness and deterioration of the underlying facial structure. Fillers are an attempt to replace what has been lost structurally, while methods of restoring intercellular lipids are aimed at improving the skin surface. Finally, reversing and preventing inflammation appear to be important to preserving youthful skin while stopping carcinogenesis.

Rethinking fundamental ideas regarding aging is the first step toward finding effective solutions.

Published October 2005 *Cosmetics & Toiletries* magazine.

Slowing Intrinsic and Extrinsic Aging: A Dual Approach

Liki von Oppen-Bezalel

IBR Ltd., Ramat Gan, Israel

KEY WORDS: *antiaging, photoaging, intrinsic aging, extrinsic aging, cell proliferation, oxidation, dormins, colorless carotenoids*

ABSTRACT: *In this dual approach to antiaging, intrinsic factors are first addressed by slowing cell proliferation in skin via a technology based on dormins—in this case, extracts from dormant Narcissus tazetta bulb. Then, to protect against extrinsic aging, colorless carotenoids are employed to absorb UV radiation and prevent oxidative stress damage.*

When designing products for maximum antiaging benefits, formulators should consider both intrinsic and extrinsic aging factors—intrinsic being naturally programmed aging, and extrinsic resulting from accumulated damage. This chapter describes a combined approach to reduce the signs of aging, the first of which focuses on intrinsic aging by slowing cell proliferation rate to preserve cells in their younger stage. The second approach aims at extrinsic factors to protect the skin from photo- and oxidative damage.

Intrinsic Aging

Intrinsic aging is, in a way, programmed aging that is regulated by a cell's internal clock that defines the number of replications a cell can endure as it ages.^{1,2} This is based on Hayflick's theory stating that

cells have a limited capacity to replicate. With recent elucidation of the respective role of telomeres and telomerase, the Hayflick theory has been strongly supported in the scientific community. Consequently, there are at least two ways to delay intrinsic aging: by slowing proliferation and extending the cell cycle, or by preventing telo- mere shortening.

The slowing of cell proliferation can be accomplished via the concept of dormancy. Dormancy is a natural state that plants and some animals enter to protect themselves from unfavorable environmental and growth conditions. Once more favorable conditions are present, these entities are able to rejuvenate themselves. During dormancy, however, growth functions are slowed and cell proliferation is inhibited. Flower bulbs are a good example; they go dormant through the winter season and emerge as beautiful, rejuvenated blossoms in the spring.

Inhibition of cell proliferation during dormancy is achieved through dormins. However, the chemical nature of these entities varies between plants and organisms, which is why it has been difficult to determine their mechanism of action beyond reversing growth arrest. Once dormins are removed, cell growth resumes; thus dormins are a favorable approach to treat intrinsic aging since they are cell proliferation inhibitors. Further, it is known³ that UV and oxidative stress can cause enhanced proliferation (i.e., cancer), leading to premature aging. Therefore, slowing this proliferation process could not only preserve the cell youth capital, but also slow the effects of premature aging.⁴⁻¹⁰

Researchers recently have developed several technologies based on a natural extract derived from plants during their dormant stage. These dormins^a were shown to slow cell proliferation in a demonstration using germinated cucumber seeds.

Dormins and seed root elongation: Extracts taken from dormant and nondormant *Narcissus tazetta* bulbs were applied to germinated cucumber seeds to examine their effects on seed root elongation (see **Figure 1**).

^a *IBR-Dormin (INCI: Water (aqua) (and) Narcissus Tazetta Bulb Extract)* is a product and registered trademark of IBR Ltd.

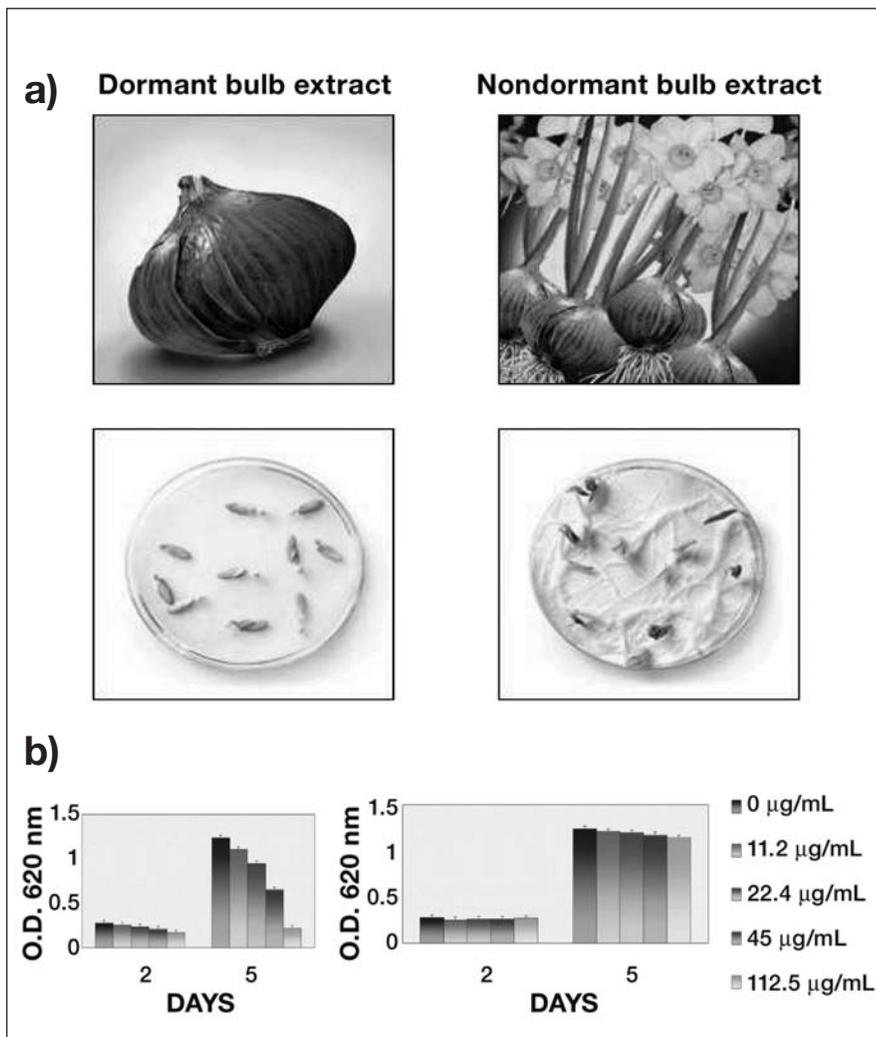


Figure 1. Effects of dormant and nondormant bulb extract on a) germinated seed elongation, and b) normal human keratinocyte proliferation

The length of roots and hypocotyls of the germinated seed were measured for several days. Results reflected a dose-response curve where the higher concentration of extract matched with the shortest length of roots and hypocotyls, indicating less growth and cell proliferation.

Similar effects were observed by counting cells in a cell culture. Methods to examine these effects are protected under copyright, but it can generally be stated that comparing dormant and nondormant extracts clearly showed the slowing of cell proliferation in the

presence of dormins, while no such effect was observed with extracts from the nondormant plant. Interestingly, a similar effect was shown on normal human keratinocytes (see **Figure 1**).

Materials and Methods

To demonstrate the antiaging benefits of a dormant extract on skin cell proliferation, a placebo-controlled, double blind study was conducted with a cream containing *Narcissus tazetta* bulb extract at 0.5% and 1.5%, and a control cream excluding the extract. An example of a similar cream is shown in **Formula 1**.

Formula 1. Antiaging and moisturizing o/w day cream

A. Cetearyl alcohol (and) polysorbate 60	8.00% w/w
Isopropyl isostearate	10.00
Caprylic/capric triglyceride	10.00
<i>Macadamia ternifolia</i> seed oil	2.00
Tocopherol (mixed) (and) β -sitosterol (and) squalane (and) glyceryl linoleate	0.05
B. Glycerin	2.00
Xanthan gum	0.20
C. Water (<i>aqua</i>)	qs to 100.00
D. Betaine	2.00
E. Glycerin (and) water (<i>aqua</i>) (and) <i>Narcissus tazetta</i> bulb extract (IBR Dormin R Organic, IBR)	2.00
Benzyl alcohol (and) dehydroacetic acid (and) benzoic acid (and) sorbic acid	1.00

Procedure: Mix B and add to warm C (45–50°C). Stir for 20–30 min. Add D to BC and heat to 75°C. Heat A to 75°C. Add A to BCD while stirring moderately (approx. 300 rpm). Homogenize batch for 1 min (Silverson: 3000 rpm). Allow to cool to 45–40°C, stirring slowly. Adjust final pH = 5.5–6.0. Add E to batch and cool to RT with low stirring; mPa·s (Brookfield LVT, Spindle D, 12 rpm) 41.350; Viscosity (20°C); pH = 5.93.

A total of 135 female panelists was divided into three groups and given one of the three sample creams to test. Subjects rated the performance of their skin in relation to various parameters on a 10-cm nonscaled ruler, as is described below, before they initiated the twice daily application of a respective cream (T0). The same rating was performed at the end of the study (day 28) on a new nonscaled ruler (T28). Researchers found that the creams containing the

extract at 1.5% were rated significantly higher than the placebo. This was particularly true for the perception of skin's resistance to the environment and protective capabilities in addition to skin sensitivity and irritability (see **Figure 2**).

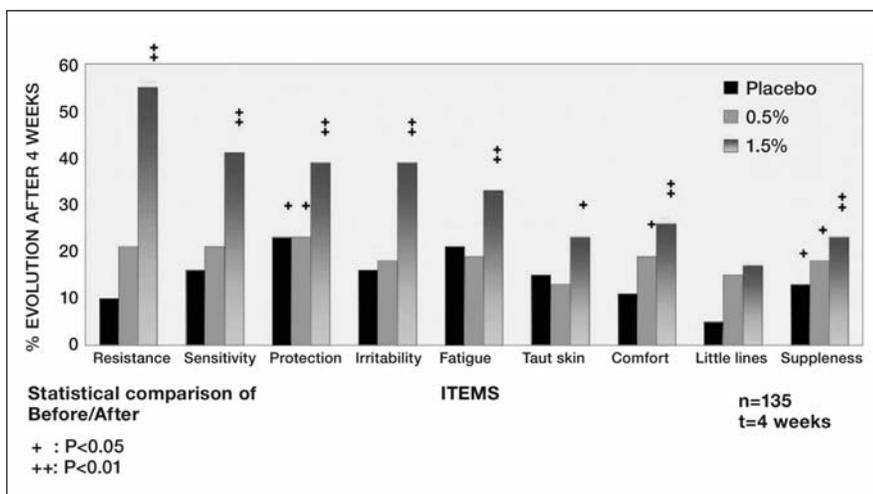


Figure 2. Sensory analysis of dormins in a cosmetic cream

These results support the hypothesis that the dormant extracts could slow the proliferation of cells to allow the epidermis time to fully develop into a healthier-looking, more complete skin layer including defenses to protect the skin against environmental stressors. Besides antiaging, slower cell turnover can provide other benefits. For instance, slowing intrinsic aging prolongs the appearance of artificially tanned skin since corneocytes shed less quickly and therefore maintain a tan for longer periods of time.¹¹ And provided that hair follicle cells may also respond to the dormant extracts, dormins could slow the growth of hair. Sebaceous cells or adipocytes may also be interesting targets.

Extrinsic Aging

Besides intrinsic aging, extrinsic factors cause skin aging. Extrinsic aging is affected by environmental stresses including reactive oxygen species (ROS), UV irradiation and free radicals that contribute to accumulated damage, and collagen and cell matrix degradation. In general, a free radical is any molecule that has a single unpaired

electron in its outer shell. While few free radicals are stable, most biologically relevant free radicals are fairly reactive and for most biological organisms, free radical damage is closely associated with oxidative damage.

Based on Harman's free radical theory of aging stating that organisms age because cells accumulate free radical damage over time,^{12–15} antioxidants are commonly used in skin care to fight aging. One such antioxidant recently was developed based on colorless carotenoids^b.

It is important to note that, while they are important to use, sunscreens can also increase an individual's exposure to environmental stresses such as free radicals that lead to premature aging. Since sunscreens protect against UVB and reduce erythema formation, they also allow consumers to stay in the sun longer without immediate visible signs of damage. Longer exposure to UV irradiation increases the chances of free radical generation.

The colorless carotenoids phytoene and phytofluene (P&P) are natural carotenoids that lack visible color due to their lower number of conjugated double bonds on the C40 carbon backbone of the molecule. This structure allows them to absorb light in the UVA and UVB range and quench the free radicals formed by UV light— with or without the presence of sunscreens (**Figure 3**).¹⁶ These colorless carotenoids have also shown good antioxidant capacity against hydroxyl radicals.^{17,18} These capabilities, among others, enable them to reduce damage to DNA and collagen degradation while reducing inflammation.^{17–20} Thus, the addition of the P&P to sun protection formulas could reduce the risk of skin damage, as was previously shown.¹⁶ Two sunscreen creams were prepared, one containing 5% *Dunaliella salina* extract, which is an extract of P&P from algae, and one without the extract used as control. The SPF of both sunscreens was measured *in vitro* by applying a 2 mg/m² sample of the test creams to a skin substrate and irradiating it. The reflected light was measured, the Minimum Protection Factor (MPF) was obtained, and the SPF was calculated, revealing a

^b *IBR Colorless Carotenoids (IBR CLC and TCLC) (INCI: Dunaliella Salina Extract; and INCI: Solanum Lycopersicum (Tomato) Fruit Extract, respectively) are products and registered trademarks of IBR Ltd.*

significantly higher SPF level in the presence of the *Dunaliella salina* extract (see **Table 1**).

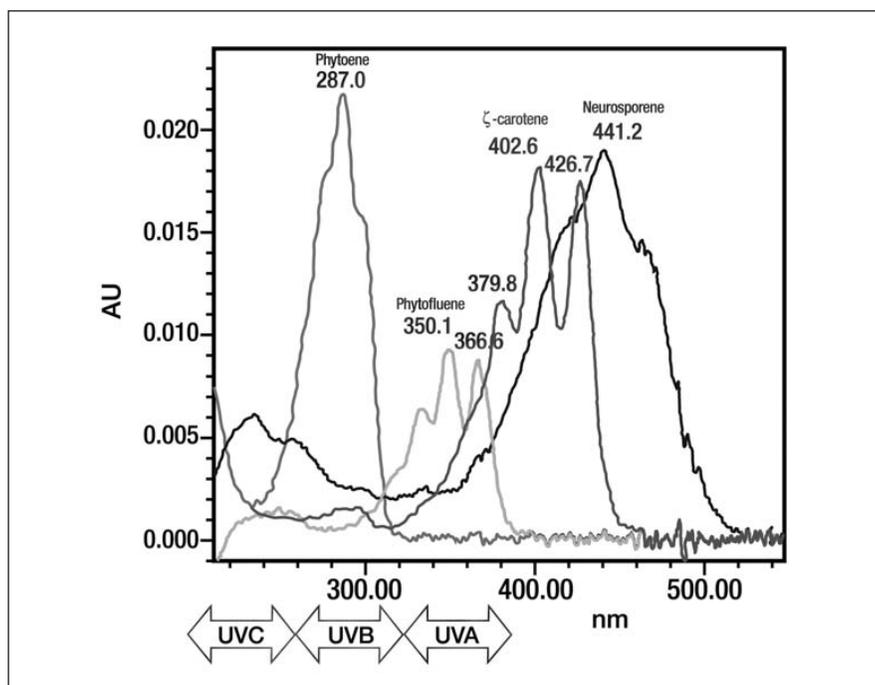


Figure 3. The colorless carotenoids phytoene and phytofluene absorption spectra

Table 1. SPF of sunscreen creams with and without P&P

Sunscreen	SPF <i>in vitro</i> (382 nm)
Sunscreen cream	18.7 ± 0.4
Sunscreen cream + 5% P&P	32.5 ± 0.1

Conclusions

The two primary mechanisms of aging—i.e., intrinsic and extrinsic—can be targeted together to provide a dual approach to antiaging. By slowing cell proliferation to maintain youth capital in cells, and protecting against UV damage and free radicals with

antioxidants, internal and external aggressions can be counteracted. This dual approach provides a tool that may slow aging more comprehensively, preventing the formation of skin disorders that result from pre-matured extrinsic as well as intrinsic aging. The skin is thus provided with a defense and prevention tools to keep it healthier, younger-looking and better able to shield the body, fitting its basic function.

Published May 2009 *Cosmetics & Toiletries* magazine.

References

1. AG Bondar et al, Extension of life span by introduction of telomerase into human cells, *Science* 279 349–352 (1998)
2. KH Buchkovich, Telomeres, telomerase and cell cycle, *Prog Cell Cycle Res* 2 187–195 (1996)
3. Ichihashi et al, UV-induced skin damage, *Toxicology* 189, 1–2: 21–39 (2003)
4. L Hayflick and PS Moorhead, The serial cultivation of human diploid cell strains, *Exp Cell Res* 25 585–621 (1961)
5. L Hayflick, Current theories of biological aging, *Fed Proc* 34 9–13 (1975)
6. L Hayflick, The cell biology of aging, *Clin Geriatr Med* 1(1) 15–27 (1985)
7. L Hayflick, *How and Why We Age*, New York: Ballantine Books (1994)
8. L Hayflick, The future of ageing, *Nature* 408(6809) 267–269 (2000)
9. L Hayflick, “Antiaging” is an oxymoron, *J Gerontol A Biol Sci Med Sci* 59(6) B573–578 (2004)
10. J Campisi, Replicative senescence and old lives’ tale? *Cell* 84 497–500 (1996)
11. E Soudant, L Bezalel, M Ziv and I Perry, EU Patent No. 0973532; US Patent No. 6342254: Anti-proliferative Preparations
12. 1. D Harman, Aging: A theory based on free radical and radiation chemistry, *J Gerontol* 11(3) 298–300 (1956)
13. D Harman, Free radical theory of aging: Affect of free radical reaction inhibitors on the mortality rate of male LAF mice, *J Gerontol* 23(4) 476–482 (1968)
14. D Harman, The biologic clock: The mitochondria? *J Am Geriatr Soc* 20(4) 145–147 (1972)
15. D Harman, The aging process, *Proc Natl Acad Sci USA* 78(11) 7124–7128 (1981)
16. L von Oppen-Bezalel, Lightening, boosting and protecting with colorless carotenoids, *Cosm & Toil* 124(3) (Mar 2009)
17. L von Oppen-Bezalel, E Lerner, DG Kern, B Fuller, E Soudant, A Shaish, IBR-CLC, Colorless carotenoids: Phytoene and phytofluene from unicellular algae—applications in cosmetics, wellness and nutrition, *Frag J*, 34 3 48–53 (2006)
18. L von Oppen-Bezalel, Colorless carotenoids, phytoene and phytofluene for the skin: For prevention of aging/photo-aging from inside and out, *SÖFW* 7 (2007)
19. L von Oppen-Bezalel, UVA, A main concern in sun damage: Protection from the inside and outside with phytoene, phytofluene the colorless carotenoids and more, *SÖFW* 11 (2007)
20. BB Fuller, DR Smith, AJ Howerton and D Kern, Anti-inflammatory effects of CoQ10 and colorless carotenoids, *J Cos Derm* 5(1) 30–38 (2006)

Sirtuins: A Breakthrough in Antiaging Research

Isabelle Imbert, PhD; Claude Dal Farra, PhD; and Nouha Domloge

Vincience, ISP Global Skin Research Center, Sophia Antipolis, France

KEY WORDS: *sirtuins, SIRT1, SIRT1 expression, skin aging, ex vivo human skin*

ABSTRACT: *This chapter reviews the first scientific evidence confirming the presence of sirtuins in the skin, as well as their role in cell survival, senescence and longevity. This vital discovery could lead the way to new and innovative types of antiaging cosmetic ingredients that activate sirtuins.*

A scientific breakthrough was made in 2001 when lifespan extension induced by caloric restriction was clearly linked to the expression of sirtuins—a recently discovered family of proteins. This discovery opened new areas of investigation in the fight against aging.

The Role of Sirtuins

SIRT1 is the human homologue of Sir2, a key regulator of cell defense and survival in response to stress involved in diverse biological functions including cell development, metabolism, gene silencing, DNA repair, cell cycle progression, apoptosis, heterochromatin formation and especially longevity.¹⁻⁵ Recent studies have demonstrated the role of sirtuin proteins (the Sir2 gene protein) in life extension induced by caloric restriction.⁶⁻¹⁰ These findings on the relationship between sirtuins and cell survival extension have attracted a great deal of attention by establishing a direct link between life extension and Sir2 gene expression. In order to better understand the role of sirtuins in aging and longevity, in the last few

years, many studies have investigated the presence of SIRT1 in different human tissues, including human skin.¹¹⁻¹³

Sirtuin Expression in Human Skin

The current authors investigated sirtuin expression in human skin to evaluate the capacity of tissue to adapt to various aging and stress conditions through the expression of sirtuins.

In fresh *ex vivo* skin samples, SIRT1 expression in the epidermis and dermis highlights the importance of the role of sirtuins in cell aging and the crucial necessity of their presence in cells. In fresh *ex vivo* skin, SIRT1 exhibited a predominant nuclear staining throughout the epidermis (**Figure 1a**). This nuclear localization of SIRT1 confirms the essential role of sirtuins in protection and cell survival against stress. The nuclear presence of SIRT1 enables an immediate response of the sirtuin, acting on stimulating or inhibiting other genes.

Interestingly, frozen skin samples (**Figure 1b**) exhibited a predominant cytoplasmic staining, probably related to the cold stress, suggesting a correlation between sirtuin expression and the type of stress. However, if frozen skin is viewed as a reliable model, it seems that the cell is using a mechanism other than sirtuin to fight against such cold stress.

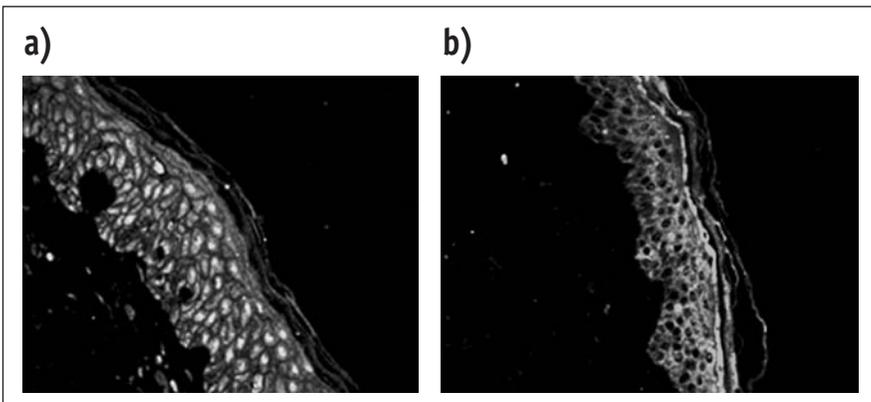


Figure 1. SIRT1 expression in fresh (left) and frozen (right) human *ex vivo* skin

Comparative studies of fresh skin samples from various donors revealed a moderate difference in degree and pattern of SIRT1

expression. Comparative studies of skin samples from donors aged 30 to 55 did not reveal any significant age-related difference in SIRT1 level, under stress-free conditions (**Figure 2**).

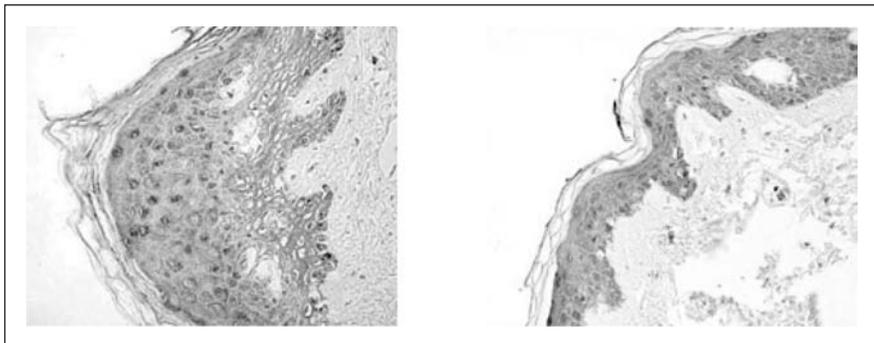


Figure 2. SIRT1 expression in skin from donors aged 30 years (left) and 50 years (right)

When skin samples were irradiated with UVB (50–200 mJ/cm²), fresh skin samples exhibited a clear dose-dependent increase in SIRT1 nuclear expression, up to 100 mJ/cm², which correlated with low tissue damage and low p53 expression, whereas high UVB doses yielded strong p53 expression. Interestingly, these results were more apparent in young skin (results not shown), showing a higher degree of adaptation of young skin to stress.

Taken together, the authors' investigations of sirtuin expression (SIRT1) in human skin samples have demonstrated a pattern of expression that is highly dependent on stress conditions.

STACS and Cellular Longevity

Cell senescence studies by the current authors have demonstrated that the induction of SIRT1 by addition of a selective sirtuin-activating compound (STAC) to aged human skin cells correlates with a decrease in the expression of a senescence marker and with an extension of the lifespan of these cells. Moreover, parallel studies on aged human skin cells have shown that the expression of the senescence biomarker β -galactosidase was considerably diminished in cells where SIRT1 was induced. Aged cells, after SIRT1 induction, exhibited a decrease in the number of stained cells and in the staining intensity of the senescence marker, compared to the control

where SIRT1 expression was not induced (**Figure 3**). This data suggests that STACs can restore the adaptive response in aged cells.

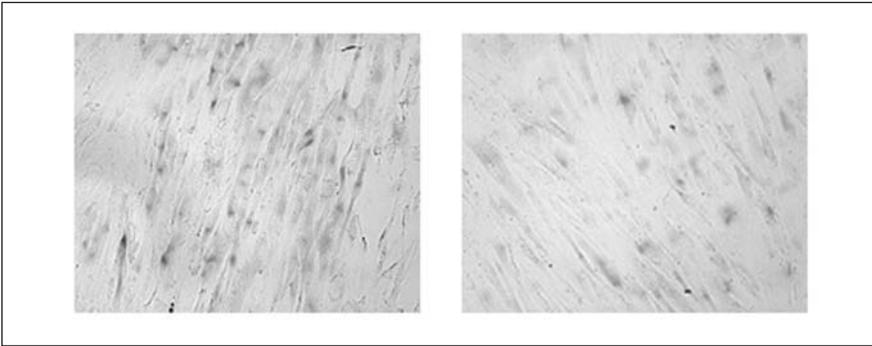


Figure 3. β -galactosidase staining in *in vitro*-aged human fibroblasts (P16) following one week during which the fibroblasts were untreated (left) or treated (right) with an active ingredient that induces SIRT1

Similarly, the authors' supplementary histological studies on human skin samples after multiple days in culture have confirmed that skin morphology, structure and integrity are clearly more preserved in skin samples in which SIRT1 was induced than in control skin where SIRT1 expression was not induced (**Figure 4**). Furthermore, viability studies have revealed an increase of 9% to 28% (depending on the cell's age) in cell viability in cells where SIRT1 was induced, compared to control cells where SIRT1 was not induced.

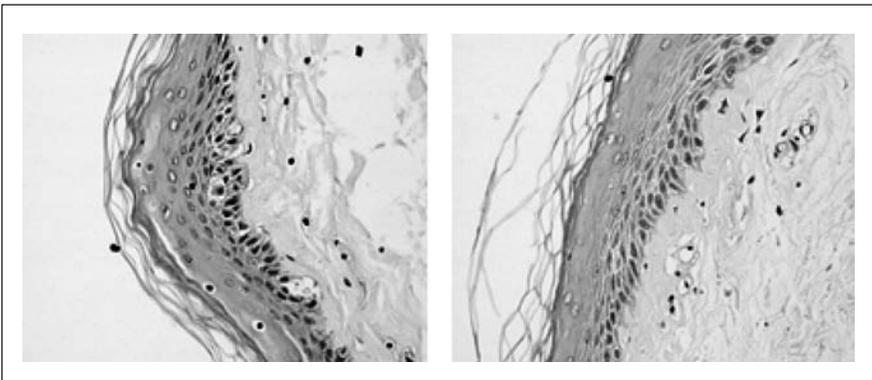


Figure 4. Hematoxylin and eosin staining of control skin (left) and skin with induced SIRT1, in each case after five days in culture

Sirtuins in the Cell Cycle

The authors studied sirtuin expression in various conditions. These studies demonstrated a significant correlation between stress, cell viability and sirtuin expression. This correlation suggests that sirtuins play a fundamental role in regulating stress damage response through stimulation of repair mechanisms (Ku70 pathway) or apoptosis (p53 pathway), depending on the dose of stress received.¹⁴ The strategic role that the SIRT1 gene plays in the cell cycle confirms its potential to regulate life extension and shows how a direct effect on sirtuin expression, by STACs treatment, can have a direct impact on cellular longevity by upstream regulation of many genes directly involved in life-regulating pathways.

It is known that different stress signals related to significant DNA damage lead to p53 protein increase in the nuclei where p53 plays a role at the level of the cell cycle. Recent data by the current authors strongly suggests a predominant role of sirtuins in regulating p53 expression.

- Under low stress conditions, sirtuins are induced and, in turn, activate Ku70 and p53 expression. In these conditions, p53 expression blocks the cell cycle, preventing proliferation of abnormal cells and promoting DNA repair mechanisms.
- Under stronger damaging UVB doses, increased p53 expression induces the apoptotic process involving Fas/CD95 expression. In parallel, a decrease in SIRT1 level shows a dominance of the p53 pathway over the action of SIRT1 on cell survival, indicating an increased likelihood of cell death to protect from the proliferation of abnormal cells in the skin.

Protective Role of Sirtuins

To demonstrate SIRT1's role in cell aging, the authors performed cell senescence studies using a specific biomarker that is absent from quiescent and differentiated cells. Indeed, a decrease in the number of cells entering into senescence was observed in cells where SIRT1 was induced. This data was confirmed by an improvement in the viability of aged cells. These studies have confirmed the fundamental role that SIRT1 plays in counteracting skin aging.

Moreover, histological studies by the authors on human skin samples have revealed that skin in which SIRT1 was induced is more preserved than in control skin that exhibits the usual stress and signs of damage that accompany multiple days in culture.

These findings further substantiate the notion that the presence of sirtuins is important for cell survival. Nevertheless, in irradiated aged skin, SIRT1 expression and its relationship to UVB dosage and p53 expression was less obvious than in younger skin samples. This interesting result correlates with the defective functioning of some key molecules that may accompany or induce aging.

Taken together, this data demonstrates that SIRT1 induction in the skin by topical application of a selective STAC would be an interesting approach in the skin care field. Interest in including such STACs in different skin moisturizers or specific antiaging products, as well as in cosmetics, is occurring with the knowledge that sirtuins play a key role in protecting the skin from stress and photoaging.

Conclusion

The key role of sirtuins as “longevity proteins” is becoming well-accepted and new data confirms the clear and strong expression of SIRT1 in human skin. As has been seen, studies also suggest that SIRT1 plays an additional protective role against environmental stresses, such as UV, and other factors that cause the skin to age. Moreover, at the molecular level, the fundamental role of sirtuins in important cellular pathways demonstrates that they could be explored as key regulators in many areas of dermatological study, including skin repair and antisenesescence research.

Overall, these studies suggest that SIRT1 expression optimization is an important strategy in the fight against aging. Its importance can be compared to other leading antiaging strategies such as DNA protection or the use of anti-free radicals, due to the central role that sirtuins play in cellular protection and repair. Indeed, it would be interesting to combine STACs with other antiaging strategies in order to provide a synergistic and multifaceted approach to the fight against aging.

References

1. D Shore, M Squire and KA Nasmyth, Characterization of two genes required for the position-effect control of yeast mating-type genes, *EMBO J* 3(12) 2817–2823 (1984)
2. HA Tissenbaum and L Guarente, Increased dosage of a sir-2 gene extends lifespan in *Caenorhabditis elegans*, *Nature* 410(6825) 227–230 (2001)
3. CB Brachmann, JM Sherman, SE Devine, EE Cameron, L Pillus and JD Boeke, The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability, *Genes Dev* 9(23) 2888–2902 (1995)
4. SC Dryden, FA Nahhas, JE Nowak, AS Goustin and MA Tainsky, Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle, *Mol Cell Biol* 23(9) 3173–3185 (2003)
5. T Finkel, Ageing: A toast to long life, *Nature* 425(6954) 132–133 (2003)
6. HY Cohen, C Miller, KJ Bitterman, NR Wall, B Hekking, B Kessler, KT Howitz, M Gorospe, R de Cabo and D Sinclair, Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase, *Science* 305(5682) 390–392 (2004)
7. EJ Masoro, Role of sirtuin proteins in life extension by caloric restriction, *Mech Ageing Dev* 125(9) 591–594 (2004)
8. JG Wood, B Rogina, S Lavu, K Howitz, SL Helfand, M Tatar and D Sinclair, Sirtuin activators mimic caloric restriction and delay ageing in metazoans, *Nature* 430(7000) 686–689 (2004)
9. A Brunet, LB Sweeney, F Sturgill, KF Chua, PL Greer, Y Lin, H Tran, SE Ross, R Mostoslavsky, HY Cohen, LS Hu, HL Cheng, MP Jedrychowski, SP Gygi, DA Sinclair, FW Alt and ME Greenberg, Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase, *Science* 303(5666) 2011–2015 (2004)
10. L Bordone and L Guarente, Calorie restriction, SIRT1 and metabolism: understanding longevity, *Nat Rev Mol Cell Biol* 6(4) 298–305 (2005)
11. A Perrin, E Bauza, C Dal Farra and N Domloge, SIRT1 expression in human skin: comparative studies of skin samples of varying age level, stress level and freshness, with one another and with culture keratinocytes, *J Invest Dermatol* 124(4s) 369 (2005)
12. C Dal Farra, E Bauza, JM Botto and N Domloge, SIRT1 the human homologue of Sir2 is expressed in human skin and in cultured epidermal and dermal cells and is closely related to stress and aging. *J Invest Dermatol* 124(4s) 367 (2005)
13. E Bauza, T Marchand, C Dal Farra and N Domloge, SIRT1 induction in human skin cells increases cell viability and decreases signs of senescence, *J Invest Dermatol* 126(4s) 345 (2006)
14. J Jeong, K Juhn, H Lee, SH Kim, BH Min, KM Lee, MH Cho, GH Park and KH Lee, SIRT1 promotes DNA repair activity and deacetylation of Ku70, *Exp Mol Med* 39(1) 8–13 (2007)

Pep Talk: Slowing Down Aging

Donald R. Owen

Chief Executive Officer and Chief Science Officer of BioSouth Research Lab

KEY WORDS: *peptides, bacteria, histidine, aging, skin lightening, tanning*

ABSTRACT: *Formulators have found new applications of peptides in the suppression and stimulation of melanocyte activity, which could leave to a new generation of skin lightening and self-tanning products.*

Peptides play many supporting roles in the pharmaceutical, cosmetic/personal care and clinical/industrial disinfectants industries. In personal care, peptides and their derivatives provide a healthy helping of nutrition to the skin. Their daily repair and antiaging properties are often touted on the labels of high-tech skin care products.

More recently, formulators have found new applications of peptides in the suppression and stimulation of melanocyte activity, which could lead to a new generation of skin lightening and self-tanning products. Beyond their uses in skin care, peptides are seen by Dr. Donald R. Owen, Chief Executive Officer of BioSouth Research Labs, as offering great potential in a new concept aimed to slow the process of biological aging.

Getting Defensive

“Peptides have been used by insects and other animals and plants for millions of years as a self-defense system,” explained Dr. Owen. These organisms do not have white blood cells so they use peptides and their derivatives to kill invading bacteria. “Indeed, bacteria use them themselves to fend off competitive species,” said Dr. Owen.

So through evolution, we have kept many of these defense peptides. For example, research has shown that we produce histidine, an antimicrobial peptide; a fragment of it is found in our saliva. Histidine can now be produced synthetically, and a natural fragment of it is being used in products to kill surface bacteria, for example, in acne.

Stimulating Conversation

These histidine fragments have also been found to be mildly stimulating when used with treatments such as microdermabrasion, intense pulse light, or dermal rolling—a needling technique that directly stimulates the dermis. According to Dr. Owen, combining these treatments and formulations enhances the stimulation of the dermis and allows for rapid entry of ingredients. “[This] can, of course, become a negative if the formulation has not been intentionally designed for use with that kind of high penetration system,” added Dr. Owen.

“High penetration” claims like this will raise the watchful eyebrows of regulatory officials because they stretch beyond a cosmetic function to a much closer drug claim. However, Dr. Owen said that regulatory restrictions will not be avoided for the industry to make progress. “[We] do not hesitate when the technology reaches the point it needs to be prescriptive—that’s where we’re going to go.”

In many formulations incorporating these newer peptides, the prescriptive/non-prescriptive delineation may be based on the concentration—the way hydroquinone or retinoic acid are regulated currently. Hydroquinone at 1% or 2% can be bought as an over-the-counter (OTC) product, but consumers need a prescription to get the 4% concentration. Likewise, consumers can buy OTC products with lower concentrations of retinoic acid that do not require prescriptions, versus levels that require prescriptions.

Action: Louder than Words

One great challenge to developing effective peptides is determining an *in vitro* model to test whether they are suitable and, when you put them into a final formulation, whether they still work. Skin constituents are offering researchers at least a primitive model of the

human skin to test the technologies being developed. “[The] DNA microarray assay allows us to look unequivocally at what the potential active ingredient is doing when it’s coming in contact with a cell component of skin,” explained Dr. Owen. He added that researchers must be able to judge whether a sample has any sort of effect—positive or negative.

“It’s important that we’re trying to get some of these skin [testing] methods [established] as a standard,” commented Dr. Owen. “As a scientist, it’s wonderful because you don’t have to take a stab in the dark and try to put it on 50 people to see what happens—you have a pretty good idea by the time you leave the laboratory that you’re better than the product that you had before.”

An Antiaging Patch?

According to Dr. Owen, peptides have demonstrated the ability to enhance daily repair and wound healing. One application of these abilities is currently under development—an adhesive patch that is a breakthrough in the delivery of growth hormone release peptide. Dr. Owen explained that as the body ages, the natural level of growth hormone drops, thus decreasing our daily repair and wound-healing capabilities. This slows the production of antioxidant enzymes, which means the body is much more susceptible to free-radical damage. “[But] if you have peptides that allow you to turn on the enzymes ... you are hundreds of thousands of times more likely to eat up free radicals,” said Dr. Owen.

The release peptides allow growth hormones and their binding proteins to produce/reproduce naturally. “We have a growth hormone release peptide that is 200 times more active than the normal human growth hormone,” explained Dr. Owen. Initially it will be used to treat individuals 55 years or older undergoing elective surgery to enhance their wound-healing abilities. However, patches are being designed that, according to Dr. Owen, could keep consumers biochemically in their forties, rather than in their sixties and seventies.

Drawing the Line on Wrinkles

Diane Bilodeau, PhD, and Isabelle Lacasse

Atrium Innovations Inc., Quebec, Canada

KEY WORDS: *antiaging, dermal-epidermal junction, collagen VII, laminin-5, fibronectin.*

ABSTRACT: *A new effective strategy to reduce the appearance of fine lines and wrinkles involves supporting the production of structural proteins involved in skin cohesion at the dermal-epidermal junction with a new biomimetic peptide created by solid phase organic synthesis.*

Wrinkling is the expression of life and an inherent part of skin aging. Wrinkles hold memories of emotions, expressions and lifestyle. If only the worried frown lines could be filtered out to preserve just the smiling fine lines. It is clear that wrinkles cannot merely be wiped away but scientists can design new technologies capable of reducing fine lines; first, however, the process of aging must be understood.

Skin Aging and Wrinkle Formation

Aging is a complex process that translates into morphological and functional changes within the skin (**Table 1**). Skin aging occurs in two different ways that interact: the intrinsic form of chronological aging and the extrinsic form of photoaging.

Chronological aging comes with the passage of time and is influenced by individual genetic makeup. Clinical manifestations at the skin level include fine wrinkling, thinning, reduced elasticity, dryness, a reduced immune response and slow formation of scar tissue, known

as cicatrization. The hallmark of aged skin is a flattening at the dermal-epidermal junction (DEJ) with loss of the dermal papillae. Also seen are a reduced number of fibroblasts as well as a general atrophy of the extracellular matrix (ECM) comprising structural proteins and glucosaminoglycans (GAGs).

Table 1. Types of Skin Aging

	Chronological aging	Photoaging
Causes	Passage of time Genetic background	Sun exposure Genetic background (skin type)
Manifestations at the skin level	Fine wrinkling Thinning Reduced elasticity Dryness Reduced immune response Slow cicatrization Deep wrinkling	Deep wrinkling Sagging Irregular pigmentation Brown spots Roughness Leathery appearance Reduced immune response
Manifestations at the cellular level	DEJ flattening Loss of dermal papillae ECM atrophy Reduced number of fibroblasts Low levels of collagen and elastin Reduced cell turnover	Dermal elastosis (accumulation of proteolyzed elastin fibers) Collagen reduction and fragmentation Reduced cell turnover

Photoaging of the skin is mainly caused by chronic sun exposure. Clinical manifestations include deep wrinkling, sagging, irregular pigmentation, brown spots, coarseness and a leathery appearance. As with chronological aging, sun exposure also affects immune response in the skin. The main characteristic of photoaged skin is dermal elastosis with the accumulation of agglomerates of useless denatured elastin fibers beneath the DEJ. The collagen content is decreased and fragmented.

Wrinkles form when a force is applied to a thin, rigid material that rests on a softer, thicker basis.¹ This description applies to human skin that consists of a rather rigid and thin epidermis laying on a deeper viscoelastic dermis. Wrinkles form in the skin in response to the application of mechanical constraints like muscle contraction or gravitational forces. As long as skin extensibility and elasticity are optimal and there is good cohesion between the epidermis and the dermis, wrinkles relax upon dissipation of the applied force. However, with the passage of time, cumulative alterations affecting both the structure and the mechanical properties of the skin contribute to the development of permanent wrinkles. Working on skin structure to reinforce mechanical properties thus appears to be a good strategy to ease wrinkles.

A major structure involved in skin cohesion is the DEJ (**Figure 1**). This is where the epidermis meets the dermis for dynamic exchanges. Composed of a complex network of interconnecting proteins, the DEJ forms the skin basal membrane. The DEJ is unique in that it holds structures forming anchoring complexes that assure mechanical stability to the skin and serve to increase frictional resistance to externally applied forces.² Better resistance results in fewer wrinkles. Any weakness at the DEJ has tremendous effects and in some tragic pathological cases may even lead to severe skin blistering and separation of the skin layers.

As part of anchoring complexes (**Figure 1**), laminins, fibronectins and collagen VII are found at the DEJ. Laminin-5 is the real anchor point between the epidermis and the dermis.³ The glycoprotein bridges basal keratinocytes with collagen VII of anchoring fibrils at the DEJ. Reduced deposition of laminin-5 with age contributes to the disorganization and the flattening of the DEJ that underlies wrinkle formation.

Collagen VII is a subtype of collagen and a key structural component of anchoring fibrils at the DEJ.² Attached to the basal membrane, these fibrils extend into the dermis to loop back, forming arcs that support dermal collagen fibers. Collagen VII plays a major role in the mechanical stability of the DEJ. Its expression is known to be reduced with aging, contributing to wrinkle formation.⁴

Fibronectin is a major adhesive protein whose role is to firmly anchor cells to extracellular materials.⁵ Through its interaction with integrins receptors at cell membrane, fibronectin also affects skin cell functions such as gene expression and cell growth. Fibronectin production is modulated by growth factors whose expression and responsiveness may be downsized with aging.

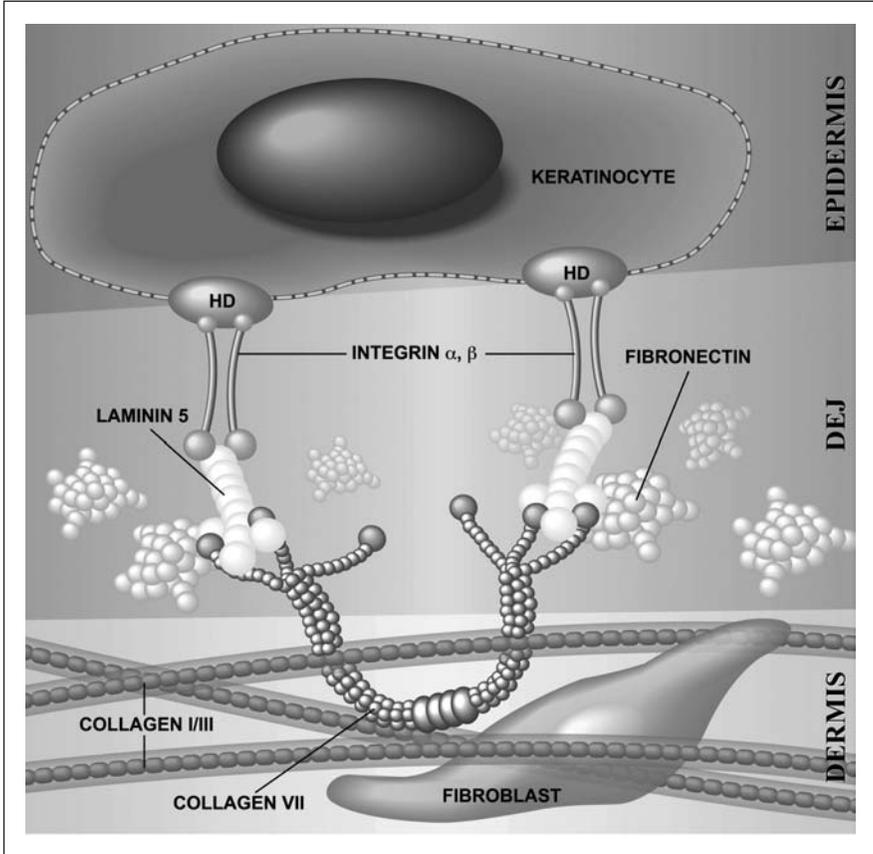


Figure 1. Structural proteins are important for skin cohesion at the DEJ.

Cell matrix interactions at the DEJ are lowered with aging. Cells need a proper matrix support to survive, to stay in place, to proliferate and to differentiate and this is one of the functions of the ECM at the DEJ. Unfortunately, aging is associated with a low level of expression and an increase destruction of supporting fibers. This results in a slowing of cellular turnover and regeneration.

The structure of the DEJ is weakened and flattens. These structural changes reduce the surface area for nutritional exchange and metabolic byproducts evacuation between the dermis and the epidermis. As a consequence, epidermal cell turnover slows down even further and harmful free radicals and metabolites accumulate. Protecting and restoring cell-matrix interactions at the DEJ ensure improved, younger looking skin. Biomimetic peptides have proven to be useful cosmetic actives for that purpose.

Biomimetic Peptides in Cosmetic Precision

Traditionally, the sources of cosmetic actives have been limited to plant extracts, essential oils and vitamins; however, biomimetic molecules can now be engineered for this purpose. The beauty of the process is that highly specific ingredients that mimic natural sources can be designed with increased efficacy and specificity, requiring lower dosages. Based on the industry's developing knowledge of the complex physiology of the skin, peptides are created and shaped in such a way that they can be substituted for natural skin factors to address various cosmetic needs. Peptides can be engineered to mimic the three-dimensional structure of beneficial skin factors, allowing them to act as such.

For example, beginning with the known sequence of a growth factor involved in wound healing, the authors produced a bank of peptides by solid phase organic synthesis. To optimize skin penetration, peptides were coupled to various lipids. Peptide conjugates were then screened *in vitro* for their ability to stimulate the production of structural proteins by fibroblasts at the DEJ. The selected peptide, caprooyl tetrapeptide-3^a, was clinically tested in a serum formulation for fine line and wrinkle reduction.

Caprooyl Tetrapeptide-3 on Skin Proteins

In vitro studies: Confluent normal human dermal fibroblasts (NHDF) were cultured for 48 hr in the presence and absence of 10^{-7} M (0.03%) or transforming-growth factor (TGF- β). The latter,

^a ChroNoline (INCI: Glycerin (and) water (aqua) (and) dextran (and) caprooyl tetrapeptide-3) is a product of Atrium Innovations.

a general stimulator of the synthesis of extracellular matrix (ECM) proteins, was used as a positive reference.⁶ At the end of the incubation period, laminin and fibronectin content were quantified in the supernatant using a highly sensitive and specific enzyme immunoassay (EIA) kit.

In the presence of caprooyl tetrapeptide-3, NHDF cells increased their expression of laminin by 26% and that of fibronectin by 60% (**Figure 2**). In the same conditions, the stimulating potential of TGF- β was 10% for laminin and 64% for fibronectin.

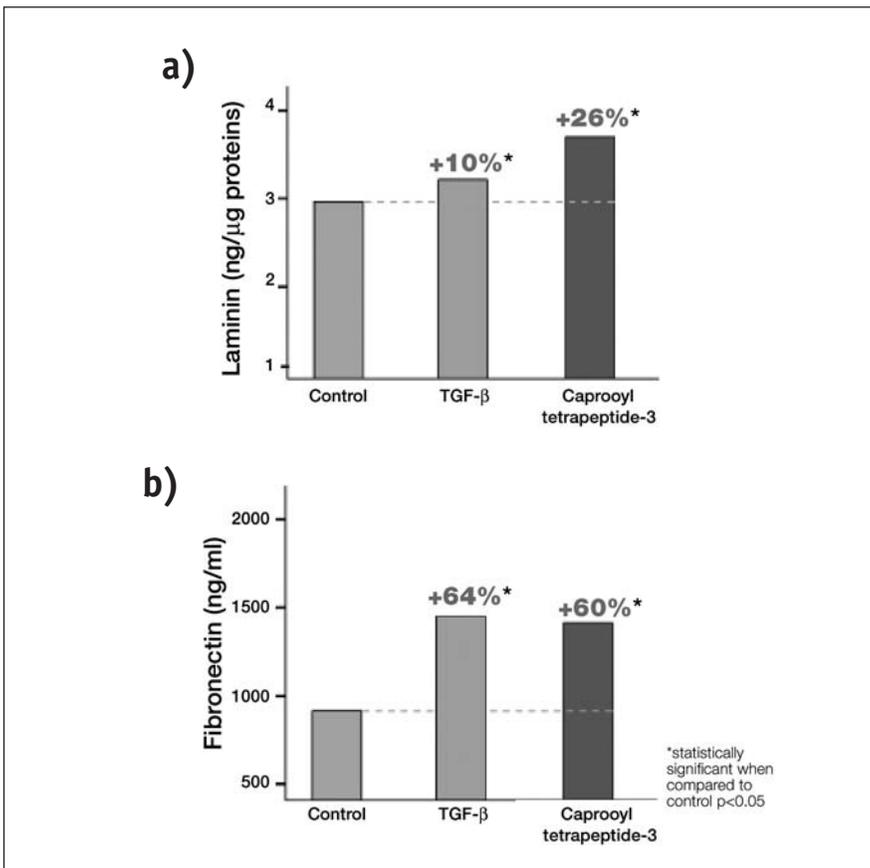


Figure 2. Effect of caprooyl tetrapeptide-3 on a) the expression of laminin and b) fibronectin by fibroblasts *in vitro*

Ex vivo model of skin aging: An additional bench study was carried out using human skin explants exposed to corticoids as an accelerated *ex vivo* model of skin aging. Topical corticoids are

infamous for inducing skin atrophy and flattening the DEJ, at least partly through an effect on collagen turnover, mimicking aging.⁷

Four human skin specimens were obtained from different patients undergoing plastic surgery and maintained in culture. Before the experiment began, the corticoid cream betamethasone^b (0.05%) was applied at the surface of some of the skin explants, leaving some explants without the cream. On the same day, caprooyl tetrapeptide-3 (0.03%) was added to the culture media of skin explants. On Day One, caprooyl tetrapeptide-3 (0.03%) treatment was repeated. On Day Three, all skin explants were frozen. The presence and localization of collagen VII and laminin-5 were assessed using indirect immunofluorescence. Visual scoring was performed by two dermatologists.

Both collagen VII and laminin-5 expressions were localized at the DEJ (**Figure 3**). Results revealed decreases of 16% and 45% respectively in collagen VII and laminin-5 staining in skin explants stressed with corticoids, compared to control level. Treatment with caprooyl tetrapeptide-3 in the presence of corticoids resulted in increases of 34% and 49% respectively in collagen VII and laminin-5 staining compared to what was seen with corticoid treatment alone. Interestingly, the flattening observed at the DEJ with corticoid treatment was prevented with the application of caprooyl tetrapeptide-3.

Clinical Antiaging Properties of Caprooyl Tetrapeptide-3

Antiwrinkle efficacy study: An *in vivo* study was conducted on 27 women ages 40 to 65, with healthy skin. Volunteers applied a placebo serum (**Formula 1**) on the crow's-feet of one randomized temple area and the same formulation containing 2.5% caprooyl tetrapeptide-3 on the crow's-feet of the other temple. Applications were repeated twice daily for 56 days. The micro relief of the eye area was assessed using special processing software from scanning by interference fringe profilometry of silicone replicas taken at days 0, 28 and 56.

^bDiprosone is a registered trademark of Atrium Innovations.

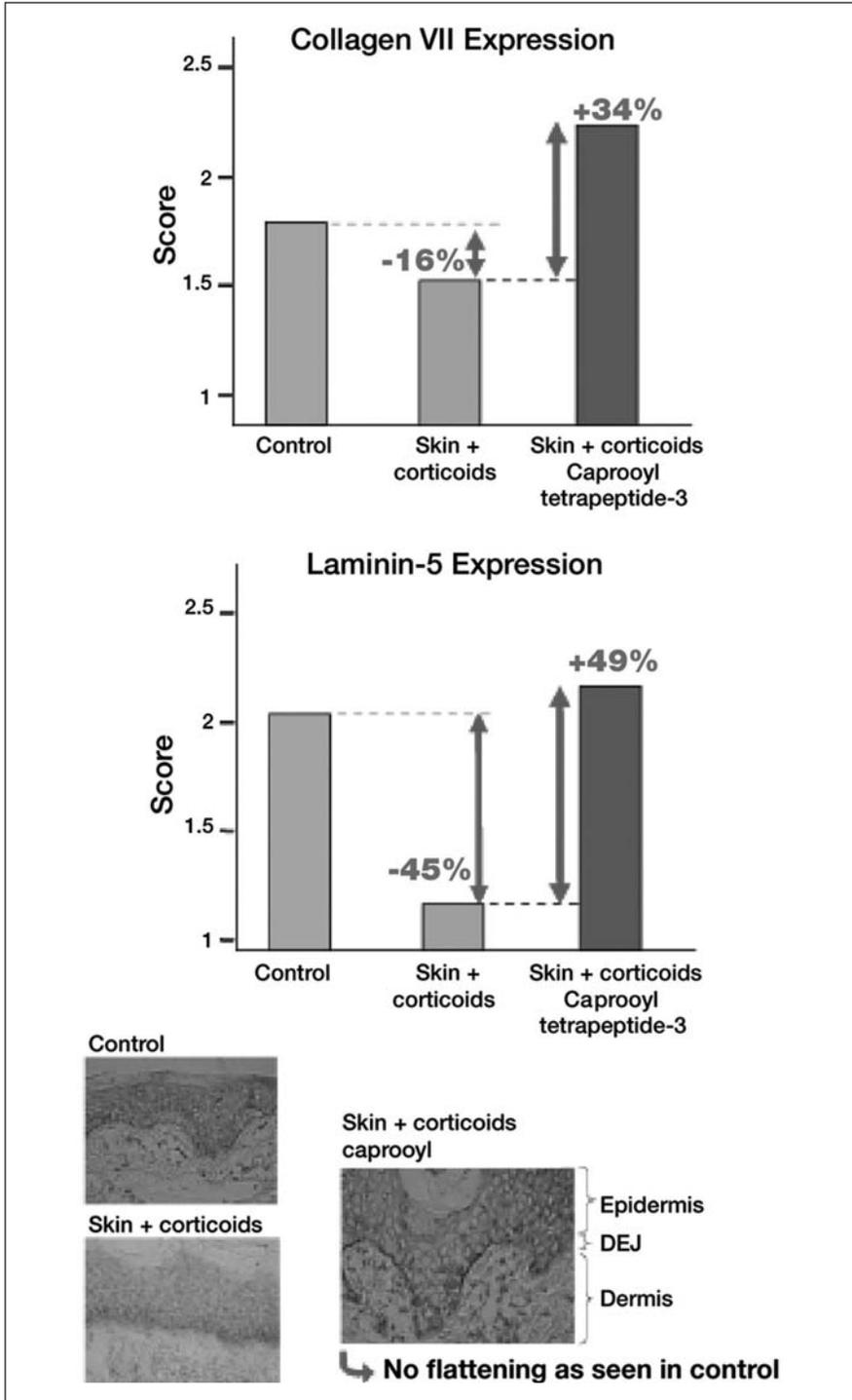


Figure 3. Effect of caprooyl tetrapeptide-3 on the expression of collagen VII and laminin-5 at the DEJ in skin explants

Formula 1. Antiwrinkle eye serum

A. Water (<i>aqua</i>)	59.65% w/w
Carbomer (Carbopol Ultrez-10, Noveon)	0.06
B. Butylene glycol	3.00
Glycerin	3.00
Phenoxyethanol (and) methylparaben (and) ethylparaben (and) butylparaben (and) propylparaben (and) isobutylparaben (Phenonip, Nipa)	0.80
C. Caprylic/capric triglyceride (Dermol M-5, Alzo)	10.00
Myristyl myristate (Saboderm MM, Sabo)	4.50
Glyceryl stearate (Cerasynt SD, ISP)	3.00
Polysorbate 60 (Tween 60, Uniqema)	3.00
<i>Butyrospermum parkii</i> (shea butter) fruit	1.50
Sorbitan stearate (Arlacel, Uniqema)	0.75
Dimethicone (Fluid 200, 300cs, Dow Corning)	0.60
D. Fragrance (<i>parfum</i>) (Fleurarôme No 6270, Fleuraôme)	0.03
Triethanolamine, 99% (Triethanolamine, Produits CCC)	0.11
Water (<i>aqua</i>)	7.50
E. Caprooyl tetrapeptide-3 (ChroNOLine, Atrium)	<u>2.50</u>
	100.00

Procedure: Add A to main tank. Start stirring and heating. Separately mix B. Add B to A. Heat to 75°C. Separately heat C under slow stirring and heat to approximately 75–80°C. Add C slowly (over 10 min) into the vortex of the AB. Stir vigorously for 15 min at 75°C. Begin cooling and decrease the stirring speed. Add D to batch at approx. 40°C. Add E into batch and continue stirring to 30°C to obtain a smooth emulsion. Add water if necessary.

Replicas appearing in as little as 28 days of application (**Figure 4**) show a significant average reduction in wrinkles with a maximum of 29%. Interestingly, for individuals aged 50–65, benefits continued to progress over two months for an average reduction in fine lines and wrinkles of 27% with a maximum of 35% (**Figure 5**).

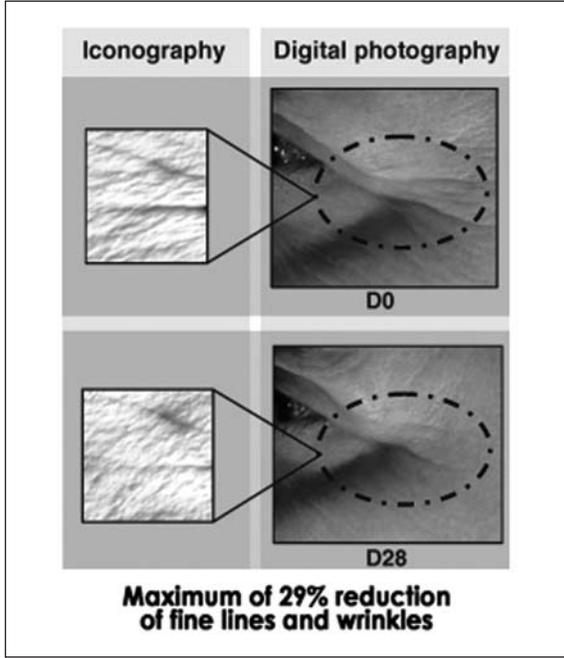


Figure 4. Improvement of crow's feet wrinkles within 28 days of treatment with caprooyl tetrapeptide-3

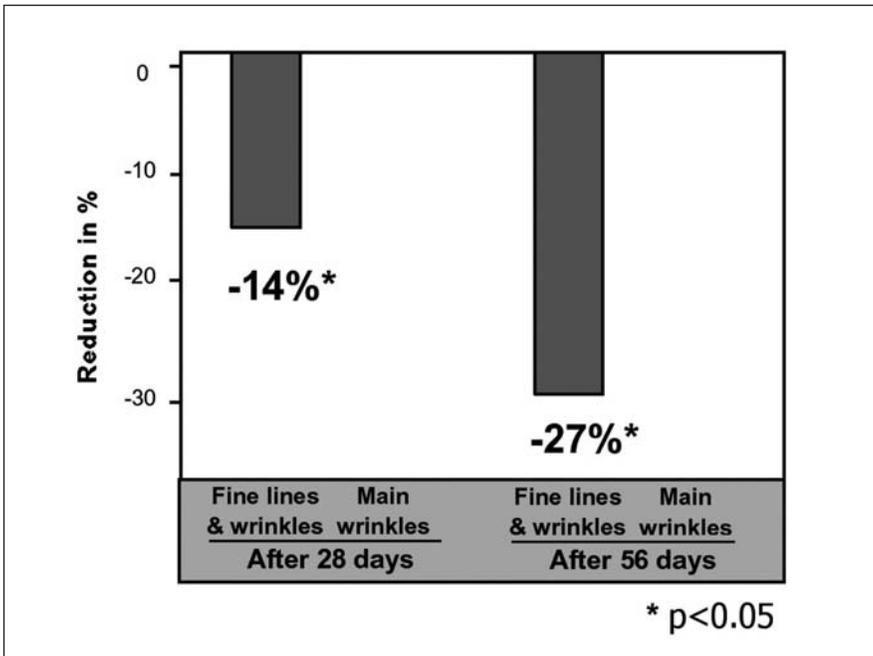


Figure 5. Fine line and wrinkle reduction for subgroup 50–65 years treated (L to R) without and with caprooyl tetrapeptide-3

Ultrasonographic study: To further document the *in vivo* effect of caprooyl tetrapeptide-3 on dermal structures, echographic measurements were taken. High-frequency ultrasound imaging known as echography can be used to identify the dermis that is visualized as a speckled pattern.⁸ The echoes of the dermis are considered to originate from the boundaries between collagen and elastin fibers, the surrounding water-rich ground substance and cells.⁹ Changes in echogenicity may reflect an altered connective tissue composition of the dermis. In particular, appearance of a superficial low-echogenic band in the dermis, immediately below the epidermal entrance echo, has been related to age.^{10,11} Dermal echogenicity has therefore been proposed as a marker for skin aging.

All 27 women volunteers, ages 40–65, applied a placebo serum on the crow’s-feet of one randomized temple area and the same formulation containing 2.5% caprooyl tetrapeptide-3 on the crow’s-feet of the other temple. Applications were repeated twice daily for 168 days. Skin echography scanning was performed on temple areas at Day 0 and Day 168. A 20-MHz ultrasound scanner^c was used to obtain cross-sectional images of the skin. This process involved an echography technique allowing for an image to be taken in two dimensions known as B mode or *brightness on display*.[”]

Results showed that caprooyl tetra-peptide-3 treatment had a positive influence on skin connective tissue. As shown in **Figure 6**, application of the peptide for six months restored the echogenic response of the upper dermis lost with aging. Most notably, the ultrasound parameter “entropy” significantly improved in the treated area versus placebo for 78% of the volunteers. Entropy represents the disorder of an image that increases with better skin hydration.

Consumer opinion: The objective of this study was to evaluate the consumer perception of caprooyl tetrapeptide-3 as an anti-wrinkle active following 56 days of treatment. A total of 30 women volunteers, ages 40–65, participated in the self-assessment study. Each consumer applied a serum of 2.5% caprooyl tetrapeptide-3 twice a day on the crow’s-feet of the temples for a period of 56 days. Subjects were asked to fill out a self-evaluation questionnaire at the end of the study in order to evaluate their overall opinion and feeling on the effectiveness of the product.

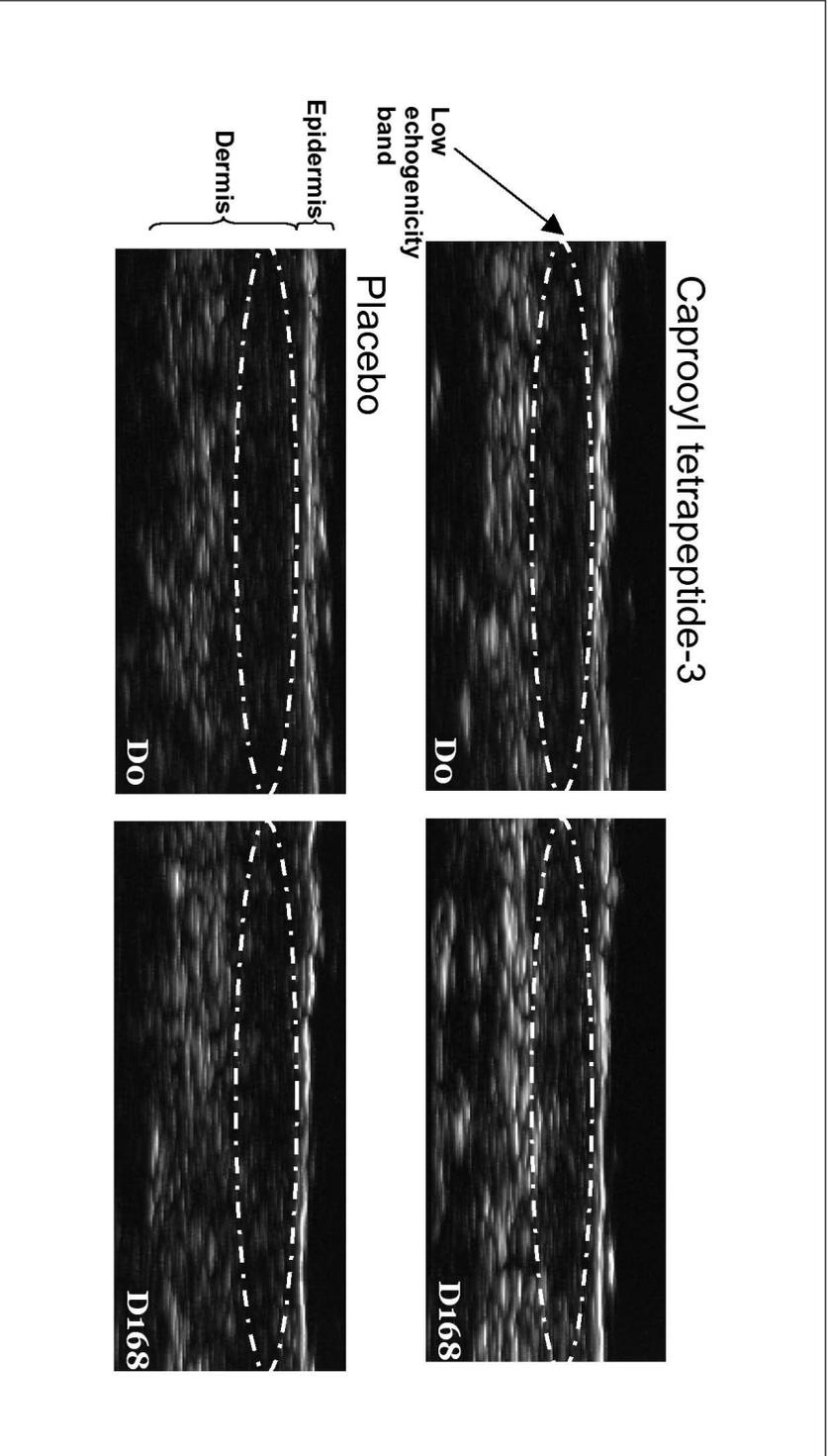


Figure 6. Restoration of upper dermis echogenicity with caprooyl tetrapeptide-3

Results condensed on **Figure 7** revealed that consumers agree that caprooyl tetrapeptide-3 renders the skin smoother, firmer and more even for a younger and more rested look. Moreover, caprooyl tetrapeptide-3 provided an immediate well-being effect and was well-tolerated around the eye.

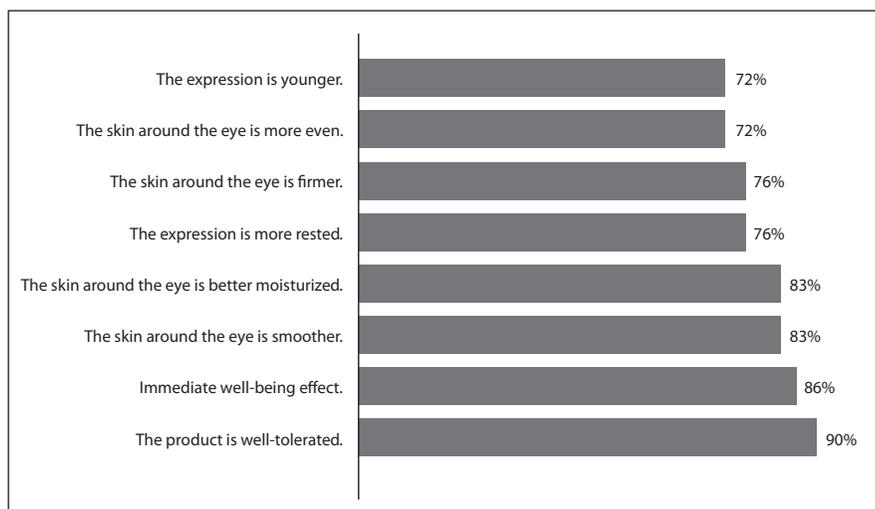


Figure 7. Consumer opinion of caprooyl tetrapeptide-3

Conclusions

Biomimetic peptides are effective instruments to restore and support skin physiology, as the authors have shown here. They mimic natural skin factors and have the potential to revive signaling pathways that have lost responsiveness with age. In the present report, evidence was presented supporting the use of the growth factor-derived peptide caprooyl tetrapeptide-3 for the reduction of fine lines and wrinkles.

In vitro, caprooyl tetrapeptide-3 stimulated the production of laminin and fibronectin by fibroblasts at a similar or greater level than TGF- β used as a positive control. *Ex vivo* treatment of skin explants with caprooyl tetrapeptide-3 in a model of corticoid-induced skin aging resulted in the protection of laminin-5 and collagen VII expression at the DEJ. While expression of these proteins was seriously impaired with corticoid treatment alone, DEJ flattening in this model of skin aging was also prevented in the presence of caprooyl tetrapeptide-3.

In clinical trial, caprooyl tetrapeptide-3 serum significantly and rapidly reduced the appearance of fine lines and wrinkles in mature skin. Interestingly, the strength of caprooyl tetrapeptide-3 seems to self-adjust to the skin needs, delivering increasing benefits with increasing age. An ultrasonographic study additionally showed that use of caprooyl tetrapeptide-3 over six months reduced the appearance of a low echogenic band that develops in the upper dermis with age. Improved echogenicity means better collagen content and integrity for improved water retention within the dermis, translating into a younger skin appearance. Volunteers also valued caprooyl tetrapeptide-3 as an antiaging agent.

Ultimately, this study demonstrates that supporting the production of structural proteins involved in skin cohesion at the DEJ is an effective strategy to provide a long-term, significant reduction in the appearance of fine lines and wrinkles in mature skin. In the end, controlling one's level of wrinkling may not be so unrealistic after all.

Published July 2008 *Cosmetics & Toiletries* magazine.

References

Send e-mail to: cosmetic@atrium-innov.com.

1. J Genzer and J Groenewold, Soft matter with hard skin: From skin wrinkles to templating and material characterization, *Soft Matter* 2(4) 310–323 (2006)
2. RE Burgeson and AM Christiano, The dermal-epidermal junction, *Curr Opin Cell Biol* 9(5) 651–8 (1997)
3. M Aumailley and P Rousselle, Laminins of the dermo-epidermal junction, *Matrix Biol* 18(1) 19–28 (1999)
4. NM Craven, RE Watson, CJ Jones, CA Shuttleworth, CM Kielty and CE Griffiths, Clinical features of photo-damaged human skin are associated with a reduction in collagen VII, *Br J Dermatol* 137(3) 344–50 (1997)
5. KA Bush, BR Downing, SE Walsh and GD Pins, Conjugation of extracellular matrix proteins to basal lamina analogs enhances keratinocyte attachment, *J Biomed Mater Res A* 80(2) 444–52 (2007)
6. Y Jiang, DW Cheng, ED Crook and LP Singh, Transforming growth factor-beta1 regulation of laminin gamma1 and fibronectin expression and survival of mouse mesangial cells, *Mol Cell Biochem* 278(1–2) 165–75 (2005)
7. S Schoepe, H Schacke, E May and K Asadullah, Glucocorticoid therapy-induced skin atrophy, *Exp Dermatol* 15(6) 406–20 (2006)
8. SM Milner, OM Memar, G Gherardini, JC Bennett and LG Phillips, The histological interpretation of high frequency cutaneous ultrasound imaging, *Dermatol Surg* 23(1) 43–5 (1997)
9. S Richard, B Querleux, J Bittoun, O Jolivet, I Idy-Peretti, O de Lacharriere and JL Leveque, Characterization of the skin *in vivo* by high resolution magnetic resonance imaging: Water behavior and age-related effects, *J Invest Dermatol* 100(5) 705–9 (1993)
10. K Tsukahara, Y Takema, S Moriwaki, T Fujimura, T Kitahara and G Imokawa, Age-related alterations of echogenicity in Japanese skin, *Dermatology* 200(4) 303–7 (2000)
11. JM Waller and HI Maibach, Age and skin structure and function, a quantitative approach (I): Blood flow, pH, thickness, and ultrasound echogenicity, *Skin Res Technol* 11(4) 221–35 (2005)

Modeling UVB-induced Formation of Photoproducts in Human Keratinocytes

C. Lenaers, D. Boudier, V. Barruche and B. Closs

SILAB, Brive Cedex, France

KEY WORDS: *photorepair, cyclobutane-pyrimidine dimer, UVB, keratinocyte, cotton extract*

ABSTRACT: *The authors describe a model to visualize the appearance of cyclobutane-pyrimidine dimers in the nuclei of normal human keratinocytes after UV irradiation. These dimers are one of the principal agents in DNA damage. The model can be used to assess the effect of a cosmetic ingredient on the formation of direct UV-induced lesions to DNA.*

In order to repair endogenous lesions or photoinduced alterations to DNA, all cells possess a complex defense system for the detection, elimination and repair of damaged DNA fragments. Although the sun is essential for human life, it can directly or indirectly induce DNA lesions when the skin is overexposed (see **Radiation Damage and the Cellular Response**). The principal damage to DNA is the formation of cyclobutane-pyrimidine dimers (CPD), pyrimidine (6-4) pyrimidone photoproducts (6-4PP) and Dewar isomers. This chapter focuses on CPD and on a model for the formation of CPD after UVB irradiation.

In order to assess the effect of a cosmetic ingredient on the formation of direct UV-induced lesions to DNA, it first was necessary to develop a model to visualize the appearance of CPD in the nuclei of normal human keratinocytes.

Radiation Damage and the Cellular Response

The sun is indispensable for life in plants, animals and humans, but it can cause biological effects responsible for chemical reactions that are often harmful to the skin and lead to conditions such as actinic erythema, heliodermia, photosensitization and skin cancers. Thus, the skin must be photoprotected against the sun's potentially destructive energy.

Ultraviolet (UV) radiation within the solar spectrum is characterized by a wavelength that expresses its energy: the shorter the wavelength, the higher the energy of the radiation and the greater the biological effects on the skin.

Potential effects of UVC: The UVC component (200–280 nm) has the shortest wavelength and the highest energy in UV radiation. No longer being absorbed as completely by an increasingly thin ozone layer, UVC may pose an increasing threat to the skin because its very short wavelength makes it biologically active.⁵

Oxidative damage caused by UVA radiation: UVA radiation (320–400 nm) is not absorbed by DNA directly but can damage it indirectly via the release of reactive oxygen species. Indirect oxidative lesions of DNA are produced by photosensitization, either by charge transfer from an excited endogenous chromophore, or by reaction with reactive oxygen species produced at these wavelengths.⁶

The major oxidative lesions resulting from UVA are oxidized purines, in particular 8-oxo-7,8-dihydroguanine (8-oxoGua). It has also been reported in the literature that exposure of cells to UVA irradiation also induces the formation of cyclobutane-pyrimidine dimers (CPD).⁶⁻⁷

Photoproducts and UVB radiation: UVB radiation (280–320 nm) was one of the first agents identified as being capable of inducing damage to DNA because its emission spectrum is the same as the DNA absorption spectrum. At these wavelengths, UVB photons can be directly absorbed by the DNA of cells and cause degradation reactions to pyrimidine bases (thymine and cytosine).⁷ This is also the reason why the major part of the oncogenic power of sunlight is attributed to this spectral band. Dimerization reactions occur at sites where there are two adjacent pyrimidine bases.

Several types of photoproducts are formed, including a majority of cyclobutane dimers and pyrimidine (6-4) pyrimidone adducts (6-4PP).

After a UVB stress, lesions occur immediately and the quantity of photoproducts accumulated in DNA depends on the UVB dose applied. The quantity of photoinduced lesions also influences their rate of repair.³⁻⁴

The cellular response: In order for cells to protect themselves from endogenous lesions to DNA in the body (about 10,000 lesions per day), or photoinduced degradation, cells are equipped with a complex system of defense to repair damaged DNA.

The principal pathway of elimination of photoproducts, especially CPD and 6-4PPs, is the nucleotide excision repair mechanism (NER). It is composed of two major parts: overall genome repair, a surveillance system of the entire DNA molecule, and transcription-coupled repair that specifically eliminates damage in transcribed strands of DNA.⁸ In the case of transcription-coupled NER, RNA polymerase is responsible for detecting damaged DNA, while in overall NER it is protein XPC. This facultative protein is rapidly mobilized after a UV stress, between 30 min and 2 hr later.⁸⁻⁹

The elimination of photoproducts also is correlated with the induction of apoptosis that occurs 24 hr after irradiation, and with the reduction of cell proliferation capacities. These two processes are set in motion to prevent division of the cell whose genome was altered by favoring either its death or the repair of its DNA, depending on the extent of the induced damage.

CPD that are formed in abundance and repaired to only a slight extent, persist in the cell's genome when it resumes its proliferation capacities. This is why they are considered as being most responsible for the mutagenic effects of UV irradiations.⁴ If these mutations are situated in key genes of cell functioning, they may cause the cancerous transformation of the cell or trigger the process of programmed cell death known as apoptosis.

The first objective was to determine and target the post-irradiation period in which the level of CPD induced in keratinocyte nuclei was maximal. This is also the period in which the cellular DNA repair process is not yet optimal. The efficiency of this process, and thus the safeguard of the stability of the cell genome, results from the balance between the extent of exposure of the organism to a variety of factors inducing DNA lesions, and its repair capacity.¹ Photoproducts were assayed at different times after exposing cells to a single dose of UVB. Once a time was established, the authors examined the influence of different UVB doses on the quantity of CPD formed.

These initial experiments enabled the authors to define standard conditions of time of UVB irradiation and intensity of UVB irradiation in their model. With these standard conditions, the authors could accomplish their second objective: to assess the effect of a cosmetic active substance on the quantity of lesions persisting after UVB irradiation.

Visualization of CPD by Immunocytofluorescence

Normal human keratinocytes were inoculated at a density of 40,000 cells per well in culture chambers^a in 500 μ L of keratinocyte serum-free medium^b (KSFM) containing 5 ng/mL of epidermal growth factor^c (EGF). After two days of growth in a 37°C incubator in a humid atmosphere containing 5% CO₂, the culture medium was eliminated after the cells reached 70% confluence. The cells were then rinsed in phosphate buffer saline (PBS)^d.

Modeling with respect to time: Cells were irradiated with UVB (312 nm) at an intensity of 80 mJ/cm² with an artificial UV source^e in 500 μ L of a noncolored medium^f that does not interact during irradiation and maintains the cells in better condition than the PBS does. After irradiation, the cells were placed in KSFM culture medium at 37°C in a humid atmosphere containing 5% CO₂ and were incubated for 1, 3, 5 and 16 hr. The controls were nonirradiated cells.

After each incubation time, fluorescence immunocytological labeling of CPD was conducted. The culture chambers were disassembled, slides were rinsed for 5 min in PBS, fixed in a 4% (v/v) solution of paraformaldehyde^g for 15 min and then permeabilized in a 0.50% (m/v) solution of saponin^h for 5 min.

The slides were then incubated for 45 min with a murine monoclonal anti-CPD^k diluted 1:250. They were rinsed for 5 min in a buffer of PBS and 1% polysorbate-20^m, then twice for 5 min in PBS. The slides were incubated for 35 min at 4°C with a FITC-coupled anti-murine immuno-globulin Gⁿ diluted at 1:400. After two 5-min rinses in PBS, a fluorescent assembly medium^p was used for assembly between slide and cover slip.

^a Falcon culture chambers, 354108, Lab-Tek Products, Westmont, IL, USA

^b Invitrogen Corp., 17005-034, Carlsbad, CA, USA

^c Invitrogen Corp., 15290-012

^d Invitrogen Corp., 18912-014

^e Biosun system, Vilber-Lourmat, Torcy, France

^f 1 X medium 199, Product Number 21157-029, Gibco, Glostrup, Denmark

^g Sigma, F-1635, Lyon, France

^h Sigma, S-4521, Lyon, France

^k Sigma, T1192, Lyon, France

^m Dako, S3306, Glostrup, Denmark. Tween is a registered trademark of Uniqema.

ⁿ A-11004, Alexa Fluor 488, Molecular Probes, Inc., a division of Invitrogen Corp. Alexa Fluor is a registered trademark of Molecular Probes, USA.

^p Dako, S3023, Glostrup, Denmark

Immunolabeling was visualized with a microscope^q coupled to an image analysis system^r. The quantity of CPD is proportional to fluorescence intensity in the nuclei of the keratinocytes. The greater the fluorescence, the more CPD have formed.

Because immunocytology results are qualitative, four immunofluorescence intensity levels were defined (–, +, ++ and +++ in **Table 1**), corresponding to four quantities of CPD formed.

Table 1. Immunofluorescence intensity levels and their correspondence to quantities of CPD formed

Code	Immunofluorescence intensity Detection of immunoreactivity	Quantity of CPD formed
–	None	None
+	Slight	Slight
++	Moderate	Moderate
+++	Strong	High

As shown in **Figure 1**, a fluorescent signal corresponding to the presence of CPD lesions was detected when keratinocytes were irradiated with 80 mJ/cm² of UVB, while a very weak signal was detected in nonirradiated control cells. The quantity of CPD formed increased between 1 and 5 hr after irradiation. After 16 hr, immunofluorescence intensity persisted but was lower than that at 5 hr, reflecting a reduction in the number of lesions in the nuclei of irradiated keratinocytes. Based on these results that the maximum quantity of photoinduced CPD was obtained after 5 hr, this time point was chosen to assess the effect of different UVB doses on the quantity of photo-induced CPD.

Modeling with respect to the intensity of UVB irradiation: Cells were irradiated with UVB at intensities of 50, 80 and 150 mJ/cm² with an artificial UVB source^e in 500 μ L of the noncolored medium^f. After irradiation, the cells were placed in KSFM culture medium and incubated at 37°C in a humid atmosphere containing 5% CO₂ for 5 h. The controls were nonirradiated cells.

After 5 hours of incubation, fluorescence immunocytological labeling of CPD was conducted using the procedure already described. Similarly, immunolabeling was visualized and read as already described.

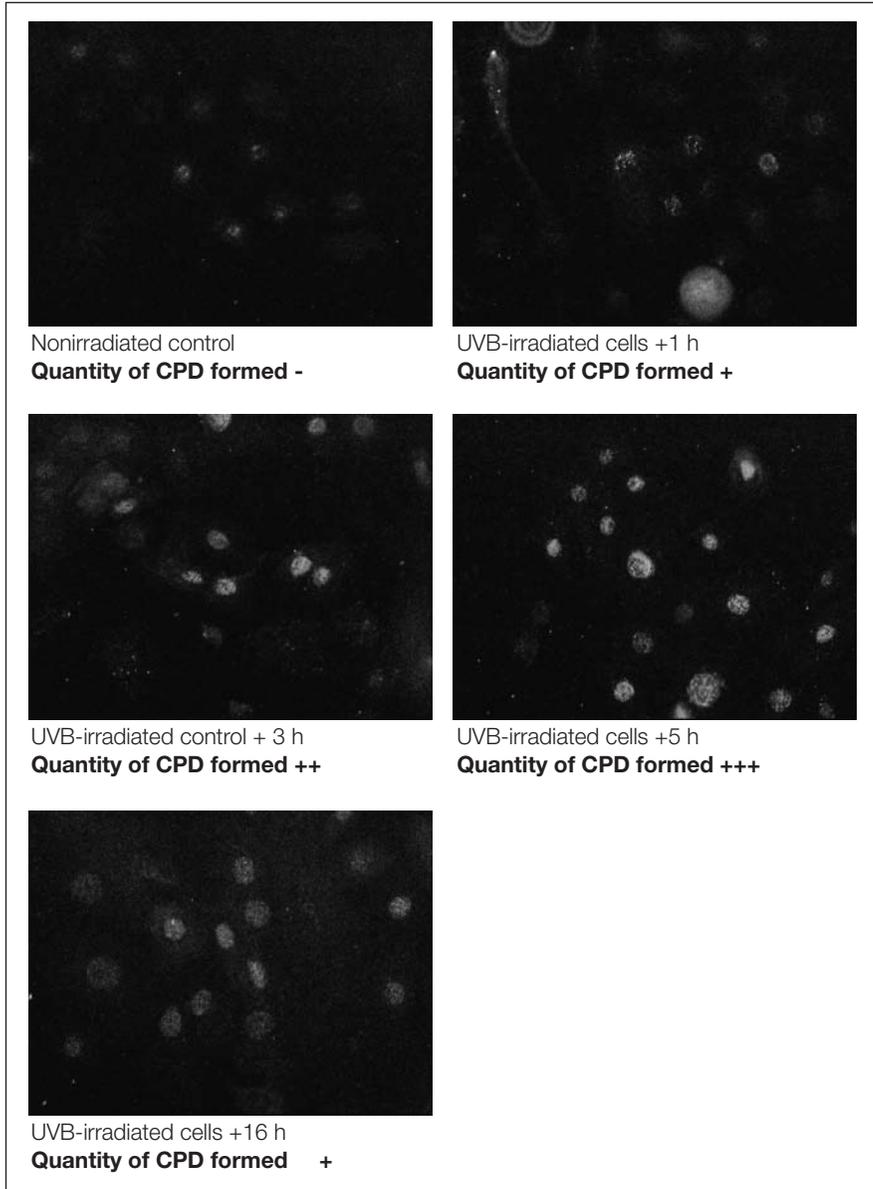


Figure 1. Kinetics of CPD formation after UVB irradiation (80 mJ/cm^2) of normal human keratinocytes [Note: Background was lightened during pre-publication to improve clarity of this image.]

When keratinocytes were exposed to increasing UVB doses (50, 80 and 150 mJ/cm²), the immunofluorescence intensity at 5 hr increased progressively (**Figure 2**). The number of photoproducts formed in keratinocyte DNA was thus dependent on the UVB dose. A clear-cut increase was noted between 50 and 80 mJ/cm², while it was lower between 80 and 150 mJ/cm². These levels of CPD formed by the two UVB doses were semiquantified by dot blot to determine if the difference was significant.

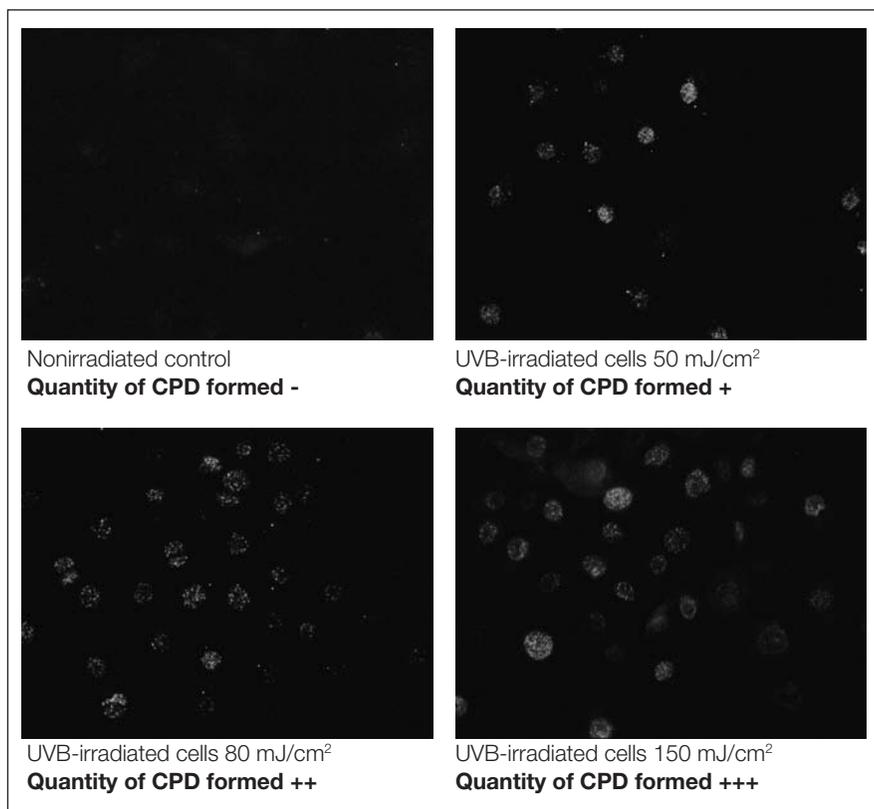


Figure 2. Formation of CPD (5 hr) vs. intensity of UVB irradiation of normal human keratinocytes [Note: Background was lightened during pre-publication to improve clarity of this image.]

Semiquantification of CPD by Dot Blot

Dot blot has been described as a technique in molecular biology used to detect biomolecules. A mixture possibly containing the molecule to be detected is applied directly on a membrane as a dot that is then immediately exposed to nucleotide probes or an antibody.

The technique can only confirm the presence or absence of a biomolecule or biomolecules that respond to the probes or the antibody, and it reveals nothing about the size of the biomolecules, but it provides faster results than alternative chromatographic methods.²

Normal human keratinocytes were inoculated in KFSM medium^b in Petri dishes 100 mm in diameter at a density of 10^6 cells per plate, followed by incubation at 37°C in an atmosphere of 5% CO₂ for four days, renewing the culture medium every two days. After incubation, when the cells reached 70% confluence, the culture medium was discarded. Cells were rinsed in PBS^d and irradiated with UVB at an intensity of 80 and 150 mJ/cm² with an artificial UV source^c in 5 mL of non-colored medium^f. After irradiation, the cells were placed in KFSM culture medium and incubated at 37°C in a humid atmosphere containing 5% CO₂ for 5 hr. Controls were nonirradiated cells.

After incubation, DNA was extracted^s. DNA was quantified by measuring absorbance at 260 nm and 280 nm with a bio-photometer^t. 200 ng of DNA from the different samples were immobilized on a membrane^u using a blotting apparatus^v. DNA was then fixed by incubating the membrane at 80°C for 30 min.

For immunolabeling, the membrane was saturated for 1 hr in a solution of PBS and polysorbate-20^m containing 5% skim milk. The membrane was incubated overnight with murine monoclonal anti-CPD^k diluted 1:250. After rinsing, the membrane was incubated with peroxidase-coupled anti-murine IgG^w. The signal was assayed by colorimetric detection using 4-chloro-1-naphtol (4CN) as substrate of the peroxidase^x.

For visualization, spots were semi-quantified by densitometry after image analysis^y. The intensity of CPD labeling is proportional to the color intensity of the spot. The more intense the spot, the more CPD had formed.

^a IX 70 microscope, Olympus, Tokyo

^b VisioLab 2000 image analysis system, Biocom, Les Ulis, France

^c QIAmp DNA Mini Kit, Qiagen, 5104, Courtaboeuf, France. QIAmp is a registered trademark.

^d Eppendorf Biophotometer, Eppendorf, Hamburg, Germany. Eppendorf is a registered trademark.

^e Zetaprobe membrane, Bio-Rad, Marne la Coquette, France

^f Biodot Apparatus, Bio-Rad, Marne la Coquette, France

^g HRB, P0447, Dako, Glostrup, Denmark

^h OPTI-4CN kit, Bio-Rad, Marne la Coquette, France

ⁱ TotalLab software, Perkin-Elmer, MA, USA

The quantity of CPD induced in the nuclei of keratinocytes irradiated at the UVB doses of 80 and 150 mJ/cm² increased by 100% and 193%, respectively, in comparison to nonirradiated control cells and these increases were significant. The quantity of lesions formed is thus dependent on the UVB irradiation dose.

Study of the Effect of Cotton Extract on Formation of CPD

Using this *in vitro* model, Silab researchers screened for an active ingredient able to reduce the CPD formation in human keratinocytes. The researchers finally selected a cotton extract obtained after selective steps of extraction and purification. The cotton extract is mainly composed of proteins consisting of oligopeptides and sugars consisting of glucose and galactose in the form of oligo- and polysaccharides.

The effect of the cotton extract on the formation of CPD was determined by immunocytology and semi-quantified by the dot blot procedures previously described. Normal human keratinocytes were irradiated with the highest UVB dose studied (150 mJ/cm²) and then treated with 0.5%, 1% and 2% cotton extract for 5 hr.

Detection of CPD by immuno cytofluorescence: As described above, the immunofluorescence intensity of UVB-irradiated cells was high, whereas no signal was detected for the nonirradiated control (**Figure 3**). When irradiated cells were treated with increasing doses of cotton extract (0.5%, 1% and 2%), immunofluorescence intensity decreased progressively, reaching a level of almost zero for the cotton extract at 2%. The CPD lesions that remained in the nuclei of keratinocytes 5 hr after the UVB irradiation were thus less numerous when the cells were treated with the cotton extract. In addition, there was an apparently inversely proportional relationship between the quantity of CPD persisting 5 hr after UVB irradiation and the dose of cotton extract used for the treatment.

In order to further study and prove or disprove this relationship, dot blot was used to determine the effect of the cotton extract on the quantity of CPD lesions remaining in the nuclei of keratinocytes 5 hr after UVB irradiation.

Semi-quantification of CPD by dot blot: The dot blot spots from these experiments are shown in **Figure 4**. The intensity of spot labeling corresponds to the quantity of CPD formed and remaining 5 hr after irradiation of the keratinocytes. The results are in agreement with those obtained by immunofluorescence and shown in **Figure 3**.

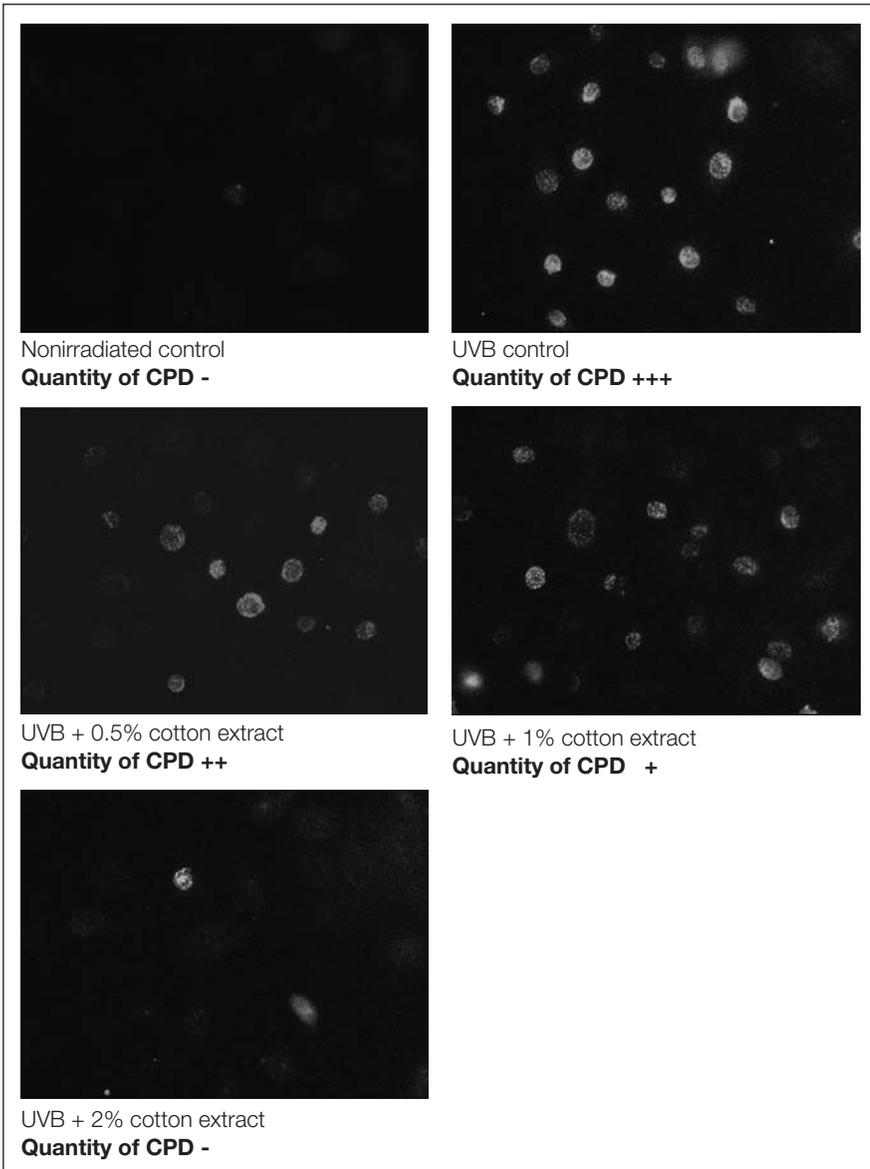


Figure 3. Effect of cotton on the quantity of CPD 5 hr after UVB irradiation (150 mJ/cm²) of normal human keratinocytes

[Note: Background was lightened during pre-publication to improve clarity of this image.]

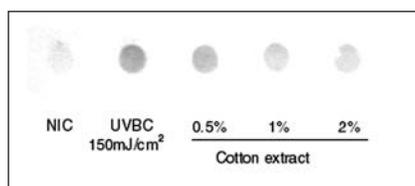


Figure 4. Effect of cotton extract on the quantity of CPD 5 hr after UVB irradiation (150 mJ/cm²) of normal human keratinocytes
 NIC = Nonirradiated control
 UVBC = UVB-irradiated control (150 mJ/cm²)
 [Note: Background was darkened during pre-publication to improve clarity of this image.]

After semiquantification of spots by densitometry, it was found that the quantity of CPD present in the nuclei of UVB-irradiated keratinocytes was 293% and the difference was significant in comparison to nonirradiated control cells. When UVB-irradiated cells were treated for 5 hr with the cotton extract at 0.5%, 1% and 2%, CPD was 218%, 185% and 160%, respectively. These differences with the irradiated control were significant. Treating UVB-irradiated keratinocytes with cotton extract at 0.5%, 1% and 2% thus reduced the quantity of CPD 5 hr after UVB irradiation by 26%, 37% and 45%, respectively, all significant differences.

By the way, chromatographic methods could be used to confirm these results and to obtain more sensitive results. According to published data, CPD lesions can be detected and assayed by high performance liquid chromatography (HPLC) and mass spectrometry or radioactivity detector. Those studies have not yet been conducted by these authors.

Conclusion

The kinetics of formation of direct lesions to DNA after UVB irradiation of human keratinocytes showed that the quantity of CPD formed reached a maximum 5 hr after the irradiation. This quantity decreased considerably 16 hr after the stress. The majority of CPD lesions in the nuclei of keratinocytes was thus repaired between 5 and 16 hr after a single UVB irradiation (80 mJ/cm²) by DNA repair mechanisms such as nucleotide excision repair.

Based on this finding, the authors decided to determine the quantity of CPD lesions induced by different UVB stress intensities 5 hr after the irradiation. After irradiating keratinocytes with increasing UVB doses (50–80 and 150 mJ/cm²), the authors observed a proportionality between the UVB irradiation dose and the quantity of CPD formed. These results are consistent with published data.³⁻⁴

These initial experiments were used to standardize the model of UVB-irradiated human keratinocytes subsequently used to study the effect of a cosmetic active substance on the quantity of UV-induced CPD. In this study, human keratinocytes were irradiated with a UVB dose of 150 mJ/cm², the highest dose used in the previous study, and then treated with the cotton extract for 5 hr. Immunofluorescence and dot blot showed that keratinocytes irradiated with UVB and then treated with the cotton extract at 0.5%, 1% and 2% exhibited significantly fewer CPD lesions in their nuclei 5 hr after irradiation than irradiated untreated controls. Because the cells were treated after induction of the UV stress, it can reasonably be supposed that the cotton extract increased the efficacy of cellular mechanisms for the repair of damaged DNA and/or accelerated their mobilization.

The method described in this paper substantiated the claim that skin care active ingredients such as the cotton extract could be used to protect the skin from UV exposure by stimulating the skin cell repair equipment to accelerate the elimination of DNA damage. Such active ingredients could thus provide a good complementary action of the use of filters and/or anti-free radicals in sun care products.

Published September 2009 *Cosmetics & Toiletries* magazine.

References

1. IM Hadshiew, MS Eller and BA Gilchrest, Skin aging and photoaging: The role of DNA damage and repair, *Am J Contact Dermat* 11(1) 19–25 (2000)
2. *Dot blot* entry, available at: http://en.wikipedia.org/wiki/Dot_blot (Accessed Jul 27, 2007)
3. T Maeda, PPS Chua, MT Chong, ABT Sim, O Nikaido and VA Tron, Nucleotide excision repair genes are upregulated by low-dose artificial ultra-violet B: Evidence of photoprotective SOS response? *J Invest Dermatol* 117(6) 1490–1497 (2001)
4. S Courdavault, C Baudouin, M Charveron, B Canguilhem, A Favier, J Cadet and T Douki, Repair of the three main types of bipyrimidine DNA photoproducts in human keratinocytes exposed to UVB and UVA radiations, *DNA Repair Amst* 4(7) 836–844 (2005)
5. T Douki and J Cadet, Formation of the spore photoproduct and other dimeric lesions between adjacent pyrimidines in UVC-irradiated dry DNA, *Photochem Photobiol Sci* 2 433–436 (2003)
6. D Perdiz, P Grof, M Mezzina, O Nikaido, E Moustacchi and E Sage, Distribution and repair of bipyrimidine photoproducts in solar UV-irradiated mammalian cells. Possible role of Dewar photoproducts in solar mutagenesis, *J Biol Chem* 1 275(35) 26732–26742 (2000)
7. M Cario-Andre, Rôle des mélanocytes dans l'unité épidermique de mélanisation reconstruite ex-vivo après une irradiation UV aiguë. Peau et rayonnement solaire, *Doctoral thesis, University of Bordeaux 2, November 23, 2000* (2000) pp 29–65
8. QE Wang, Q Zhu, MA Wani, G Wani, J Chen, AA Wani, Tumor suppressor p53 dependent recruitment of nucleotide excision repair factors XPC and TFIIH to DNA damage, *DNA Repair* 2 483–499 (2003)
9. S Adimoolam and JM Ford, P53 and DNA damage-inducible expression of the xeroderma pigmentosum group C gene, *Proc Natl Acad Sci USA* 99 20 12985–12990 (2002)

Mutations in Mitochondrial DNA as Principal Aging Factor

Daniel Schmid and Fred Züllli

Mibelle AG Biochemistry, Buchs, Switzerland

KEY WORDS: *coenzyme Q10, nanoemulsion, premature skin aging, mitochondrial DNA, common deletion*

ABSTRACT: *Irradiation of normal human fibroblasts with UV was shown to increase the frequency of mutations in the mitochondrial DNA. Treatment of the fibroblast culture with a nanoemulsion containing coenzyme Q10 and vitamin E acetate was found to protect the mitochondrial DNA against UV-induced mutations.*

Ultraviolet irradiation has negative effects on mitochondrial DNA mutations. These effects are shown in this chapter to be an indirect result of the formation of OH radicals. Also shown in this chapter is the protective effect of a nanoemulsion containing antioxidants that scavenge those radicals.

Structure and Function of Mitochondria

Mitochondria are the cellular organelles that generate energy from aerobic metabolism. Glucose and other food molecules are oxidized to carbon dioxide and water. The energy released is stored in the form of adenosine triphosphate (ATP).

Mitochondria are enclosed by a double membrane. The inter-membrane space between the two membranes is separated from the cytosol outside and the mitochondrial matrix inside. Pyruvate that is

produced by glycolysis in the cytosol is transported into the matrix where it enters into the Krebs cycle to form nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH_2). Several protein complexes of the inner membrane form an electron transfer chain where electrons from NADH and FADH_2 are transferred to oxygen. This releases energy that is used by the protein complexes to transport protons into the intermembrane space. The ATP synthetase complexes use the flow back of these protons into the mitochondria to produce ATP (Figure 1).

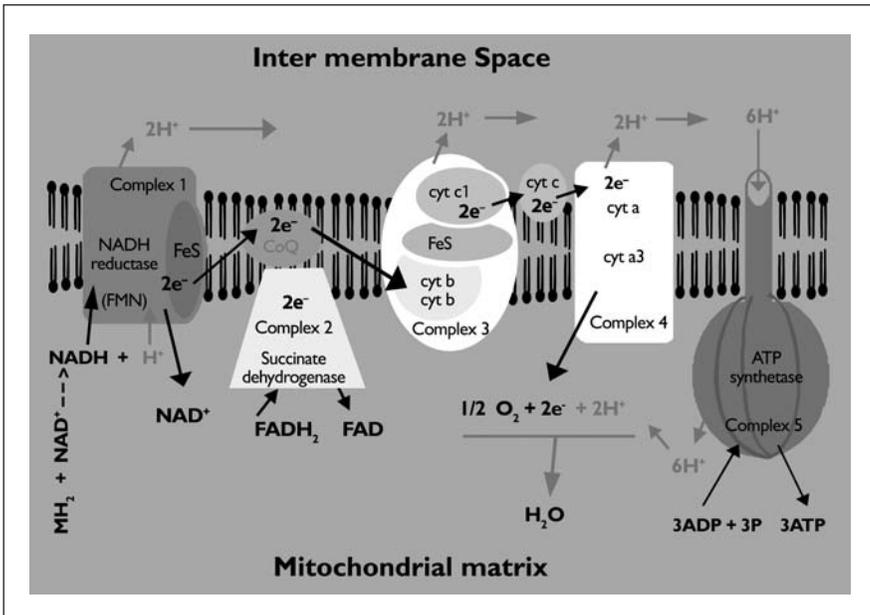


Figure 1. Electron transport chain in the inner mitochondrial membrane

Mitochondria contain their own DNA (mtDNA). It is a circular DNA molecule consisting of 37 genes that are made up of 16,569 base pairs. Thirteen of those genes encode proteins of the electron transfer chain. However, each of these protein complexes in the inner mitochondrial membrane also contains proteins that are encoded by nuclear genes and transported into the mitochondria after synthesis in the cytosol.

Reactive Oxygen Species in Aerobic Metabolism

The process of oxidative phosphorylation where electrons from NADH and FADH_2 are transferred to oxygen generates a significant

amount of reactive oxygen species (ROS) that can damage the mtDNA. At several sites along the electron transfer chain, electrons derived from NADH and FADH₂ can directly react with oxygen or other electron acceptors and generate free radicals. Since mtDNA lacks the protection of histones and generally is repaired less efficiently than nuclear DNA, mtDNA mutates much more frequently. The increased mutations impair the mitochondrial function, which decreases energy production and induces a vicious cycle. A defective respiratory chain generates even more ROS that in turn damages the whole cells. Mutations and deletions of mtDNA are thought to be a principal cause of aging and age-related diseases. Numerous studies have found increased mitochondrial mutations and deletions in a variety of tissues from older persons.

Activity of Coenzyme Q10

For elimination of ROS, mitochondria contain scavenging enzymes such as superoxide dismutase and catalase and different antioxidants. One of these antioxidants is coenzyme Q10 (CoQ10), also known as ubiquinone. CoQ10 is a lipophilic molecule consisting of a quinone with an isoprenoid side chain. Because of its lipophilic nature, CoQ10 is present in cellular membranes, especially in the inner membrane of mitochondria where it has an essential role as a carrier of electrons in the electron transport chain. There it passes electrons from complex 1 or 2 to complex 3, known as coenzyme Q - cytochrome c reductase complex (**Figure 1**). The oxidized quinone form of CoQ10 can accept a single electron to form a semiquinone, and then a second electron and two protons to form the fully reduced form, dihydroubiquinone (CoQ10H₂) (**Figure 2**).

In its reduced form, CoQ10H₂ is an effective fat-soluble antioxidant. Together with vitamin E it is the principal antioxidant in membranes. Although the human body can synthesize CoQ10, deficiencies of it have been reported to occur frequently. In addition, CoQ10 levels decline rapidly under stress or with advancing age. In case of deficiency, CoQ10 has to be supplemented to guarantee the body's energy production and its essential antioxidant protection.¹

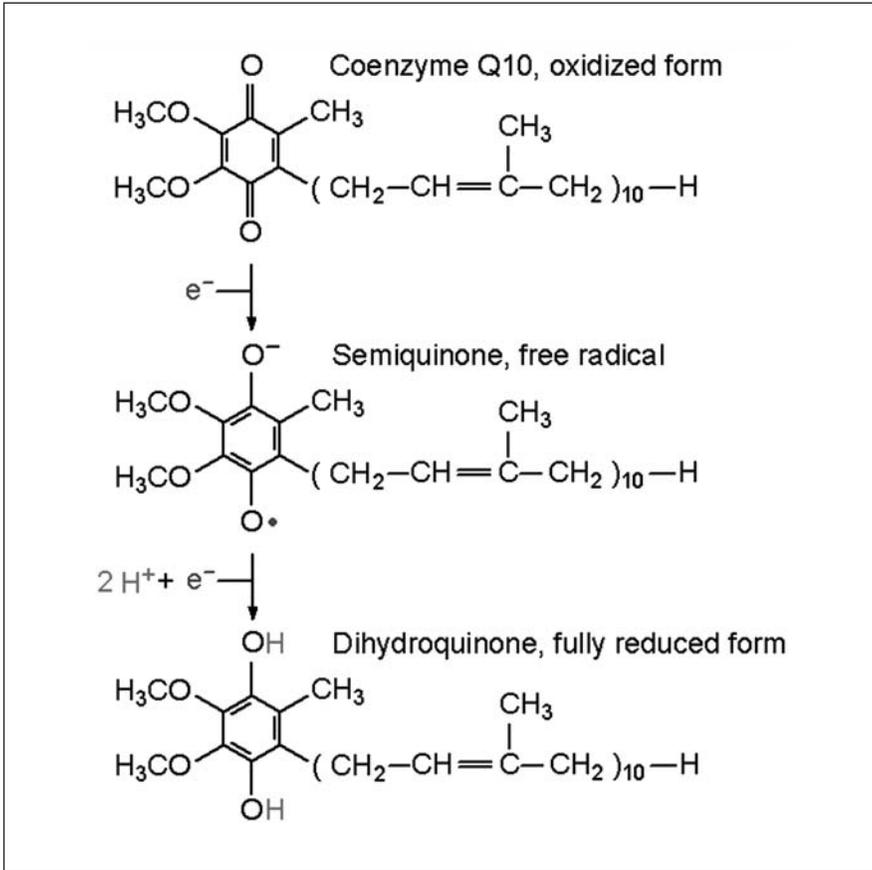


Figure 2. Oxidized, partially and fully reduced state of coenzyme Q10

Mechanisms in Premature Skin Aging

That mtDNA mutations are involved in the premature aging of skin is especially well-documented. Studies showed that for the same individual, photoaged skin contained up to 10 times more mtDNA mutations or deletions than skin protected from the sun.² Repetitive exposure of keratinocytes, fibroblasts or human skin to UVA at physiological doses was found to induce mutations of mtDNA.³ Histologic analysis of photoaged skin shows an atrophy of matrix components such as elastin and collagen. This characteristic reduction in the formation of matrix fibers could be a sign of a slower metabolism of keratinocytes and fibroblasts because of a deficiency in energy production as consequence of impaired mitochondrial func-

tion. On the other hand, ROS are produced to a greater extent after UV exposure and mtDNA mutations or deletions. These ROS are known to stimulate the expression of collagen- and elastin-degrading enzymes, known as matrix metalloproteinases.

Study on UV-induced mtDNA Mutations in Fibroblasts

In aging and mitochondrial degenerative diseases, a frequent occurrence of a deletion of 4977 bp was detected in mtDNA. This deletion, referred to as the “common deletion” (Cdel) is used as a marker of mtDNA mutations and deletions.⁴

In an experiment with normal human fibroblasts, the authors analyzed the degree of mutations in mtDNA before and after irradiation with UVB. The experiment was used to study the protective effect of a nanoemulsion containing 1% CoQ₁₀ and 3% vitamin E acetate. The nanoemulsion encapsulating CoQ₁₀ and vitamin E acetate was stabilized by lecithin and had a particle size of only 50 nm. Because of the small particle size, the nanoemulsion is transparent and is suitable for cell culture studies.

Real time polymerase chain reaction (RT-PCR) was used to analyze the Cdel frequency in different cell extracts. The method normally is used for quantifying messenger RNA, but also can be used for quantitative analysis of amplifications or deletions in genomic DNA. The oligonucleotide of interest is amplified with the aid of two primers that define the ends of the piece. A fragment of 262 bp out of a conserved

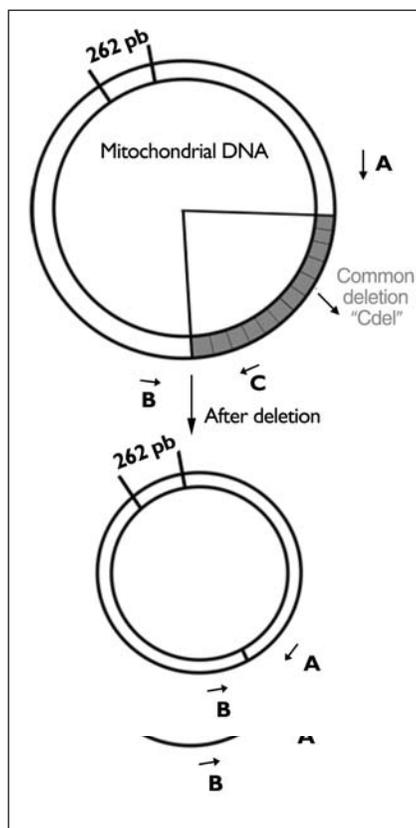


Figure 3. Fragments of mtDNA amplified by RT-PCR

area was amplified for quantification of total mtDNA (**Figure 3**). For quantification of the frequency of Cdel, primers A and B were used that defined a 470 bp oligonucleotide. The amount of intact mtDNA was measured by amplification of a 755 bp fragment using the primers B and C.

Normal human fibroblasts were grown to confluence in a standard growth medium. Upon reaching confluence, the cells were pretreated for 72 hr with 0.4% and 2% of the nanoemulsion. Then the cells were irradiated twice daily for 5 days with 50 mJ/cm² of UVB. For irradiation, the cells were put into phosphate-buffered saline (PBS) buffer to prevent the formation of phototoxic products. Between irradiations, the cells were placed in the standard medium containing the CoQ₁₀ nanoemulsion. At the end of the irradiation period, the cells were incubated for a further 72 hr in the standard medium with the tested nanoemulsion. Extraction of total DNA and RT-PCR were performed according to standard protocols.

The results of the experiment are summarized in **Figures 4** and **5**. Irradiation of normal human fibroblasts in the standard growth medium without the tested nanoemulsion with sublethal repetitive UVB doses induced a 2.72-fold increase of Cdel (**Figure 4**).

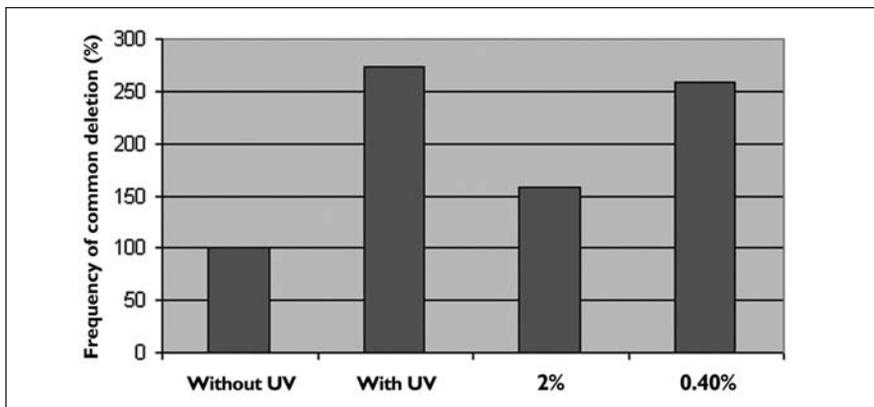


Figure 4. Frequency of the common deletion in mitochondrial DNA after UVB irradiation

Figure 6 shows an agarose gel where the amplified oligonucleotides had been separated. Formation of the 470 bp fragment corresponding to the amount of Cdel clearly was enhanced in the irradiated fibroblast cultures compared to the control. In presence of 2% of

the CoQ10 nanoemulsion, the increase of Cdel after irradiation was only 1.58-fold.

The same tendency was found regarding the number of complete mtDNA (**Figure 5**). Irradiation induced a 35% decrease of complete mtDNA. When the fibroblasts were incubated with 2% or 0.4% of the nanoemulsion, the decrease of intact mtDNA after irradiation was only 9% or 22%, respectively. In real life, one might argue that several

difficulties would prevent the delivery of 2% of a formulation to the fibroblasts and limit the penetration of actives into deeper skin layers. However, a cosmetic product is normally applied two times every day. After several weeks of application, there will be a depot effect, and the actives also will concentrate in the deeper skin.

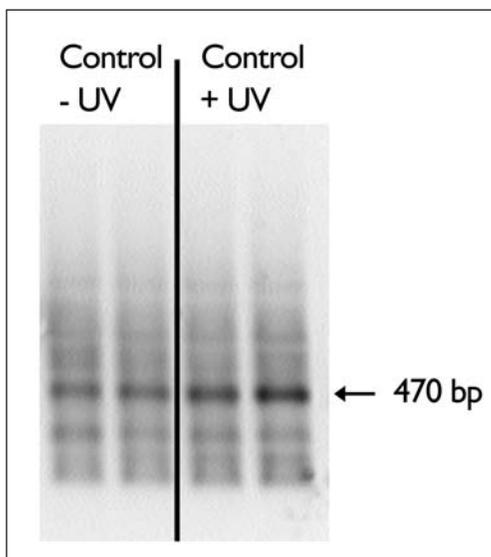


Figure 6. Amplified 470 bp oligonucleotide, corresponding to the amount of common deletions, separated on agarose gel

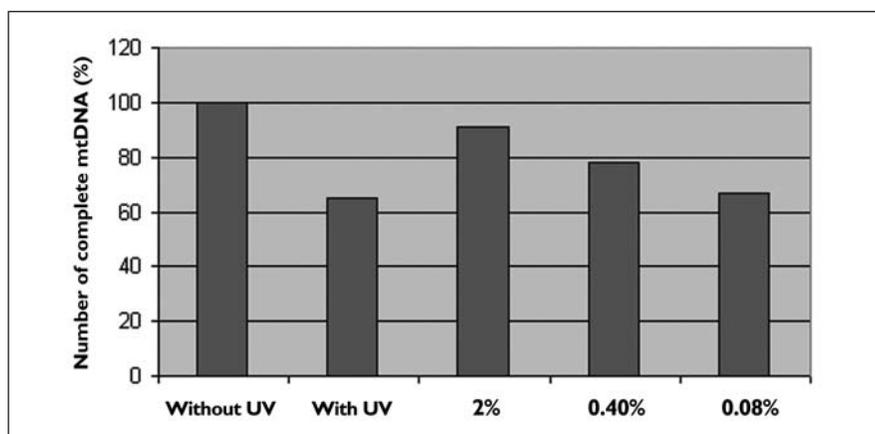


Figure 5. Amount of complete mitochondrial DNA after UVB irradiation

The results of the study confirm previous scientific reports about the negative effect of UV irradiation on mitochondrial DNA mutations.

The UVB-induced deletions could to a great extent be prevented by a nanoemulsion containing CoQ10 and vitamin E acetate with an extremely small particle size. This shows that the deleterious effect of UVB is not primarily because of direct interactions with the mitochondrial DNA, but rather a consequence of the formation of OH radicals. The tested nanoemulsion of CoQ10 and vitamin E acetate therefore is a promising cosmetic ingredient to prevent premature skin aging.

Published January 2007 *Cosmetics & Toiletries* magazine.

References

1. FL Crane, Biochemical functions of coenzyme Q10, *J Am Coll Nutr* 20(6) 591–598 (2001)
2. AJ Ray, R Turner, O Nikaido, JL Rees and MA Birch-Machin, The spectrum of mitochondrial DNA deletions is a ubiquitous marker of ultraviolet radiation exposure in human skin, *J Invest Dermatol* 115 674–679 (2000)
3. M Berneburg, H Plettenberg, K Medve-König, A Pfahlberg, H Gers-Barlag, O Gefeller and J Krutmann, Induction of the photoaging-associated mitochondrial common deletion *in vivo* in normal human skin, *J Invest Dermatol* 122 1277–1283 (2004)
4. S Prithivirajsingh, MD Story, SA Bergh, FB Geara, KK Ang, SM Ismail, CW Stevens, TA Buchholz and WA Brock, Accumulation of the common mitochondrial DNA deletion induced by ionizing radiation, *FEBS Letters* 571 227–232 (2004)

Mitochondrial Nourishment and Protection for Antiaging Effects

KG Sabarinathan, PhD

CoValence Inc., Chandler, Ariz. USA

KEY WORDS: *mitochondria, antioxidant, nutrition, antiaging, DNA*

ABSTRACT: *Multiple factors affect the integrity of cell mitochondria, leading to loss of cell function, aging and apoptosis. In skin, this is expressed in the form of wrinkles, loss of tone, etc. To combat these effects, the author describes a technology that contains mitochondria-nourishing compounds to deliver antiaging benefits.*

Virtually everything that human cells need to maintain health requires energy. Each cell contains hundreds to thousands of mitochondria, and each mitochondrion contains multiple copies of mitochondrial DNA (mtDNA). Mitochondrial proteins take food molecules and combine them with oxygen to create chemical energy. This chemical energy produced by the mitochondria through the cellular respiration process is called adenosine triphosphate (ATP) (see **Figure 1**).

Generally the duration of life varies inversely with the rate of energy expenditure during life. Bio-gerontologist Denham Harman, PhD, the “father of the free radical theory of aging,” once suggested that mitochondria are the crucial component of cells whose rate of decline dictates the overall rate of aging.¹ In addition to supplying cellular energy, mitochondria are involved in a range of other

processes such as signaling, cellular differentiation and cell death, as well as controlling the cell's cycle and growth.²

In human cells and eukaryotic cells in general, DNA is found in two cellular locations: inside the nucleus and inside the mitochondria. The generation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radicals as by-products of mitochondrial oxidative phosphorylation (see **Figure 2**) damages mitochondrial macromolecules including the mtDNA, leading to deleterious mutations.

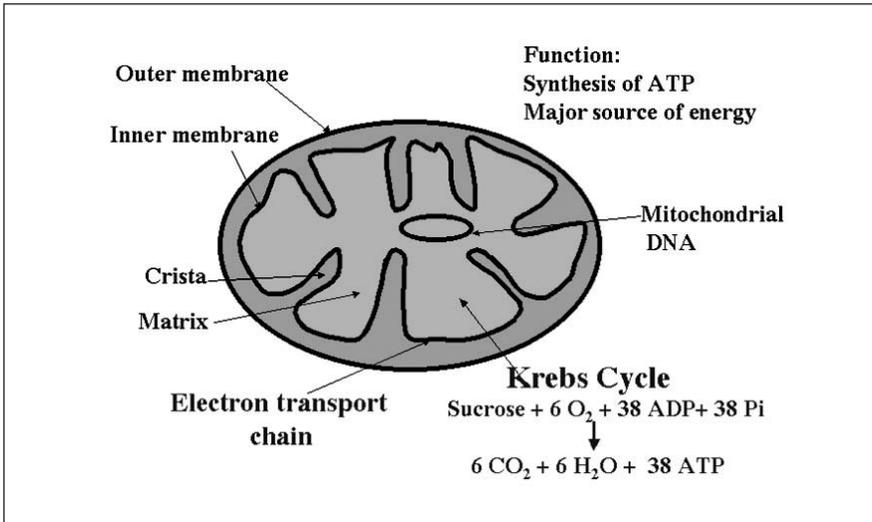


Figure 1. The chemical energy produced by the mitochondria through the cellular respiration process is called adenosine triphosphate (ATP).

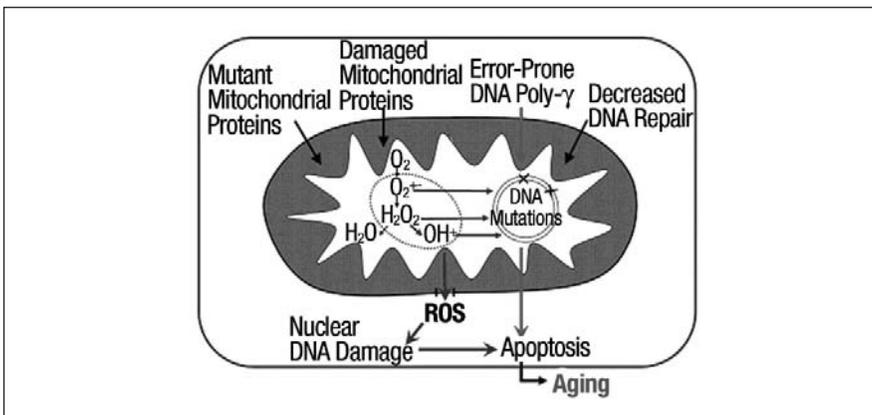


Figure 2. By-products of mitochondrial oxidative phosphorylation damage the mitochondrial macromolecules, including the mtDNA, and lead to deleterious mutations.

MtDNA damage is more extensive and persists longer than nuclear DNA damage in human cells,³ so when ROS have damaged the mitochondrial energy-generating apparatus beyond a functional threshold, rather than restoring it to a healthy condition, the mitochondria releases proteins that activate the caspase pathway, leading to aging and apoptosis.⁴ In skin, this aging is expressed in the form of wrinkles, loss of tone, thinning and abnormal collagen accumulation.

The human mitochondrial genome was sequenced in 1981, facilitating the isolation of mutant mtDNA and eventually its quantification. These experiments confirmed that mtDNA damage increases aging in humans and other mammals.⁵ Recent work by Trifunovic et al.⁶ also supports the mitochondrial theory of aging, showing that in prematurely aged mice, defective mitochondrial DNA polymerase is expressed. This work provides a causative link between mtDNA damage and aging phenotypes in mammals.⁶

Delivering Mitochondria Nutrition

An approach to the mitochondrial theory of aging, as will be described, is a technology focused on mitochondria-nourishing compounds. However, since the efficacy of functional ingredients fundamentally is determined by their delivery and influenced by the vehicle and molecules themselves,⁷ it is important first to consider the delivery aspect; in this case, the technology was encapsulated for controlled delivery.

A free-flowing powder of solid, hydrophobic nanospheres containing the mitochondria-nourishing compounds was further encapsulated in moisture-sensitive microspheres averaging 100–300 nm in diameter to deliver them to the epidermal level. These spheres adhere to skin as a result of lipophilic properties and electrostatic forces. While they are unable to penetrate the stratum corneum (SC), once they are deposited on skin, they form a reservoir, and moisture in the skin triggers the release of the nanospheres, which penetrate the lower layers of the SC. The incorporation of this controlled release system into anhydrous cosmetic formulations was found to provide moisture-triggered and prolonged release of the active ingredients (data not shown).

Mitochondria-nourishing Components

The nano-encapsulated, chirally active ingredients^a at the core of the described technology (see **Chirality in Chemistry**) were used to nourish mitochondria and stimulate the mitochondria support enzymes,^{8–10} to protect against endogenous and exogenous ROS, in turn slowing the aging of skin cells and other cellular material.

Chirality in Chemistry

Chirality or *handedness* refers to a molecule that is not superimposable on its mirror image. This positioning is described in chemistry using *L* to define the left side of a molecular twin—i.e., the *L-isomer*, and *D* to define the right side, or *D-isomer*. One version of the molecule may interact with a cell receptor to produce the desired outcome, while the other may have no useful application, or even unwanted effects.

In the present invention, the company used chirally active ingredients by selecting the effective isomer from the racemic mixture of the ingredient. The efficiency of the percutaneous absorption of cosmetic products is also influenced by the chirality of the ingredients; this is well-documented by Heard and Brain (1995) and Heard et al. (1993).

Spin-trap phenyl-butyl-nitrone (PBN): Research has shown that spin-trap PBN provides neuroprotective, cytoprotective, anti-inflammatory, oxidative stress recovery and free radical scavenging properties.¹¹ Spin-traps originally were used to measure free radical activity both *in vivo* and *in vitro* by their ability to form stable complexes. Reactive free radicals are attracted and bound to the beta carbon atom in the spin trap, forming a spin adduct and effectively “trapping” the free radical, allowing the structure of the trapped radical to be deduced, and returning it to a normal orbit before it causes damage to the mitochondria.¹² The material acts to prevent free radical damage caused endogenously through normal metabolic processes or exogenously by sources such as UV radiation, NO and other air pollutants by absorbing electrons as they spin out of control.

^a Mitoprotect (INCI: Phenyl-butyl-nitrone (PBN) (and) Ubiquinone (and) 1,2-Dithiolane-3-Pentanoic Acid (and) Adenine (and) Acetyl Carnitine (and) antioxidants) is a product and registered trademark of CoValence.

Coenzyme Q10 (CoQ10): CoQ10 or ubiquinone is a naturally occurring compound found in all cells in the human body that plays a key role in the mitochondria by converting food into energy. Ninety-five percent of all human body energy requirements are converted to ATP with the aid of CoQ10, and the depletion of CoQ10 levels in skin cells leads to the premature aging as well as a decrease in the formation of new cells.^{13,14} Studies have shown that low doses of CoQ10 applied topically reduce oxidation and DNA double-strand breaks. In addition, CoQ10 supplements have been shown to extend the lifespan of human cells. In the described technology, CoQ10 stabilizes the CoQ10 content in the skin cells to increase their longevity.¹⁵

R-lipoic acid: Normally only the R-enantiomer of lipoic acid occurs naturally and in miniscule amounts in animal and plant tissues. Due to the difficulty and high cost of isolating natural R-alpha lipoic acid (ALA), studies and products were initially conducted and produced using synthetic lipoic acid. Unlike natural R-ALA, however, synthetic lipoic acid contains a 50/50 mixture of both R-ALA and S-ALA. Thus, most cosmetic products on the market containing ALA in fact contain both forms of lipoic acid—the synthetic S form and the natural R form.

However, Loffelhardt and co-workers have shown¹⁶ the S-enantiomer to have an inhibiting effect on the R-enantiomer since they are isomers, and with their atomic arrangements reversed, the biological activity of the R-enantiomer is substantially reduced, creating oxidative stress in human cells.

R-lipoic acid is water- and fat-soluble⁸ and therefore can neutralize free radicals both in membranes and within cells; it can also mimic other antioxidants and improve their performance by replenishing them.¹⁷ When an antioxidant neutralizes a free radical, it turns the radical into a stable form. In the chemical reaction that follows, the free radical is eventually passed off to lipoic acid or a glutathione molecule, which allows the original antioxidant to regenerate and continue to neutralize more free radicals while ALA washes out the offending free radical.¹⁸ This provides protection to the mitochondria.

In addition to its antioxidant function, R-lipoic acid has been

shown to remodel collagen synthesis; to inhibit the abnormal attachment of sugar to protein and collagen, which makes skin inflexible and stiff (glycosylation); and to provide an anti-inflammatory function.^{19,20} Research also has shown that R-lipoic acid increases the mitochondrial membrane potential of aged rats by up to 50%, compared with unsupplemented, aged rats.⁸

Adenine: Adenine is one of the two purine nucleobases used in forming nucleotides of the nucleic acids. In DNA, adenine binds to thymine via two hydrogen bonds to assist in stabilizing the nucleic acid structures. Some scientists have proposed that during the origin of life on Earth, the first molecule formed was adenine by polymerization reaction.²¹ Adenine performs a variety of roles in cellular biochemistry including cellular respiration in the form of energy-rich ATP and other cofactors such as nicotinamide adenine dinucleotide and flavin adenine dinucleotide, thus increasing the longevity and proper functioning of the mitochondria.²²

Acetyl-L-carnitine (ALC): ALC regulates the mitochondrial cytochrome-C oxidase level in the human body, including skin cells; cytochrome-C oxidase is a vital component of cellular energy processes and is responsible for virtually all oxygen consumption in mammals.²³ ALC transports long-chain acyl groups from fatty acids into the mitochondrial matrix so they can be broken down to acetate via β -oxidation to obtain usable energy through the citric acid cycle. Throughout human aging, carnitine concentration in cells diminishes, affecting energy production by the mitochondria. Recently reported data clarifies the role of ALC and the carnitine transport system in the interplay between peroxisomes and mitochondrial fatty acid oxidation.²⁴

Thus, the combination of R-lipoic acid with ALC can significantly improve metabolic function; at the same time, this combination lowers oxidative stress and free radical production.²⁵

Antioxidant Activity

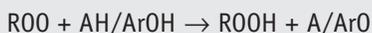
The antioxidant potential and activity of the enzymes in the complex^a were evaluated through the Oxygen Radical Absorbance Capacity (ORAC) assay^b developed at the National Institute on

^b The ORAC assay is a patented technology from Brunswick Laboratories.

Aging in the National Institutes of Health (NIH). This assay is based on a hydrogen atom transfer (HAT) reaction mechanism, which is relevant to human biology (see **HAT Assays**). The stimulation by antioxidant enzymes thus served as an indicator of the nutritional effects of the complex on the mitochondria.

HAT Assays

HAT-based assays measure the capability of an antioxidant to quench free radicals (generally peroxy radicals) by H-atom donation. The HAT mechanisms of antioxidant action in which the hydrogen atom (H) of a phenol (Ar-OH) is transferred to an ROO radical can be summarized by the reaction:



The ORAC assay based on Glazer's study measures the oxidative degradation of a fluorescent molecule (either β -phycoerythrin or fluorescein) after being mixed with free radical generators such as azo-initiator compounds. Azo-initiators are considered to produce peroxy free radicals by heating, which damages the fluorescent molecule, resulting in the loss of fluorescence. Antioxidants protect the fluorescent molecule from oxidative degeneration, and this degree of protection is quantified using a fluorometer.

The water-soluble vitamin E analog Trolox^c was used as a calibration standard and the ORAC result is expressed as micromole Trolox equivalent (TE)/g (see **Figure 3**).¹ Caffeic acid also served as a calibration standard and the hydroxyl radical antioxidant capacity (HORAC) result is expressed as μ mole caffeic acid equivalent (CAE)/g.² The acceptable precision of the ORAC assay is 15% relative standard deviation.

Results of the ORAC assay indicated that the water-soluble or hydrophilic antioxidant capacity and the lipid-soluble or lipophilic antioxidant capacity of the text complex were 30 and 13 μ mole TE/g, respectively. In addition, the HORAC and nitrogen radical absorbance capacity was found to be 7 μ mole CAE/g and 0.3 μ mole TE/g, respectively (see **Figure 3**).

^c 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Hoffman-LaRoche) is a water-soluble derivative of vitamin E.

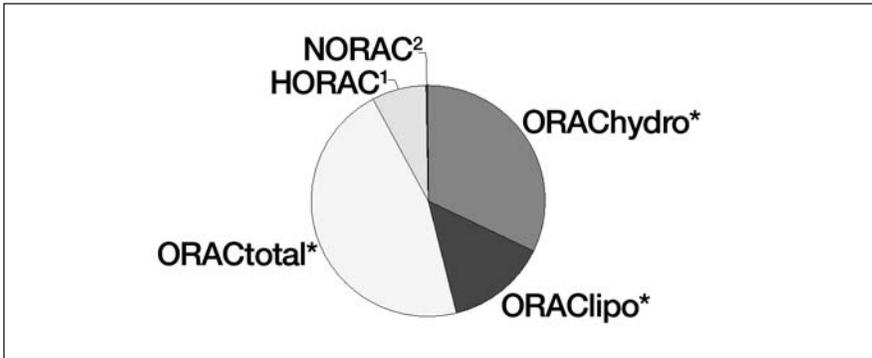


Figure 3. The radical absorbance capacity of HORAC and nitrogen was found to be 7 $\mu\text{mole CAE/g}$ and 0.3 $\mu\text{mole TE/g}$, respectively.

The Singlet Oxygen Absorbance Capacity (SOAC) using α -tocopherol as a calibration standard showed that the tested complex possessed the antioxidant power of 198 $\mu\text{mole VtE/g}$ —higher antioxidant power than anti-oxidants currently used in the cosmetics industry. As a comparison, apples, evaluated for their ORAC activity by the USDA, generally have measured 22.10 to 42.75 micromoles TE/g.

Stimulating SC Antioxidant Enzymes

The skin is constantly exposed to environmental sources, producing reactive oxygen species (ROS). To protect against oxidative damage, the skin is equipped with a large network of enzymatic antioxidant defense systems such as catalase, superoxide dismutase (SOD), and tissue GSH.²⁶ In human SC, SOD and catalase are considered major antioxidant enzymes.²⁷ In various skin disorders, the decreased antioxidant enzyme activity could be considered a marker for the increased susceptibility of the skin to external stimuli.²⁸

The ability of the test complex to induce SC antioxidant enzymes was evaluated by the protocol standardized by Hellemans et al.²⁷ In this study, 40 albino guinea pigs of the same age weighing 350–430 g were selected. The dorsal skin of the guinea pigs was washed and a 35 cm^2 area was shaved before exposing it to a single dose of UVB (290–320 nm) irradiation. The total energy exposure of the guinea pig was 0.9 J/cm^2 . The irradiation time was approximately 30 sec.

The test complex was applied in the irradiated area and the antioxidant enzyme expression was studied by taking noninvasive tape strippings to determine SOD and catalase activity in guinea pig SC

in vivo. In each study, 5 successive tape strippings were collected on the abdomen region, and stored at -80°C until analysis.

Detection of the catalase and SOD activity on the tape strippings from the SC was estimated as described by Giacomoni et al.²⁹ and Hellemans et al.,²⁷ respectively. The total protein amount on the tape stripping was quantified as the total amount of amino acids after acid hydrolysis at elevated temperature.

The GSH activity was estimated through Beutler et al.³⁰ and showed an increase in SOD (see **Figure 4a**), catalase (see **Figure 4b**), and tissue GSH level (see **Figure 4c**) in skin cells, up to 3.33-, 4.3- and 2.7-fold higher than the control treatment.

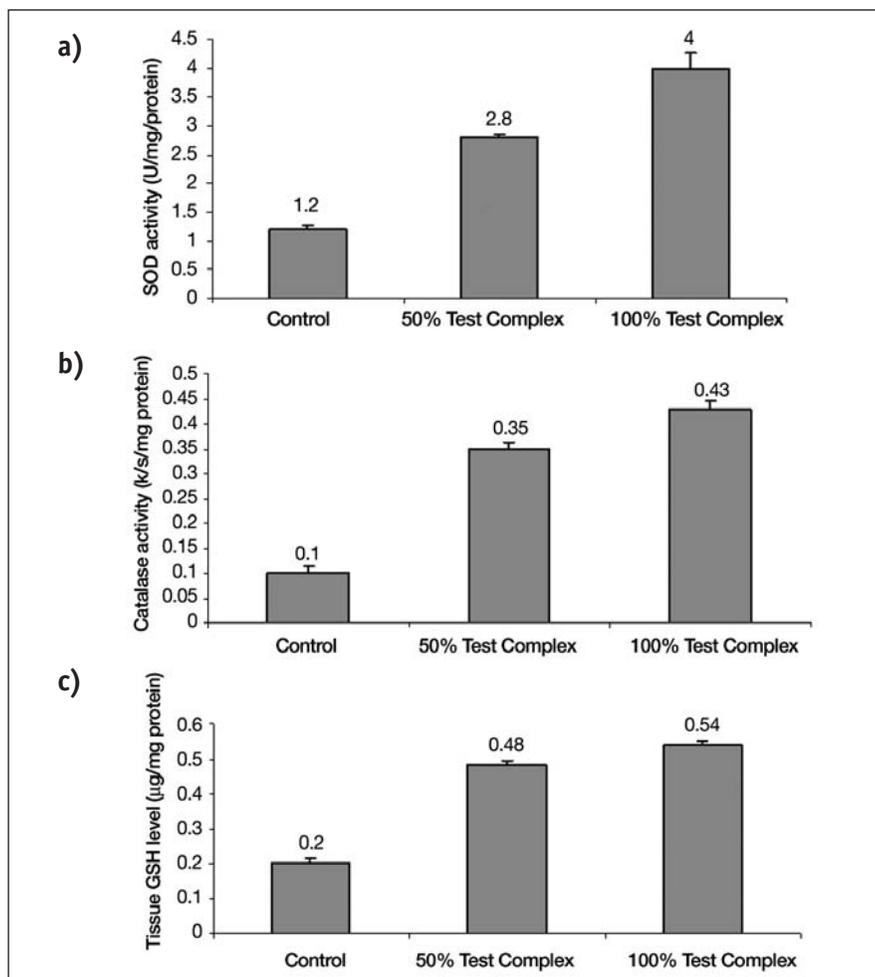


Figure 4. The GSH activity showed an increase in a) SOD, b) catalase, and c) tissue GSH levels in skin cells.

Conclusion

Mitochondria are a key organelle to the health of skin, producing important proteins necessary to control the cell energy release process. Oxidative damage has been implicated as a major factor in the decline of physiological functions of the mitochondria, which leads to the aging process.

The free radical scavenging and antioxidant abilities shown in the data for the test complex^a suggest its ability to scavenge a range of deleterious free radicals and stimulate antioxidant enzymes to delay the aging symptoms. In addition to its antioxidant mechanisms, the complex prompts DNA repair abilities and mitochondrial nutrition through mobilization of acyl groups from fatty acids, and enhanced ATP production. In short, these effects and formulation benefits suggest a new, mitochondrial approach for targeting young and mature skin alike.

Published June 2009 *Cosmetics & Toiletries* magazine.

References

1. D Harman, A biologic clock: The mitochondria? *J of the Amer Geriatrics Soc* 20 (4) 145–147 (1972)
2. HM McBride, M Neuspiel and S Wasiak, Mitochondria: More than just a powerhouse, *Curr Biol* 16(14) 551–560 (2006)
3. FM Yakes and B Van Houten, Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress, *Proc Natl Acad Sci USA* 94(2) 514–9 (Jan 21, 1997)
4. LA Loeb, DC Wallace and GM Martin, The mitochondrial theory of aging and its relationship to reactive oxygen species damage and somatic mtDNA mutations, *PNAS* 102(52) 18769–18770 (2005)
5. A Trifunovic, Mitochondrial DNA and aging, *Biochim Biophys Acta* 1757(5-6) 611–617 (2006)
6. A Trifunovic et al, Premature aging in mice expressing defective mitochondrial DNA polymerase, *Nature* 429 357–359 (2004)
7. S Richert, A Schrader and K Schrader, Transdermal delivery of two antioxidants on different cosmetic formulations, *Intl J Cos Sci* 25 (1–2) 5–13 (2003)
8. TM Hagen et al, (R)- α -Lipoic acid-supplemented old rats have improved mitochondrial function, decreased oxidative damage, and increased metabolic rate, *The FASEB J* 13 411–418 (1999)
9. B Cohen and D Gold, Mitochondrial cytopathy in adults: What we know so far, *Cleveland Clinic J Medicine*, 68 7 625–642 (2001)
10. M Sugrue and W Tatton, Mitochondrial membrane potential in aging cells, *Biol Signals Recept* 10 3–4, 176–188 (2001)
11. CE Thomas et al, Characterization of the radical trapping activity of a novel series of cyclic nitron spin traps, *J Biol Chem* 271 3097–3104 (1996)

12. N Perricone, Spin traps: Stopping free-radical damage before it begins, in *The Wrinkle Cure*, NY: Warner Books, (2001) 181-183
13. L Ernster and G Dallner, Biochemical, physiological and medical aspects of ubiquinone function, *Biochim Biophys Acta* 1271:195-204 (1995)
14. PL Dutton et al, Coenzyme Q oxidation reduction reactions in mitochondrial electron transport, in *Coenzyme Q: Molecular mechanisms in health and disease* VE Kagan and PJ Quinn (eds), Boca Raton: CRC Press (2000) 65-82
15. J Herschthal and J Kaufman, Cutaneous aging: A review of the process and topical therapies, *Expert Review of Derm* 2(6) 753-761(2007)
16. S Loffelhardt, C Bonaventura, M Locher, HO Borbe and H Bisswanger, Interaction of alpha-lipoic acid enantiomers and homologues with the enzyme components of the mammalian pyruvate dehydrogenase complex, *Biochem Pharmacol* 50(5) 637-46 (1995)
17. JH Suh et al, Oxidative stress in the aging rat heart is reversed by dietary supplementation with (R)-(alpha)-lipoic acid, *FASEB J* 15(3) 700-706 (2001)
18. S Jacob, K Rett, EJ Henriksen and HU Haring, Thioctic acid—effects on insulin sensitivity and glucose-metabolism, *Biofactors* 10(2-3) 169-174 (1999)
19. H Beitner, Randomized, placebo-controlled, double blind study on the clinical efficacy of a cream containing 5% alpha-lipoic acid related to photo-aging of facial skin, *Br J Dermatol* 149(4) 841-9 (Oct 2003)
20. RM Moore et al, Alpha-lipoic acid inhibits tumor necrosis factor-induced remodeling and weakening of human fetal membranes, *Biol of Reproduction*, 80 781-787 (2009)
21. Shapiro and Robert, The prebiotic role of adenine: A critical analysis, *Origins of Life and Evolution of Biospheres* 25 83-98 (1995)
22. Adenine, Genetics Home Reference, available at: <http://ghr.nlm.nih.gov/ghr/glossary/adenine> (accessed Apr 29, 2009)
23. TM Hagen, J Liu, J Lykkesfeldt and BN Ames, Feeding acetyl-L-carnitine and lipoic acid to old rats significantly improves metabolic function while decreasing oxidative stress, *Proc Natl Acad Sci USA*, 19; 99(4)1870-1875 (2002)
24. A Steiber, J Kerner and CL Hoppel, Carnitine: A nutritional, biosynthetic and functional perspective, *Molecular Aspects of Medicine* 25 (5-6) 455-473 (2004)
25. J Liu, DW Killilea and BN Ames, Age-associated mitochondrial oxidative decay: Improvement of carnitine acetyltransferase substrate-binding affinity and activity in brain by feeding old rats acetyl-L- carnitine and/or R-alpha -lipoic acid, *Proc Natl Acad Sci USA* 19 99(4) 1876-1881 (2002)
26. HO Yang, G Stamatas, C Saliou and N Kollias, A chemiluminescence study of UVA-induced oxidative stress in human skin *in vivo*, *J of Investigative Derm* 122 1020-1029 (2004)
27. L Hellemans, H Corstjens, A Neven, L Declercq and D Maes, Antioxidant enzyme activity in human stratum corneum shows seasonal variation with an age-dependent recovery, *J of Investigative Derm* 120 434-439 (2003)
28. S Briganti, A Cristaudo and V D'Argento, Oxidative stress in physical urticarias, *Clin Exp Dermatol* 26 284-288 (2001)
29. PU Giacomoni, L Declercq, L Hellemans and D Maes, Aging of human skin: Review of a mechanistic model and first experimental data, *IUBMB Life* 49 259-263 (2000)
30. E Beutler, O Duron and BM Kelley, Improved method for the determination of blood glutathione, *J Lab Clin Med* 61 882 (1963)

It's Never Too Late: DNA Repair and Photoaging

Alan M. Walfield and Daniel B. Yarosh, PhD

AGI Dermatics

Mindy Goldstein, PhD

Estee Lauder

KEY WORDS: *DNA, photoaging, sun, UV*

ABSTRACT: *The delivery of DNA repair enzymes provides protection from DNA damage.*

It has been known for many years that continued sun exposure (UV radiation) leads to skin damage. The damage caused by UV radiation is manifested in a number of ways including cyclobutane pyrimidine dimers found in the DNA and oxidative damage to the DNA, proteins and lipids of the skin. Much of this damage is repaired by internal enzymes; however, some damage escapes repair and accumulates over time, resulting in accelerated skin aging, also known as photoaging.

The effects of photoaging, as manifested by wrinkling, are produced by chronic exposure to solar ultraviolet (UV) radiation. UV irradiation also causes DNA damage. Not only does DNA damage increase the long-term risk for cancer, but it also increases the risk for photoaging through cytokine signaling between the keratinocytes and fibroblasts of the epidermis and dermis. These cytokines then elicit stress responses such as the secretion of matrix metalloproteinase-1 (MMP-1) into the dermis, leading to the degradation of collagen and wrinkling. It is shown that the delivery of DNA repair enzymes using liposomes provides protection from DNA damage *in vitro* and *in vivo*, reduction in MMP-1 responses as a marker for photoaging and lessened wrinkling in a clinical trial.

Photoaging and Wrinkling

Photoaging is the damage that accumulates from life-long exposure to both UVA and UVB solar light.¹ It produces wrinkling, loss of elasticity, erythema, hyperpigmentation and increased risk of skin cancer. Signs of photo-damage in Asian skin most prominently include discoloration and wrinkling, and both changes appear to correlate with the frequency and duration of sun exposure.²

UV radiation acts on the epidermis through direct exposure and on the underlying dermis through cell-to-cell molecular signaling. Cell-to-cell molecular signaling happens when a damaged cell produces small protein messengers called cytokines that travel to their target cell, bind to the surface and trigger responses. Collagen fibrils in the extracellular matrix of the dermis are responsible for the strength and resiliency of the skin. Chronic exposure to solar UV results in the scarring and loss of dermal collagen fibrils, eventually causing wrinkles.¹ This occurs by both direct action and molecular signaling.

Molecular Mechanisms of Photoaging

UV irradiation leads to elevated levels of matrix metalloproteinases (MMPs) in human skin. One of these enzymes, MMP-1, also known as collagenase-1, cleaves collagen type 1, the primary constituent of the collagen fibrils in the extracellular matrix of the dermis. The imbalance of MMP-1 promotes the scarring of the collagen fibril structures and it is this disorganization of collagen fibrils in the dermal connective tissue that leads to loss of skin tone and wrinkling.¹

MMP-1 up-regulation in fibroblasts is stimulated by cytokine signaling from irradiated keratinocytes in the epidermis.^{1,3} DNA damage has been suggested as an upstream event in this chemical signaling pathway.⁴ How DNA damage is linked to the signaling of stress to neighboring cells through cytokine gene expression is becoming clearer. For example, a signal transduction pathway between DNA damage and cytokine signaling seems to involve phosphatidylinositol 3-kinase-related kinase (PIKK) cascades.⁵

UV radiation directly damages DNA in epidermal keratinocytes by causing the formation of cyclobutane pyrimidine dimers (CPDs). If not repaired, these lesions in skin cell DNA can result in either

cell death or mutagenesis. The UV-induced DNA damage also activates immunosuppressive pathways that inhibit the cutaneous immune system from reacting to challenges from the environment and tumors. The immunosuppressive cytokines' tumor necrosis factor alpha (TNF α) and interleukin-10 (IL-10) have been observed among the repertoire of cellular responses following UV irradiation.⁵

Irradiation by UV damages DNA indirectly through the formation of oxygen radicals, to oxidize guanine to 8-oxo-guanine in nuclear and mitochondrial DNA. The oxidative events of UV irradiation, including metabolic stress associated with loss of mitochondrial DNA, also are associated with the inflammatory responses that lead to the release of collagen-degrading MMPs into the dermal layer.^{6,7}

DNA Repair Enzymes and Delivery by Liposomes

DNA repair enzymes have been encapsulated in specially formulated pH-sensitive liposomes called microscopic lipid vesicles in order to effectively reach sites of DNA damage. The externally applied enzymes are internalized by cells within an hour and delivered to the nuclei and mitochondria of the cells for the repair of DNA. They supplement the endogenous processes that would excise only about half of daily damage by themselves.⁸ The repair enzymes localize within the epidermis with little delivery beyond the dermis or into circulation. By means of these pH-sensitive liposomes, DNA repair enzymes can be delivered directly to the affected cells.

Liposomes carrying T4 endonuclease V, a CPD glycosylase derived from the bacteriophage T4 (T4N5 liposome lotion), excise CPD lesions from DNA and accelerate repair in mammalian cells.^{9,10} A T4N5 liposome lotion (1 $\mu\text{g}/\text{mL}$ T4N5 liposomes in a 1% hydrogel lotion) reduced the incidence of actinic keratoses in patients with xeroderma pigmentosum (XP)—a genetic condition characterized by pigment abnormalities and multiple skin cancers in body areas exposed to the sun—through this repair process.¹¹ UV endonuclease and photolyase are other useful CPD glycosylases that carry out the excision-repair of dimerized pyrimidines.^{12,13}

The DNA repair enzyme 8-oxo-guanine glycosylase-1 (OGG1) reverses oxidative damage to nuclear and mitochondrial DNA, caused by UV exposure, an environmental pollutant, or as a result of normal metabolism by the oxidation-intensive mitochondria.¹⁴

The direct association of UV-induced DNA damage with photoaging was further tested by investigating whether the topical delivery of DNA repair enzymes by proprietary liposomes could influence the expression of MMP-1 and reduce wrinkling in *in vitro* and *in vivo* models for UV-exposed skin. Whether the topically applied liposomal repair enzymes could smooth the fine lines of photo-aged skin were further investigated in a study, described here.

Cell Culture

Neonatal normal human epidermal keratinocytes (NHEK)^a and normal human dermal fibroblasts (NHDF)^b were obtained and cultured according to the manufacturer's recommendations at 37°C in a 5% CO₂ humidified incubator.

NHEK and NHDF were irradiated with 500 J/m² UVB in 3 mL of 1X PBS through a UVC filter^c with two FS40 UV-B bulbs that emit 65% of their energy in the 290–320 nm range with a peak emission at 313 nm. Cells were treated immediately after irradiation with 1 µg/mL liposome-encapsulated T4 endonuclease V (T4N5 liposome lotion^d)¹⁰ for 24 hr. DNA and RNA were extracted from cells at 0, 6, and 24 hr for measurement of CPD repair or MMP-1 mRNA expression, respectively.

Similarly, NHEK were irradiated and treated with either the T4N5 lotion or 5% of the antioxidant magnesium ascorbyl phosphate (MAP) for 24 hr. The media from the NHEK were transferred to NHDF. Media and cells were collected after the incubation with NHEK-conditioned media and analyzed for MMP-1 protein by the enzyme-linked immunosorbent assay (ELISA) and for MMP-1 mRNA by real time reverse transcriptase polymerase chain reaction (RT-PCR).

^aNHEK is a product of Cascade Biologics.

^bNHDF is a product of Lonza Walkersville, Inc.

^cKodacel UVC Filter is a product of Kodak.

^dDimericine is a product of AGI Dermatics.

Artificial Skin Model

The artificial tissues of the epidermal full-thickness human skin model^e were grown and maintained on standard culture plate inserts^f at the air liquid interface to facilitate direct application of topical agents. They were exposed to 3,000 J/m² UVB, as described above, followed by treatment with T4N5 liposome lotion applied with the end of a glass rod for 0, 6, and 24 hr. In the second study, artificial tissues were treated with a DNA repair formula (1% Ultrasomes containing UV endonuclease, 1% Photosomes containing photolyase and 1% Roxisomes containing OGG1, in a proprietary formula) for 6 hr before UVB irradiation. Tissues of control groups were treated with maintenance medium only, or a popular skin moisturizer.

RNA was extracted and MMP-1 mRNA content determined by real time RT-PCR. DNA was extracted and CPD content determined by dot blot anti-thymine dimer antibody^g.

Clinical Study

Following a one-week preconditioning period, 32 Caucasian females between the ages of 39 and 60 applied the DNA formula^h to their faces twice daily for four weeks.

Before starting the treatment and after four weeks of using DNA repair formula, the skin near the outer edge of the right eye in the crow's-feet area was evaluated for fine lines and wrinkles by producing silicon replicas of the facial skin and analyzing the surface roughness of the replicas. The number of fine lines and wrinkles was calculated from the shadows produced under collimated light projected onto the replicas at an oblique horizontal 25-degree angle. Digital images were taken of the shadowing when the replicas were positioned at both 90-degrees and parallel to the major expression wrinkles on the replicas. These are standard industry techniques for measuring fine lines and wrinkles and they were performed according to good clinical practice (GCP) and International Conference on Harmonization (ICH) guidelines.

^e EpiDermFT is a product of MatTek Corp.

^f Millipore Millicell plate inserts are a product of Millipore.

^g Anti-Thymine Dimer, clone KTM53 is a product of Kamiya Biomedical Company.

^h Remergent DNA Repair Formula is a product of AGI Dermatics.

MMP-1 Gene Expression in Irradiated Fibroblasts

Cultured human fibroblasts were irradiated with UVB and the effect on MMP-1 gene transcription, a marker for photoaging, was determined. A 370% increase in MMP-1 mRNA synthesis was observed 24 hours following UV irradiation. This increase was reduced by approximately half in the samples treated with the T4N5 liposome lotion.

Signaling Between Skin Cells

As a model for analyzing the *in vivo* interaction of keratinocytes with fibroblasts, cultured NHEK were irradiated with 500 J/m² UVB and incubated for 24 hr with or without T4N5 liposome lotion (1 µg/mL) or 5% magnesium ascorbyl phosphate. Media from these NHEK were then transferred to unirradiated NHDF for 48 hr.

The secretion of MMP-1 by fibroblasts treated with keratinocyte-conditioned media was measured by ELISA after 48 hr. Exposure of the NHEK to 500 J/m² UVB caused MMP-1 production in NHDF to significantly increase by 56% over untreated cells ($p < 0.001$; $N = 9$). Treating the NHEK cells with T4N5 liposomes after UVB exposure caused the UVB-induced MMP-1 production in NHDF to decrease by 96% from the MMP-1 levels produced by UVB alone ($p < 0.001$). Treatment of the keratinocytes by the MAP antioxidant had no protective effect ($p < 0.001$).

MMP-1 Gene Expression in Artificial Skin

The epidermal full-thickness human skin model^e models the human epidermis and consists of NHEK and NHDF cells that have been cultured to form a multilayered, highly differentiated tissue equivalent to the human epidermis. When UVB-irradiated tissues were treated for 24 hr with T4N5 liposome lotion before irradiation, there was a 30% reduction in MMP-1 mRNA synthesis in comparison to an untreated control.

Irradiated artificial tissues^e, as described, also were treated with the DNA repair formula having UV endonuclease, photolyase and OGG1 liposomes. The treatment was administered for 24 hr before irradiation, so as to model a preventive skin care regimen. Nucleic

acids were extracted 6 hr after irradiation and the tissues were evaluated for DNA damage by an ELISA for CPD content, and for MMP-1 mRNA synthesis by real time RT-PCR. The samples pre-treated with the repair formula had a 23% reduction in CPD from the untreated control, and a 67% reduction in MMP-1 gene expression. The placebo skin moisturizer had no protective effect.

Clinical Study

The repair formula applied by the female subjects twice daily for four weeks produced a significant reduction of the lines in the crow's-feet area ($p \leq 0.05$). The comparisons to each subject's baseline under parallel oblique lighting indicated an increase in the spacing and reduction in the quantity of detectable fine lines and wrinkles per unit area (**Figure 1**). Improvement was noted in 72% of the subjects.

Conclusions

MMP-1 gene expression was used as a marker for photoaging in the *in vitro* models. The increasing levels of MMP-1 gene transcription observed for fibroblasts following UV irradiation, and the inhibition of increased transcription seen following the application of T4N5 DNA repair liposomes supports the correlation between DNA photo-damage and photoaging (**Figure 1**).

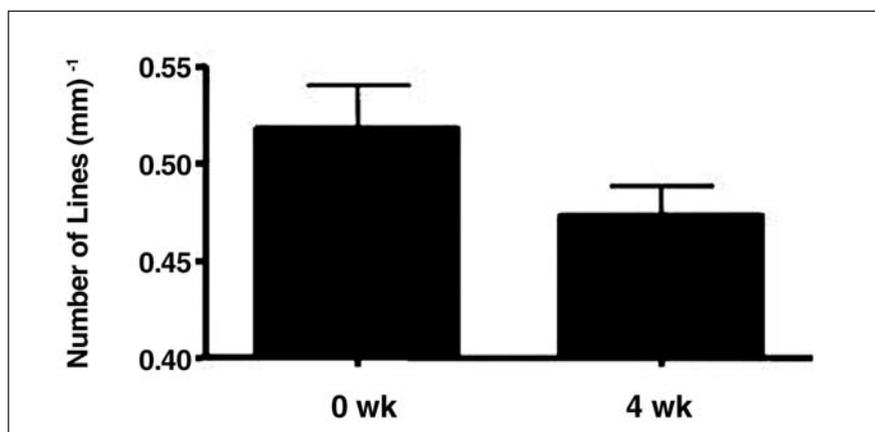


Figure 1. Thirty-two Caucasian female subjects ages 35–60 with fine lines in eye area, applied DNA repair formula. Silicon replicas of the crow's-feet area taken before and after

That MMP-1 transcription by fibroblasts was stimulated by medium transferred from irradiated keratinocytes, and the smaller increase was less when the keratinocytes had been treated with T4N5 liposomes demonstrating that keratinocytes in the epidermis can signal collagen reduction by fibroblasts that have not been directly exposed to UV radiation. These *in vitro* results suggest that UV-induced DNA damage could be linked to a response that leads to collagen degradation and wrinkles. The studies performed on the artificial epidermis model provided supporting results that may be more reflective of conditions within actual human skin.

The accumulation of DNA lesions was monitored in the irradiated artificial tissues in addition to MMP-1 gene expression. It was found that a reduction of merely 23% in CPD lesions by the repair enzyme was sufficient to reduce MMP-1 expression by two-thirds. Thus, DNA repair may exert a disproportionately potent effect on an adverse response to UV exposure.

The clinical trial showed the antiwrinkling effect of liposomal repair enzymes in human subjects. The liposomes reversed photo-damage in the subjects. The DNA repair formula used for the clinical trial and for an artificial skin study included three DNA repair enzymes (1% each of UV endonuclease, photolyase, and OGG1 encapsulated in liposomes, as described above) to target several types of DNA lesions. It can be readily applied to studies on photoaging having other clinical endpoints such as immunosuppression and hyperpigmentation.

Published July 2007 *Cosmetics & Toiletries* magazine.

References

1. GJ Fisher, Z-Q Wang, SC Datta, J Varani, S Kang and JJ Voorhees, Pathophysiology of premature skin aging induced by ultraviolet light, *N Engl J Med*, 337(20) 1419–28 (1997)
2. JH Chung, SH Lee, CS Youn, BJ Park, KH Kim, KC Park, KH Cho and HC Eun, Cutaneous photodamage in Koreans: influence of sex, sun exposure, smoking, and skin color, *Arch Dermatol*, 137(8) 1043–51 (2001)
3. D Fagot, D Asselineau and F Bernerd, Matrix metalloproteinase-1 production observed after solar-simulated radiation exposure is assumed by dermal fibroblasts but involves a paracrine activation through epidermal keratinocytes, *Photochem Photobiol*, 79(6) 499–505 (2004)
4. B Stein, HJ Rahmsdorf, A Steffin, M Liftin and P Herrlich, UV-induced DNA damage is an intermediate step in UV-induced expression of human immunodeficiency virus type 1, collagenase, c-fos, and metallothionein, *Mol Cell Biol* 9(11) 5169–81 (1989)

5. DB Yarosh, Enhanced DNA repair of cyclobutane pyrimidine dimers changes the biological response to UV-B radiation, *Mutat Res*, 509(1-2) 221–6 (2002)
6. K Kuwamoto, H Miyauchi-Hashimoto, K Tanaka, N Eguchi, T Inui, Y Urade and T Horio, Possible involvement of enhanced prostaglandin E2 production in the photosensitivity in xeroderma pigmentosum group A model mice, *J Invest Dermatol* 114(2) 241–6 (2000)
7. K Obayashi, K Kurihara, Y Okano, H Masaki and DB Yarosh, L-Ergothioneine scavenges superoxide and singlet oxygen and suppresses TNF-alpha and MMP-1 expression in UV-irradiated human dermal fibroblasts, *J Cosmet Sci*, 56(1) 17–27 (2005)
8. DB Yarosh, Liposomes in investigative dermatology. *Photodermatol Photoimmunol Photomed*, 17(5) 203–12 (2001)
9. D Yarosh, C Bucana, P Cox, L Alas, J Kibitel and M Kripke, Localization of liposomes containing a DNA repair enzyme in murine skin, *J Invest Dermatol*, 103(4) 461–8. 1994
10. D Yarosh, J Kibitel, L Green and A Spinowitz, Enhanced unscheduled DNA synthesis in UV-irradiated human skin explants treated with T4N5 liposomes. *J Invest Dermatol*, 97(1) 147–50 (1991)
11. D Yarosh, J Klein, A O'Connor, J Hawk, E Rafal and P Wolf, Effect of topically applied T4 endonuclease V in liposomes on skin cancer in xeroderma pigmentosum: a randomised study. Xeroderma Pigmentosum Study Group, *Lancet*, 357(9260) 926–9 (2001)
12. DB Yarosh, J Kibitel, A O'Connor, V Hejmadi, P Bennett and BM Sutherland, *J Environ Pathol Toxicol Oncol* 16 287 (1997)
13. S Stege, L. Roza, AA Vink, M Grewe, T Ruzicka, S Grether-Beck and J Krutmann, Enzyme plus light therapy to repair DNA damage in ultraviolet-B-irradiated human skin. *Proc Natl Acad. Sci. USA*, 97(4) 1790–5 (2000)
14. DB Yarosh, J Galvin J S Nay, A Peña, M Canning and D Brown, Anti-inflammatory activity in skin by biomimetic of *Evodia rutaecarpa* extract from traditional Chinese medicine, *J Dermatol Sci*, 42(1) 13–21 (2006)

Understanding Extracellular Proteolytic Enzymes

Sanford Simon, PhD

State University of New York

KEY WORDS: *extracellular proteolytic enzymes, MMPs, proteinases, cells, enzymes*

ABSTRACT: *Describes the mechanisms by which the activity of proteinases is controlled and suggests a goal for their use.*

Matrix metalloproteinases (MMPs), along with the serine proteinases, constitute the two major classes of secreted extracellular proteolytic enzymes; other families of proteinases, such as aspartyl, cysteinyl and threonyl proteinases, can be localized to the cytosol of cells or to specialized intracellular structures such as the proteasome.¹

This chapter describes the mechanisms by which the activity of these proteinases is controlled, and suggests a modest goal for their safe and effective use in therapies for inflammatory injury to the skin.

MMPs

Types: MMPs are a class of proteolytic enzymes with a broad range of architectures and functions; indeed, this family is growing—there are more than two dozen distinct MMPs—and provoking active debate over the significance of the impressive multiplicity of member enzymes. Some of the identified MMPs preferentially degrade structural proteins of the extracellular matrix, especially the native triple helical structure of the fibrillar types of collagen (e.g., types I and III collagen found in the dermal stroma). Other so-called

gelatinolytic MMPs further degrade the products generated by the initial cleavages of the collagenolytic MMPs as well as the type IV collagen-containing basement membrane at the dermal-epidermal junction.

Another group of MMPs, known as the stromelysins, are especially effective at degrading proteoglycans such as aggrecan, and adhesion glycoproteins such as fibronectin in the skin and other tissues.¹⁻³ As new members of the MMP family are identified and as their functions are being elucidated, it is becoming clear that, in spite of their name, MMPs are not at all restricted to targeting structural components of the extracellular matrix. For example, still other MMPs can attack soluble molecules in the plasma or extracellular fluid of tissues, including cytokines and chemokines, growth factors and even inhibitors of other proteinase classes. Yet other MMPs can function as “sheddas,” detaching membrane-bound proteins such as the inflammatory cytokine TNF- α from their anchors on cell membranes. With this broad range of substrate specificities, it is not surprising that the architectural complexities of the MMPs provide a structural basis for their multiple functional roles.^{4,5}

Domains: Even the simplest MMPs have a so-called catalytic domain, in which is located at least one zinc atom that is essential for proteolytic activity. Synthetic inhibitors of active MMPs typically target this zinc atom, along with additional interactions stabilizing the association of the inhibitor with the catalytic domain. In virtually all cases, the catalytic domain is occluded in the enzymatically inactive MMP precursor zymogens or “pro-MMPs,” by interacting with a second domain, the so-called pro-domain, located on the N-terminal side of the catalytic domain in the pro-MMP amino acid sequence. The inactive pro-MMP structure is maintained specifically through an interaction between the zinc atom and the thiol side chain of a cysteine residue in the pro-domain that acts as a ligand for the metal. This thiol can be oxidized or otherwise modified, or the entire pro-domain can be proteolytically severed from the catalytic domain, releasing the now enzymatically active MMP.⁶

In addition to proteolytic activation of pro-MMPs by other

already activated MMPs, several members of a second family of proteolytic enzymes, the serine proteinases, can remove the pro-domain of many MMPs. For activation of other MMPs, removal of the pro-domain requires the action of a member of another proteinase family, the furins. The furins and the subfamily of MMPs that are activated by them share a common localization on the cell membrane surface. These furin-activated, cell surface-associated MMPs are known as membrane type MMPs (MT-MMPs).⁷

In addition to the catalytic and pro-domains, most MMPs have additional domains that are located on the C-terminal side of the catalytic domain. The most common element that is incorporated into these C-terminal domains has a sequence homology to the hemopexin family of proteins; in the MMPs the hemopexin or PEX domains play major roles in conferring substrate specificity as well as localization of the different MMPs within tissues. In the case of the MT-MMPs, other additional domains on the C-side of the PEX domains are responsible for attaching the enzymes to the cell surface, either via a typical hydrophobic amino acid sequence that functions as a trans-membrane domain and leaves a short C-terminal amino acid sequence remaining within the cytosol, or via a so-called glycosylphosphatidylinositol-, or GPI-, linkage on the outer leaflet of the plasma membrane bilayer.

Yet other additional domains are found in some of the MMPs as apparent insertions within the catalytic domain, or as so-called “linker” regions between the catalytic and PEX domains. The apparent purposes of these extra domains, which resemble amino acid sequences found in collagen or in fibronectin, are to enhance further the localization and substrate specificity of the different MMPs.⁸

Pathology: While the MMPs are associated with a range of functional roles, their participation in tissue injury associated with inflammatory processes is a source of significant pathology and has prompted vigorous efforts to develop specific inhibitors to diminish proteolytic destruction.

Serine Proteinases

It is useful to consider the similarities and differences between the multiplicities of serine proteinases and the MMPs. The serine

proteinases display remarkable preferences for cleaving certain amino acid sequences in their protein substrates. These proteinases all share a common “catalytic triad” of three amino acids—serine, histidine, and aspartic acid—and they all have very similar conformations of their catalytic domains. But the explanation for their cleaving preferences can be traced to distinctive arrangements of amino acid side chains in a so-called “specificity pocket.” In addition, several of the serine proteinases, especially those involved in clotting and fibrinolysis, have additional domains that have relatively little effect on substrate specificity but have marked effects on localization, activation and modulation of expression of the enzymes.

The presence of a distinct specificity pocket spatially separated from the catalytic site of the serine proteinases makes it relatively easy to design highly specific synthetic inhibitors for each of the members of this family. The MMPs employ a more complex strategy to control the range of preferred substrates, using multiple domains to control not only substrate specificity, but also an extremely diverse range of sites of action. Because they lack a distinct specificity pocket, the individual members of the MMP family are less easily targeted by synthetic inhibitors with high selectivity; MMP inhibitors are more likely to block the entire family of zinc metalloproteinases or to show preferential, but rarely highly specific, affinity for a single member of the family.⁹

Because the serine proteinases and the MMPs attack amino acid sequences that may be found in a number of substrates within healthy tissues, it is necessary that both classes of enzymes be maintained under an elaborate system of controls (see Controlling the Extracellular Proteolytic Enzymes) to avoid inappropriate or excessive cleavage events. When these controls break down, the consequences can be life-threatening, but it is clear that the purpose of the multiple layers of modulation of proteolytic activity all combine to impose some homeostatic regulation over proteolytic activities that could otherwise produce catastrophic damage to tissues.

These multiple levels of regulation include a number of interactions between the two proteinase families that only add to the need for layers of regulatory controls. For example, the MMPs and serine proteinases in the neutrophil, the key leukocyte involved in the acute

inflammatory response, have such potential for destruction they are packaged in separate granules within the cell, either as zymogens in the case of the MMPs, or bound to other granule constituents in the case of the neutrophil azurophilic granule serine proteinases.

When the granules are released in response to an inflammatory stimulus, neutrophil elastase, which simply diffuses away from the negatively charged glycosaminoglycans that keep it inhibited within azurophilic granules, can proteolytically activate the MMP precursors. The active MMPs then proteolytically inactivate alpha-1-PI, the major endogenous plasma inhibitor of neutrophil elastase, while the now unopposed elastase proteolytically inactivates the tissue inhibitors of metallo-proteinases (TIMPs) so that the MMPs can now also attack their substrates unopposed.¹⁰ This example serves to illustrate how, in spite of the multiple layers of regulatory controls designed to prevent unrestrained proteolysis, the MMPs and the serine proteinases can still synergize to produce massive tissue destruction when these controls are inadequate to restrain excessive enzyme activity.

Even in an environment in which fluid-phase TIMPs can control excessive activity of secreted MMPs, some of these proteinases can elude the antiproteinase defenses by associating with the cell surface, in some aspects mimicking the membrane-associated MT-MMPs. The associations of cells with secreted MMPs involve noncovalent interactions with cell adhesion molecules, or with so-called “scaffold”

Controlling the Extracellular Proteolytic Enzymes

Controlling the proteolytic activity of serine proteinases and MMPs is a multilayered process involving the following:

- Changes in levels of protein expression
- Use of highly regulated proteolytic cascades required to activate the zymogen precursors of the MMPs as well as analogous inactive zymogen precursors of several of the serine proteinases
- Modulation of levels of endogenous specific antiproteinases that can inhibit each class of proteinase
- Additional modifications of the enzymes that affect their localization

molecules such as CD44 on which multiple secreted MMPs can be assembled (MMP-2, MMP-9, and MMP-7 can all be bound to a single CD44 molecule). These cell associations achieve two goals:

Resist antiproteinases. The proteinases are less easily inhibited by large antiproteinases when they are associated with cell surfaces.

Achieve synergy. When multiple proteinases are assembled on a scaffold, their multiple selective activities can synergize to accomplish more proteolysis than would be achieved if multiple fluid phase cleavage events were to occur as a result of random diffusion.¹¹

It is noteworthy that the serine proteinase, neutrophil elastase, can also be bound noncovalently but very tightly to negatively charged glycosaminoglycans on cell surfaces, where it retains its enzymatic activity while eluding its own specific fluid-phase antiproteinase, alpha1-PI.

It may be surmised that an especially great challenge to inhibition of excessive proteolysis may be to block the concerted actions of surface-bound MMPs and serine proteinases on inflammatory cells. A very similar mechanism of synergistic complicity of surface-bound proteinases in attacking the surrounding extracellular matrix has been suspected to account for the invasiveness of metastasizing tumor cells. Thus, synthetic inhibitors of the MMPs and the serine proteinases that have been developed as agents for controlling inflammatory tissue injury have also been considered for control of cancer.¹²

Low molecular weight inhibitors, which can insinuate themselves between the cell surface and the matrix components to which the cells have adhered, may be more successful in reaching their intended targets than large endogenous antiproteinases, which may be more than twice the size of the enzymes they are intended to inhibit.

It is noteworthy that the mechanisms involving excessive or unopposed proteolysis mediated by the MMPs and the serine proteinases can be responsible for the devastating massive inflammatory damage of multiple organ failure and the Systemic Inflammatory Response Syndrome (SIRS) in conditions such as sepsis, invasive surgery or polytrauma.¹³ Remarkably, the same mechanisms can be seen in the highly localized settings of individual acne lesions.¹⁴ These lesions have been shown to be associated with the generation

of inflammatory cytokines, especially IL-1 and TNF- α , which in turn trigger signal transduction pathways culminating in the relocation of the transcription factors NF- κ B and AP-1 to the nucleus of monocytes and macrophages.

These two transcription factors bind to promoters for expression of the MMPs as well as chemotactic cytokines or “chemokines” in the mononuclear cells. The lesions then become infiltrated with additional mononuclear cells as well as neutrophils, all of which are stimulated by the inflammatory cytokine “cocktail” to secrete their MMPs and, in the case of the neutrophils, their serine proteinases as well. Even the reactive oxygen species that are also generated at the sites of inflammation contribute to the process of proteolytic destruction by further stimulating NF- κ B and AP-1 activation and by oxidizing the cysteine in the MMP pro-domains, thereby converting the zymogens to the active proteinases.

It is now generally acknowledged that the roles of these proteinases in producing destruction of the connective tissue of the skin in acne lesions or in chronic wounds differ from the sources of tissue injury in SIRS only with respect to scale, and that therapies that have been contemplated for systemic inflammatory injury may be equally effective in diminishing the less catastrophic tissue destruction that accompanies focal inflammatory processes in the skin.

Conclusion

Because the MMPs and the serine proteinases clearly have essential roles in normal tissue remodeling, total inhibition of these enzymes is not required to prevent tissue damage and, indeed, may provoke unintended pathologic side effects. Rather, the levels of the active forms of these proteinases must only be diminished to the point that the endogenous antiproteinases can re-establish a balance with the proteinases. The more modest goal of restoring such homeostasis is becoming recognized as an achievable objective and can be anticipated to result in development of safe and effective therapies for inflammatory injury to the skin.

Researchers are continuing to discover that the proteinase-antiproteinase balance in inflamed skin can be restored by adding natural products that complement and reinforce the activities of

endogenous antiproteases. By taking full advantage of the natural homeostatic mechanisms for controlling inflammatory injury, cosmetic chemists can use these natural materials in novel ways to formulate personal care products without compromising a commitment to achieve authentic efficacy.

Published August 2007 *Cosmetics & Toiletries* magazine.

References

1. TE Cawston and AJ Wilson, Understanding the role of tissue degrading enzymes and their inhibitors in development and disease, *Best Pract Res Clin Rheumatol* 20 983–1002 (2006)
2. NTV Le, M Xue, LA Castelnoble and CJ Jackson, The dual personalities of matrix metalloproteinases in inflammation, *Frontiers Biosci* 12 1475–1487 (2007)
3. H Nagase and JF Woessner, Matrix metalloproteinases, *J Biol Chem* 274 21491–21494 (1999)
4. AJ Gearing et al, Processing of tumor necrosis alpha precursor by metalloproteinases, *Nature* 370 555–557 (1994)
5. Q Yu and I Stamenkovic, Cell surface localized matrix metalloproteinases-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis, *Genes Devel* 14 163–176 (2000)
6. EB Springman, EL Angleton, H Birkedal-Hansen and HE Van Wart, Multiple modes of activation of latent human fibroblast collagenase: evidence for a role of the Cys73 active-site zinc complex in latency and a “cysteine switch” mechanism for activation, *Proc Natl Acad Sci USA* 87 364–368 (1990)
7. Y Itoh, A Takamura, N Ito, Y Maru, H Sato, N Suenaga, T Aoki and M Seiki, Hemophilic complex formation of MT1-MMP facilitates proMMP-2 activation on the cell surface and promotes tumor cell invasion, *EMBO J* 20 4782–4793 (2001)
8. G Murphy and V Knauper, Relating matrix metalloproteinase structure to function: why the “hemopexin” domain? *Matrix Biol* 15 511–518 (1997)
9. JT Peterson, The importance of estimating the therapeutic index in the development of matrix metalloproteinase inhibitors, *Cardiovasc Res* 69 677–687 (2006)
10. AE Mast, JJ Enghild, H Nagase, K Suzuki, S Pizzo and G Salvesen, Kinetics and physiologic relevance of the inactivation of alpha 1-proteinase inhibitor, alpha 1-antichymotrypsin, and antithrombin III by matrix metalloproteinases-1, -2, and -3, *J Biol Chem* 266 7313–7316 (1991)
11. WWM Van Hinsbergh, MA Engelse and PJA Quax, Pericellular proteases in angiogenesis and vasculogenesis, *ArteriosclerThromb Vasc Biol* 26 716–728 (2006)
12. AH Drummond et al, Preclinical and clinical studies of MMP inhibitors in cancer, *Ann NY Acad Sci* 30 228–235 (1999)
13. J Steinberg, J Halter, H Schiller, L Gatto, D Carney, HM Lee et al, Chemically modified tetracycline prevents the development of septic shock and acute respiratory distress syndrome in a clinically applicable porcine model, *Shock* 24 348–356 (2005)
14. E Papakonstantinou et al, Matrix metalloproteinases of epithelial origin in facial sebum of patients with acne and their regulation by isotretinoin, *J Invest Dermatol* 125 673–684 (2005)

RNA Interference and Therapeutic Applications

Mindy S. Goldstein, PhD

The Estée Lauder Companies Inc

KEY WORDS: *RNA interference, genes, molecular biology, RNAi, siRNA*

ABSTRACT: *RNAi has become an integral tool for geneticists and molecular biologists in understanding gene function.*

RNA interference (RNAi) is an evolutionarily conserved mechanism for sequence-specific post-transcriptional gene silencing in animals and plants. Since its discovery in the late 1990s,^{1,2} RNAi has become an integral tool for geneticists and molecular biologists in understanding gene function by knockdown techniques; i.e., suppressing gene expression. Small interfering RNAs (siRNAs) offer great promise as a new class of therapeutics with potential advantages over small molecule drugs and antibodies owing to high specificity and relatively low toxicity.³

In RNAi mechanism, short double-stranded RNA (dsRNA) that shares sequence homology to the target message initiates mRNA cleavage or translation arrest.⁴ Two kinds of short dsRNAs of 21–23 nucleotides can initiate RNAi. The active molecules that mediate mRNA degradation are small interfering RNAs (siRNAs) and share perfect homology to mRNA, while dsRNAs that arrest translation are microRNAs (miRNAs) and usually contain 2–3 base mismatches and bind at the 3′-UTR (untranslated region) (**Figure 1**).

siRNAs are generated from the random cleavage of the long precursor dsRNAs by the Dicer class of RNase III enzymes. Alternatively, siRNA can also be generated by chemical synthesis and introduced into cells with the help of carrier molecules. Dicer

processed siRNA duplexes contain 2-3 nt 3'-overhanging ends and contain 5'-phosphate and 3'-hydroxyl termini. After production from long double-stranded RNA by Dicer cleavage, the siRNA duplexes enter into a multi-protein-component RNA Interference Silencing Complex (RISC) that contains an endonuclease Argonaute 2 (AGO2).

In the RISC duplex, RNA is unwound and the guide strand (antisense strand) enters the RISC to generate a catalytically active RISC while the passenger strand is cleaved and degraded. The guide strand generally shares perfect complementarity with the target mRNA. Active RISC guides the sequence-specific recognition of the target mRNA and catalyzes its cleavage (see upper portion of **Figure 1**). The mRNA cleavage is carried out by the catalytic domain of AGO2.

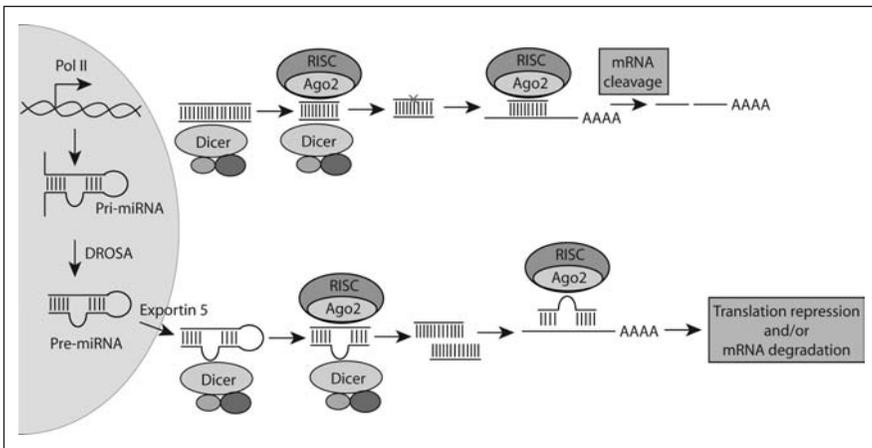


Figure 1. siRNAs are active molecules that mediate mRNA degradation and share perfect homology to mRNA. miRNAs are dsRNAs that arrest translation; they usually contain 2-3 base mismatches and bind at the 3'-UTR (untranslated region).

Either strand of the siRNA duplex is capable of inducing RNAi. If the antisense strand of the siRNA duplex enters into RISC, it directs the cleavage of sense RNA target, and vice versa.

Which strand is selected for RISC formation is predominantly a function of siRNA duplex-asymmetry.⁵ Because RISC containing sense strand of siRNA duplex may engage in off targeting, it is suggested that the design of highly asymmetric siRNAs that allow

loading of predominantly antisense strand into RISC may reduce sense strand directed off-target silencing.

The benefits of this entire effect could include specifically down-regulating genetic-based disorders such as muscular dystrophy and multiple sclerosis.

In contrast to siRNAs, miRNAs are encoded by endogenous genes and are increasingly being implicated in regulating many important genes, including those involved in cell cycle regulation, cancer and viral replication, in a range of organisms and cell types.⁶ miRNAs originate from long stem-loop structures that are part of noncoding RNA transcripts (Pri-miRNA) (see lower portion of **Figure 1**). These primary noncoding transcripts are processed in the nucleus by Drosha into hairpins of ~60–70 nucleotides (pre-miRNAs).

Pre-miRNAs are exported to cytoplasm by a protein Exportin 5 where Dicer processes them to mature miRNAs of 21–23 base pairs. Finally, one of the miRNA strands is incorporated into the RISC complex in a similar manner as siRNAs. miRNAs usually contain 2–3 bp mismatch compared to a target mRNA and direct translation arrest. If miRNA shares perfect complementarity to target RNA, it can mediate RNA cleavage akin to siRNA. Targeting miRNAs involved in various disease conditions, such as cancer, opens novel avenues in disease management.

RNAi in Therapeutics

In a relatively short span of time since the discovery of RNAi, siRNAs have become popular for the development of potential therapeutics against various diseases such as cancer, genetic disorders and viral infections.^{7,8} siRNAs are attractive as drugs because these molecules are specific and easily synthesized, and siRNA drugs may all have similar pharmacokinetic profiles. Similarly, miRNAs have emerged as attractive therapeutic targets due to their role in various cellular mechanisms such as regulation of development, cellular differentiation and programmed cell death. However, advances in delivery technologies will be required to alter the siRNA/miRNA pharmacokinetic profile and for targeted delivery of these drugs to a desired tissue or cell type.

Several applications of siRNA technology have been demonstrated recently both *in vitro* and *in vivo* in numerous experimental models.

ApoB protein silencing: siRNA *in vivo* efficacy has been demonstrated in medically relevant nonhuman primates. In a recently published study, Zimmerman et al. systemically delivered liposome formulated siRNA in cynomolgus monkeys that effectively silenced apolipoprotein B (ApoB).⁹

ApoB protein is required for the assembly and secretion of low-density lipoprotein (LDL). Elevated levels of ApoB protein have been found in association with increased risk of coronary artery disease.

In the Zimmerman study, a dose-dependent silencing of ApoB mRNA was demonstrated with maximum silencing of > 90%. Silencing was maintained for up to 11 days following administration of ApoB siRNAs. Treated animals had significantly reduced levels of ApoB protein in the serum as well as reduced total cholesterol and LDL.

Huntington, viral infection and drug safety: A Huntington gene specific short hairpin RNA (shRNA) encoded by adeno-associated virus-encoded was administered locally by intrastriatal injection, demonstrated motor, pathological and molecular improvement in mouse models of Huntington's disease.¹⁰ Administration of siRNAs directed against the enzyme heme oxygenase-1 via intranasal route has been shown to increase the ischemia-reperfusion-induced cell death in lungs.¹¹ This study is important in that it demonstrated the delivery of siRNA in lungs without the aid of viral vectors or transfection agents, indicating the clinically relevant siRNAs may be delivered directly to the lung.

Intranasal administration of siRNA nanoparticles was achieved for targeting the nonstructural gene 1 of the respiratory syncytial virus (RSV). Plasmid-borne siRNAs complexed with a nanochitosan polymer administered before or after infection with RSV were shown to result in significant reduction of viral titers in the lung as well as in decreased inflammation and airway reactivity.¹²

Pain management, spinal cord injuries: Intrathecal delivery of siRNA directly into the spinal fluid targeting the pain-related P2X₃ in a rat model of neuropathic pain showed diminished pain. This

study indicated that RNAi can block a pathological pain response and provide relief from neuropathic pain. In this study a minimally modified siRNA containing 3'-overhangs of two phosphorothioated 2'-O-(2-methoxyethyl)-ribonucleotides were used without the aid of a delivery vehicle.¹³ This study indicates that RNAi technology can be useful in pain management and treating diseases such as spinal cord injury without delivery vehicles that may induce undesired toxic effects.

Challenges

The major challenges for effective siRNA/miRNA therapeutics include their stability on a therapeutically relevant time scale and their delivery to target tissues. It is well-established that the stability of biologically active nucleic acid molecules can be improved while retaining potency via chemical modifications.¹⁴

Another challenge is effective delivery of RNA molecules to achieve proper pharmacokinetics and action at a target site. One possible option is to deliver therapeutic RNA molecules directly to the target site such as in topical applications.¹⁵ Some impressive advances have been made on this front by using RNA conjugated to delivery agents such as cell-penetrating peptides, noncovalent complexes and lipid nano-particles.¹⁶

As these and other challenges are worked through, RNAi therapeutics will likely be developed in many more areas of medicine. Even at this early stage of development, three siRNAs—one each for VEGF, VEGF-R (both for AMD), and respiratory syncytial virus—are in various stages of clinical trials.^{7,8}

While it augurs well that many of currently untreatable diseases may find cure in RNAi therapeutics, caution must be exercised in monitoring the safety of these drugs for long-term use in humans. One possible option for its use in personal care could be to deliver therapeutic RNA molecules directly to the target site, such as in topical applications, but as of yet this technology is still young and untested.

References

1. A Fire, S Xu, MK Montgomery, SA Kostas, SE Driver and CC Mello, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, *Nature* 391 806–811 (1998)
2. SM Elbashir, J Harborth, W Lendeckel, A Yalcin, K Weber and T Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, *Nature* 411 494–498 (2001)
3. SC Quay and PH Johnson, RNAi therapeutics: The future of medicine, *Pharma* 3 18–21 (2007)
4. A Fougerolles, H Vornlocher, J Maraganore and J Lieberman, Interfering with disease: A progress report on siRNA-based therapeutics, *Nature reviews* 6 443–453 (2007)
5. A Reynolds, D Leake, Q Boese, S Scaringe, WS Marshall and A Khvorova, Rational siRNA design for RNA interference, *Nat Biotechnol* 22 326–330 (2004)
6. L He and GJ Hannon, MicroRNAs: Small RNAs with a big role in gene regulation, *Nature Rev Genet* 5 522–531 (2004)
7. F Eckstein, The versatility of oligonucleotides as potential therapeutics, *Expert Opin Biol Ther* 7 1021–34 (2007)
8. K Jeyaseelan, WB Herath and A Armugam, MicroRNAs as therapeutic targets in human diseases, *Expert Opin Ther Targets* 11 1119–29 (2007)
9. TS Zimmermann et al, RNAi-mediated gene silencing in non-human primates, *Nature* 441 111–114 (2006)
10. SQ Harper et al, RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model, *Proc Natl Acad Sci USA* 102 5820–5825 (2005)
11. X Zhang et al, Small interfering RNA targeting heme oxygenase-1 enhances ischemia-reperfusion-induced lung apoptosis, *J Biol Chem* 279 677–684 (2004)
12. W Zhang et al, Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene, *Nat Med* 11 56–62 (2005)
13. G Dorn et al, siRNA relieves chronic neuropathic pain, *Nucl Acids Res* 32 e49 (2004)
14. H Zhang, Q Du, C Wahlestedt and Z Liang, RNA interference with chemically modified siRNA, *Curr Top Med Chem* 6 893–900 (2006)
15. PB Cserhalmi-Friedman, AA Panteleyev and AM Christiano, Recapitulation of the hairless mouse phenotype using catalytic oligonucleotides: Implications for permanent hair removal, *Exp Dermatol* 13 155–162 (2004)
16. Johnson et al, in press

Stimulating Stem Cells for Younger Skin

Katie Schaefer

Cosmetics & Toiletries *magazine*

KEY WORDS: *stem cells, antiaging, cell renewal*

ABSTRACT: *This chapter reviews stem cell research.*

Agree or disagree with it—stem cell research is a hot topic that cannot be ignored in today’s scientific realm. Its research may lead to the reversal of Parkinson’s and diabetes, to the growth of new organs, to strengthening bones and to healing damaged skin. The research of stem cells in conjunction with burn victims has led to innovation in another field—skin care.

Although it temporarily remained in the shadows, Voss Laboratories’ (Salt Lake City, Utah, USA) stem cell anti-aging serum is coming into the sun. The serum initially was available through private channels, only to be introduced to Sephora’s Champs-Élysées location in Paris; however, it has traveled to the United States and its creators are finally talking.

One of the main problems surrounding the serum’s release was confusion about exactly how it is made and how it works. Louis Rinaldi, chief cosmetic development officer for Basic Research (Salt Lake City, Utah, USA), the development company behind products for Voss Laboratories and Klein-Becker, had a hand in the product’s creation and in this report, he clears up the confusion surrounding this stem cell serum.

Exogenous and Endogenous Stem Cells

Most who protest the research of stem cells believe it is unethical to

use exogenous stem cells, also known as fetal material or embryonic tissue, for research. The described innovation, however, does not contain exogenous stem cells but rather a material that stimulates adult stem cells.

“Many are given the erroneous impression that the product contains stem cells. It does not contain stem cells nor does it contain any embryonic tissue. It is a peptide, a chain of amino acids,” said Rinaldi.

The stem cells that are stimulated by the use of the product are endogenous stem cells or adult stem cells.

“Endogenous stem cells are in your body already. They are in a stem cell reservoir from the time you are young until the day you die,” added Rinaldi, who noted that endogenous stem cells are always available for rejuvenation, given the correct stimulus. That stimulus, according to Rinaldi, is polypeptide 153, or the main ingredient in the company’s anti-aging serum.

Polypeptide 153

The serum was formulated by Basic Research for Voss Laboratories based on polypeptide 153, an ingredient that Russian scientists in Moscow and St. Petersburg discovered could help trigger stem cell rejuvenation in severe burns and scarred skin.

“Polypeptide 153 designates the 153 sequenced amino acids that comprise the peptide. It is purely a protein molecule,” said Rinaldi.

The peptide was not available for cosmetic use until late 2003, when Voss Laboratories was granted rights by the Russian scientists to use the ingredient. First, however, a large amount of testing had to be performed on the product to determine its efficacy.

Marker Activity

Polypeptide 153 underwent a series of tests before it was used commercially in the serum. Its marker activity on adult stem cells was measured.

“When the product stimulates these markers, you get a popcorn effect; once a couple are rejuvenated, more and more are rejuvenated,” said Rinaldi.

Highlighting stem cell markers boosts cellular renewal, according to Rinaldi, thereby reducing the appearance of wrinkles and the discoloration associated with photoaging.

After the product was tested *in vitro* for its activity on stem cells, it was tested *in vivo* by performing biopsies both before and after application of the product.

Although polypeptide 153 is the chief ingredient in the serum, additional ingredients are reported to help increase the product's antiaging benefits. Among them are hydrolyzed soy extract to visibly reduce the appearance of wrinkles, a smaller peptide to reduce the appearance of forehead wrinkles, and Scotch pine extract to reduce hyperpigmentation and blotchiness.

According to Rinaldi, the company plans to expand its use of polypeptide 153 from just a face emulsion to a whole line of products for the neck, décolleté, eyes and body. Some have questioned the appropriateness of utilizing the technology for a cosmetic product but Rinaldi finds the usage completely appropriate.

“This product promotes the health of the skin, which in turn can prevent a number of health problems,” said Rinaldi.

Cutaneous Blood Flow in Aging Skin

Jeanette M. Waller and Howard I. Maibach, MD

University of California School of Medicine

KEY WORDS: *blood perfusion, skin, glucose, cutaneous perfusion, photoaging*

ABSTRACT: *This chapter reviews the effects of decreased cutaneous perfusion, especially in photo-exposed skin, and the effects on aging.*

Changes in cutaneous blood perfusion with age, if present, could have profound effects on skin physiology, including alterations in concentration gradients for percutaneous penetration of compounds, alterations in diffusion of glucose and other nutrients to the epidermis, and implications for wound healing. While a decrease in blood flow to the skin is often an assumed effect of aging, little direct evidence-based knowledge exists.

This article reviews the research and attempts to provide a foundation for future study. Distinctions are not always easily made between intrinsic physiologic aging and extrinsic aging due to photo-exposure, wind, relative humidity and other environmental factors. Distinctions that can be made will be reported here.

Methods

This review results from a literature search on age-related changes in cutaneous blood perfusion conducted in *PubMed*, *Em-Base*, *Science Citation Index* and the UCSF dermatological library's collection of books on the topic of aging skin. The review includes brief descrip-

tions of commonly used quantitative methods and a discussion of research data.

Techniques

Cutaneous blood perfusion has been quantitatively studied *in vitro* as well as *in vivo*. *In vitro* histologic methods include the study of frozen sections stained with alkaline phosphatase for microscopic analysis. However, this technique is older and gives only a two-dimensional view, tending to yield overestimates of actual cutaneous perfusion.¹ A more recent immunohistochemical method studies specimens of skin stained for the CD31 antigen, the platelet endothelial cell adhesion molecule.²

In vivo methods include the use of intravital capillaroscopy, including native microscopy and fluorescein angiography, for non-invasive three-dimensional visualization of cutaneous blood flow.¹ Native microscopy uses an epi-illuminated microscope for *in vivo* viewing of superficial vasculature in immobilized, paraffin-treated skin.¹ Fluorescein angiography visualizes capillaries after intravenous administration of a bolus of sodium fluorescein solution.¹

Laser Doppler flowmetry (LDF) and laser Doppler velocimetry (LDV) are commonly used *in vivo* methods that involve a helium-neon laser light being transmitted to the skin via an optical fiber. The laser Doppler signal penetrates to an estimated depth of more than 1 mm, giving information about deeper vessels and arteriovenous anastomoses, not seen by capillaroscopy methods.¹ Light reflected from nonmoving tissue contains radiation at the same frequency as the incident source. Light reflected off of moving red blood cells, however, is Doppler-shifted.

The instrument extracts the frequency-shifted signal and derives a numerical reading proportional to blood flow.^{3,4} LDF and LDV involve essentially the same principles, but the instruments process the information slightly differently such that LDF results in an improved signal-to-noise ratio compared to LDV.⁴ Estimates of cutaneous blood flow obtained using LDF and LDV are reported in arbitrary units and are not absolute; they are instrument- and laboratory-specific.⁵

Photoplethysmography, another optical method, provides a measurement of arterial pulsations of the dermal plexus. Infrared radiation is directed into the skin, scattered, absorbed and reflected, primarily by hemoglobin in the tissue. Backscattered radiation is measured and the change of blood volume in the illuminated tissue is correlated to the amount of light absorbed by the blood. Greater blood flow results in greater absorption and less backscattered radiation.⁴

Data and Discussion

Results of studies using LDV or LDF are somewhat mixed; this often appears to be the result of varying age ranges and smaller sample sizes. Fluhr et al. found no significant difference in children vs. adults, but this did not allow for an assessment of changes in elderly skin because the average age of the older group was only 44.⁶ Likewise, Kelly et al. found little difference in blood flow between young (age 18–26) and elderly (age 65–88) subjects; however, the presence of only 10 subjects in each age group makes the study less than conclusive.¹ Another LDV study found that skin's vasodilation response to heat stress and vasoconstriction in response to cold challenge appears delayed in elderly (age 70–83) subjects compared to younger (age 20–30) subjects, indicating a possibly reduced vessel density in aged skin.⁷ Again, this study included only 10 elderly subjects and nine younger subjects, so it may not provide the most conclusive answer to questions about the effect of age on cutaneous perfusion.

Intravital capillaroscopy measurements of 26 subjects using fluorescein angiography and native microscopy suggest a decrease in dermal papillary loops and little change in horizontal vessels (post-capillary venules, ascending arterioles, and part of subpapillary plexus) with increasing age.¹ An immunohistochemical study of 19 individuals aged 20–84 revealed little effect of intrinsic aging of buttock skin on blood perfusion, but progressive and marked decrease in cutaneous perfusion in the photoaged eye corners.² A photoplethysmographic study including 69 individuals, aged 3–99, revealed significantly decreased capillary circulation in forehead skin with advancing age. Specific numbers, however, were not given.⁸

Conclusion

A review of this data makes a clear conclusion difficult because so much variation exists between studies. It appears possible that increased age may be associated with decreased cutaneous perfusion, especially in photo-exposed areas. However, this certainly is not an obvious or universal effect of aging. Future studies with more subjects and greater standardization of technique and body site studied may be helpful in further clarifying our understanding of the effects of age on cutaneous perfusion.

Published January 2007 *Cosmetics & Toiletries* magazine.

References

1. RI Kelly, R Pearse, R Bull, JL Leveque, J de Rigal and P Mortimer, The effects of aging on cutaneous microvasculature, *J Am Acad Dermatol* 33 749–756 (1995)
2. JH Chung, K Yano, MK Lee, CS Youn, JY Seo, KH Kim, KH Cho, HC Eun and M Detmar, Differential effects of photoaging vs. intrinsic aging on the vascularization of human skin. *Arch Dermatol* 138 1437–1442 (2002)
3. KV Roskos, The effect of skin aging on the percutaneous penetration of chemicals through human skin, PhD Dissertation, University of California, San Francisco (1989)
4. A Bircher and HI Maibach, Laser Doppler velocimetry and photoplethysmography, In *Cutaneous Aging*, A Kligman and Y Takase, eds, Japan: University of Tokyo Press (1988) pp 521–540
5. N Montiero-Riviere, Y Banks and L Birnbaum, Laser Doppler measurements of cutaneous blood flow in ageing mice and rats, *Toxicology Letters* 57 329–338 (1991)
6. JW Fluhr, S Pfistener and M Gloor, Direct comparison of skin physiology in children and adults with bioengineering methods, *Pediatric Dermatology* 17(6) 436–439 (2000)
7. MA Tolino and JK Wilkin, Aging and cutaneous vascular thermoregulation responses, *J Invest Dermatol* 90(4) 613 (1988)
8. JL Leveque, P Corcuff, J de Rigal and P Agache, *In vivo* studies of the evolution of physical properties of the human skin with age, *International J Dermatol* 23(5) 322–329 (1984)
9. E Berardesca, JL Leveque, P Masson and the EEMCO group, EEMCO guidance for the measure of skin microcirculation, *Skin Pharmacol Appl Skin Physiol* 15 442–456 (2002)

Cutaneous Biochemistry in Aging Skin

Jeanette M. Waller and Howard I. Maibach, M.D.

University of California School of Medicine

KEY WORDS: *biochemistry, aging skin, elastin*

ABSTRACT: *This chapter discusses elastin and its effects on aging.*

Methods

Age-related transformations in skin's appearance inevitably inspire curiosity about internal structural changes. In addition to the generally accepted histological changes, recent technological advances have increased markedly the understanding of specific biochemical changes that occur in aging skin.

Based on a literature search of *PubMed*, *Em-Base*, *Science Citation Index* and the University of California at San Francisco dermatological library, this article reviews some of the structural modifications in skin that underlie the visible effects of aging. Where possible, the discussion addresses differences between intrinsic, physiologic aging and extrinsic aging due to photo-exposure, wind, relative humidity and other environmental factors, although the authors acknowledge that this distinction is not always easily made.

Techniques for Protein Study

Raman spectroscopy: Raman spectroscopy is a nondestructive analytical method for determining the structure and conformation of molecular compounds such as proteins. It does not require sample preparation or pretreatment and thus eliminates much potential interference. Recently, near-infrared Fourier transform Raman spectroscopy has emerged as being specially suited for investigations

of biologic material.¹ This method gives highly reproducible results with only minor differences seen in spectra of different skin types.¹

Racemization: Another method of protein study involves measurement of racemized aspartic acid. Racemization represents one of the major types of nonenzymatic covalent modifications of proteins initially synthesized using only L-amino acids. Aspartic acid racemization (AAR) leads to an age-dependent accumulation of D-aspartic acid in more long-lived human proteins.²

Racemization in collagen is slow because of conformational constraints of the triple helix, but occurs more quickly in elastin.² Skin samples can be purified such that, if indicated, the AAR due to elastin alone can be studied and its longevity can be measured.² Studies of elastin in various tissues including the aorta and lungs reveal high levels of AAR.³ This indicates a lack of turnover and accumulation of elastin damage in diverse aging tissues, possibly as part of programmed aging.²

Skin Proteins

Collagen: Collagen, which comprises approximately 70–80% of the dry weight of the dermis, is primarily responsible for skin's tensile strength. A collagen molecule consists of three polypeptide chains, each containing about 1,000 amino acids in its primary sequence. In each collagen molecule the α -chains are wrapped around each other to make a triplehelical conformation.⁴

Chronologically aged skin shows a decrease in all of the following characteristics: collagen solubility, the rate of collagen synthesis, the activity of enzymes that act in the post-translational modification, and the thickness of collagen fiber bundles in the skin.^{5,6} Also, the ratio of type III to type I collagen has been shown to increase with increasing age.^{6,7} In photoaged skin, however, collagen fibers were shown to be fragmented, thickened and more soluble.⁶ It is plausible that reduced collagen deposition in elderly skin could explain development of dermal atrophy and might relate to poor wound healing in the elderly.⁵

Histological data reveals important information about orientation and arrangement of collagen fibers in skin. Lavker et al. compared skin from the upper inner arm of older (age 70–85) and younger

(age 19–25) individuals using light, transmission electron and scanning electron microscopy.⁸

Interestingly, Lavker et al. suggested that the upper inner arm might be an optimal site for analyzing sun-protected skin because it is not exposed to the pressure deformations and reformations occurring in the buttock. They found that in young adults, collagen in the papillary dermis forms a meshwork of randomly oriented thin fibers and small bundles. The reticular dermis consists of loosely interwoven, large, wavy, randomly oriented collagen bundles. However, the collagen within each bundle is packed together closely. In aged skin the density of the collagen network appears to increase. This likely reflects a decrease in ground substance that would otherwise form spaces between the collagen. Also, rather than appearing in discrete rope-like bundles of tightly packed fibers, collagen forms aggregates of loosely woven, mostly straight fibers. As fibers become straighter in aged skin, there is less room for the skin to be stretched, so tensile strength increases.⁸

Using immuno-electron microscopy, Vitellaro-Zuccarello et al. found similar age-related trends in skin collagen. They also noted greater intensity of collagen III staining in subjects over 70 years of age.⁹

Elastin: The skin's intact elastic fiber network, which occupies approximately 2–4% of the dermis by volume, provides resilience and suppleness. This network shows definite changes associated with aging, especially between the ages of 30 and 70. In sun-exposed skin, an excessive accumulation of elastotic material occurs. Accumulation of new elastin in response to photoaging also is apparent from upregulation of the elastin promoter activity and increased abundance of elastin mRNA.^{10,11}

Bernstein et al. compared photoaged skin to intrinsically aged skin and found a 2.6-fold increase in elastin mRNA, a 5.3-fold increase in elastin expression and a 5-fold increase in elastin promoter activity in photodamaged skin.¹¹ However, these increases in elastin synthesis do not account for the massive accumulation of elastotic material seen histologically in photoaged skin.¹¹ Some attribute this to elastin degradation being slower than synthesis, leading to an accumulation of partially degraded fibers. In purified skin

elastin, the amount of racemized aspartic acid increases rapidly and shows a high correlation with age ($r = .98$).² This indicates that skin's elastin, like elastin in the aorta and lung, is long-lived and accumulates damage over time.^{3,12}

In innate aging, fragmentation of elastic fibers decreases their number and diameter. Computerized image analysis of elastin-stained skin biopsies from the buttock and upper inner arm reveal an age-related increase in mean elastin fiber length and percentage surface area coverage in the dermis, but these fibers are thought to be enriched abnormally in polar amino acids, carbohydrates, lipids and calcium.¹²

Through different mechanisms, photoaging and intrinsic aging ultimately result in a deficiency of functional, structurally intact elastic fibers.⁵ The finer oxytalan fibers in the papillary dermis are depleted or lost altogether; eluanic and elastic fibers become progressively abnormal. These alterations largely account for the widely recognized decrease in skin's physiological elasticity with increased age.¹² (See **Assessing Tensile Properties of Skin.**)

Examination of intrinsically aged skin elastin and fibrillin with immunohistochemical staining revealed that elastin was located in the papillary dermis just below the basement membrane, as small fibers mostly oriented perpendicular to the epidermis. In the deeper dermis, fibers were thicker and oriented differently. Areas surrounding adnexal structures and larger vessels in the deep dermis were also intensely stained.¹¹ Photoaged skin demonstrated similar small-diameter fibers just below the basement membrane within a zone lacking excessive staining, which was of variable thickness.¹¹ This may correspond to the subepidermal non-echogenic band (SENEB) seen in ultrasound imaging. Beneath this area of relatively sparse staining was a region of poorly formed, clumped, thick fibers. This staining pattern occupied the superficial to mid dermis, below which staining again resumed its well-defined pattern as seen in sun-protected skin.¹¹

Assessing Tensile Properties of Skin

Setting standards for product claims and efficacy is an important task under the EU Cosmetics Directive. The European Group on Efficacy Measurement of Cosmetics and Other Topical Products (EEMCO) was formed in 1994 to develop industry-wide test methodologies and universal standards for cosmetic product claims substantiation.

The EEMCO guidance for the assessment of tensile functional properties of the skin was written by Gérald E. Piérard and published in *Skin Pharmacology and Applied Skin Physiology* in 1999.¹⁵

Piérard offers a four-layered model to explain how the integument deforms appropriately to withstand and transmit loads. The stratum corneum, the association between the living epidermis and papillary dermis, the reticular dermis and the hypodermis have each their own intimate structures whose tensile functions are balanced ideally to respond adequately to the casual mechanical demands on the skin. A series of physiological variables, aging and skin diseases alter the tensile functions of the skin.

In the overall analysis, truly comprehensive multidisciplinary approaches in this field have brought advances in the understanding of functional skin biology, according to Piérard. The assessment of tensile functions of skin also provides incentives for progress in skin care.

Primary and tertiary protein structure: A more general discussion of primary and tertiary protein structure in skin is informative. Through Raman spectroscopy, little difference is seen between photo-exposed areas and protected areas in young individuals; the majority of proteins in young skin are in helical conformation. Intrinsically aged skin shows slightly altered protein structure, and photoaged skin reveals markedly altered protein conformation, with increased folding and less exposure of aliphatic residues to water.^{1,6}

Aged skin versus young skin differs significantly in free amino acids and in the amino acid composition of proteins; furthermore, aged skin shows an increase in overall hydrophobicity of amino acid fractions.¹³ Because free amino acids are believed to play a key role in stratum corneum water binding, this shift in their composition—combined with the evidence of altered tertiary

protein structure—provides insight into the increased incidence of xerosis in aged individuals.

Glycosaminoglycans

Glycosaminoglycans (GAGs) are composed of specific repeating disaccharide units. Those attached to a core protein are referred to as proteoglycans and are distributed widely throughout the skin. GAGs most often present in human skin are hyaluronic acid (not attached to a protein core) and the proteoglycan family of chondroitin sulfates, including dermatan sulfate.¹⁴ GAGs are especially important in skin because they bind up to 1,000 times their volume in water. Therefore, skin hydration is highly related to the content and distribution of dermal GAGs, especially hyaluronic acid.¹⁴

GAG content increases in photoaged skin compared to young or intrinsically aged skin.^{6,14} This seems paradoxical because photoaged skin appears leathery and dry, unlike newborn skin, which also contains high levels of GAGs. Confocal laser scanning microscopy reveals that GAGs in photodamaged skin are deposited abnormally on elastotic material, rather than diffusely scattered as in young skin.¹⁴ This aberrant localization may interfere with normal water binding by GAGs, despite their increased number.

Water Structure

In young skin, most of the water is bound to proteins and, appropriately, is called bound water.¹ This is important for the structure and mechanical properties of many proteins and their mutual interactions. Water molecules not bound to proteins bind to each other and are called tetrahedron or bulk water.¹

Intrinsic aging does not appear to alter water structure significantly.⁶ However, in photoaged skin, Raman spectroscopy reveals an increase in total water content.⁶ Again, this seems paradoxical because aged skin is often dry and weathered. However, significantly more of the water in aged skin is in tetrahedron form. Thus, because proteins are more hydrophobic and folded, and GAGs are clumped on elastotic material, proteins and GAGs interact less with water, and water in aged skin binds to itself instead. This lack of interaction between water and surrounding molecules in photoaged skin likely contributes to its characteristically dry and wrinkled appearance.

Conclusions

Collagen becomes less soluble, thinner and sparser in intrinsically aged skin, but is thickened, fragmented and more soluble with photoaging.⁶ The ratio of type III to type I collagen is reported to increase with age.^{6,7,9} Histologically, young collagen is randomly organized into a meshwork of loosely interwoven bundles. Age leads to a loosening within these bundles and straightening of collagen fibers, increasing skin's tensile strength.⁸

Elastin is a long-lived protein in human skin; it appears to accumulate damage with age and sun exposure. New elastin is synthesized in greater quantities in aged skin, but it is thought that this synthesis results in abnormally structured elastin.^{10,11} Also, elastin degradation does not appear to keep pace with new synthesis in aged skin. This results in massive accumulations of elastotic material, especially in photoaged skin. The abnormal structure of this elastin prevents it from functioning as it does in young skin.

Studies of primary and tertiary skin protein structure in aged skin reveal an environment unfriendly to water, with an overall increase in hydrophobic amino acids and greater folding such that aliphatic residues are more hidden from water.^{6,13} Also, although total amounts of GAGs appear to be increased in aged skin, these are localized abnormally on the elastotic material in the superficial dermis; thus they are unable to bind water as well as if they were scattered appropriately throughout the whole dermis.¹⁴ Hence it is not surprising that, although aged skin contains increased amounts of water, most of this water is bound to itself in tetrahedral form, rather than being bound to proteins and GAGs as it is in young skin.⁶ These factors together likely contribute to increased xerosis and withered appearance of aged skin.

Published September 2006 *Cosmetics & Toiletries* magazine.

References

1. M Gniadecka, OF Nielsen, DH Christensen and HC Wulf, Structure of water, proteins, and lipids in intact human skin, hair, and nail, *J Invest Dermatol* 110 (4) 393–398 (1998)
2. S Ritze-Timme, I Laumier and MJ Collins, Aspartic acid racemization—evidence for marked longevity of elastin in human skin, *Br J Dermatol* 149 941–959 (2003)
3. JT Powell, N Vineand M Crossman, On the accumulation of D-aspartate in elastin and other proteins of the ageing aorta, *Atherosclerosis* 97(2–3) 201–208 (1992)

4. A Oikaren, Aging of the skin connective tissue: how to measure the biochemical and mechanical properties of aging dermis, *Photodermatol Photoimmunol Photomed* 10 47–52 (1994)
5. J Uitto, Connective tissue biochemistry of the aging dermis, *Clin Geriatric Medicine* 5 (1) 137–147 (1989)
6. M Gniadecka, OF Nielsen, S Wessel, M Heidenheim, DH Christensen and HC Wulf, Water and protein structure in photoaged and chronically aged skin, *J Invest Dermatol* 11 1129–1133 (1998)
7. CR Lovell, KA Smolenski, VXC Duance, ND Light, S Young and M Dyson, Type I and III collagen content and fibre distribution in normal human skin during aging, *Br J Dermatol* 117 419–428 (1987)
8. RM Lavker, P Zheng and G Dong. Aged skin: a study by light, transmission electron micro-scopy, and scanning electron microscopy, *J Invest Dermatol* 88 44s–53s (1987)
9. L Vitellaro-Zuccarello, R Garbelli and VD Rossi, Immunocytochemical localization of collagen types I, II, IV, and fibronectin in the human dermis: modifications with aging, *Cell Tissue Res* 268 505–511 (1992)
10. S Ritze-Timme and MJ Collins, Racemization of aspartic acid in human proteins, *Age Res Rev* 1 43–59 (2002)
11. EF Bernstein, YQ Chen, K Tamai et al, Enhanced elastin and fibrillin gene expression in chronically photodamaged skin, *J Invest Dermatol* 103 182–186 (1994)
12. C Robert, C Lesty and AM Robert, Ageing of the skin: study of elastic fiber network modifications by computerized image analysis, *Gerontology* 34 91–96 (1988)
13. T Jacobson, Y Yuksel, JC Geesin, JS Gordon, AT Lane, and RQ Gracy, Effects of aging and xerosis on the amino acid composition of human skin, *J Invest Dermatol* 95(3) 296–300 (1990)
14. EF Bernstein, CB Underhill, PJ Hahn, DB Brown and J Uitto, Chronic sun exposure alters both content and distribution of dermal glycosaminoglycans, *Br J Dermatol* 135 255–262 (1996)
15. Gérald E. Piérard, EEMCO guidance to the *in vivo* assessment of tensile functional properties of the skin, part 1: relevance to the structures and ageing of the skin and subcutaneous tissues, *Skin Pharmacol Appl Skin Physiol* 12 352–362 (1999)

Thickness of Aging Skin

Jeanette M. Waller and Howard I. Maibach, M.D.

University of California School of Medicine

KEY WORDS: *stratum corneum, ceramides, SAXD, RT-PCR, mature skin*

ABSTRACT: *This chapter reviews the available methods and results of studies of age-related changes in skin strata thickness.*

This review examines the medical literature on the thickness of aging human skin and the common assumption, based on appearance, that elderly skin is thinner. In fact, among dermatological researchers there is great controversy about the effects of aging on the thickness of skin strata. Even a single aspect of aging—the presence or absence of various sex steroids—may have an impact on the thickness of various skin strata (see **Effect of Sex Steroids on Skin Thickness**).

Comparing measures of skin layer thickness between individuals, and between studies, is especially challenging due to significant variation in measurements between individuals and between sites within each individual. Light and electron microscopic studies have provided important evidence for morphological changes in skin strata with age. Many quantitative techniques are also commonly used in measurement of skin layer thickness.

Based on a literature search of *PubMed*, *Em-Base*, *Science Citation Index* and the University of California at San Francisco dermatological library, this chapter reviews the available methods and results of studies of age-related changes in skin strata thickness. Where possible, the chapter reviews differences between intrinsic, physiologic aging and extrinsic aging due to photo-exposure, wind, relative humidity and other environmental factors, although the authors acknowledge that this distinction is not always easily made.

Effect of Sex Steroids on Skin Thickness

Do sex steroids play an important role in skin morphology and physiology? Yes, they do—at least in mice—as demonstrated by measuring the thickness of each skin layer in intact and gonadectomized (GDX) male and female mice, as well as in GDX animals treated for three weeks with 17β -estradiol (E_2), dihydrotestosterone (DHT), or their precursor dehydroepiandrosterone (DHEA).

Morphological analysis showed the following:

- The dorsal skin of the intact male is thicker than in the female, whereas the epidermis and hypodermis are thicker in the female.
- Epidermal thickness after GDX decreases only in the female to become similar to that of the intact male. Epidermal thickness in GDX animals of both sexes increases after E_2 treatment to a value similar to that of intact females, whereas an increase is observed only in females after DHEA treatment.
- Dermal thickness was increased by both DHEA and DHT.
- Hypodermal thickness was markedly reduced by E_2 , DHT and DHEA in GDX animals of both sexes.
- Under all conditions, the hypodermis remains thicker in females.
- This data shows that DHT, E_2 and DHEA exert specific effects on the different skin layers in mice.

For more on this research, see L Azzi, M El-Alfy, C Martel and F Labrie, Gender differences in mouse skin morphology and specific effects of sex steroids and dehydroepiandrosterone, *J Invest Dermatol* 124 22–27 (2005).

Techniques

Historical or questionable: Some histological methods are questioned due to possible artifacts caused by tissue preparation.

An older *in vivo* method, using the skin caliper instrument, measures the thickness of skin in a fold. This technique has lost popularity because it cannot fully eliminate the influence of the hypodermis and does not give the most accurate measurements of skin thickness.¹

Microscopy: Morphometric analyses of images taken from light microscopy, scanning electron microscopy and transmission electron microscopy, among other methods, have been used to determine the thickness of various skin layers *in vitro*.

The more recent use of confocal laser scanning microscopy allows a direct measurement on unmodified skin, and is considered to be a gold standard for measurement of stratum corneum (SC) and epidermal thickness.² Various radiographic methods are also commonly used. Pulsed ultrasound has been widely used in dermatology since 1982.^{3,4}

Ultrasound: For the determination of whole skin thickness, ultrasound enables the most exact determination without any restriction concerning site and without disadvantage for the patient.⁵⁻⁹ Incident ultrasonic energy is partially transmitted and partially reflected at the boundary between adjacent structures; this generates echoes, whose amplitudes are characteristic of the nature of the media. The image formed can be measured either manually or with computer assistance to determine the thickness of the skin.

Ultrasonography is available in two forms: A mode and B mode. Use of B mode ultrasonography requires more time and attention than A mode.⁴ While A scan measurements have, at least in many cases, produced similar results as B scan measurements,⁷⁻¹⁰ B mode assessment is more reproducible because it enables cross-sectional imaging. This allows for more reliable determination of skin thickness, even where the dermis-hypodermis interface is unclear. An A scanner only gives a unidimensional representation of skin echogenicity and can be problematic where the dermis-subcutis interface is ambiguous.^{4,11}

Seidenari points out the importance of carrying out ultrasound skin thickness measurements under standardized conditions, because a single skin site can produce different results if the measurements employ different gain swept curves; use of a gain curve at a higher level produces higher measurements.⁵ Differences in frequencies used by different researchers may also contribute to variation in results.

Data and Discussion

Changes in thickness of skin strata: Qualitative histologic data from young and old skin provide a foundation for discussing skin strata thickness quantitatively.

In its microscopic appearance, aged skin reveals a thinner epidermis than young skin, although the intrafollicular epidermis maintains a constant thickness. The epidermal thinning is primarily due to a retraction of the epidermal projections into the dermis, resulting in a flattened interface between the epidermis and dermis. One of the consequences is that aged epidermis becomes less resistant to shearing forces and tears more easily after trauma.^{2,12}

Flattening of the interface also is observed histologically at the ultrastructural level. In young skin, basal cells display numerous villous cytoplasmic projections into the dermis, resulting in a highly convoluted dermal/epidermal interface. In contrast, basal cells from aged skin lack these serrations, and the dermal/epidermal junction is flattened. Using scanning electron microscopy, Hull et al. determined that the corrugated papillary interface between the dermis and epidermis is visible up through the sixth decade; flattening occurs in the sixth through tenth decades.¹³ This flattening also may be associated with a decreased proliferative potential of aged epidermis and could also affect absorption.^{12,13}

Aside from increased basal cell atypia, keratinization does not appear markedly different in aged epidermis. Keratin filaments, lamellar bodies and keratohyalin granules are present histologically in usual amounts. The appearance and number of horny cells does not appear to change or diminish with age, so the SC retains its normal thickness of approximately 14–17 layers.¹²

Quantitative data provides less conclusive evidence. Ultrasound and microscopy studies focusing specifically on changes in SC thickness with age show little or no appreciable change, even up to age 97. These studies involve large numbers of individuals and give generally accepted conclusions.^{14,15} However, note that there is significant variation in SC thickness between individuals and between sites within each individual. Therefore, any future studies of SC thickness should include as large a sample size as possible, and should assess multiple sites.

Studies of epidermal thickness agree less. A confocal microscopy study of 34 women aged 18–69 found that the living epidermis thins on the back of the arm with increased age.¹⁵ A morphometric analysis of histological sections from 64 adults aged 20–80 found

progressive and significant (6.4% per decade) thinning of the epidermis, beginning in subjects as young as 30.¹⁶ However, a microscopic study of punch biopsies from the dorsal forearm, buttock and shoulder included 71 people aged 20–68 and found no significant change in epidermal thickness with age.¹⁷

It is difficult to explain these differences between studies, other than to attribute them tentatively to differences in site. Branchet et al. analyzed the upper inner arm, whereas the other studies looked at different areas. For researchers to reach a consensus on this matter, it may be important for future studies to focus on standardized skin sites so that their results can be compared more easily.

Analyses regarding dermal thickness reveal similar results: no change on the back of the arm¹⁷ and progressive thinning on the upper inner arm.¹⁶ Again, skin site may be the main explanatory factor for these contradictory results.

Changes in the thickness of whole skin: While studies are conflicting on whether changes occur in epidermal and dermal thickness, studies of whole skin thickness clearly are just as challenging to interpret.

An elegant ultrasound study by Richard et al. analyzed the effects of aging on two anatomically similar areas of neck, one exposed to the sun throughout life, the other covered. This experiment included 30 women aged 81 ± 6 and found thinning of the skin (approximately 0.1 mm difference) in the photoaged region compared to the covered area.¹⁸ In a larger study of 170 females aged 17–76 using A mode ultrasound, Takema et al. found significant thinning with age on sun-protected skin, and thickening with age on sun-exposed skin.¹⁹ The differences between these studies might be accounted for by the older population in the smaller study by Richard, or by the use of different modes of ultrasound. Richard et al. suggest that perhaps solar elastosis causes thickening in photoaged skin of younger individuals, but that this ceases to occur in older people.¹⁸

Several other studies find an increase in skin thickness over the first 20 years of life, then a period of either constant skin thickness or progressive thinning, followed by more marked thinning in older individuals, such that a diagram of skin thickness versus age might look like a bell curve.^{1,4,9,20} However, using B mode ultrasonography,

Pellacani et al. found an increase in facial skin thickness with age in 40 people aged between 25 and 90.¹¹ Yet other ultrasound studies show thinning of forehead skin with age.^{21,22} One B mode ultrasound study of 61 women aged 18–94 found that skin thickness increased on the forehead and buttock but decreased in extremity skin with increasing age.²³

Conclusions

Despite extensive data, it is difficult to define the effects of aging on skin thickness. It appears that the SC likely maintains its thickness during aging, but it is by no means obvious whether the other strata or the whole skin change in thickness with age. Individual and regional variations likely play a large role in the answer to this question. Also, elastoic effects of chronic sun exposure probably are involved. More concordant future results might be obtained by greater standardization of body site and ultrasound method, such that different laboratories use the same frequency and gain curves.

Published November 2006 *Cosmetics & Toiletries* magazine.

References

1. C Escoffier, J de Rigal, A Rochefort, R Vasselet, JL Leveque and PG Agache, Age-related mechanical properties of human skin: An *in vivo* study, *J Invest Dermatol* 93(3) 353–357 (1989)
2. P Agache, Metrology of the stratum corneum, In: *Measuring the Skin*, P Agache and P Humbert, eds, Berlin: Springer-Verlag (2004) pp 101–111
3. PA Payne, Medical and industrial application of high resolution ultrasound, *J Phys E Sci Instrum* 18 465–473 (1985)
4. J de Rigal, C Escoffier, B Querleux, B Faivre, P Agache and JL Leveque, Assessment of aging of the human skin by *in vivo* ultrasonic imaging. *J Invest Dermatol* 93 621–625 (1989)
5. S Seidenari, A Pagnoni, AD Nardo and A Giannetti, Echographic evaluation with image analysis of normal skin: Variation according to age and sex, *Skin Pharmacol* 7 201–209 (1994)
6. JE Wahlberg, Assessment of skin irritancy: Measurement of skin fold thickness, *Contact Derm* 9 21–26 (1983)
7. RA Booth, BA Goddard and A Paton, Measurement of fat thickness in man: A comparison of ultrasound, Harpenden calipers and electrical conductivity, *Br J Nutr* 20 719–725 (1966)
8. H Alexander and DL Miller, Determining skin thickness with pulsed ultrasound, *J Invest Dermatol* 72 17–19 (1979)
9. CY Tan, B Statham, R Marks and PA Payne, Skin thickness measurement by pulsed ultrasound: Its reproducibility, validation, and variability, *Br J Dermatol* 106 657–667 (1982)
10. PJ Dykes and R Marks, Measurement of skin thickness: A comparison of two *in vivo* techniques with a conventional histometric method, *J Invest Dermatol* 1 418–429 (1976)
11. G Pellacani and S Seidenari, Variations in facial skin thickness and echogenicity with site and age, *Acta Derm Venereol* 79 366–369 (1999)

12. RM Lavker, P Zheng and G Dong. Aged skin: A study by light, transmission electron microscopy, and scanning electron microscopy, *J Invest Dermatol* 88 44s–53s (1987)
13. MT Hull and KA Warfel, Age-related changes in the cutaneous basal lamina: scanning electron microscopic study, *J Invest Dermatol* 81 378–380 (1983)
14. Z Ya-Xian, T Suetake and H Tagami, Number of cell layers of the stratum corneum in normal skin: relationship to the anatomical location on the body, age, sex, and physical parameters, *Arch Dermatol Res* 291 555–559 (1999)
15. D Batisse, R Bazin, T Baldegeck, B Querleux and JL Leveque, Influence of age on the wrinkling capacities of skin, *Skin Research and Technology* 8 148–154 (2002)
16. MC Branchet, S Boisnic, C Francis and AM Robert, Skin thickness changes in normal aging skin, *Gerontology* 36 28–35 (1990)
17. J Sandby-Moller, T Poulsen and HC Wulf, Epidermal thickness at different body sites: Relationship to age, gender, pigmentation, blood content, skin type, and smoking habits, *Acta Derm Venereol* 83 410–413 (2003)
18. S Richard, J de Rigal, O de Lacharriere, E Berardesca and JL Leveque, Noninvasive measurement of the effect of lifetime exposure to the sun on the aged skin, *Photodermatol Photoimmunol Photomed* 10 164–160 (1994)
19. Y Takema, Y Yorimoto, M Kawai and G Imokawa, Age-related changes in the elastic properties and thickness of human facial skin, *Br J Dermatol* 141 641–648 (1994)
20. S Shuster, M Black and E Mc Vitie, The influence of age and sex on skin thickness, skin collagen, and density, *Br J Dermatol* 93 639–642 (1979)
21. M Denda and M Takahasi, Measurement of facial skin thickness by ultrasound method, *J Soc Cosmet Chem Jpn* 23 316–319 (1990)
22. M Nishimura and T Tuji, Measurement of skin elasticity with a new suction device, *Jpn J Dermatol* 102 1111–1117 (1992)
23. M Gniadecka and GBE Jemec, Quantitative evaluation of chronological aging and photoaging *in vivo*: Studies on skin echogenicity and thickness, *Br J Dermatol* 139 815–821 (1998)

Stimulation of Dermal and Epidermal Metabolism: An Approach to Antiaging

Maud Jouandeaud-Le Guillou, Sylvie Bordes, Catherine Soulie and Brigitte Closs
Silab, Brive, France

Patrice Andre

LVMH Parfums & Cosmétiques, St. Jean de Braye, France

KEY WORDS: *Retinol-like, epidermal metabolism, extracellular matrix protection, mallow extract, keratinocytes*

ABSTRACT: *Aging results in a number of modifications to cells and tissues. Here the authors describe an active ingredient from common mallow whose active fraction containing polyphenols and galacturonic acids is able to regulate both dermal and epidermal metabolism much like retinoids do.*

Skin aging is a complex process, controlled by both genetic determination (chronologic aging) and the influence of modifications resulting from external factors (extrinsic aging). Intrinsic or chronologic aging is genetically programmed. It is a progressive phenomenon that occurs with a variable rapidity depending on the individual. It affects the skin in the same way it affects other organs. Environmental aging is superimposed over chronologic aging, primarily in skin areas chronically exposed to the sun.

Skin aging results in a number of modifications to both cells and tissues. A number of studies have shown the reduced proliferation capacity of keratinocytes and fibroblasts¹ resulting from the aging process. In addition, the synthetic capacities of cultured fibroblasts

decrease—old fibroblasts synthesize smaller quantities of collagen I and III than young fibroblasts.² Skin aging thus results in a general reduction of cell metabolism and protein synthesis capacities.

All these variations in the biology of the dermis and epidermis are shown by a visible change in the surface appearance of the skin. It is less firm and less elastic and wrinkles appear, initially around the eyes and on the face.

Retinoids, in particular retinoic acid, have been shown to be very effective for limiting aging. The use of retinoids as topical applications improves the characteristics of old skin with lines and wrinkles, as well as skin texture and color. A significant improvement in general appearance, as well a dose-dependent effect that increases with the duration of treatment, have been observed.¹ The use of retinoids, however, sometimes causes irritating side effects.

Research conducted at Silab has thus been developed to prepare an active ingredient to replace retinoids. We initially determined efficacy markers for the selection of our active ingredient. After this and based on toxicologic, analytic and regulatory criteria, we screened a large number of molecules extracted from such diverse origins as fruits or plants, as well as plant parts as varied as stems, flowering tops or leaves.

This screening led to the discovery that an active ingredient obtained from the flowering tops of common mallow (*Malva sylvestris*) regulates both dermal and epidermal metabolism just as retinoids do. Purification of *Malva sylvestris* extract (MSE)^a yielded a fraction rich in polyphenols and galacturonic acids whose behavior *in vitro* and *in vivo* and the gene expression profile were comparable to retinoic acid.

MSE and Epidermal Metabolism

Stimulating metabolic activity: Aging is accompanied by a reduction in cell renewal and substantial modifications of biosynthesis that are responsible for the reduced production of various constitutive molecules of the epidermis. We initially investigated the effect of MSE on the metabolic activity of keratinocytes, either aged artificially by successive transfers or obtained from donors 26 and 51 years old. The cells

^a The discussed *Malva sylvestris* extract is a patented extract from Silab

were incubated for 72 hours with 2% mallow extract in keratinocytes serum free medium (KSFM)^b.

Metabolic activity was assessed by measuring total cell proteins with the bicinchoninic acid (BCA) method^c.

The proliferation capacity of human keratinocytes grown *in vitro* as well as their metabolic activity decreased as the cells underwent an increasing number of cell divisions. The use of MSE favors the restitution of this activity in both young and aging cells. Tested at 2%, MSE improved the metabolic activity of cells by +24% in cells from a 51-year-old donor and by +14% in cells that underwent 5 successive transfers (**Figure 1**).

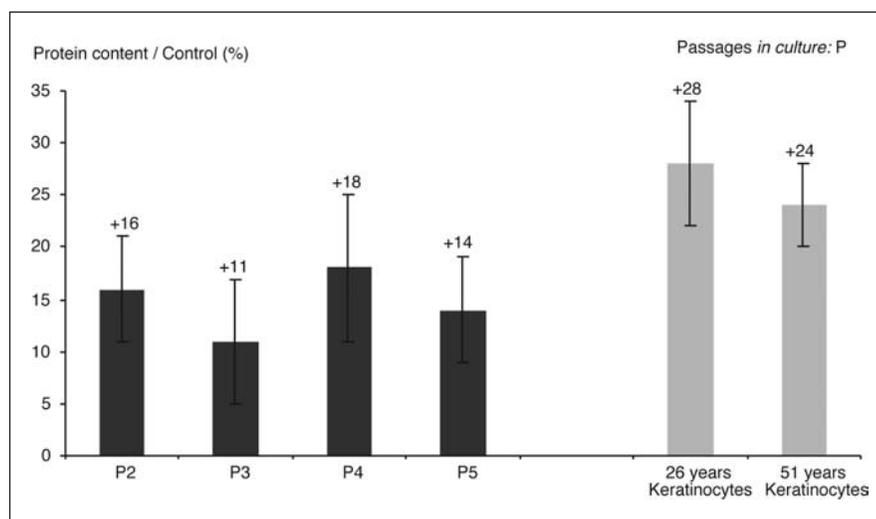


Figure 1. Effect of 2% MSE on the metabolic activity of keratinocytes artificially aged by successive passages or from donors of different ages

Keratinocyte differentiation: The effect of MSE on keratinocyte differentiation was also studied by using filaggrin as marker. This protein stimulates the aggregation of cytokeratin filaments (**see sidebar**). The study of the expression of mRNA coding for filaggrin was carried out with RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) on human keratinocytes incubated for 72 hours with 4% MSE. The results were confirmed using reconstructed human epidermis^d by immunostaining of filaggrin.

^b KSFM medium is a product of Invitrogen, USA.

^c The bicinchoninic acid (BCA) method is registered to Pierce, USA.

^d Reconstructed epidermis is a product of SkinEthic Laboratories, France.

Filaggrin in Keratinocyte Differentiation

Keratinocytes in the epidermis originate in a proliferative basal layer, then undergo growth-arrest and migrate upward in a carefully-orchestrated differentiation program. This program involves the sequential expression of a number of tissue-specific genes encoding specialized cytoskeletal proteins and other keratinocyte-specific proteins (loricrin, involucrin and filaggrin) that become cross-linked to each other, forming a tough latticework beneath the keratinocyte plasma membrane. The expression of these products leads to characteristic phenotypic changes in the epidermis.

For example, hematoxylin-positive blue granules in the upper epidermal layers (the stratum granulosum) are called keratohyaline granules. They contain filaggrin, a filament-aggregating protein, that is synthesized from a large profilaggrin precursor, which is subsequently cleaved to release small peptides. These filaggrin peptides intersperse between keratin filaments and cause them to aggregate and collect at the periphery of the keratinocyte.

The keratinocyte differentiation program ultimately ends with a tough outer layer, the stratum corneum, which can be likened to a brick wall - held together by intercellular lipids ("mortar") to form an impenetrable barrier.

Source: Dartmouth College Web site. Available at: <http://www.dartmouth.edu/~pdt/p1res.htm>. Accessed Mar. 31.

The results (**Figure 2**) show that MSE in the culture medium leads to an increase in the expression of filaggrin mRNA, even in aging cells. Tested at 4%, MSE increases the expression of filaggrin mRNA by 39% in cells from a 51-year-old donor and by 33% in artificially aged cells by 5 successive transfers (P5).

In addition, a study on reconstructed epidermis showed that filaggrin is more intensely labeled when the epidermis is treated with 4% MSE and that the stratum corneum is thicker when the tissue is treated with 10^{-6} M retinoic acid (**Figure 3**).

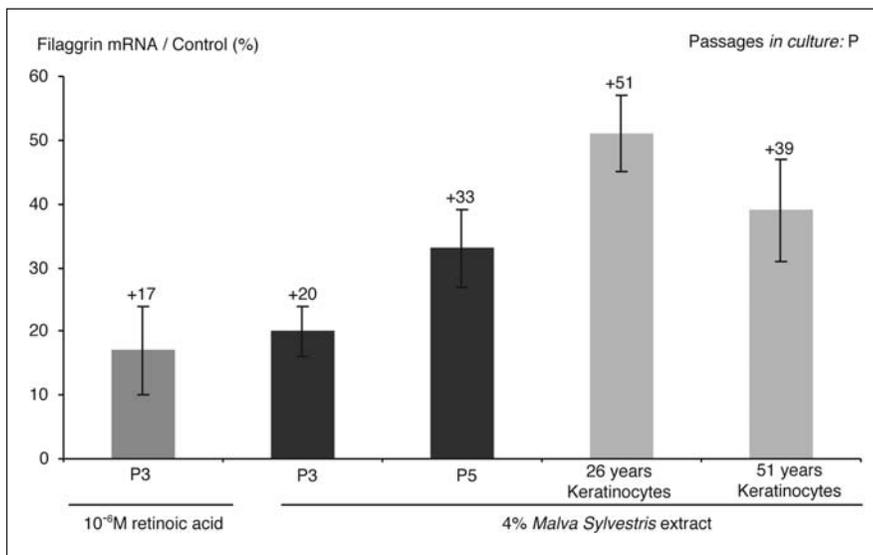


Figure 2. Effect of 4% MSE on the expression of mRNA coding for filaggrin

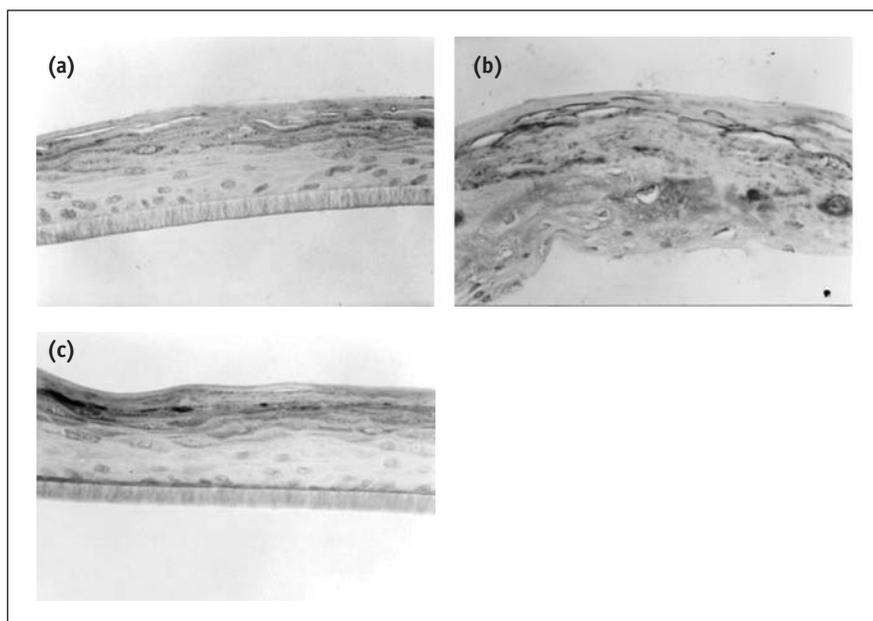


Figure 3. Effect of 4% MSE on keratinocyte differentiation using a marker: filaggrin

(a) Untreated reconstructed epidermis

(b) Reconstructed epidermis treated with 10^{-6} M retinoic acid

(c) Reconstructed epidermis treated with 4% MSE

MSE and Dermal Metabolism

Synthesis of collagen I: According to published data, the density of different types of collagen fibers decreases with age.^{3,4} In our work, special attention was paid to collagen I, the major collagen in the dermal matrix.

The influence of MSE on the synthesis of collagen I was studied using cultures of normal human fibroblasts. The cells were treated with 2% MSE or 10^{-6} M retinoic acid as positive control. After 72 hours of incubation, the cells were recovered and the expression of mRNA coding for collagen I was assayed with RT-PCR.

Tested at 2%, MSE increases the expression of mRNA coding for the synthesis of collagen I after 72 hours of treatment (+18%). This effect is comparable to that of retinoic acid (**Figure 4**).

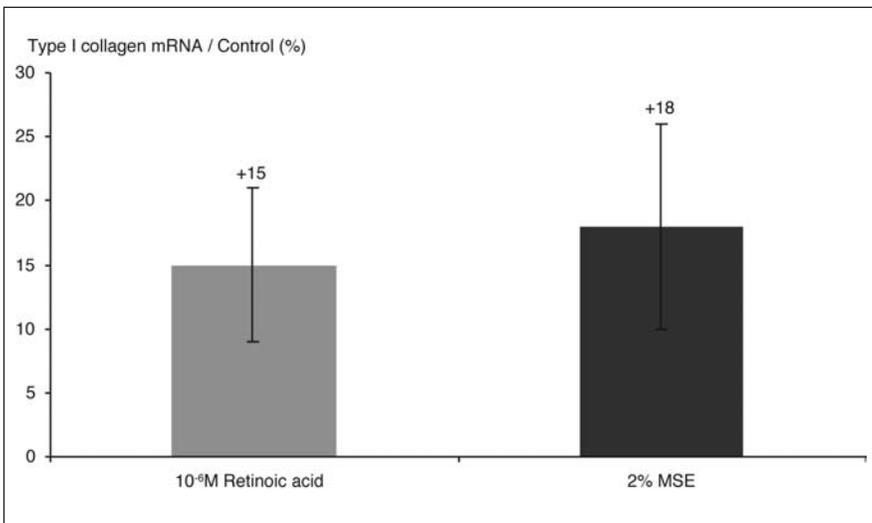


Figure 4. Effect of MSE or retinoic acid on the expression of mRNA coding for collagen I

Limiting extracellular matrix degradation: The influence of MSE on the equilibrium between MMP-1, an enzyme that can degrade certain proteins such as collagen I, and TIMP-1, inhibitor molecules of MMP-1 was investigated. Human fibroblasts were incubated for 48 hours MEM medium, without fetal calf serum and with 2% MSE or 10^{-6} M retinoic acid and were subjected to a UVA stress (20 J/cm^2). The MMP-1 and TIMP-1 concentrations in cell-free supernatants were determined with an ELISA method.

MSE and retinoic acid reduce the synthesis of MMP-1 by 66% and 4% when the cells are not irradiated.

When the cells are subjected to a UVA irradiation, the level of MMP-1 increases considerably (+195%). MSE at 2%, as well as 10^{-6} M retinoic acid, reduce the synthesis of MMP-1 by 19.6% and 15.1%, respectively, when the skin is subjected to a UVA solar stress (**Figure 5**).

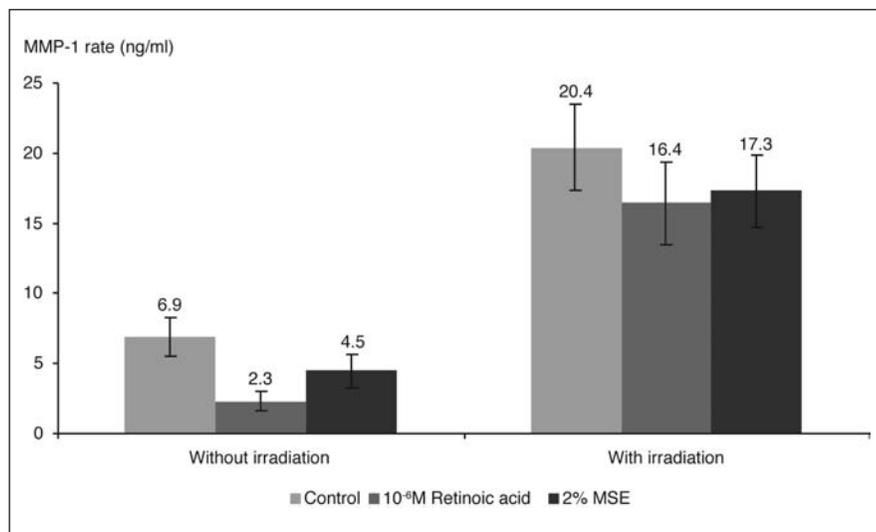


Figure 5. Effect of 2% MSE or 10^{-6} M retinoic acid on the MMP-1 activity of human fibroblasts

MSE is also a powerful stimulant of TIMP-1 activity (+88%) in cells subjected to a UVA irradiation and compensates the harmful effects of UV irradiations on the synthesis of these MMP inhibitors (results not shown).

MSE effect on the gene expression profile: The antiaging properties of MSE were investigated by studying and comparing the influence of retinoic acid on the differential expression of genes in reconstructed human epidermis, using the cDNA macroarray method.

The effects of different treatments were studied in terms of the expression of selected genes coding for major structural proteins of the epidermis or the extracellular matrix, coding for cytokeratins and their receptors or for other proteins of cosmetic interest.

The study was conducted on reconstituted epidermis^d, untreated or treated with 3% MSE or 10^{-6} M retinoic acid for 8 or 24 hours.

The cDNA macroarray analysis of gene expression was conducted^e containing 600 genes selected for their importance in skin physiology.

The results cDNA macroarray showed that genes reported as being modulated by retinoic acid were also regulated by MSE at 3% (**Table 1**). It is thus seen that MSE, just as retinoic acid, modulates:

- genes coded for markers of the differentiation process—cytokeratin 1, 10, 2E, filaggrin or encore desmoplakin I, calgranulin A and B; and
- genes coded for certain cytokines, chemokines, growth factors such as IL-1RA, heparin-binding EGF-like growth factor (HBEGF) and platelet-derived growth factor (PDGFA)

Table 1. Summary of the principal genes regulated by retinoic acid and MSE

Genes down-regulated by MSE and retinoic acid	Genes up-regulated by MSE and retinoic acid
Bullous pemphigoid antigen 1 (BPAG1; BPA)	S100 calcium-binding protein A4
Bullous pemphigoid autoantigen 180 (BPAG2)	S100 calcium-binding protein A7
Catalase	Calgranulin A
Cytokeratin 1	Calgranulin B
Cytokeratin 10	Elafin
Cytokeratin 2E	Epithelial-derived neutrophil-activated peptide 78
Desmocollin 1A/1B	Granulocyte chemotactic protein 2 (GCP 2)
Desmoglein 1	Heparin-binding EGF-like growth factor (HBEGF)
Desmoglein 3	Interleukin-1 receptor antagonist protein (IL-1RA)
Desmoplakin I & II	Platelet-derived growth factor A subunit (PDGFA)
Estrogen-regulated LIV-1 protein	Potassium transporting ATPase alpha-like chain
Epidermal filaggrin (FLG)	
Fatty acid transporter (FAT); CD36 antigen	

^e The array system study was founded by BIOalternatives.

Nevertheless, and in contrast to retinoic acid, MSE did not modulate the expression of the genes for cellular retinoic acid-binding protein II (CRABP-II) that mediate the effects of retinoids. CRABP-II is a specific protein for retinoic acid transport into nuclei and whose synthesis is induced by a direct action of retinoic acid on the skin.⁵ This suggests that the two treatments have a different mechanism of action.

Conclusion

Retinoids are recognized antiaging molecules but cause a number of side effects in the form of irritation and intolerance reactions when used; they are also difficult to stabilize in formulation. To overcome this problem, we have investigated an extract whose behavior is comparable to that of retinoic acid in terms of epidermal and dermal metabolism.

Both retinoic acid and MSE favor the metabolic activity of epidermal cells and regulate the process of keratinocyte differentiation. Tested at 4% on aging cells, MSE leads to a 39% increase in the expression of mRNA coding for filaggrin, a marker protein of this process. This active ingredient also favors the protection of the extracellular matrix by stimulating the expression of mRNA coding for the synthesis of collagen I (+18%) and also by limiting the expression of MMP-1 induced by a UVA irradiation (-19.6%).

Finally, the gene expression profile in the presence of MSE was compared to that of retinoic acid. This cDNA microarray assay showed that a large number of genes modulated by retinoic acid are also regulated by MSE.

Compared with retinoic acid, however, MSE does not modulate the expression of the genes of CRABP II. This suggests a different mechanism of action although they both lead to the same gene expression profile.

Published June 2005 *Cosmetics & Toiletries* magazine.

References

1. M Yaar and BA Gilchrist, Skin aging: Postulated mechanisms and consequent changes in structure and function, *Clin Geriatr Med* 17 617-630 (2001)

2. J Varani, GJ Fisher, S Kang and JJ Voorhees, Molecular mechanisms of intrinsic skin aging and retinoid-induced repair and reversal, *J Invest Dermatol Symp Proc* 3 41-44 (1998)
3. M Dumas, C Chaudagne, F Bonte and A Meybeck, *In vitro* biosynthesis of type I and III collagens by human dermal fibroblasts from donors of increasing age, *Mech Ageing Dev* 73 179-187 (1994)
4. CA Carrino, JM Sorrell and AI Caplan, Age-related changes in the proteoglycans of human skin, *Arch Biochem Biophys* 373 91-101 (2000)
5. A Astrom, A Tavakkol, U Pettersson, M Cromie, JT Elder and JJ Voorhees, Molecular cloning of two human cellular retinoic acid-binding proteins (CRABP). Retinoic acid-induced expression of CRABP-II but not CRABP-I in adult human skin *in vivo* and in skin fibroblasts *in vitro*, *J Biol Chem* 266 17662-17666 (1991)

In vitro Approaches to Antiaging Testing

Robert Holtz

BioInnovation Laboratories

KEY WORDS: *antiaging, senescent, keratinocytes, skin cells*

ABSTRACT: *Using proper methods, it is possible to screen materials for potential antiaging effects.*

Is it possible to maintain the beautiful skin of youth throughout a lifetime? Unfortunately, the smooth and supple skin of young adulthood gradually declines with age in both its physical attractiveness and functionality. Fine lines and wrinkles begin to appear; and while these can be considered as markers of maturity and wisdom, most consumers prefer their formation to be delayed as long as possible.

So is it possible to slow the aging process in the skin? Under circumstances the answer may be *yes*—with help from science. Using proper methods, it is possible to screen materials for potential antiaging effects; in turn, these materials can be incorporated into cosmetic or personal care products. To properly screen antiaging materials, an understanding of the aging process is required.

Aging Skin

Aging is associated with an accumulation of senescent cells in the skin; i.e., cells that are in a state of growth arrest. While senescent cells are still viable, they will no longer replicate DNA or go through the process of cell division. In addition to this lack of replicative ability, senescent cells can also become damaging to their local environment. For instance, senescent fibroblasts will shift from producing extracellular matrix proteins,¹ which are essential to

maintaining the structure of the dermal layer of the skin, and will instead produce proteolytic enzymes,² which can shift their focus toward degradation of the dermis.

In addition, senescent keratinocytes can release significant amounts of IL-1 α ,³ that can promote areas of focal inflammation in aged skin. While the number of senescent skin cells in aged individuals can vary, it has been theorized that only a small population of senescent cells could negatively and significantly influence the appearance and structure of the skin.⁴

Senescent Cells

Cells can become senescent via two basic means. The first is due to replicative senescence. Normal cells (i.e., noncancerous or nonimmortalized cells) can only undergo a certain number of cell divisions. The number of cell divisions will vary depending upon the cell type and the culture conditions; however, as the cells approach this limit, their rate of division will slow and eventually stop. At the point where there is no longer any measurable cell division in the culture, the entire population is said to be senescent.

One of the main triggering events in replicative senescence is telomere shortening. Telomeres are repeating DNA sequences (TTAGGG) at each end of linear chromosomes that function to assist DNA polymerase in replicating the 5' end of the lagging strand during DNA synthesis. They also function to cap the end of the chromosome such that it does not appear to be a double-strand break. However, each time a cell undergoes the process of DNA replication before cell division it loses a number of telomeres. Once the number of telomere repetitions is shortened to a certain critical length, the cell will no longer undergo replication and becomes senescent. Telomere shortening is considered to be one of the main mechanisms for intrinsic aging.

While replicative senescence was one of the first methods used by researchers⁵ to induce cellular senescence, it can take many months of culturing for cells to reach the point of senescence. An alternative means by which cells can become senescent—and a much faster method—is by exposure to certain types of sublethal stress. The most common stresses are exposure to either peroxides⁶

or UV light,⁷ although recently, high concentrations of glyoxal have also been used.⁸ Upon exposures, often repeat exposures, to such stresses made during a period of a few days, the cells become senescent. This type of senescence has been termed Stress-induced Premature Senescence (SIPS).

With SIPS, exposure to the peroxides, UV or glyoxal can induce oxidative damage to cell DNA. Once the DNA is damaged to a critical yet nonlethal level, the cell goes into growth arrest and becomes senescent. Oxidative damage is considered to be one of the factors contributing to extrinsic aging or photo-aging. Interestingly, both the oxidative DNA damage associated with SIPS and extrinsic aging, and the critically short telomeres associated with replicative senescence and intrinsic aging, can induce senescence via a common pathway: the p53/p21/p16^{ink4a} pathway.

The p53/p21/p16^{ink4a} Pathway

The p53 protein functions as a transcription factor. When DNA damage such as oxidative damage or short telomeres are detected, p53 is activated and can enter the cell nucleus, binding to special regions in the genomic DNA and promoting the expression of several aging-related genes including p21. The p21 protein functions as a cyclin-dependent kinase (CDK) inhibitor, as is p16^{ink4a}. CDKs control the cell cycle by regulating when the cell can make the transition from the G1 phase of the cell cycle to the S phase (see **Cell Cycle**).

Cell Cycle

Gap Phase 1 (G1): Gap phases in the cell cycle are primarily characterized by cell growth and an increase in cellular organelle content such that the cell will have enough mass and organelles to support the formation of two daughter cells. Gap Phase 1 is also a time where the cell evaluates the environment both inside and outside of the cell to make sure that conditions are suitable for cell division before making the transition to the S phase.

S Phase (S): This is the phase in cell division where DNA is replicated.

Gap Phase 2 (G2): Similar to Gap Phase 1.

M Phase (M): This is the phase of the cell cycle where the actual process of cell division (mitosis) occurs, resulting in the formation of two daughter cells from a single original cell.

When p21 and p16^{ink4a} inhibit the CDKs, the cell remains parked in the G1 phase and will not enter into the S phase—the phase during which it would normally replicate its DNA in preparation for cell division. The timing with which these three proteins are active is important and it appears that an initial increase in p53 activity and p21 expression is required to start cells on the path to senescence, after which it is p16^{ink4a} that is required to maintain cells in the senescent state.⁹

Because of their central role in cellular senescence, p53, p21 and p16^{ink4a} can be useful markers for screening materials for antiaging effects. If a replicative senescence model is utilized, it is necessary to culture the cells in the presence of potential antiaging materials for the entire duration of treatment, which could span months. Either at set time intervals during the treatment or once the control cells become senescent, p53, p21 and p16^{ink4a} can be measured and their levels of expression/activity compared between untreated cells and cells treated with the potential antiaging material. If the level of expression is found to be lower in the treated cells, the material may have antiaging properties.

To screen potential antiaging models using a SIPS model, cells can be cultured in the presence of the potential anti-aging material during the few days that the cells are periodically exposed to UV light or peroxides. At the end of the SIPS induction, the untreated cells should be senescent and expressing various levels of p53, p21 and p16^{ink4a}. In contrast, if the potential antiaging material was effective, the levels of p53, p21 and p16^{ink4a} expression in the treated cells should be much lower.

Senescence-associated β -galactosidase

Replicative senescence and SIPS share another common marker for senescence. This marker is based on the induction of senescence-associated β -galactosidase (SA- β -gal) enzyme activity in aged cells. β -galactosidase activity is normally found in the acidic environment of the lysosome. Normal cellular β -galactosidases are optimally active at a pH of 4.0; however in aging cells, another form of β -galactosidase activity becomes apparent, which is optimally active at a pH level of 6.0. This latter form of galactosidase has been termed SA- β -gal.

SA- β -gal activity can be observed by staining the cells with 5-bromo-4-chloro-3-indolyl- β -D-galacto-pyranoside or *X-gal*, a substrate for β -galactosides. When *X-gal* stain is added to cultured cells at a pH level of 6.0, SA- β -gal—not normal lysosomal β -galactosidase—can convert the *X-gal* into an insoluble blue-green chromagen. Thus the colored chromagen will accumulate in senescent cells while nonsenescent cells will not be stained.

The application of SA- β -gal staining to screen potential antiaging materials would be similar to the method outlined above for the use of p53, p21 and p16 expression/activity. The cells could be treated with potential test materials during the induction of senescence and at the end of the induction, the extent of SA- β -gal staining could be compared between treated and untreated cells. If there is less staining in the treated group, then the test material may have antiaging properties.

Conclusions

It should be reiterated that in addition to the loss of replicative capability, there are also negative functional changes that occur when cells make the transition to senescence. These negative functional changes include both the production of harmful products that can damage the local environment, and also the loss of some or all of the beneficial functions the cell had. If the transition to the senescent state can be prevented or delayed by an antiaging material, then the negative functional changes that occur within the cell could be delayed as well. The methods described in the chapter can provide a means by which antiaging materials can be initially screened for efficacy *in vitro*. This can lead to the development of finished cosmetic products that have the potential of slowing the aging process in the skin, thus effectively maintaining the youthful appearance of the skin over a longer period of time.

Published September 2007 *Cosmetics & Toiletries* magazine

References

1. T Quan et al, Elevated cysteine-rich 61 mediates aberrant collagen homeostasis in chronologically aged and photoaged human skin, *Amer J of Pathology* 169(2) 482–490 (2006)

2. VB Swope and ST Boyce, Differential expression of matrix metalloproteinase-1 *in vitro* corresponds to tissue morphogenesis and quality assurance of cultured skin substitutes, *J of Surgical Research* 128(1) 79–86 (2005)
3. M Okazaki, K Yoshimura, G Uchida and K Harii, Correlation between age and the secretion of melanocyte-stimulating cytokines in cultured keratinocytes and fibroblasts, *Brit J of Derm*, 153 suppl 2, 23–29 (2005)
4. J Campis, The role of cellular senescence in skin aging, *J of Invest Derm Symposium Proceedings* 3(1) 1–5 (1998)
5. L Hayflick and PS Moorhead, The serial cultivation of human diploid cell strains, *Experimental Cell Research* 23 14–20 (1961)
5. P Dumont et al, Induction of replicative senescence biomarkers by sublethal oxidative stresses in normal human fibroblasts, *Free Radical Biology and Medicine* 28(3) 361–373 (2000)
6. F Chainiaux, JP Magalhaes, F Eliaers, J Remacle and O Toussaint, UVB-Induced premature senescence of human diploid skin fibroblasts, *Intl J of Biochemi and Cell Biology* 34, 1331–1339 (2002)
7. H Sejersen and SI Rattan, Glyoxal-induced premature senescence in human fibroblasts in *Annals of the New York Academy of Science* 1100 518–523 (2007)
8. GH Stein, LF Drullinger, A Soulard, and V Dulic, Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanism of senescence and differentiation in human fibroblasts, *Molecular and Cellular Biology* 19(3) 2109–2117 (1999)

Tetrapeptide Targets Epidermal Cohesion

G. Pauly, MD; P. Moussou, PhD; J.-L. Contet-Audonneau, MD; C. Jeanmaire, PhD; O. Freis, PhD; M. Sabadotto; L. Danoux; V. Bardey, PhD; I. Benoit; and A. Rathjens, PhD

Cognis France, Division de Laboratoires Sérobiologiques, Pulnoy, France

KEY WORDS: *glycosaminoglycans, proteoglycans, syndecan-1, aging, type XVII collagen*

ABSTRACT: *Most antiaging products claim to act on the dermis; however, the epidermis, a key element of cutaneous aging, is often forgotten. In the present study, researchers selected an acetylated tetrapeptide for its effect on epidermis cohesion, triggered by activity on syndecan-1 and collagen XVII; these effects are confirmed in vivo.*

Actives proposed for skin care generally are focused on wrinkle prevention—but another sign of aging is fragile skin. During aging, the epidermis becomes thinner, the cohesion of the epidermal cells diminishes and the epidermis loses its resistance to environmental aggressors. The skin becomes dry and slack and consequently is easily damaged even with the lightest friction or shock.

To enhance epidermal cohesion, Laboratoires Sérobiologiques has developed an antiaging active designed to target two proteins that affect epidermis cohesion: syndecan-1 and type XVII collagen.

Syndecan-1

In the skin, proteoglycans (PG) and glycosaminoglycans (GAG) are present not only in the extracellular matrix of the dermis, but also in the epidermis. Syndecans represent the major form of PG synthesized by the epidermis with syndecan-1, a small PG with a MW

<60,000 da, located in supra-basal layers of the epidermis. Syndecan-1 plays an important role in keratinocyte activation during wound healing.¹ In addition, it has diverse functions including the regulation of cell signaling such as by fibroblast growth factors, participation in cell-to-cell and cell-to-laminin adhesion,² and in the organization of cell matrix adhesion. According to Carey,³ syndecan-1 may link the intracellular cytoskeleton to the interstitial matrix.

Much data exists on the alteration of the synthesis and the structure of GAG and some PGs during skin aging,⁴ but little information is available concerning small PGs in the epidermis, in particular syndecan-1. One recent study on cell cultures from donors of different ages showed a reduced synthesis of syndecan-1 by keratinocytes during aging.⁵ This data has been confirmed by immunohistochemistry (IHC) on skin biopsies from donors of different ages (**Figure 1**).

In **Figure 1a**, syndecan-1, revealed in yellow-green in the epidermis (E), is not very visible in skin from a 3-year-old donor. It is more strongly visible in skin from a 41-year-old donor (+222%, $p < 0.01$), yet greatly reduced in skin from a 55-year-old donor (-88%, $p < 0.05$) (**Figure 1b**). This type of level evolution, according to aging, is usually observed for proteoglycan skin components.⁶

Type XVII Collagen

Type XVII collagen is a component of hemidesmosomes, participating in the adhesion of basal keratinocytes to the extracellular matrix of the dermo-epidermal junction (**Figure 2**).

Hemidesmosomes are composed from different proteins such as plakin BP230, $\alpha 6 \beta 4$ integrins, tetraspanin/CD151, plectin and type XVII collagen, also called BP180 (**Figure 3**).

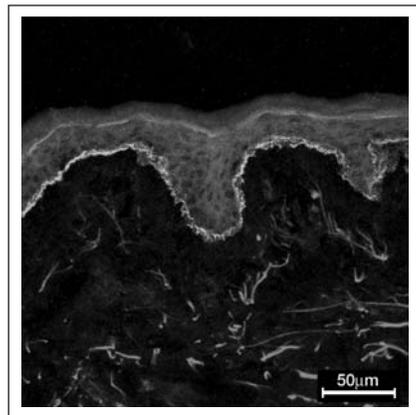


Figure 2. Visualization, in green, of type XVII collagen on human skin by immunohistochemistry and confocal microscopy

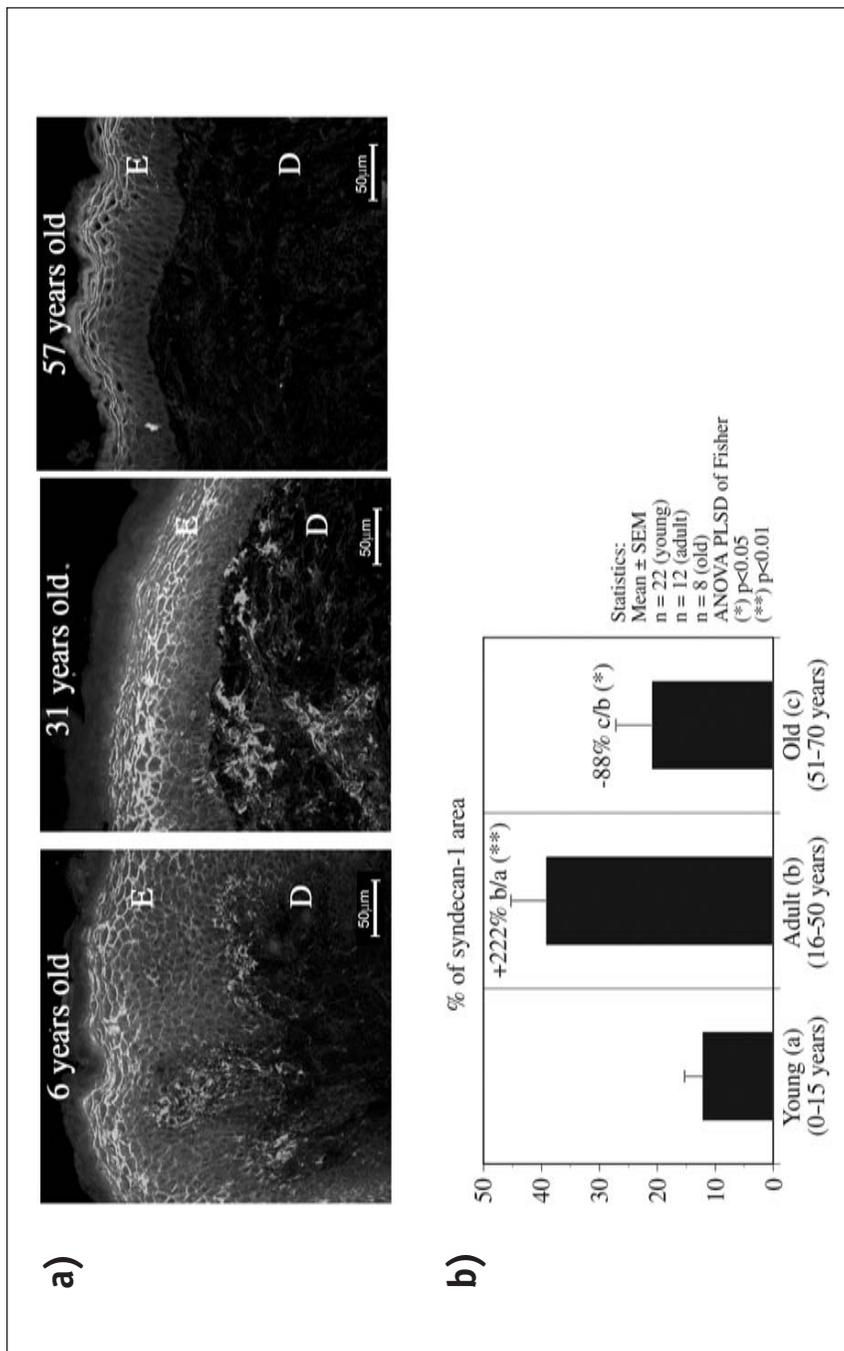


Figure 1. Variation of syndecan-1 expression in the epidermis with aging (E = epidermis, D = dermis); a) visualization, in yellow-green, of skin sections; b) quantification

Mutations in the type XVII collagen encoding gene (COL17A1) led to a decrease in epidermal adhesion, as well as skin blistering in response to minimum mechanical deformation.⁷ This underlines the fundamental importance of stabilizing interactions between type XVII collagen and laminin-5 and integrins to maintain the correct cohesion between different skin layers.

During the aging process, skin becomes more fragile and presents an increased blistering response to weak physical constraints such as external shock.⁸ Because type XVII collagen plays a role in anchoring and cohesion, any alteration of this protein may at least be partly responsible for skin weakness.

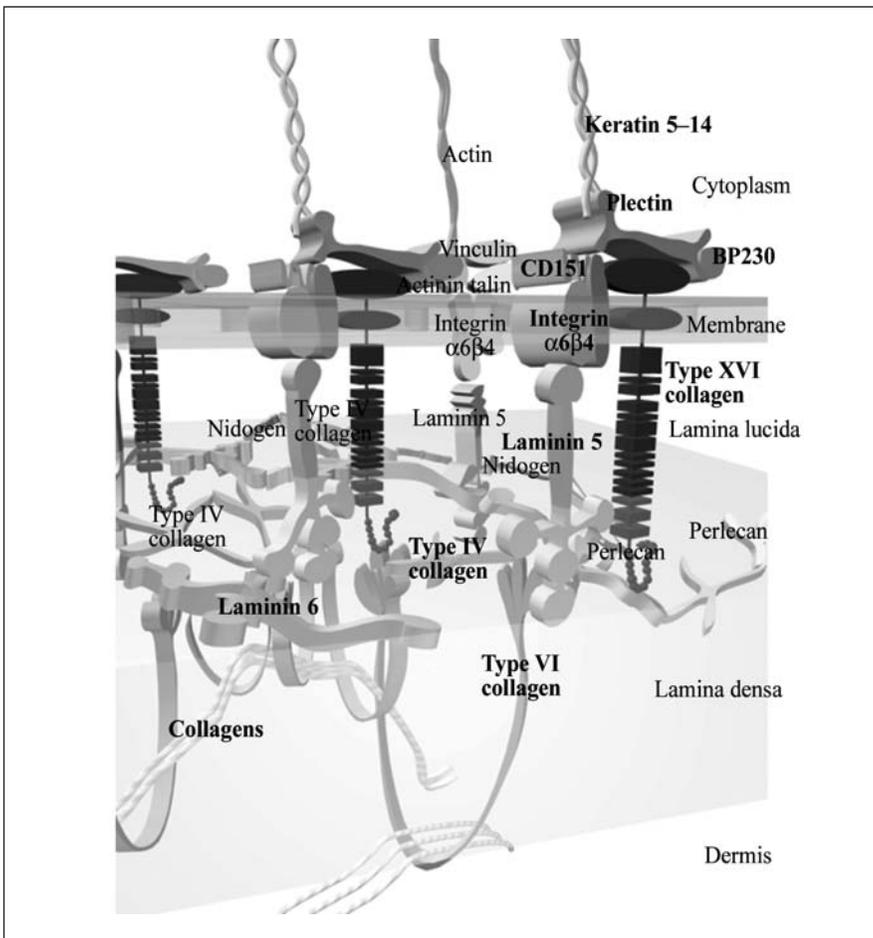


Figure 3. Schematic representation of the hemidesmosome structure between basal keratinocytes and the extracellular matrix; type XVII collagen is represented in green.

N-Acetyl-tetrapeptide-11

With the aim of reducing consequences of the aging process and maintaining the level of syndecan-1 and type XVII collagen in the epidermis, an active ingredient was developed based on an acetylated tetrapeptide N-acetyl-Pro-Pro-Tyr-Leu (AcTP11) (**Figure 4**)^a.

The peptide sequence corresponds to four amino acids of an anti-microbial polypeptide, cathelicidin PR-39, that is expressed during wound healing and induces syndecan-1 expression.⁹

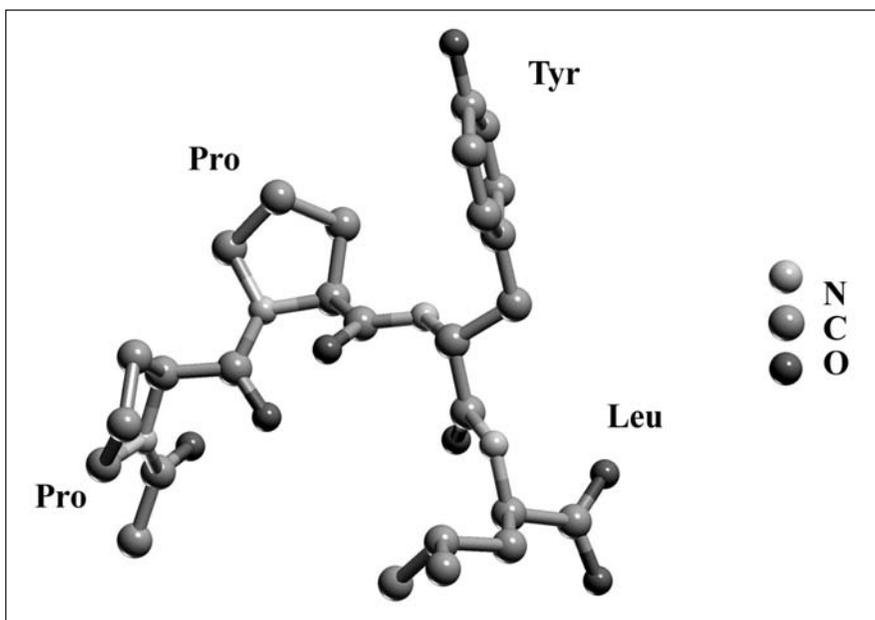


Figure 4. Chemical structure of AcTP11 (N-acetyl-Pro-Pro-Tyr-Leu)

In vitro Efficacy

Keratinocyte cultures: Human keratinocyte suspensions were prepared by trypsinization of human skin biopsies from adult donors. Keratinocytes were cultured at 37°C, CO₂ 5%, within medium containing fetal calf serum (FCS) at 5%.

- *Keratinocyte culture for syndecan-1:* After incubation for two days, the growth medium was changed for medium (DMEM

^a SYNiorage (INCI: Glycerin (and) acetyl tetrapeptide-11) is a product of Cognis.

without FCS) but containing AcTP11 (at 0.87 and 2.6mg/mL) or KGF as reference substance at 0.01mg/mL.¹⁰ The cultured cells were processed five days later for visualization of syndecan-1 by immunocytochemistry (ICC)^b and quantification by image analysis.

- *Keratinocyte culture for type XVII collagen:* After incubation for three to five days, the growth medium was changed for medium (DMEM without FCS) containing AcTP11. The cultured cells were processed three days later for visualization of type XVII collagen by ICC^c and quantification by image analysis.

Reconstructed full thickness skin models: Reconstructed full-thickness skin models^d contain growing keratinocytes overlaying a pseudo-dermis that is formed by viable fibroblasts dispersed in collagen matrix. The skin models were treated by AcTP11 or KGF at the beginning of the differentiation. After nine days of incubation, models were analyzed by IHC.

DNA-array analysis: DNA-array analyses were performed on human keratinocyte cultures treated or untreated with AcTP11. Four strains taken from different donors were mixed to take into account inter-individual variabilities.

After incubation for 3 hr or 24 hr, to identify shorter and moderate time-lapse effects, total RNAs were extracted from cell cultures^e. During the reverse transcription step, complementary DNAs (cDNAs) were synthesized and labeled either with cyanine-3 (Cya3) for total RNA extracted from nontreated cultures, or with cyanine-5 (Cya5) for AcTP11 treated cultures. Competitive hybridizations of the two labeled cDNAs were performed on the same specific DNA-array^f.

^b Primary polyclonal rabbit antibody antihuman syndecan-1, SC-5632, was obtained from Tebu, France; also, secondary sheep antibody anti-rabbit—FITC, was obtained from BIO-RAD, France.

^c Primary polyclonal rabbit antibody antihuman collagen XVII, Ab28440, was obtained from Abcam, Cambridge, UK; and secondary monoclonal goat antibody anti-rabbit, 4010-2, was obtained from Southern Biotechnology Associates, Birmingham, USA.

^d Epiderm FT Kit EFT 200 was obtained from MatTek, USA.

^e The Nucleospin RNA II kit from Macherey-Nagel, France, was used for this test.

^f 1300 genes PIQOR Skin by Memorec Biotec GmbH, Germany, was used to perform this test.

After washing with stringent buffer to eliminate nonspecifically bound cDNA, the red and green fluorescence of Cya3 and Cya5 were measured to evaluate the gene expression rates in the two tested conditions—control and AcTP11 treatment. Each gene was analyzed four times in the slide and statistical analyses were elaborated to identify significant gene expression modifications.

qRT-PCR: A quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for type XVII collagen encoding gene (COL17A1) was realized on total RNAs extracted from keratinocytes cultured for two days with or without AcTP11.

Microscopy and image analysis: After staining by ICC or IHC, cell cultures and tissue sections were observed by a confocal laser scanning microscope^g and images were quantified by an image analyzer^h.

For skin sections, the percentage of stained PG area was quantified (% of stained epidermis). For cell cultures, the results were expressed as: expression index 1 = number of stained pixels x fluorescence intensity in green channel in arbitrary unit. For the full-thickness skin model sections, the result was expressed as: expression index 2 = number of stained pixels x fluorescence intensity in green channel/epidermal thickness in arbitrary unit.

Results

Global effect of AcTP11 on DNA array: The expression of two strategic genes was potentially modified by AcTP11 treatment: the expression of the Discoidin Domain Receptor 1 (DDR1) gene was down-regulated, and the type XVII collagen encoding gene expression was increased.

The DDR1 gene codes for a cellular receptor, which is activated by numerous extracellular collagens.¹¹ Signals transduced from this receptor induce a cell growth decrease¹² and a repression of the expression of some genes of the extracellular matrix (ECM), including the syndecan-1.¹³ Since AcTP11 seemed to decrease DDR1 expression, it could also induce a keratinocyte growth stimulation and thus limit syndecan-1 gene repression.

^g The CLSM 310 is a device of Zeiss, France.

^h The TCS-SP2 is a device of Leica, France.

The collagen XVII or BP180 protein is implicated in the hemidesmosome structure that forms a bridge between cytoskeleton of basal epidermal keratinocytes and the extracellular matrix of the dermo-epidermal junction. This collagen is strongly implicated in the cohesion of the different skin layers.

AcTP11 on syndecan-1, keratinocytes, full-thickness models: The stimulation of keratinocyte cell growth by AcTP11 was confirmed on *in vitro* cell cultures (+13%, $p < 0.05$), and the decrease of syndecan-1 gene repression by AcTP11 was confirmed by *in vitro* tests. On keratinocyte cultures, AcTP11 significantly increased the rate of syndecan-1 in keratinocyte cultures (**Figure 5a**), with a dose dependent and significant effect, comparatively to the control (**Figure 5b**).

On the full-thickness skin model, AcTP11, introduced in the culture medium at 2.60 $\mu\text{g}/\text{mL}$, significantly increased the rate of syndecan-1 (**Figure 6a**), comparatively to the control (+38%, $p < 0.02$) (**Figure 6b**).

AcTP11 on type XVII collagen, keratinocyte cultures: The increase of type XVII collagen encoding gene expression also has been confirmed by *in vitro* tests. AcTP11 at 2.60 $\mu\text{g}/\text{mL}$ significantly increased the COL17A1 gene expression by human epidermal keratinocytes in culture (+18%, $p < 0.05$) (**Figure 7**).

This result has been confirmed by visualizing the production of type XVII collagen protein (shown in green) on cell culture (**Figure 8**).

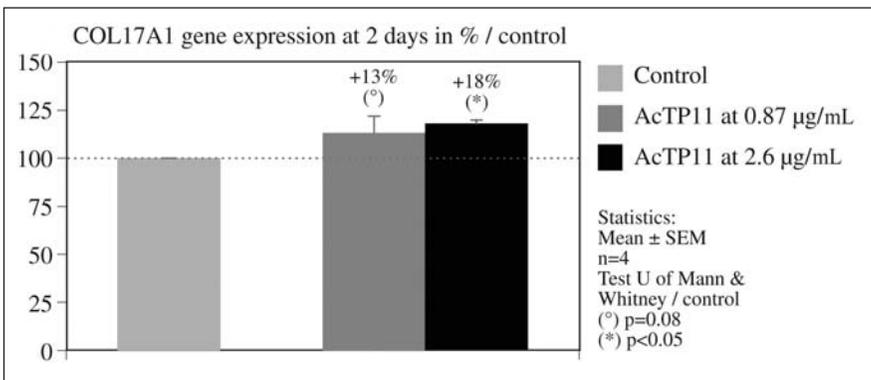


Figure 7. Quantification of AcTP11 effect on the expression of type XVII collagen encoding gene (COL17A1) in human epidermal keratinocytes by qRT-PCR

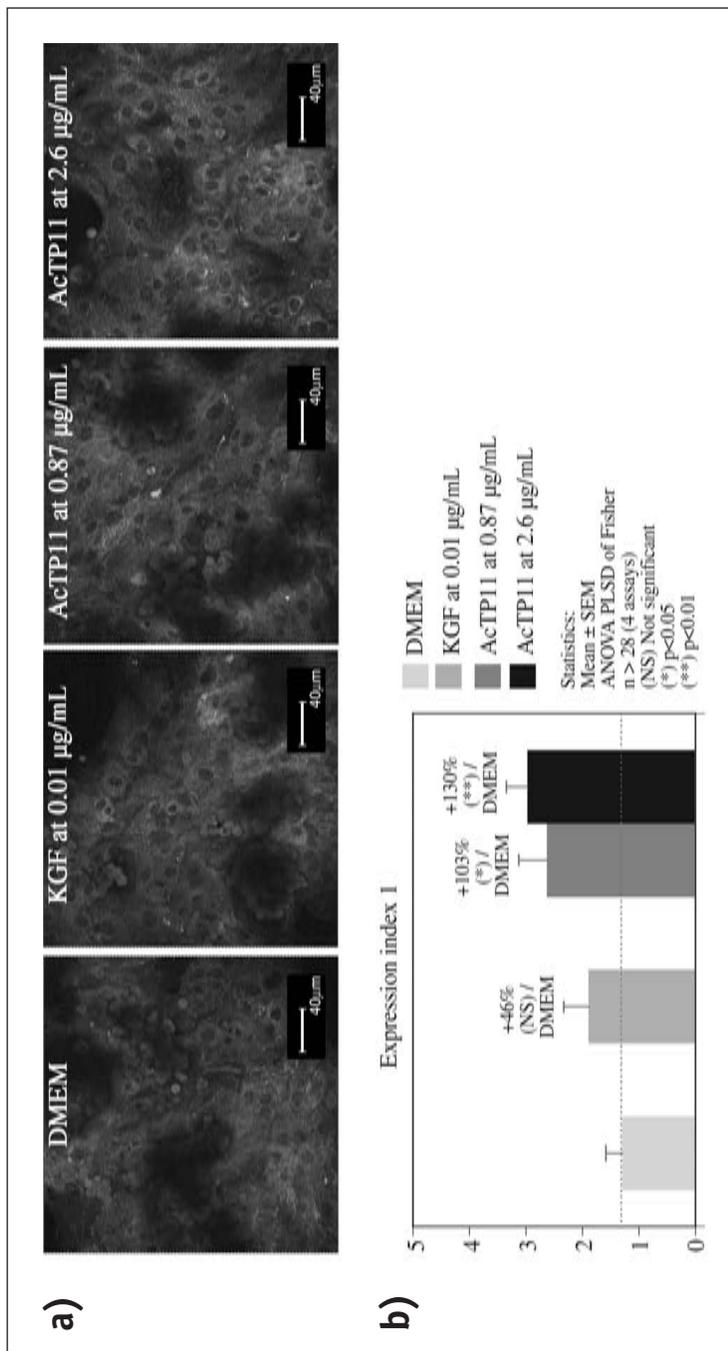


Figure 5. Effect of AcTP11 on the syndecan-1 synthesis by human keratinocytes in primary cell culture; a) visualization of the cells, in green; b) quantification

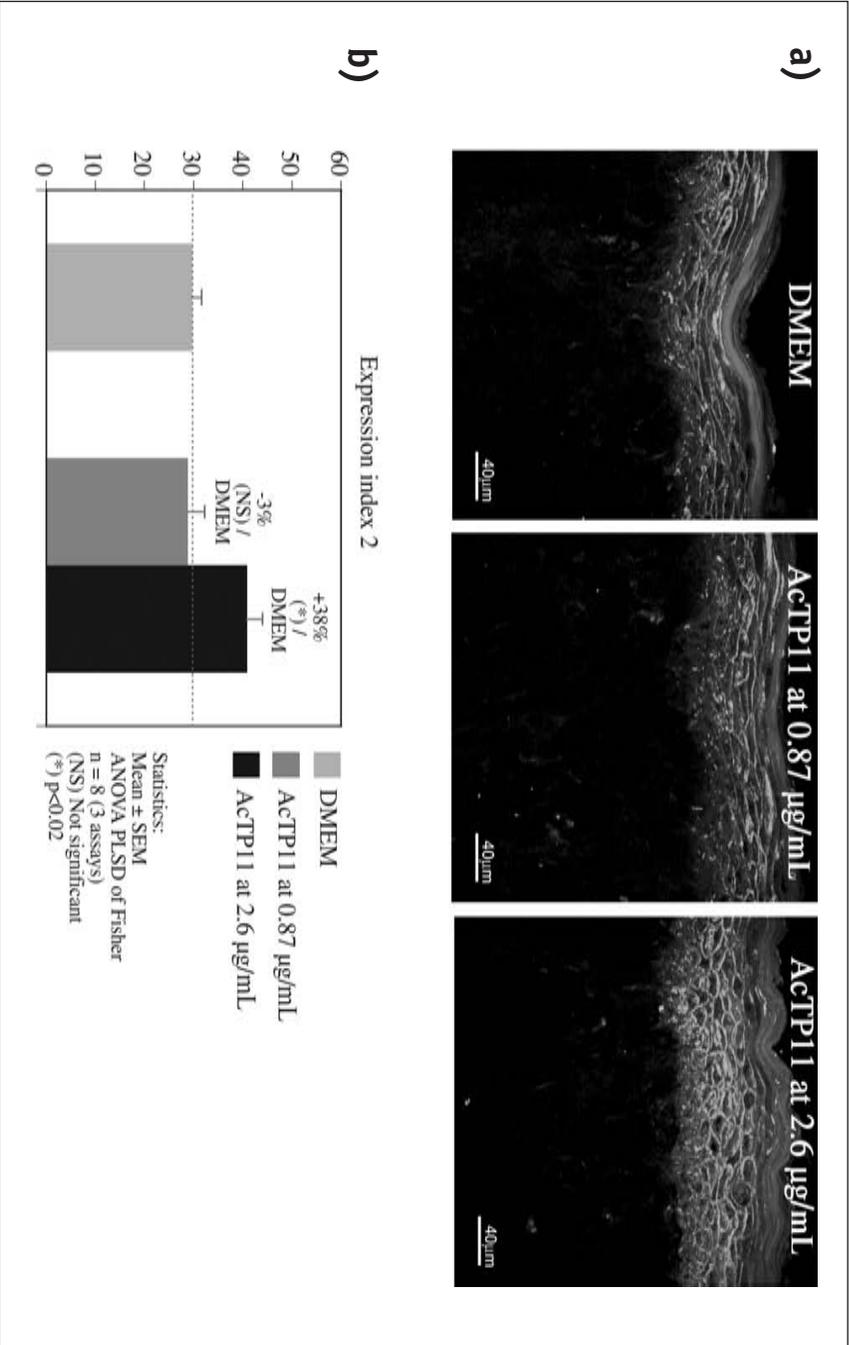


Figure 6. Effect of ActP11 on the syndecan-1 synthesis by human keratinocytes in reconstructed full-thickness skin model; a) visualization of the sections, in green; b) quantification

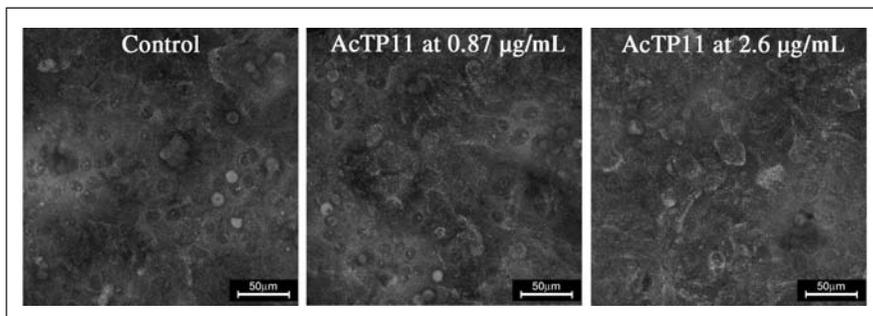


Figure 8. Visualization of AcTP11 effect on type XVII collagen, shown in green, in human epidermal keratinocytes by ICC

In vivo Efficacy

A clinical study was conducted on 19 healthy female volunteers aged 60 to 70 years who were experiencing loss of elasticity on the face. This study evaluated the antiaging effects of a cream incorporating 3% (13.5 ppm) of the active ingredient based on AcTP11 at 60mg/cm² (see **Formula 1**), after 56 days of twice-daily use, versus a placebo cream.

Formula 1. Antiaging test cream incorporating AcTP11 active

A. Lauryl glucoside (and) polyglyceryl-2 dipolyhydroxystearate (and) glycerin	4.00% w/w
Behenyl alcohol	2.00
Ethylhexyl stearate	4.00
Hexyldecanol (and) hexyldecyl laurate	4.00
Dicaprylyl ether	2.00
Dimethicone	1.00
Sodium polyacrylate	1.00
B. Water (<i>aqua</i>)	qs to 100.00
Propylene glycol (and) phenoxyethanol (and) chlorophenesin (and) methylparaben (Elestab 388, Laboratoires Sérobiologiques)	2.50
Xanthan gum	0.25
Glycerin	3.00
Sodium cocoyl glutamate	0.80
C. Glycerin (and) acetyl tetrapeptide-11 (SYNiorage LS 9748, Cognis)	<u>3.00</u>
	100.00

The antiaging activity in the skin was evaluated by biomechanical measurements since some biomechanical parameters such as firmness and elasticity decrease with skin aging.¹⁴

The biomechanical properties of the skin were measured on the upper cheek by a torquemeter before treatment (D0) and after 28 (D28) and 56 (D56) days of treatment. Macrophotographs of the temples were taken at the same time.

The torquemeter is designed to measure the angular displacement of skin in response to torsional forces applied by the torque motor incorporated into the probe. The gap between the central rotating disk and the external stationary ring of the probe determines the depth of the skin solicitation. A gap of 1 mm was used for the characterization of the biomechanical behavior of the superficial layers of epidermis.¹⁵ The parameters measured are shown in **Figure 9**.

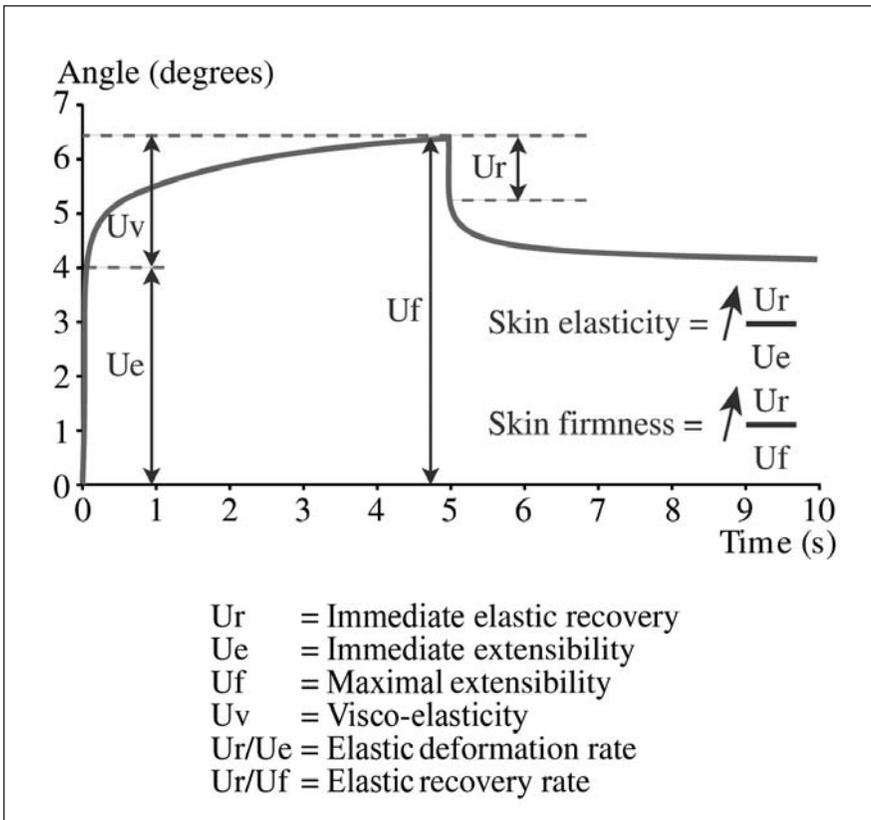


Figure 9. Curve of skin deformation obtained with a torquemeter

After 56 days of treatment, a significant increase was observed in the biomechanical properties of firmness and elasticity in the superficial layers of the epidermis. Skin texture was improved, skin relief was smoothed and skin radiance was increased (**Figure 10a**).

A sample cream with 3% AI based on AcTP11 had a 5–10% greater effect than the placebo cream (**Figure 10b**).

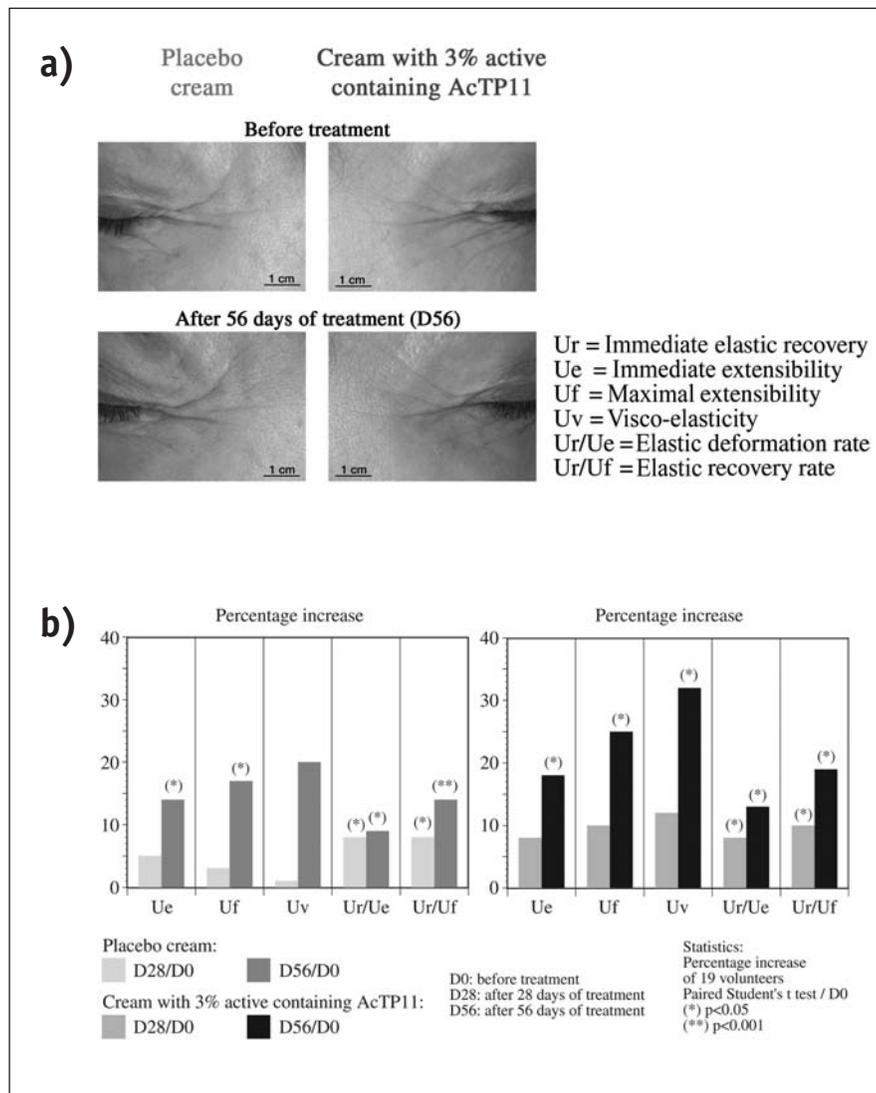


Figure 10. Evolution of cutaneous relief *in vivo* after treatment (D56); a) with 3% AI cream with AcTP11, versus placebo cream (macrophotography); and b) effect on biomechanical properties of the skin (torquemeter)

The significant increase in firmness and elasticity reflects an improvement of epidermal cohesion and consequently an antiaging effect.

Conclusions

The newly developed tetrapeptide AcTP11 was shown to have an *in vitro* stimulating effect on keratinocyte growth via a DNA array and growth test, and on syndecan-1 synthesis by keratinocytes via a DNA array, ICC and IHC. This increase of syndecan-1 level may induce a better homeostasis of the epidermis due to its multiple functions on skin organization, regulation and intercellular adhesion.

By stimulating the synthesis of type XVII collagen, shown by DNA array, q-RT PCR and ICC, AcTP11 also allowed for the improvement of cohesion between epidermis and dermis.

There is a discrepancy between the spirit of seniors and the appearance of their skin. Whereas they acquire strength and serenity with age, their skin on the contrary becomes more fragile and blemished as a consequence of epidermis weakening. By targeting two specific constituents of the epidermis responsible for its cohesion, syndecan-1 and type XVII collagen, an AI-based AcTP11 can help the skin to recover resistance, a refined texture and consequently better radiance, a growing market request in an aging society.

Published January 2008 *Cosmetics & Toiletries* magazine.

References

1. MA Stepp et al, Defects in keratinocyte activation during wound healing in the syndecan-1-deficient mouse, *J Cell Sci* 115 4517–31 (2002)
2. O Okamoto et al, Normal human keratinocytes bind to the 3LG4/5 domain of unprocessed laminin-5 through the receptor syndecan-1, *J Biol Chem* 278(45) 44168–11477 (2003)
3. DJ Carey, Syndecans: Multifunctional cell-surface co-receptors, *Biochem J* 327 1–16 (1997)
4. B Vuillermoz, Y Wegrowski, JL Contet-Audonneau, L Danoux, G Pauly and FX Maquart, Influence of aging on glycosaminoglycans and small leucine-rich proteoglycans production by skin fibroblasts, *Mol Cell Biochem* 277 63–72 (2005)
5. Y Wegrowski, L Danoux, JL Contet-Audonneau, G Pauly and FX Maquart, Decreased syndecan-1 expression by human keratinocytes during skin aging, *J Invest Dermatol* 125(5), Abstracts 029 (2005)
6. L. Robert, Tissue conjonctif I Introduction, in *Précis de physiologie cutanée*, France La porte verte Eds, (1980) pp131–136
7. AM Powell, Y Sakuma-Oyama, N Oyama and MM Black, Collagen XVII/BP180: A collagenous transmembrane protein and component of the dermoepidermal anchoring complex, *Experimental Dermatol* 30 682–687 (2005)

8. NA Fenske and CW Lober, Structural and functional changes of normal aging skin, *J Am Acad Dermatol* 15 571–585 (1986)
9. RL Gallo et al, Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds, *Proc Natl Acad Sci USA* 91 11035–9 (1994)
10. P Jaakkola and M Jalkanen, Transcriptional regulation of Syndecan-1 expression by growth factors, *Progress in Nucleic Acid Research & Molecular Biology*, 63 109–138 (1999)
11. W Vogel, Discoidin domain receptors: Structural relations and functional implications, *FASEB J* 13(Suppl.) S77–S82 (1999)
12. CA Curat and WF Vogel, Discoidin Domain Receptor 1 Controls Growth and Adhesion of Mesangial Cells, *J Am Soc Nephrol* 13 2648–2656 (2002)
13. E Faraci, M Eck, B Gerstmayer, A Bosio and WF Vogel, An extracellular matrix-specific microarray allowed the identification of target genes downstream of discoidin domain receptors, *Matrix Biol* 22 373–381 (2003)
14. C Escoffier, J De Rigal, A Rochefort, R Vasselet, PG Agache and JL Leveque, Age-related mechanical properties of human skin: An *in vivo* study, *J Invest Dermatol* 93(3) 353–357 (1989)
15. PG Agache, Twistometry measurement of skin elasticity, in *Noninvasive Methods & Skin*, J Serup eds, Denmark: GBE Jemec (1995) pp 319–328

Chitin Nanofibrils: A Natural Compound for Innovative Cosmeceuticals

Pierfrancesco Morganti and Gianluca Morganti

Mavi Sud, Aprilia (LT), Italy

Riccardo A.A. Muzzarelli and Corrado Muzzarelli

Polytechnic University, Ancona, Italy

KEY WORDS: *chitin nanofibrils, skin hydration, cutaneous aging, biofilm, corneocyte cohesion*

ABSTRACT: *Compared to larger-sized chitin particles, chitin nanofibrils can be hydrolyzed more easily by cutaneous enzymes, leading to applications such as rehydrating dry skin, augmenting cohesion of cells in the stratum corneum, and forming a protective biofilm that supports wound healing.*

Recent improvements in methods to isolate chitin nanofibrils from crustacean chitin suggest cutaneous applications in areas such as skin rehydration, wound healing and maintenance of cutaneous homeostasis.

Chitin (from the Greek χιτων for “protective coat”) represents the most important compound of the crustacean and insect cuticle, but it also occurs in the fungal cell wall.

Chemically, chitin is a polysaccharide formed by glucosamine and N-acetylglucosamine units having β -(1 \rightarrow 4) bonds. These same bonds are found in cellulose. They also are found in the hyaluronic acid of human skin and in other mammalian organs.

Although cellulose is a neutral polysaccharide, chitin is weakly cationic and chitosan is strongly cationic because of the glucosamine

effect. On the other hand, hyaluronic acid is an anionic polysaccharide made of *d*-glucuronic acid and N-acetyl glucosamine. The structural similarity among chitin, cellulose and hyaluronic acid suggests that plants, insects and mammals evolved from a single primordial bacterium.¹

Crystalline Chitin Nanofibrils

Microstructural hierarchy: Crystalline chitin nanofibrils have a microstructure typical of crustacean and insect cuticles. In the chitin structures present in nature, native chitin crystallizes following crystallographic patterns, giving origin to polysaccharide chains assembled precisely one after another by hydrogen bonds, as in the two edges of a zipper.

The resulting rigid substance is immersed in a matrix of proteins and calcium carbonate, which forms microfibers. The microfibers form layers of a few grades on one another. These staggered layers form a structure like plywood (**Figure 1**). Remarkable is the fact that the crystalline microfibers contain nanofibrils approximately 300 nm long and 10 nm in diameter with high structural and crystalline precision.^{2,3}

Recently, chitin nanofibrils have been studied in view of elucidating their structure, but only now it is possible to isolate them in large quantity and make them useful for practical applications. In acidic environments both beta- and gamma-crystalline conformations transform rapidly into the most stable alpha form. This alpha-nanocrystalline form is extracted from crustacean chitin according to a recently patented industrial process⁴ developed by the Mavi Sud company (**Figure 2**).

General characteristics: Nanofibrils are extremely small objects, observable only under the transmission electronic microscope; each of them is made of less than 20 polysaccharide chains that recognize each other. Their specific surface area is large: one gram of nanofibrils develops a surface of 180 m². Upon slow evaporation of their suspensions, they align side by side to form a film, as on a desktop covered entirely by well-aligned pencils. These films over a certain thickness are opaque, but when the thickness is less than 1 mg/cm² they are transparent.

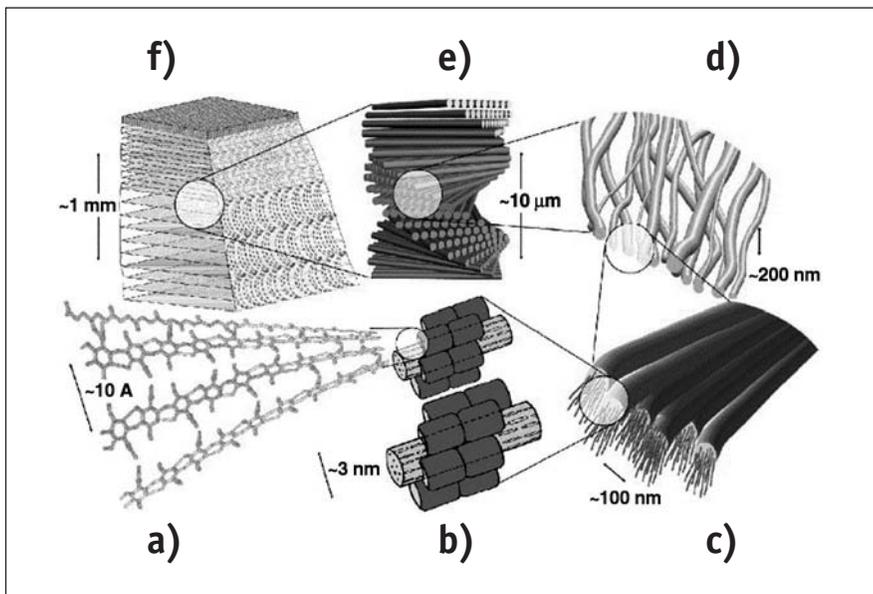


Figure 1. Hierarchy organization comprising six structural levels of chitin in crustaceans (from Reference 2)

- a) assembly of the chitin chains to form alpha crystals;
- b) nanofibrils (clear cylinders) surrounded by proteins (dark);
- c) settlement of nanofibrils in microfibrils of chitin and proteins;
- d) lamina of a net of fibers of chitin and proteins; calcite crystallizes in the openings;
- e) disposition of laminae with a rotating orientation visible under the optical microscope;
- f) structure of the cuticle (exo and endo) whose plane section shows the typical arched pattern as a consequence of the grades of rotation of the laminae.

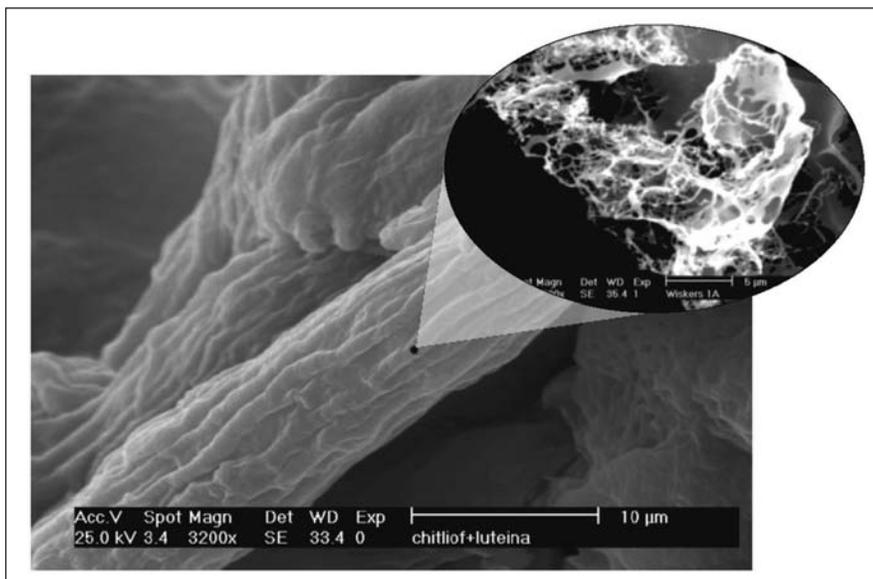


Figure 2. Chitin nanofibrils

Because of their small dimensions and of the large surface area, these nanocrystals are recognized by the enzymes as a more easily attackable material than solid substrates. In other words, an ubiquitous enzyme, such as human lysozyme, can hydrolyze chitin nanofibrils, but it is less active on the same chitin in flake form.

Applications: From a biological point of view, the crystalline nanofibrils applied on a wounded skin are able to form a protective biofilm, thin and elastic, capable of favoring tissue healing. In fact, they stimulate keratinocytes to grow fast and fibroblasts to produce the right amount and quality of collagen fibrils.⁵

For these reasons, chitin nanofibrils used in aesthetic surgery or in cosmetic formulations or to heal wounds can be considered N-acetyl glucosamine reservoirs able to release progressively due to the hydrolytic enzymes active on chitin.

Moreover, it should be recalled that during the last 10 years, human chitinases deemed not to exist in human tissues have been discovered: it has been observed that the defenses of the human organism include the ability to secrete chitinases called chitotriosidases.⁶

Chitin nanofibrils consist of a single substance; therefore, they are pure and organized and disposed in a regular way. This fact enables proteins to recognize chitin easily, as is amply demonstrated by the use of fluorescent lectins.

From a pharmaceutical or cosmetic point of view, nanofibrils represent a carrier able to favor the transcutaneous penetration of many active principles.

Because of the chemical bonds that can be established with many molecules, these nanofibrils are able to carry linked molecules in the different skin areas and at different times according to their typology and the formulation studied. For instance, coenzyme Q10 can be carried as on amorphous compound that can be easily assimilated by tissues.

An important evidence of this phenomenon of superficial adsorption is the capacity of chitin (and chitosan) to induce a fast blood coagulation following adsorption of some enzymes and blood platelets on its surface. This accelerated blood coagulation explains chitin's interesting surgical use in operations performed on varices, lungs, the esophagus and spleen.

The induction mechanism of hemostasis by chitin is redundant because it exploits simultaneously different biological phenomena, such as the interaction of blood platelets with chitin, a catalytic surface favoring the production of thrombin, and the accelerated formation of a fibrin coagulation.⁷ Moreover, nanofibrils induce the agglutination of the erythrocytes.^{8,9} Approximately 10 years ago the intravenous administration of fagocitable chitin particles of sizes 1–10 micron was suggested for the stimulation of alveolar macrophages, while chitin nanofibrils seemed advisable to obtain the production of interferon and a consequent improvement of the immune defenses.¹⁰

In veterinary practice, the positive clinical results obtained in the healing of animal wounds by the use of chitin in the form of sponges, flakes and powders, could be improved by the use of the more active chitin nanofibrils.¹¹

The metabolic activity of stratum corneum: The skin barrier, localized mainly at the level of the stratum corneum (SC), is formed by a particular tissue composed by corneocytes embedded into the extracellular matrix organized in lipidic unities with a lamellar nature (Figure 3).

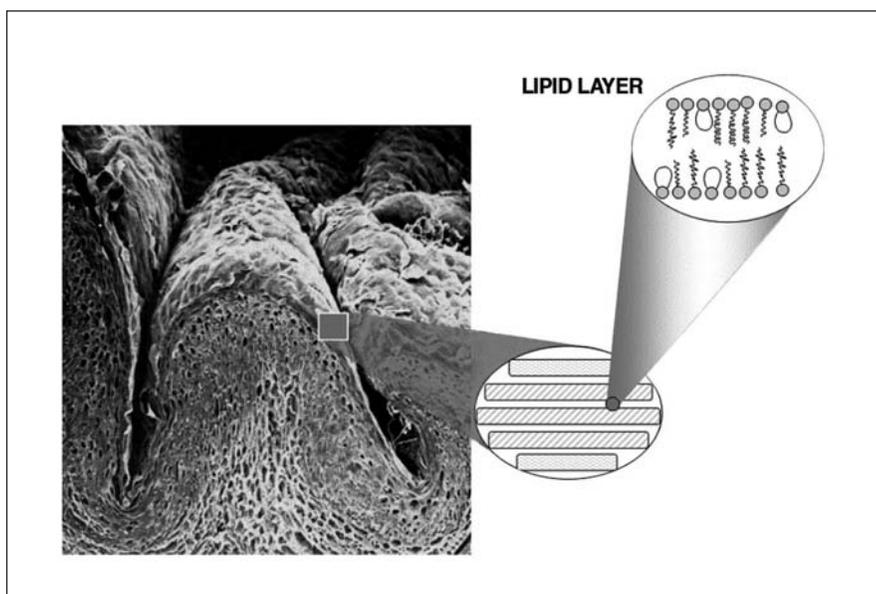


Figure 3. Organizational structure of the stratum corneum (composed skin)

By now, it is known that the SC is active metabolically in the production of hydrolase, proteases and other enzymes. These enzymes convert phospholipids, glycosphingolipids and other polar lipids into a nonpolar mixture of ceramides, cholesterol and free fatty acids that are necessary for the desquamating process (**Figure 4**).¹²

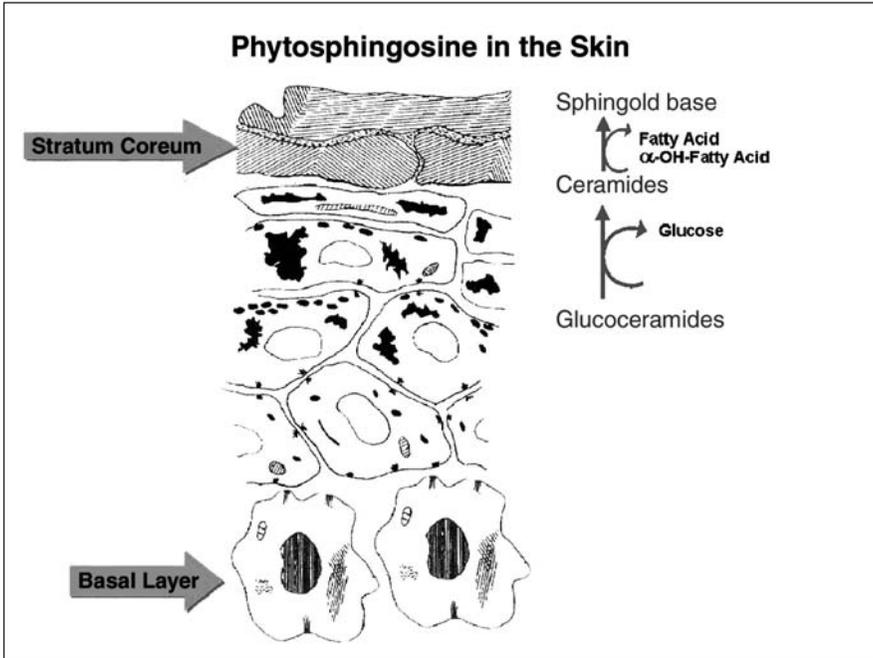


Figure 4. Conversion of glycosphingolipids in ceramides

These biochemical transformations cause such skin structural changes to lead to the formation of well-organized lipidic lamellae necessary to protect against an outer hostile environment (**Figure 5**).¹³⁻¹⁵ Any barrier alterations linked to the outer environmental modifications induced for example by cutaneous aggressions can cause a prompt response aimed to normalize this important function in hours or days.¹⁶

The initial response of the first 30 min is linked to the secretion of a pool of preformed lipidic lamellae followed within hours by the synthesis of cholesterol and fatty acids.¹⁷⁻¹⁹ If this physiological process is opposed by changes in stress or environmental humidity, the formation of keloids or hypertrophic scars may occur.²⁰

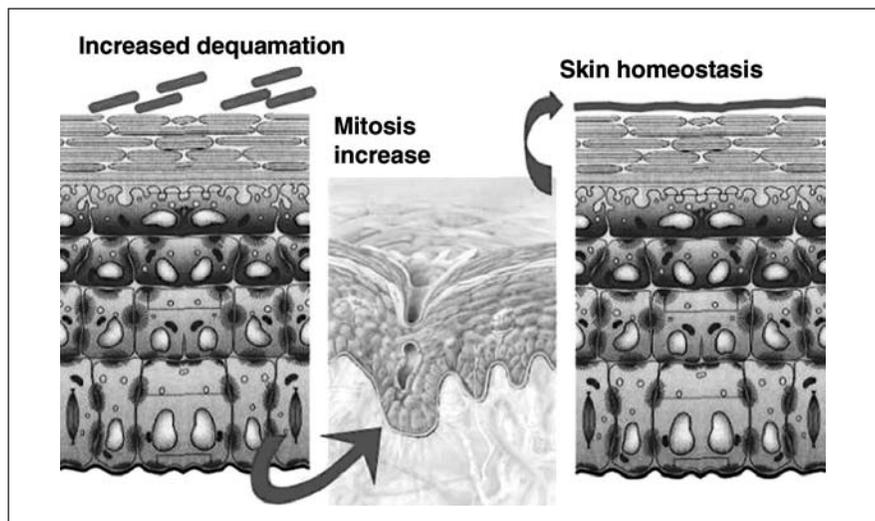


Figure 5. Skin turnover

Mechanism of Action

Chitin is recognized easily by the cutaneous enzymes and hydrolyzed. In this way, N-acetylglucosamine is able to regulate the collagen synthesis at the level of fibroblasts, facilitating also the granulation and the repair of the altered skin tissues.^{21,22}

On the other hand, glucosamine diffuses easily through all the biological membranes and enters into the cellular metabolism, working also as a molecule that is able to retain water at the level of the dermal extracellular matrix.^{23–25} In fact, some water present in the dermis is strongly linked to GAGs (glycosaminoglycans), which form the supporting gel for both the collagen fibers and the elastic ones.

Interestingly, the crystalline nanofibrils can be used to rehydrate the skin affected by more or less intense forms of xerosis (dry skin).

Moreover, as previously mentioned, these nanofibrils have the ability to connect cells such as corneocytes. In this way, nanofibrils augment the cohesion at the SC level, regulating perspiration. It is known that when the skin structure is damaged, the skin transpiration increases with a possible consequent appearance of psoriasis, atopic dermatitis and other signs of pathological dryness. Mavi's studies showed that the artificial barrier disruption induces or increases the

expression of several cytokines in the skin at the levels of mRNA and protein.

Thus the chitin nanofibrils' activity can be useful also in the presence of inflammatory processes caused, for example, by an excessive production of cytokines.

On the other hand the biological functions of cytokines involve the regulation of cell proliferation and function but can be both stimulatory and inhibitory. Therefore it now seems that the homeostatic control mechanism in the skin is a complex network of cytokines mediating interactions between resident cells (keratinocytes, Langerhans cells, endothelial cell and fibroblast) and T-lymphocytes, neutrophils and macrophages.

Their biological activity is, in fact, pleiotropic and flowery (**Figure 6**) and can be represented as a series of cascades in which the production of a cytokine stimulates the secretion of many cytokines that can interact among them in a synergic or antagonistic way.

Chitin nanofibrils act on the cells by modulating their production. Hence they have an interesting anti-inflammatory activity²⁶ and regulate at the same time the production of both collagen and cutaneous lipids present at the intercorneocitary lamellae level.²⁷

Healthy Aging

Effect of aging on cells: The aging process is not so much linked to the passage of time as to the overstock of negative events that deteriorate the body, mind and appearance.²⁸

Aging reduces the number of healthy cells of the body. The most destructive event in aging is represented by the loss of the reservoirs due to the reduction of number and functions of the cells of any organ, such as the skin. For example, the level of sugar in the blood remains almost constant during the entire lifetime, but the glucose tolerance decreases. Glucose tolerance measures the ability of human organisms to oppose the numerous stress phenomena.

With aging, the body loses the ability to maintain global homeostasis. This decadence is caused by the accumulation of free radicals that with their reactivity give rise to cellular death and accelerate the general and cutaneous aging process. In fact, they link to the skin proteins, collagen and elastin, damaging the walls of blood capillaries and altering the immune system.

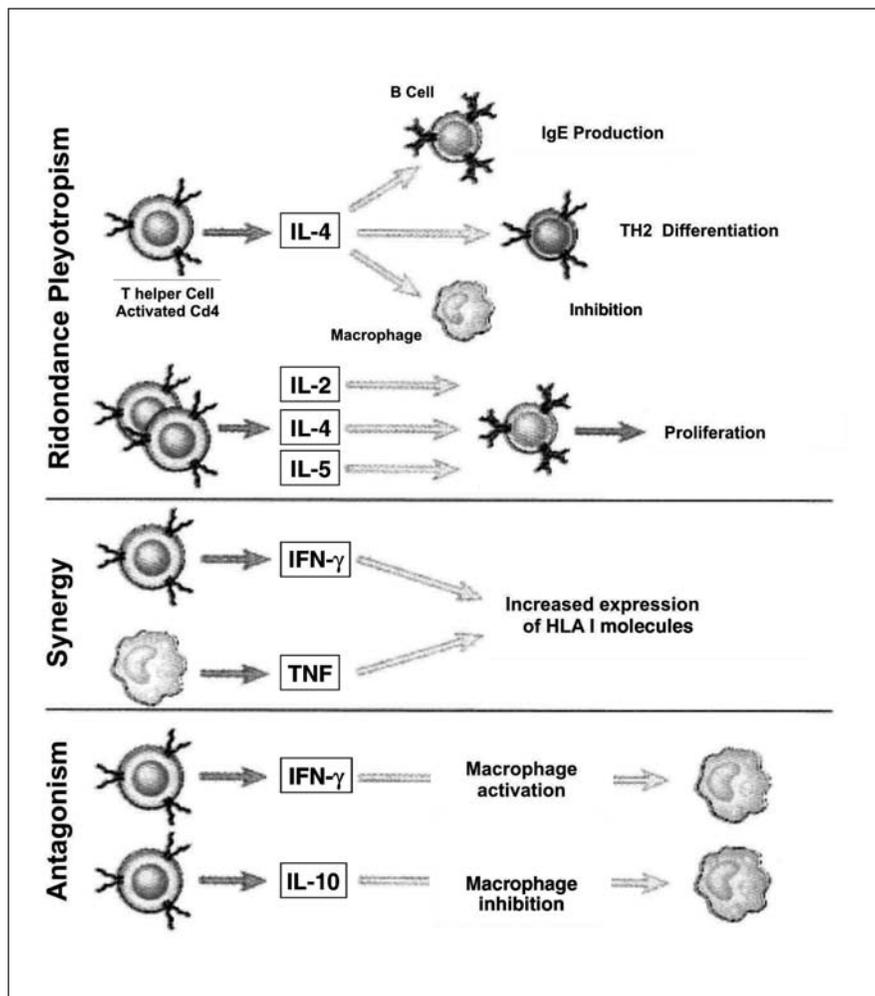


Figure 6. Cytokine cascades

Reintegration of cellular function: It is known that one of the negative effects of free radicals is to interfere at the level of the cellular membrane. The membrane alteration causes damage throughout the cell, compromising hundreds of biochemical reactions taking place continuously in its inner part.

Chitin nanofibrils, protecting both corneocytes and intercorneocitary lamellae, help to maintain the cutaneous homeostasis, neutralize the activity of free radicals and entrap them in their structure, and regularize the correct cellular turnover (Figure 7).

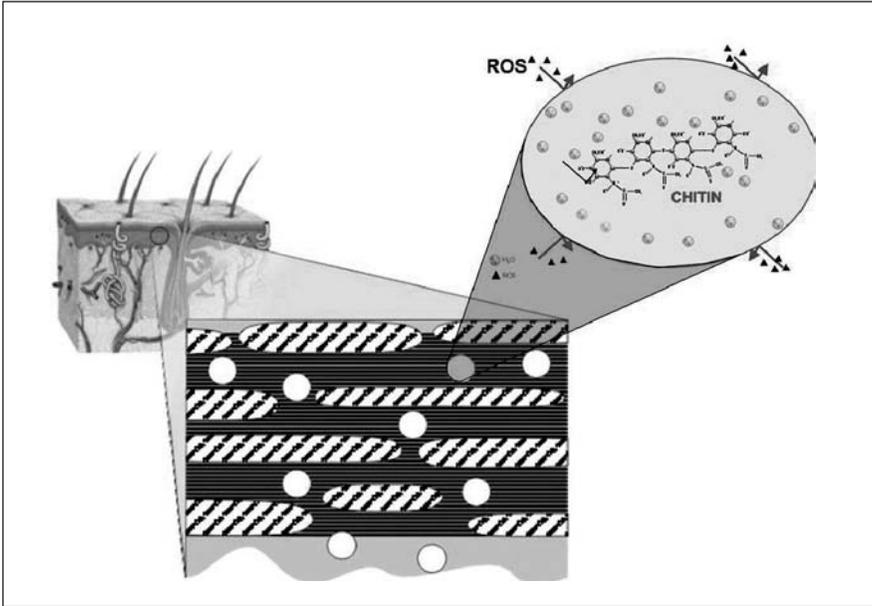


Figure 7. Protective activity of chitin

Summary

Seventy percent of the human body is water, an essential ingredient for performing all the body's functions. Water is everywhere. To appear healthy and beautiful, the skin must be always well-hydrated. A good hydration favors cellular metabolism and helps to give the skin firmness and suppleness.^{29,30}

Water as a natural element is necessary for the production of cosmetics, drugs and dietary products. Water as a basic health element is indispensable for cutaneous metabolism. A beautiful skin is also a healthy skin.

Chitin nanofibrils are useful as natural active principles able to promote health and beauty. Their inclusion in cosmetic and/or pharmaceutical solutions or emulsions generates the formation of a hygroscopic molecular film that slows down water evaporation and contributes to keep the skin perfectly hydrated as well. Soon-to-be-published studies⁵ on cell cultures and humans also demonstrate the safety and efficacy of formulations based on the use of chitin nanofibrils.

Their mechanism of action can be considered *active* because they repair the intercorneocitary cement that joins the ceramides and

joins the phospholipids to form of the lipidic lamellae. Moreover, they seem to help to protect the molecules of the natural moisturizing factor (NMF) present at the level of the corneocytes' membrane, forming a film around their membranes.

The use of the chitin nanofibrils as components of cosmetic or pharmaceutical carriers helps to restore the integrity of the skin barrier and to increase the capacity to link and retain the corneocytes' own water. In this way it is possible to obtain two results:

- A passive strategy linked to the formation of a film able to retard perspiration.
- An active strategy that reinforces the skin capacities to fix water and to protect itself against environmental assaults.

Finally, these nanofibrils have interesting applications in the production of special textiles to which chitin imparts peculiar characteristics of biocompatibility. These yarns can be largely used in the production of hypoallergenic clothing but also for manufacturing innovative bandages and gauzes to be used in the field of advanced medications.³⁰

It is of fundamental importance to use these nanofibrils appropriately, including them in carriers that maximize both the physicochemical properties and the biological properties in the setting of the cutaneous ecosystem.

Published February 2007 *Cosmetics & Toiletries* magazine.

References

1. BA Neudecker, AB Csóka, K Mio, HI Maibach and R Stern, In *Cosmeceutical: Drugs vs Cosmetics*, P Elsner and HI Maibach, eds, New York: Marcel Dekker (2000) pp 319–355
2. D Raabe, P Romano, C Sachs, A Al-Sawalmih, H-G Brokmeier, S-B Yi, G Servos and HG Hartwig, Discovery of a honeycomb structure in the twisted plywood patterns of fibrous biological nanocomposite tissue, *J Crystal Growth* 283 1–7 (2005)
3. D Raabe, P Romano, C Sachs, H Fabritius, A Al-Sawalmih, S-B Yi, G Servos and HG Hartwig, Microstructure and crystallographic texture of the chitin-protein network in the biological composite material of the exoskeleton of the lobster, *Homarus americanus*. *Materials Science and Engineering A* 421 143–153 (2006)
4. Patent Pending, Mavi (2006)
5. RAA Muzzarelli, P Morganti, P Palombo, M Palombo, G Bigini, M Mattioli-Belminite, F Giantomassi and C Muzzarelli, Wound care preparations based on chitin nanofibrils and chiosa, In print: *Biomaterials* (2007)
6. A Erikson, H Forsberg, M Nilsson, M Astrom and JE Mansson, Ten years' experience of enzyme infusion therapy of Norrbottnian (type 3) Gaucher disease. *Acta Paediatrica* 95(3) 312–317 (2006)

7. TK Fischer, HS Thatte, TC Nichols, DE Bender-Neal, DA Bellinger and JN Vournakis, Synergistic platelet integrin signaling and factor XII activation in poly-N-acetyl glucosamine fiber-mediated hemostasis *Biomater* 27 543 (2005)
8. D Kulling, JN Vournakis, S Woo, MV Demcheva, DU Tagge, G Rios, S Finkielstein and RH Hawes, Endoscopic injection of bleeding esophageal varices with a poly-N-acetyl glucosamine gel formulation in the canine portal hypertension model, *Gastroint Endos* 49 764–771 (1999)
9. MW Chan, SD Schwaizberg, M Demcheva, J Vournakis, S Finkielstein and RJ Connolly, Comparison of poly-N-acetyl glucosamine with absorbable collagen (Actifoam), and fibrin sealant (Bolheal) for achieving hemostasis in a swine model of splenic hemorrhage, *J Trauma* 48 454–457 (2000)
10. Y Shibata, LA Foster, WJ Metzger and QN Myrvik, Alveolar macrophage priming by intravenous administration of chitin particles, polymers of N-acetyl-D-glucosamine, in mice. *Infection and Immunity* 65 1734–1741 (1997)
11. Okamoto, S Minami, A Matsushashi, H Sashiwa, H Saimoto, Y Shigemasa, T Tanigawa, Y Tanaka and S Tokura, Application of polymeric N-acetyl-D-glucosamine (chitin) to veterinary practice, *J Vet Med Sci* 55 743–747 (1997)
12. L Williams, Lipid in normal and patho, In *Advances in Lipid Research: Skin Lipids*, PM Elias, ed, New York: Academic Press (1991) pp 211–252
13. PM Elias and GK Menon, Structural and lipid biochemical correlates of the epidermal permeability barrier, In *Advances in Lipid Research: Skin Lipids*, PM Elias, ed, New York: Academic Press (1991) pp 1–26
14. WM Holleran, Y Takagi, KR Feingold et al, Processing of epidermal glucosylceramide is required for optimal mammalian permeability barrier function, *J Clin Invest* 91 1656–1664 (1993)
15. M Mao-Qiang, M Jain, KR Feingold and PM Elias, Secretory phospholipase A2 activity is required for permeability barrier homeostasis, *J Clin Invest* 106 57–63 (1996)
16. T Mauro, WM Holleran, S Greyson et al, Barrier recovery is impeded at neutral pH, independent of ionic effects: implications for extracellular lipid processing. *Arch Dermatol Res* 290 215–222 (1998)
17. M Elias and R Ghadially, Geriatric dermatology, Part II: The aged epidermal permeability barrier: Basis for functional abnormalities, *Clin Geriatr Med* 18(1) 103–120 (2002)
18. PM Elias, Stratum corneum architecture, metabolic activity, and interactivity with subjacent cell layers, *Exp Dermatol* 5 191–201 (1996)
19. GK Menon, KR Feingold and PM Elias, The lamellar body secretory response to barrier disruption, *J Invest Dermatol* 98 279–289 (1992)
20. M Denda, T Tsuchiya, T Hirao et al, Stress alters cutaneous permeability barrier homeostasis, *Am J Physiol Regul Integr Comp Physiol* 278 R367–R372 (2000)
21. KR Feingold, BE Brown, SR Lear et al, Effect of essential fatty acid deficiency on cutaneous sterol synthesis, *J Invest Dermatol* 87 588–591 (1986)
22. KR Middleton and D Seal, Sugar as an aid to wound healing, *Pharm J* 235 757–759 (1985)
23. J Kössi, M Vähä-Kreula, S Peltonen, J Risteli and M Laato, Effect of sucrose on collagen metabolism in keloid, hypertrophic scar, and granulation tissue fibroblast cultures, *World J Surgery* 25(2) 142–146 (2001)
24. M Adams, About glucosamine, *Lancet* 354 353–354 (1999)
25. B Berra, Glucosamina, In *Nutraceuticals*, C Rapport and B Lockwood, eds, Milan: MDM Medical Media (2003) pp 17–29
26. Mavi, data on file (2006)
27. M Mao-Qiang, L Wood, PM Elias and KR Feingold, Cutaneous barrier repair and pathophysiology following barrier disruption in Il-1 and TNF Type 1 receptor deficient mice, *Exp Dermatol* 6 98–104 (1997)
28. B Gilchrist and J Krutmann, *Skin Aging*, Berlin: Springer Verlag (2006)
29. JJ Leyden and AV Rawlings, *Skin Moisturization*, New York: Marcel Dekker (2002)
30. P Morganti, RAA Muzzarelli and C Muzzarelli, Multifunctional use of innovative chitin derivatives for skin care, *Proceedings: Tessile & Salute, Biella, 4–5 May 2006* (2006)

Biomimetic Tripeptides for Improved Dermal Transport

Hugo Ziegler and Marc Heidl

Pentapharm Ltd., Basel, Switzerland

KEY WORDS: *peptides, tripeptides, biomimetics, cosmetic ingredients, antiaging, anti-wrinkle*

ABSTRACT: *Two novel biomimetic tripeptides, described here, have been found to enhance the delivery of antiaging benefits including collagen stimulation and treatment of mimic wrinkles. The authors describe their application as cosmetic ingredients.*

Proteins and peptides play an important role in skin health, not only as cutaneous structural components such as collagens, elastin, keratin, fibronectin, integrins and laminin, but also as inhibitors (proteases), stimulating agents (growth factors such as TGF-beta) or regulators (protein kinase C or interleukin IL-1) of biochemical processes in the skin. Small peptides often are responsible for a biochemical effect—they are either released from larger units by proteolysis or they exert their effect as a partial sequence of a protein due to a conformational change.

Probably the best known example of a tripeptide released by proteolysis after tissue or skin injury is H-Gly-His-Lys-OH (GHK). This tripeptide binds copper (II) ions as a ligand and accelerates wound healing as a copper complex.¹ The synthetic copper peptide is marketed as a cosmetic active ingredient.

The carboxy terminal tripeptide Lys-Pro-Val-OH (KPV) of the alpha-melanocyte-stimulating hormones (alpha-MSH) is described

as the shortest sequence that is responsible for the anti-inflammatory effect of alpha-MSH.² As can be seen from the different published patent applications,³⁻⁵ the cosmetic industry is attempting to develop active ingredients by optimizing the structure of the KPV tripeptide to promote epidermal renewal, improve the skin barrier function, or prevent cutaneous inflammation.

Thrombospondin-1 (TSP), a high molecular weight glycoprotein, contains a tripeptide sequence -Arg-Phe-Lys- (RFK) that is essential for activating the transforming growth factor beta (TGF-beta).^{6,7} TGF-beta is a multifunctional cytokine that regulates cell proliferation and differentiation, tissue remodelling and repair, thus inducing the synthesis of extracellular matrix proteins.⁸ Based on this knowledge, an active ingredient for reducing age-related and photo-induced wrinkles was successfully developed.

Palm-Lys-Val-Lys-OH

Few experiments were necessary to reveal that the original sequence pattern—basic amino acid, hydrophobic amino acid and basic amino acid—is required for the efficacy of such substances. Replacing one of the basic amino acids by a nonbasic amino acid leads to totally inactive compounds.

By maintaining this pattern, the most differing variations were tried on the three individual amino acids as well as on the *N*- and *C*-terminus. Part of the results obtained from the different test series previously has been presented.⁹ The compound found to be the most effective, safest and least expensive as far as preparation costs, Palm-Lys-Val-Lys-OH^a, was developed as a cosmetic antiaging ingredient. **Figure 1** shows the result of a molecular-modelling calculation of the lowest energy conformation.

Figure 2 illustrates, in a simplified manner, how TSP binds to the LAP protein of the TGF-beta LAP complex, leading to a conformational change: The LAP-TSP complex cleaves off and TGF-beta is released in the mature form. Further studies have shown that Palm-Lys-Val-Lys-OH has the ability to bind in the same way.¹⁰

^a SYN-COLL (INCI: palmitoyl tripeptide-5) is a product of Pentapharm Ltd., Switzerland.

The permeation rate of a substance through the stratum corneum mainly depends on two parameters; namely the distribution coefficient, P , between *n*-octanol and water—generally termed $\log P$, and the molecular weight (MW).¹¹ Of course, other molecular properties such as number of donor and acceptor hydrogen bonds, ionization potential and water solubility are not totally insignificant, but can be neglected when comparing permeation rates within a similar substance class.

The higher the MW, the more difficult the penetration in skin, whereby the permeation rate strongly decreases at a MW >500 Dalton.¹² The optimal skin permeation rate can be expected within the $\log P$ range between +0.5 and +1.5, as shown for alkylnicotinates.¹³

Unsubstituted tripeptides are very hydrophilic with partly highly negative $\log P$ values, which can amount to -3 depending on the amino acids.¹⁴ The substitution of such peptides with fatty acids increases their $\log P$ and palmitic acid allows an optimal permeation of Palm-Lys-Val-Lys-OH.

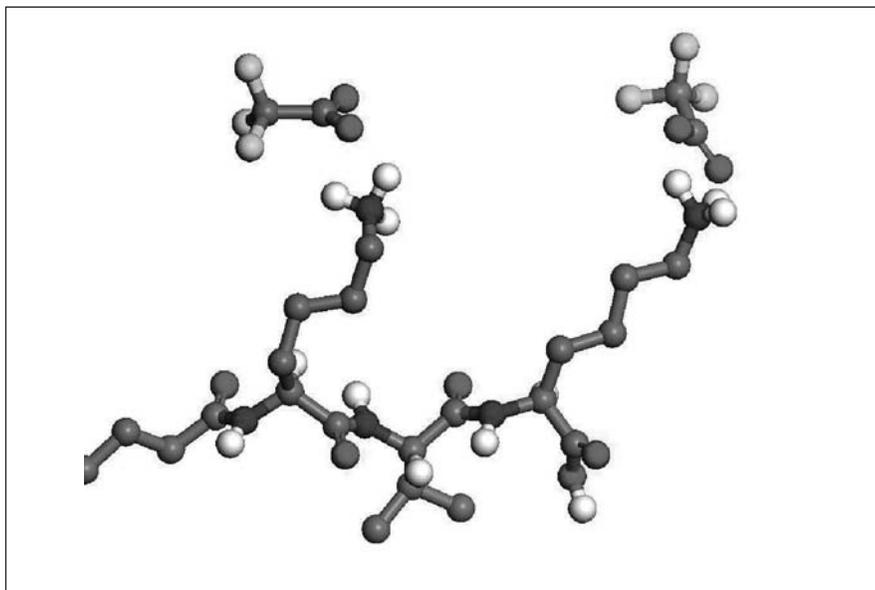


Figure 1. Calculated geometry of the lowest-energy conformation of the peptide part of palmitoyl tripeptide-5 in ball-and-stick representation

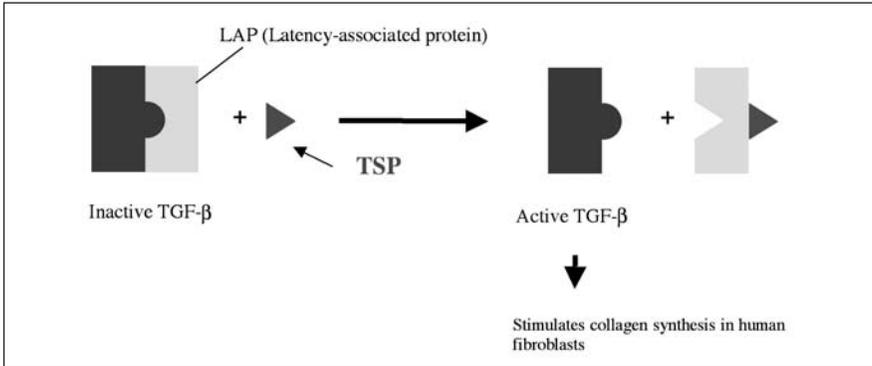


Figure 2. Simplified model of TGF-beta activation, according to literature⁷

***In vitro* Efficacy**

Setup: Type I collagen of *in vitro* cultured skin fibroblasts is detected with an enzyme-linked immunosorbent assay (ELISA). The human skin fibroblasts isolated from foreskin are incubated until confluent at a density of approx. 5000 cells per well in 96 well-plates in culture medium (37°C/5% CO₂) for three days. The medium is replaced with test medium with three different concentrations of test substance in triplicate. The quantitative determination of collagen production in the presence of the peptidic actives is performed using an antibody specific for collagen I.

Results: **Figure 3** clearly illustrates the dose-dependent effect of the peptide. The untreated cells (negative control) correspond to 100%. TGF-beta in a concentration of 0.4 nM acts as the positive control.

In view of the excellent *in vitro* results, this particular sequence was developed further as a cosmetic ingredient.

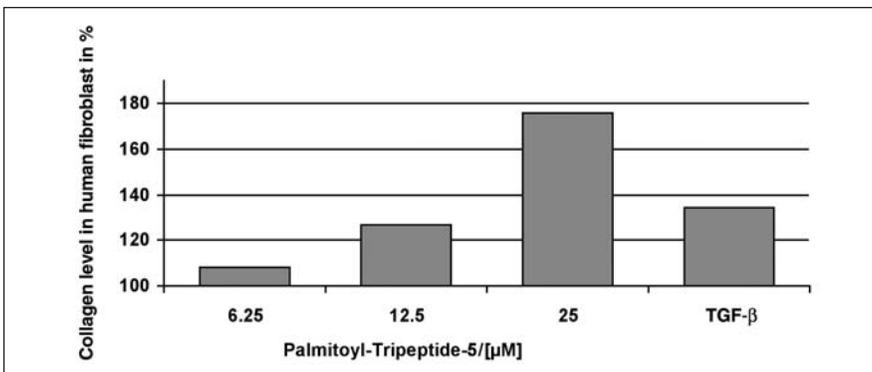


Figure 3. Dose-dependent effect of palmitoyl tripeptide-5 on collagen I synthesis

In vivo Efficacy

Setup: Four groups of 15 volunteers each applied a test cream on one-half of the face twice daily for 84 days. The test cream consisted of: 1.0% of a 1000 ppm solution of palmitoyl tripeptide-5 in 70% glycerine; 2.5% of a 1000 ppm solution of palmitoyl tripeptide-5 in 70% glycerine; the peptide content equivalent to 10% of a competitor's product; and no active ingredient (placebo). The effects were analyzed by 3D *in vivo* digital profilometry measurement scans^b and macro-photographs.

From the scans, the following three cutaneous parameters were calculated:

Ra: The average roughness; the ratio between the integrated surface around the mean value and the length of skin evaluated. A decrease of the Ra parameter expresses a smoothing effect.

Rt: The maximum relief amplitude; the maximum difference between the highest peak and the lowest valley registered over the entire profile.

Rz: The average relief on five regions of the profile; the mean value of these different maxima, obtained on five successive regions of the profile. This parameter reflects local differences. A decrease of the Rt and Rz parameter expresses an anti-wrinkle effect.

Results: It could be demonstrated that deep wrinkles were significantly reduced within three months and that the overall skin appearance was improved (see **Figure 4**). The color-coded 3D-profile after the treatment showed no more area coded in black, which corresponded to wrinkles that were deeper than 220 micrometers. The areas coded in yellow corresponded to hills in the profile and clearly were reduced, thus confirming the observation that the overall profile height was reduced after treatment with the peptide.

From the data shown in **Figure 5** it was concluded that the anti-wrinkle effects of Palm-Lys-Val-Lys-OH outperformed the effects of the well-known collagen synthesis inducing peptide Palm-Lys-Thr-Thr-Lys-Ser-OH in all three measured parameters after 84 days.

^b PRIMOS imaging system is a device of GFM Teltow, Germany

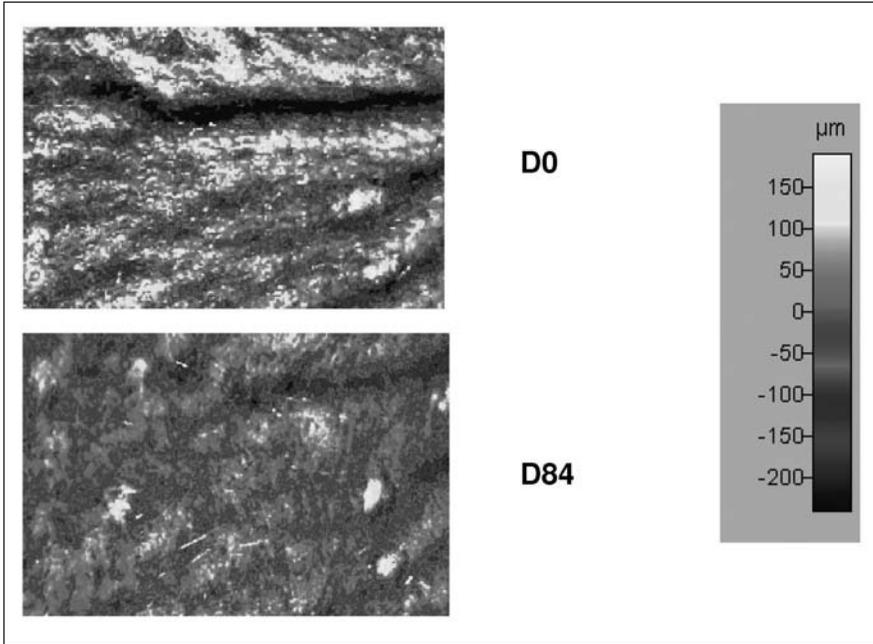


Figure 4. 3-D scans of volunteer 16 treated with palmitoyl tripeptide-5 (25 ppm) during 3 months

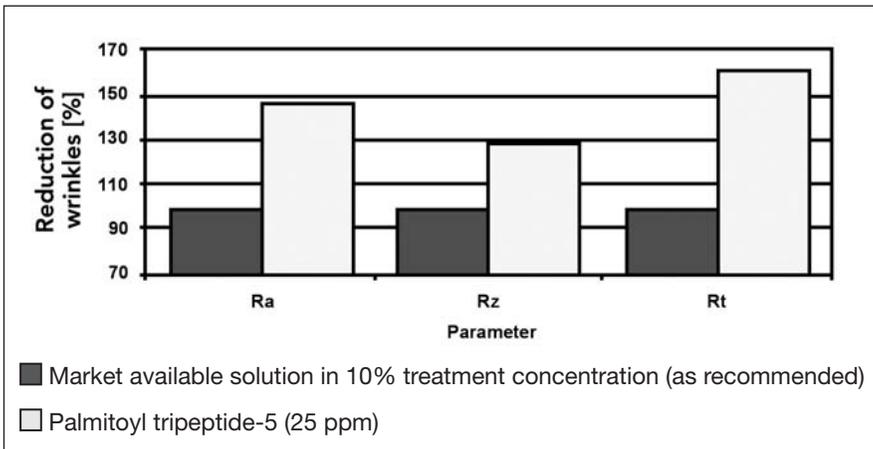


Figure 5. Comparison of palmitoyl tripeptide-5 and the well-known collagen synthesis-inducing peptide Palm-Lys-Thr-Thr-Lys-Ser-OH

H-beta-Ala-Pro-Dab-NH-benzyl diacetate

Human peptides and peptide sequences are not the only ones that are useful in cosmetic applications. Those from foreign organisms can also be used as models for the development of cosmetic actives.

Researchers from Pentapharm have applied thorough knowledge of the mode of action of snake venom components to explore fully new pathways.

Mimic wrinkles—wrinkles on the forehead, around the mouth and crow's-feet—could until recently only be reduced medically through the subcutaneous injection of Botox^c, a highly efficient neurotoxin and metabolite of *Clostridium botulinum*. Its effect is based on the relaxing of wrinkle-producing muscles by inhibiting the acetylcholine release.

Some snake venoms cause muscle relaxation as well. Waglerin-1 is known to selectively block the epsilon-subunit of the muscular nicotinic acetylcholine receptor.¹⁵ Waglerin-1 is a peptidic component of the venom of the arboreal pit viper *Tropidolaemus wagleri* from Southeast Asia; it is composed of 22 amino acids. Noticeable in this sequence is the frequent apparition of the units: basic amino acid-proline or proline-basic amino acid or basic amino acid-proline-basic amino acid. (See **Figure 6**.)

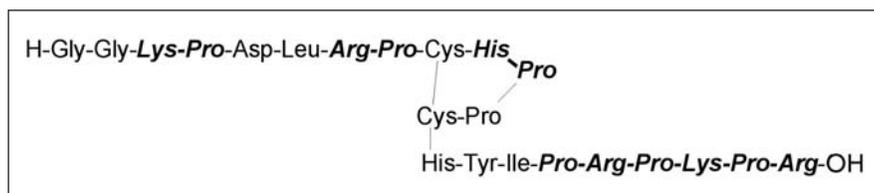


Figure 6. Structure of Waglerin-1; successive prolines and basic amino acids are bold-typed

The corresponding synthesized dipeptides and their derivatives did not show muscle-relaxing properties. This effect could, however, be surprisingly found with the tripeptide sequence of the type basic amino acid-proline-basic amino acid.

As each amino acid situated at the amino terminus of a sequence—the amino function of which is not acylated—shows basic properties, the *N*-terminal basic amino acid may be replaced by any other amino acid. A large number of effective peptides could be synthesized in this way and have been filed in a patent application.¹⁶

The *in vitro* and *in vivo* effects are illustrated by using the compound H-beta-Ala-Pro-Dab-NH-benzyl diacetate^d (**Figure 7**),

^c Botox is a registered trade name of Allergan, Inc., Irvine, CA.

^d SYN-AKE (INCI: Diaminobutyroyl benzylamide diacetate) is a product of Pentapharm Ltd., Switzerland.

which was found to imitate the most typical structural attribute of the 22 amino acid peptide Waglerin-1 best while maintaining an excellent muscle-relaxing efficacy. This compound has been developed as an anti-wrinkle product.

Within this compound class, the benzyl group proved to be the most useful modification for a favorable dermal uptake of the active ingredient. This is because unsubstituted tripeptides with basic side chains are highly water soluble compounds with a low n-octanol/water partition coefficient ($\log P < 0$) for a successful penetration into the stratum corneum. The introduction of a benzyl group increases the $\log P$ of the molecule for a favorable dermal uptake.

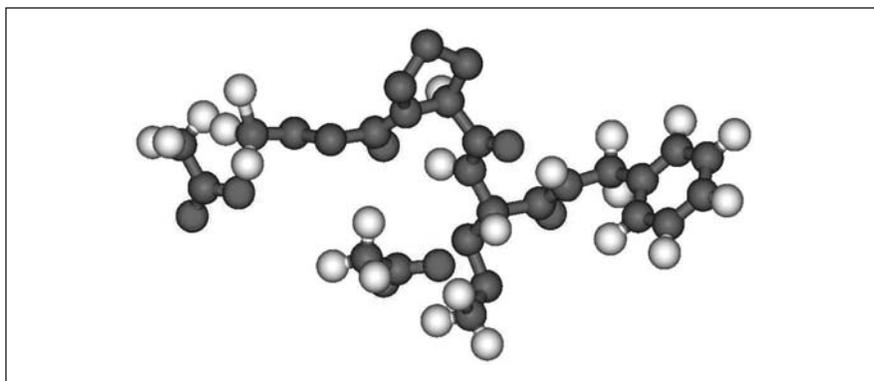


Figure 7. Chemical structure of H-beta-Ala-Pro-Dab-NH-benzyl diacetate in the lowest energy conformation—in ball-and-stick representation

***In vitro* Efficacy**

Setup: The test model was established¹⁷ as a co-culture of human muscle cells and neurons derived from the spinal cord of rat embryos. Active compounds theoretically should block the neuromuscular contraction signal. The contraction frequency was measured with an inverted microscope equipped with a video camera during 30 sec. The peptides were diluted in culture medium and added at different concentrations.

Again, the contraction frequency was measured during 30 sec, 1 min, 2 hr, 2 days and 4 days, after addition of the peptides. After two days the co-culture was checked visually and the contraction frequency was measured before and after addition of glucose to the

cells (final concentration 1 g/L) to exclude muscle blockage due to possible glucose depletion.

Results: As shown in **Figure 8**, H-beta-Ala-Pro-Dab-NH-benzyl diacetate reduced muscle cell contraction after 1 min. The maximal contraction decrease (82%) was measured after 2 hr, which meant that the compound was fast-acting. With a contraction decrease of 67% after two days, the effect was demonstrated to be long-lasting. After four days, total reversibility was determined.

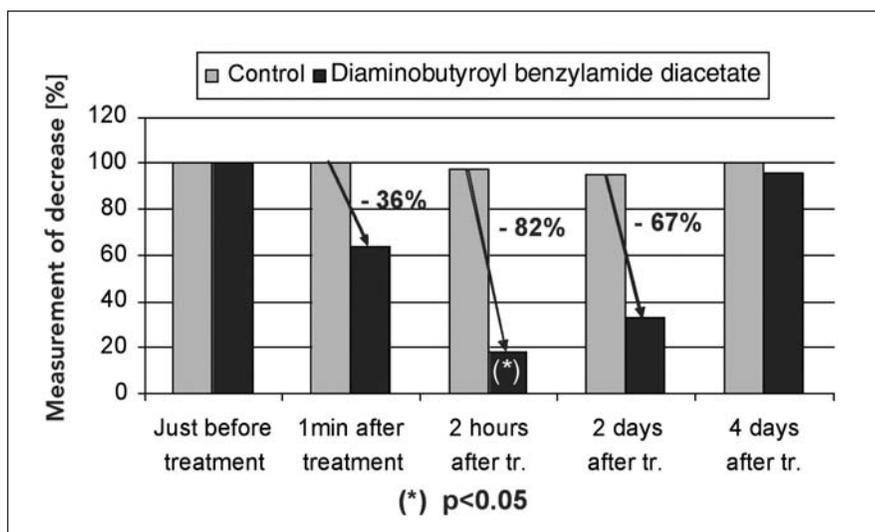


Figure 8. Frequency of contraction of the innervated muscle cells as a function of the incubation time (Peptide concentration: 0.5 mM; p = significance level)

***In vivo* Efficacy**

Measurements were taken of the smoothing (R_a) and anti-wrinkle effects (R_z and R_t) of 4% of a solution of H-beta-Ala-Pro-Dab-NH-benzyl diacetate in glycerol (70%) after 28 days.¹⁸ The 4% H-beta-Ala-Pro-Dab-NH-benzyl diacetate solution corresponds to 100 ppm (~ 0.2 mMol) peptide.

Setup: The anti-wrinkle efficacy was evaluated by a double blind and intra-individual study on 45 volunteers using the scanning technique previously described^b. Three groups of 15 volunteers each applied a cream with 4% H-beta-Ala-Pro-Dab-NH-benzyl diacetate solution, a cream with 10% of a reference product, or a placebo

on the face (forehead and crow's feet) twice daily for 28 days. The three cutaneous parameters described previously—Ra, Rt and Rz—could were analyzed.

Results: H-beta-Ala-Pro-Dab-NH-benzyl diacetate exhibited a significant anti-wrinkle effect measured on the “horizontal wrinkles” of the forehead (see **Figure 9**); a decrease of the Ra, Rt and Rz parameters was noted: -21%, -20% and -15%, and was about five times more effective than the reference compound Acetyl-Glu-Glu-Met-Gln-Arg-ArgNH₂.

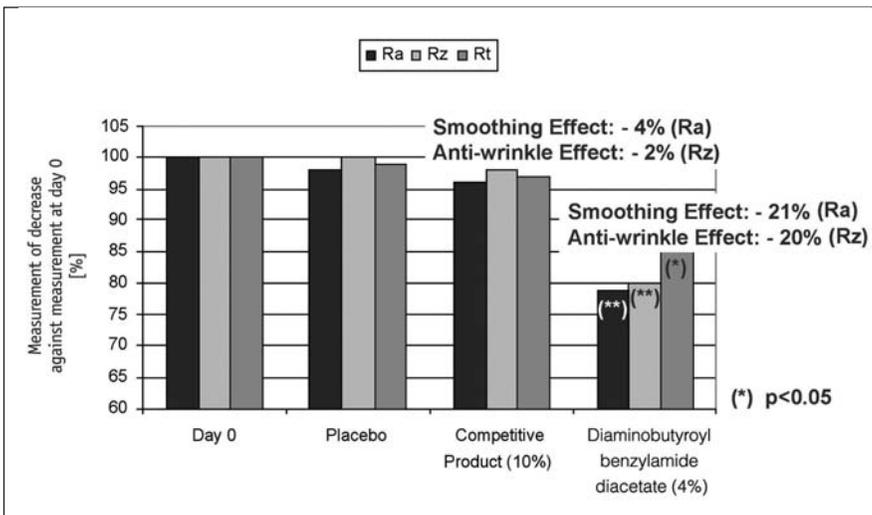


Figure 9. Measurement of the smoothing (Ra) and anti-wrinkle effect (Rz and Rt) of H-beta-Ala-Pro-Dab-NH-benzyl diacetate (4%) measured on the forehead after 28 days, compared with a competitive product (10%) and with a placebo; p = significance level

Moreover, an improvement of the cutaneous relief of the crow's-feet was observed for a majority of volunteers—68% for Ra, 77% for Rz and 69% for Rt, respectively (see **Figure 10**).

In view of its originality and high degree of innovation, this product was awarded the Swiss Technology Award 2006.

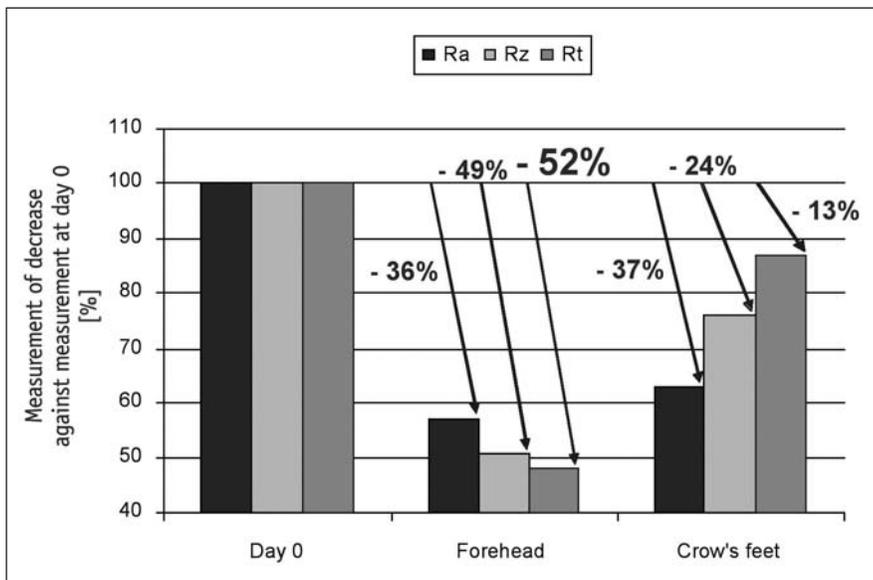


Figure 10. Measurement of the smoothing (Ra) and anti-wrinkle effect (Rz and Rt) of an emulsion containing 4% of a solution of 2500 ppm H-beta-Ala-Pro-Dab-NH-benzyl diacetate in 70% glycerol for volunteer #29, measured on the forehead and on crow's-feet after 28 days

Conclusions

Understanding the biochemical processes of natural active ingredients has led to the development of two biomimetics to improve the dermal transport of effects such as wrinkle prevention and reduction. These materials have been synthetically optimized in two aspects: economically—the natural structural complexity was simplified while maintaining or improving the biological efficacy; and efficaciously—substituents were introduced to increase the stability and improve the dermal and cellular transport.

Combining Palm-Lys-Val-Lys-OH—which stimulates collagen synthesis and thus acts against accelerated collagen degradation induced by endogenous and exogenous skin aging—with H-beta-Ala-Pro-Dab-NH-benzyl—which reduces mimic wrinkles by muscle relaxation—allows for the complete rejuvenation of the skin.

Lipophilic substituents, either at the terminal amino group or at the terminal carboxyl group, increase the logP and make the peptides more lipophilic, allowing them a much better skin delivery.

References

1. L Pickart, Copper peptides for tissue regeneration, *Specialty Chemicals* 29 (Oct 2002)
2. RJ Elliott et al, alpha-Melanocyte-stimulating hormone, MSH 11-13 KPV and adrenocorticotrophic hormone signalling in human keratinocyte cells, *J Invest Dermatol* 122 1010–1019 (2004)
3. WO 2003002086 and WO 2003002087, Y Mahé (Jan 09, 2003)
4. WO 2002080858, JM Lipton and AP Catania (Oct 17, 2002)
5. WO 8800833, JM Lipton (Feb 11, 1988)
6. J Lawler, Thrombospondin-1 as an endogenous inhibitor of angiogenesis and tumor growth, *J Cell Mol Med* 6 1 1–12 (2002)
7. JE Murphy-Ullrich and M Poczatek, Activation of latent TGF-beta by thrombospondin-1: Mechanisms and physiology, *Cytokine Growth Factor Rev* 11 59–69 (2000)
8. L Yin, A Morita and T Tsuji, The crucial role of TGF-beta in the age-related alterations induced by ultraviolet a irradiation, *J Invest Dermatol* 120 4 703–705 (2003)
9. M Heidl, D Imfeld, M Stöckli, H Ziegler, Structure activity relationship of peptide derivatives stimulating collagen synthesis in human fibroblasts, 23rd IFSCC, Orlando, FL, USA (Oct 24–28, 2004)
10. D Imfeld, M Stöckli, M Heidl, H Ziegler, T Schreier, Stimulation of collagen I synthesis by tripeptide via activation of latent transforming growth factor beta 1, 24rd IFSCC, Osaka, Japan (Oct 16–19, 2006)
11. RO Potts and RH Guy, Predicting skin permeability, *Pharm Research* 9 663–669 (1992)
12. JD Bos and MMHM Meinardi, *Exp Dermatol* 9 165–169 (2000)
13. J Arct and E Starzyk, *SÖFW Journal* 129 9 2–9 (2003)
14. IB Golovanov and IG Tsygankova, Quantitative structure-activity relationship: IX. Estimation of logP for some peptides, *Russ J General Chem* 72 1 146–152 (2002)
15. JJ McArdle et al, Waglerin-1 selectively blocks the epsilon form of the muscle nicotinic acetylcholine receptor, *J Pharmacol Exp Ther* 289 1 543–550 (1999)
16. WO Patent 047900 (2006)
17. R Steinschneider, Technical Report No RS040801V2: Bioalternatives (2004)
18. C Jossan, Technical Report No #04E1729: Laboratoires DermScan (2005)

Strategies of Antiaging Actives in Sunscreen Products*

Claire Mas-Chamberlin, Philippe Mondon, François Lamy, Olivier Peschard and Karl Lintner

Sederma, Paris, France

KEY WORDS: *antiaging, sunscreens, photoaging, vitamins, superoxide dismutase, glutathion peroxidase, catalase, barrier repair, hydroxy acids, ceramides, tissue repair, matrikine*

ABSTRACT: *The formulation of sunscreen products becomes more and more sophisticated by the addition of classical skin care benefits. Relevant active ingredients can help prevent sun damage or treat the UV-induced age symptoms.*

Antiaging actives have joined sunscreen actives in sunscreen formulations, where they enable real benefits and broader claims. But their definitions are less precise and their regulatory status is more complex. Formulators and marketers who understand the two strategies of antiaging actives in sunscreen products will be able to make better products and supportable claims.

Definitions

In cosmetics, as in so many other domains, a blurring of frontiers exists, a mixing of categories, a gradual disappearance of clear distinctions and definitions. What are sunscreens? What are antiaging actives?

* Adapted from Chapter 33 in *Sunscreens: Regulations and Commercial Development*, 3rd edition, edited by Nadim Shaath, published in 2005 (ISBN 068247-5794-7) by Taylor & Francis in Boca Raton, Fla., USA.

Sunscreen: The term *sunscreen* usually designates the finished cosmetic consumer product that bears a clear message of “protection against solar radiation.” This includes product classifications such as sun tan lotions, sun care products and sun blocks. The chemicals contained in these sunscreens are called UV filters or UV reflectors. (See **Why Add Antiaging Actives to Sunscreen Products?**)

More and more often, today’s sun product contains additional specific skin or body care active ingredients accompanied by a corresponding claim. On the other hand, an increasing number of classical skin care products for face care, lip care, makeup or body care boast SPF’s in the 5–15 range. These products have primary skin care claims (such as moisturizing, wrinkle diminishing and firming) and offer the sun protection as an additional benefit. So where is the borderline between the two?

Active: The term *active* has been defined as any cosmetic ingredient with the following characteristics:¹

- It has demonstrated cosmetic activity on human skin or its appendages.
- A substantiated claim has been made for it.
- It has a plausible story to go with it.

Why Add Antiaging Actives to Sunscreen Products?

If modern UV filters are so well-suited to protect skin against the sun’s dangerous rays, for what reason, other than a marketing or label claim, should the formulator of a modern sunscreen product add antiaging actives?

The first and most important reason is that UV filters are not absolute: any well-chosen additional active in the product will help decrease the damages that are not prevented by the UV filter. Secondly, the trend in all cosmetic formulas and products goes that way: makeup, mascaras, foundations, lipsticks, powders, cleansers, body care and scalp care SKUs all contain actives for additional benefits.

True skin care needs an approach that is global and continuous. Humans need sunlight for the synthesis of vitamin D and for psychic well-being (i.e., a healthy tan), and they desire silky, youthful skin: for this they need the optimum combination of sunscreen filters and skin care actives.

Actives under this definition encompass the wide field of ingredients from various types of plant extracts known as botanicals to pure chemical entities. These ingredients, such as those listed in the *International Cosmetic Ingredient Dictionary and Handbook*, possess a function that clearly is different from the galenic purpose of ingredients such as emulsifiers, texturizers, thickeners, preservatives or fragrances.

Antiaging: Finally, the term *antiaging* is catchy and seductive, but very vague. Some countries have regulations forbidding the use of this claim in cosmetic advertising or on the packaging.

The concept of antiaging has two strategies: prevention and treatment. Prevention implies that a consumer product helps “reduce the speed of the appearance of the clinical signs of cutaneous aging,” based on the protective active(s) contained in the product. Treatment promises to reverse some of the visible signs of skin aging, based on actives that “restore, regenerate, repair ...” skin items such as barrier, extracellular matrix, collagen fibers and hydrolipid balance.

This article describes both strategies of antiaging actives and their respective merits in sunscreens.

Prevention of Damage

Prevention of damage can be described as slowing down the aging process. It can be accomplished in sunscreen products with antiaging actives such as vitamins, botanicals and enzymes.

Vitamins: One of the first and most widely used categories of actives formulated in sunscreens is the one comprising the vitamins C and E, sometimes A (retinoids) or a few of the B group.

An impressively large body of literature documents the effects of vitamins C and E as photoprotective agents on animals and *in vitro* in cell cultures. Although general consensus is expressed that the protective effect afforded by these molecules might be beneficial to human skin, there is astonishingly little documentation of the benefits of vitamin use in cosmetic finished products, especially sunscreens, *in vivo* on human skin. Pehr and Forsey concluded in 1993, that “after 44 years of research there is still scant proof of vitamin E’s effectiveness [...]; it is of no use in [...] skin damage induced by ultraviolet light.”² A quick overview of the literature, however, shows

that research into the effects of topical vitamin application continues, with many papers focusing on combinations of vitamins such as C and E or A and E.

Vitamin E, or alpha-tocopherol, is a ubiquitous, liposoluble molecule. Its major activity is as an antioxidant. Although more powerful antioxidants can be found in nature, vitamin E is most accessible, by synthesis or extraction. It is colorless and well-documented as toxicologically safe. Ritter et al.³ showed the beneficial effects of tocopherol applied *before* UV irradiation on mice. These authors noted an increase in epidermal thickness, which might contribute to the decrease in the number of sunburn cells. The concentration of tocopherol in the vehicle (50% in ethanol) is however quite unrealistic in cosmetic and sunscreen applications.

Saral et al.⁴ studied vitamin E acetate (the more stable ester form of vitamin E that is preferentially employed in sunscreens) by applying it topically for three weeks on guinea pigs before a single UVB dose. Measuring lipid peroxide levels and enzyme scavenging activity (discussed later on page 48 in the section on enzymes), these authors found that tocopherol acetate did prevent the UVB-induced effects.

Trevithic et al.⁵ studied the application of tocopherol acetate immediately *after* UVB irradiation on mice and found decreases in sunburn cell number, inflammatory infiltration and edematous swelling. Even delayed application at 8 h after irradiation afforded some protection, although again high concentrations (5%) were required.

Vitamin C has several properties that make it attractive to the sunscreen formulator. Its cosmetic use is based primarily on two claims: the stimulation of collagen, found particularly in antiwrinkle creams; and the inhibition of melanogenesis in skin whitening products.

Darr et al.⁶ investigated topical use of vitamin C on pigskin and found protection against UVB damages as measured by erythema and sunburn cell formation. In another pigskin study, Moison et al.⁷ achieved a broad spectrum protection from a combination of vitamin C and vitamin E. The combination had a synergistic effect between the two molecules in protecting the lipids; moreover, the skin's inherent vitamin C and vitamin E levels were not depleted by UVB radiation.

Human *in vivo* studies were carried out by Dreher et al.⁸ using three antioxidant molecules, alone or in combination: vitamin C, vitamin E and melatonin. Slight synergistic results were obtained by combining the ingredients two by two or in a threesome and applying them in a vehicle 30 min *before* UV exposure. Skin color and skin blood flow were used as markers for UV-induced damage. The same authors then studied the effects of these combinations when applied 30 min, 1 h and 2 h *after* UV radiation (UVR) exposure, using the same end-points.⁹ Because no protective effect whatsoever was noted, the authors concluded that UVR-induced skin damage is rapid; antioxidants can alleviate or prevent damages only when present before or during sun exposure.

And what about vitamin A and its derivatives? Kligman¹⁰ in 1987 recommended the use of retinoic acid to replenish the inherent pool of this molecule in the skin after its depletion by UV light. Together with Schwartz¹¹ he also demonstrated that post-UV-irradiation treatment with 0.05% retinoic acid stimulated collagen synthesis *in vivo* in albino hairless mice.

Because retinoic acid is considered a prescription drug in most countries, the cosmetic industry became interested in retinol, retinol esters and retinaldehyde. Thus, more recently, Boisnic et al.¹² published a study with a retinaldehyde cream applied to an *ex vivo* human skin model. Eighteen days of regular UVA exposure simulated photoaging; this was followed by application of the retinaldehyde cream for two weeks. The retinaldehyde treatment reversed the UVA-induced alterations of collagen and elastic fibers. It also restored collagen synthesis rates to the levels of unexposed skin.

More data on human volunteers in studies on retinoids in conjunction with UV radiation can be found in Sorg et al.^{13,14} and Kang et al.¹⁵ One opposing opinion¹⁶ is expressed under this aggressive title: "Tretinoin and cutaneous photoaging: new preparation. Guaranteed adverse effects!"

In summary, most studies surveyed support a finding that "vitamins are good for you." Their use in sunscreens is widespread; based on the numerous studies, even if most of them are animal or *in vitro* studies, their claim to antiaging activity is not far-fetched. Their main drawback is the difficulty in formulating stable vehicles, such

that the right amount of efficacious vitamins can be guaranteed for sufficient shelf life.

Botanicals: Botanicals are increasingly used in sunscreens. These plant or herbal extracts cover the spectrum from hydroglycolic solutions of analytically ill-defined nature to pure, isolated, chemically identified molecules and all products that present intermediate stages of purification. Various products among them show antioxidant activity (polyphenols, vitamins, flavonoids), anti-inflammatory properties (nonsteroidal enzyme inhibitors) and tissue-repair molecules (di- and triterpenes, isoflavones).

All of these activities can be employed for antiaging claims. All of them make sense in the context of sunscreen formulation. A few references gleaned from the peer-reviewed literature shall illustrate this concept because it is impossible to list here all the commercially available plant-derived cosmetic ingredients that are claimed to be antiaging based on some or another *in vitro*, *ex vivo* or even *in vivo* test with or without UV irradiation included in the test protocol.

Green tea is a favorite among the botanicals. It has a well-known reputation. Wei et al.¹⁷ described *in vitro* scavenging of hydrogen peroxide and prevention of UV-induced oxidative damages on skin cells in culture by various fractions of green and black teas, including purified epigallocatechin gallate (EGCG). The pure molecule enhanced the observed activity and is considered the major active substance in these preparations.

An *in vivo* study by Vayalil et al.¹⁸ on hairless mice confirmed the prevention of UV-induced lipid peroxidation by green tea polyphenols, such as EGCG. Interestingly, the authors also quantified the amount of inherent antioxidant enzymes such as catalase and glutathion peroxidase: whereas UV irradiation depleted these enzymes in the skin, EGCG application before single UVB doses prevented this loss by 50–90% (discussed in the section on enzymes).

Less well-known botanical extracts, such as those obtained from methanolic maceration of *Capparis spinosa* L. buds¹⁹ and crude ethanol extracts from *Culcitium reflexum* H.B.K.²⁰ or *Chromolaena odorata*,²¹ to cite just a few exotic ones, all contain flavonoids, phenolic acids, coumarins and the like. They all show *in vitro* antioxidant activity that can be used for antiaging claims in sunscreens. Wasabi

(*Wasabia japonica*), the green mustard of sushi, possesses superoxide dismutase and peroxidase activity that may be of use in sunscreens to reduce the impact of free radicals. In dosing the ingredient, however, care must be taken to avoid vasodilatation and skin redness, rashes and allergy. Certain plant-derived molecules are prone to exhibit photoallergies, phototoxicity and photo-instability.

Afaq and Mukhtar²² reviewed photochemoprevention by botanical antioxidants in view of their use in sunscreens.

Enzymes: As fundamental research into the innate enzyme defense system of the skin has progressed, and a number of *in vitro*, animal and human *in vivo* studies point to the delicate balance that is required between the various enzymes in the skin.

Reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical, singlet oxygen and hydrogen peroxide cause numerous deleterious effects on structural and functional (enzyme) proteins, lipid membranes, tissue polysaccharides and genetic material (DNA). In skin, the molecules that are supposed to protect us against these damages are the vitamins already described, a few other antioxidants (melanin, urocanic acid, glutathion and ubiquinone) and specific enzymes: essentially superoxide dismutase (SOD), glutathion peroxidase (GPO) and catalase.

It now has become evident that these inherent antioxidant defense systems of the skin are rapidly overwhelmed by the amount of sun exposure stressing them in today's lifestyle. Vitamins C and E are depleted in the skin by UV irradiation, and the same occurs with the enzymes.²³⁻³¹

The enzymes catalase and SOD do not react in similar ways to long-term UV exposure. A thorough investigation on humans, carried out over winter and summer seasons, confirms this fact: catalase, easily destroyed by UVA light in summer, is more active in winter, whereas SOD is much more resilient.³² This then leads to a possibly harmful potential buildup of hydrogen peroxide in the skin during the summer. The need for a balanced antioxidant enzyme system thus becomes apparent.

Two approaches are possible:

- Stimulate or protect the innate enzyme system, so that even under UV exposure, it retains its efficacy; and

- Supply the lacking enzymes by topical application, for instance within sunscreen, pre-sun or post-sun products.

Hoppe and colleagues³³ and Maes and coworkers³⁴ present examples of the first strategy. They show that molecules such as salicin in skin fibroblasts³³ and vitamin D derivatives or betulinic acid in keratinocytes³⁴ are able to stimulate the synthesis of heat shock proteins that protect the catalase against UV-induced degradation. These molecules could therefore be used advantageously in sunscreens as antiaging actives inasmuch as they induce protection of the human antiaging defense systems.

The second approach has a few limitations. Available enzymes such as SOD and catalase, which is extracted from yeast or other biotech sources, usually are not easy to stabilize in cosmetic formulas, to say the least, because of their sensitivity to heat, oxygen and UV light. Furthermore, SOD alone on the skin would lead, at least theoretically, to a buildup of hydrogen peroxide, already decried by Maes³⁴ as being the natural problem of seasonal variations of these enzyme activities. Adding the fragile catalase is not only difficult, it is also forbidden in any formula sold in Europe because of an archaic prohibition of catalase use in cosmetic products.³⁵

A solution to this problem is afforded by antioxidant enzymes originating from organisms that live and thrive under extreme conditions of heat. These are the extremophiles. Discovered a bit more than a decade ago, these bacteria live close to the hydrothermal vents at the bottom of the ocean at temperatures that can reach 80°C–100°C. It is possible today to cultivate these organisms at sea level in industrial fermenters and to extract heat-stable antioxidant enzymes^a that mimic the skin's SOD, catalase and glutathion peroxidase activity. Additionally, the enzymes are more active at increasing temperatures up to 100°C. They are thus ideal for incorporation into sunscreens where the exposure to UV and the sun's heat not only preserves their defensive activity, but actually increases it: the higher the temperature, the more active the enzymes.

^a Extremozymes is a generic term that can be applied to a variety of enzymes active under "extreme" conditions. INCI: *Thermus thermophilus* ferment (Venuceane) is a product and trademark of Sederma.

An active ingredient based on this concept is described by Lintner et al.^{36,37} *Thermus thermophilus* bacteria, harvested 6000 feet below the California coast, are fermented at 75°C, then extracted and concentrated to yield a high-potency solution containing SOD with activity for converting hydrogen peroxide and mimicking glutathione peroxidase. *In vitro* tests carried out on this cosmetic ingredient include protection of human fibroblasts in culture, lipoperoxidation inhibition, collagen contraction, and protection of DNA against the formation of 8-oxo-guanidine. Studies on human volunteers show the persistence of cutaneous catalase against UVA irradiation and a decrease in *in vivo* lipoperoxidation of the stratum corneum. A recent vehicle-controlled study on volunteers living more than six months in a tropical climate demonstrated the protective effect of this enzyme cocktail against premature sun-induced skin aging [to be published].

Treatment of UV-induced Age Symptoms

As mentioned already, “reversing” some of the signs of aging also is considered antiage activity. Is it realistic? Can anything but retinoic acid reduce some of the wrinkles, the sagging skin, the dryness and the loss of tonus that comes with aging, especially photoaging?

It is not possible to review here the enormous mass of antiaging and wrinkle-repair ingredients of synthetic, marine, botanical or biotechnological origin proposed on the market, but they all might be considered, based on their merit, for inclusion in sunscreens. Therefore, discussion will be limited to two aspects: barrier repair of the skin surface and tissue repair in the deeper layers. The discussion also will identify some actives that appear to have clearly perceivable, demonstrated benefits.

Barrier repair: UVB irradiation initially stimulates barrier synthesis. The epidermis thickens, ceramide synthesis is increased, involucrine (a distinct marker protein of cell differentiation and cornification) increases.^{38–40} Long-term effects of UV exposure, however, clearly lead to a diminished barrier function.^{41,42}

Which barrier enhancement actives might be used in a sunscreen? Two possibilities are hydroxy acids and ceramides.

- Among the hydroxyl acids is lactic acid, one of the most widely used actives in skin care. It is known to stimulate many processes in skin, in particular the proliferation of keratinocytes and barrier repair. A four-week *in vivo* study by Rawlings et al.⁴³ showed that L-lactic acid increases ceramide synthesis by 38% over baseline. This is confirmed by a similar study using TEWL as a measure of barrier repair. However, a more recent study by Kaidbey et al.⁴⁴ in 2003 suggests that alpha hydroxy acids can increase the skin's sensitivity to UV light. After pretreatment for four weeks (24 applications of a 10% glycolic acid product or placebo on the back of 29 Caucasian subjects), the skin was irradiated with 1.5 MED. Kaidbey and colleagues observed increased sunburn-cell induction and lowered MEDs and concluded that 10% glycolic acid sensitizes the skin to the damaging effects of UV light. Thus, clearly more systematic studies are needed to determine the benefits of hydroxy acids in sunscreens for antiaging purposes.
- The improvement of barrier function by ceramides in general has been described in numerous papers⁴⁵ and references therein, though rarely in conjunction with UV irradiation. Various studies report the stimulation of keratinocyte differentiation and ceramide synthesis by substances such as niacinamide (vitamin B3),^{46,47} avocadofuran,⁴⁸ vitamin C,⁴⁹ calcium,⁵⁰ mevalonic acid,⁵¹ ursolic acid liposomes⁵² and others. The *Thermus thermophilus* ferment^{36,37} already described also has barrier repair functionality; it stimulates keratinocyte differentiation, involucrin synthesis and barrier repair by increased ceramide and cholesterol production. *In vivo*, this translates to greater resistance of the skin barrier against aggression (**Figure 1**) and to better moisture retention; both are important antiaging concepts.⁵³

Tissue repair: From a cosmetic point of view, the most important antiaging activity is to reduce wrinkles. Wrinkles are of course a major, visible consequence of the actinic damages excessive sunlight generates in the skin.

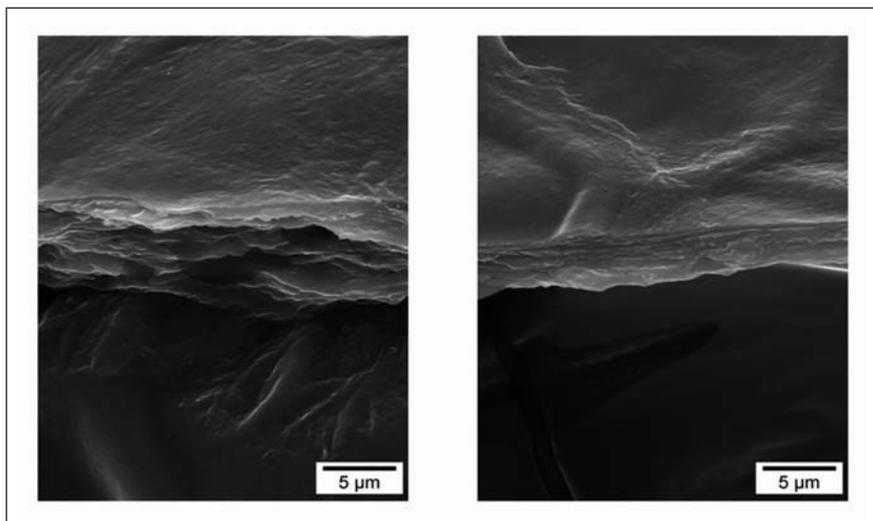


Figure 1. Electronic microscopy of the stratum corneum before treatment (left) and after 28 days of enzyme treatment (right) with a **Thermus thermophilus** ferment

Consumers who use a sunscreen during outdoor activities hardly ever use other skin care products at the same time. But the benefits of truly active, tissue-repair antiaging molecules lie in extended use, in regular exposure to their action, in constancy. A good modern skin care or face care product should offer at least some sun protection factor, even if the product is not positioned as a sunscreen. In the same way, a sunscreen may offer tissue-repair ingredients to bridge the periods between morning face preparation and the night cream.

A whole new concept in tissue repair, and thus in antiaging strategy, is offered by the discovery of certain messenger molecules called matrikines^b. The term was coined by Maquart⁵⁴ to designate protein fragments (peptides) of small size that are generated by the gradual hydrolysis of natural, structural proteins in the connective tissue. But not just any breakdown product of proteins will be a matrikine. During wound healing or inflammation, proteolytic enzymes break down collagen, elastin, fibronectin and other structure proteins into smaller pieces. Certain peptide sequences, thus released, possess mediator (“kinin”) or messenger (ορμone=hormone) function: they act on nearby cells (fibroblasts) to stimulate them into neosynthesis

^b Matrikines is a generic name found in scientific literature. It is also a trade name for a large family of products. The best known matrikine from Sederma is Matrixyl (INCI: Palmitoyl pentapeptide-4). Matrixyl is a trademark of Sederma.

of tissue macro-molecules or to attract them to the damaged tissue site (chemotaxis). Like all mediators or signal molecules, these peptides of specific amino acid sequence act at very low concentration in the nano to micromolar range, but they achieve dramatic effects in the rapid regeneration of tissue. These peptides, derived from the natural sequence of the damaged proteins, are usually in the tri- to hexamer range.

Some of these matrikines have found cosmetic use, for which it was necessary to attach a lipophilic, fatty acid chain in order to assure skin diffusion and bioavailability.⁵⁵ Thus, it has been shown that a few ppm of the tripeptide palmitoyl-Gly-His-Lys (a serum protein fragment) is able to stimulate collagen and glycosaminoglycans synthesis *in vitro*, which translates into skin thickening and antiwrinkle effects *in vivo*.⁵⁵ Equally low concentrations of palmitoyl-Val-Gly-Val-Ala-Pro-Gly (a fragment of elastin) or palmitoyl-Gly-Gln-Arg-Pro (a fragment of immunoglobulin IgG) have potent skin repair activities^{56,57} that may be used in antiaging compositions.

As a concrete example of cosmetic use of this concept, the well-known matrikine peptide palmitoyl-Lys-Thr-Thr-Lys-Ser can be cited. This peptide was discovered by Katayama⁵⁸ during wound healing-related research on lung cells. It was investigated for skin applications in *in vitro* studies on normal human fibroblasts, then on full-thickness skin tissue and in clinical, vehicle- or benchmark-controlled studies using 3–5 ppm of the Pal-KTTKS peptide in topical antiwrinkle creams.^{59–61} The benchmark was retinol or a moisturizer. Treatment with this formula for up to six months led to overall improvement of the facial skin and to significant, measurable and consumer-perceivable benefits in reducing the volume, depth and density of wrinkles. Matrikines such as Pal-KTTKS are thus ideal candidates for cosmetic tissue repair.

A recent variant combines the activities of the matrikines Pal-GHK and Pal-GQPR, which are fragments of collagen and immunoglobulin IgG, respectively. Their antiwrinkle activity is demonstrated in **Figure 2**.

Nothing in the nature, activity or mechanism of action of these molecules with true antiaging properties prevents them from being

used in sunscreens. They are compatible with any kind of formulation, they are stable and their mode of action is unperturbed by UV. They are clearly an additional benefit to sunscreens because they demonstrably can repair some of the actinic photodamage.

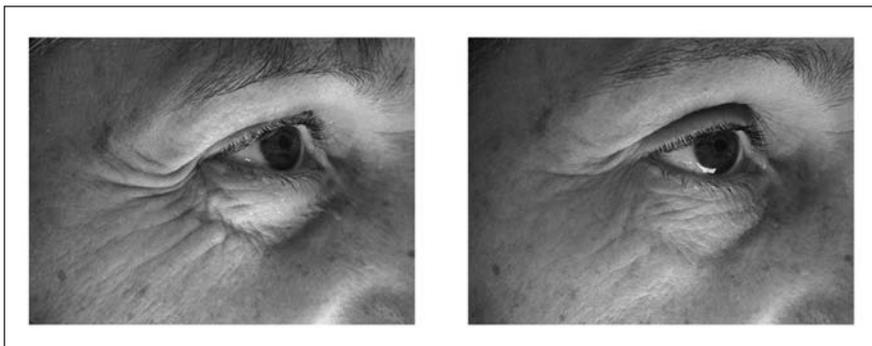


Figure 2. Crow's feet wrinkles before (left) and after two months of treatment (right) with a combination of the matrikines Pal-GHK and Pal-GQPR for tissue repair

Formulation Issues

Sunscreen formulations often present important challenges to the formulating chemist. More- or less-soluble UV filters at high concentrations must be included into textures that must be acceptable to the consumer, while at the same time the water resistance of the formulas must be improved. These are difficult tasks in themselves. How can the addition of antiaging actives such as those described in this article be achieved?

In truth, it should not be a major problem. Skin care active ingredients are used mostly at low concentrations (1–3% w/w). Most of the botanical, enzyme or biopeptide preparations are aqueous or hydroglycolic solutions that can be incorporated into the aqueous phase of the emulsion. Depending on the heat stability of the active, they are added to the emulsion at a stage compatible with their chemistry: the heat-stable enzymes can be added before the oil phase; peptides, vitamins and botanicals are more heat-sensitive and preferably should be incorporated toward the end of the cooling phase of the forming emulsion, a recommendation that is given for all types of lotions, creams and emulsions that need a heating step.

Are there any specific incompatibilities of the actives cited here with UV filters or reflectors? It should be noted that legislation in the United States, Japan and Korea views the UV filter or UV reflector as the active substance in products regulated as over-the-counter (OTC), quasidrug or functional cosmetic products; other ingredients are deemed to be inert. Theoretically, vitamins, peptides, enzymes or antioxidants should not have any adverse effect in the sunscreen formula; no specific interaction (synergy or interference) with UV filters is to be expected. Again, this expectation is explained by the great difference in high concentrations (several percent, sometimes in double digits) for the UV filter versus the low concentrations (fractions of a percent down to levels of ppm) for the anti-aging actives. The only caveat concerns the toxicology: substances that absorb significantly in the UVA or UVB region need to be tested for potential phototoxicity and photoallergy, especially in the case of botanicals.

Conclusion

Whereas UV-absorbing molecules are clearly designated as sun filters and constitute a well-defined category of chemicals, the notion of antiaging actives or cosmeceuticals is not as well characterized. This article has tried to demonstrate that the notion of antiaging actives in sunscreens opens many possibilities to the formulator to improve the basic sunscreen products, to add real benefits and to allow for variety in claims and marketing positioning.

Prevention of sun damage on the skin can be reinforced by some of the antioxidant and photoprotective agents; treatment of sun damages with repair actives during or immediately after sun exposure is also justified. Prevention goes beyond using sunscreens and includes behavioral modifications such as wearing adequate clothes and avoiding the hottest hours of the day. Teaching the consumer how to manage the sunlight has become part of the marketer's obligation.

Depending on the country, however, from the United States to Europe to East Asia, the regulations on sunscreens, claims and formulations are quite different and complex. Adding antiaging actives to these sunscreens makes the legal situation even more

complex with respect to advertised claims. Careful wording of any antiaging claims for sunscreen products is thus recommended, no matter how many studies one cites in support of this added benefit.

Published June 2006 *Cosmetics & Toiletries* magazine.

References

1. K Lintner, The role of actives in face care, *Proceedings of the PCIE Conference February 4–6, 2003, in Düsseldorf*, Augsburg: Ziolkowsky Verlag, published on CD ROM
2. K Pehr and RR Forsey, Why don't we use vitamin E in dermatology? *CMAJ* 149(9) 1247–1253 (1993)
3. EF Ritter, M Axelrod, KW Minn, E Eades, AM Rudner, D Serafin and B Klitzman, Modulation of ultraviolet light-induced epidermal damage: beneficial effects of tocopherol, *Plast Reconstr Surg* 102(5) 1785–1786 (1998)
4. Y Saral, B Uyar, A Ayar and M Naziroglu, Protective effects of topical alpha-tocopherol acetate on UVB irradiation in guinea pigs: importance of free radicals, *Physiol Res* 51(3) 285–290 (2002)
5. JR Trevithick, DT Shum, S Redae, KP Mitton, C Norley, SJ Karlik, AC Groom and EE Schmidt, Reduction of sunburn damage to skin by topical application of vitamin E acetate following exposure to ultraviolet B radiation: effect of delaying application or of reducing concentration of vitamin E acetate applied, *Scanning Microsc* 7(4) 1269–1281 (1993)
6. D Darr, S Combs, S Dunston, T Manning and S Pinnell, Topical vitamin C protects porcine skin from ultraviolet radiation-induced damage, *Br J Dermatol* 127(3) 247–253 (1992)
7. RM Moison and GM Beijersbergen van Henegouwen, Topical antioxidant vitamins C and E prevent UVB-radiation-induced peroxidation of eicosapentaenoic acid in pig skin, *Radiat Res* 157(4) 402–409 (2002)
8. F Dreher, B Gabard, DA Schwindt and HI Maibach, Topical melatonin in combination with vitamins E and C protects skin from ultraviolet-induced erythema: a human study *in vivo*, *Br J Dermatol* 139(2) 332–339 (1998)
9. F Dreher, N Denig, B Gabard, DA Schwindt and HI Maibach, Effect of topical antioxidants on UV-induced erythema formation when administered after exposure, *Dermatology* 198(1) 52–55 (1999)
10. LH Kligman, Retinoic acid and photocarcinogenesis—a controversy, *Photodermatol* 4(2) 88–101 (1987)
11. E Schwartz, FA Cruickshank, JA Mezick and LH Kligman, Topical all-trans retinoic acid stimulates collagen synthesis *in vivo*, *J Invest Dermatol* 96(6) 975–978 (1991)
12. S Boisnic, MC Branchet-Gumila, Y Le Charpentier and C Segard, Repair of UVA-induced elastic fiber and collagen damage by 0.05% retinaldehyde cream in an ex vivo human skin model, *Dermatology* 199 Suppl 1 43–48 (1999)
13. O Sorg, C Tran and JH Saurat, Cutaneous vitamins A and E in the context of ultraviolet- or chemically-induced oxidative stress, *Skin Pharmacol Appl Skin Physiol* 14(6) 363–372 (2001)
14. O Sorg, C Tran, P Carraux, L Didierjean, F Falson and JH Saurat, Oxidative stress-independent depletion of epidermal vitamin A by UVA, *J Invest Dermatol* 118(3) 513–518 (2002)
15. S Kang, GJ Fisher and JJ Vorhees, Photoaging: pathogenesis, prevention and treatment, *Clin Geriatr Med* 17(4) 643–659 (2001)
16. Anonymous, Tretinoin and cutaneous photoaging: new preparation. Guaranteed adverse effects! *Prescrire Int* 8(43) 139–140 (1999)
17. H Wei, X Zhang, JF Zhao, ZY Wang, D Bickers and M Lebowitz, Scavenging of hydrogen peroxide and inhibition of ultraviolet-light induced oxidative DNA damages by aqueous extracts from green and black teas, *Free Radic Biol Med* 26(11–12) 1427–1435 (1999)

18. PK Vayalil, CA Elmets and SK Katiyar, Treatment of green tea polyphenols in hydrophilic cream prevents UVB-induced oxidation of lipids and proteins, depletion of antioxidant enzymes and phosphorylation of MAPK proteins in SKH-1 hairless mouse skin, *Carcinogenesis* 24(5) 927–936 (2003)
19. F Bonina, C Puglia, D Ventura, R Aquino, S Tortora, A Sacchi, A Saija, A Tomaino, ML Pellegrino and P de Caprariis, *In vitro* antioxidant and *in vivo* photoprotective effects of a lyophilized extract of *Capparis spinosa* L buds, *J Cosmet Sci* 53(6) 321–335 (2002)
20. R Aquino, S Morelli, A Tomaino, M Pellegrino, A Saija, L Grumetto, C Puglia, D Ventura and F Bonina, Antioxidant and photoprotective activity of a crude extract of *Culcitium reflexum* HBK leaves and their major flavonoids, *J Ethnopharmacol* 79(2) 183–191 (2002)
21. TT Phan, L Wang, P See, RJ Grayer, SY Chan and ST Lee, Phenolic compounds of *Chromolaena odorata* protect cultured skin cells from oxidative damage: implication for cutaneous wound healing, *Biol Pharm Bull* 24(12) 1373–1379 (2001)
22. F Afaq and H Mukhtar, Photochemoprevention by botanical antioxidants, *Skin Pharmacol Appl Skin Physiol* 15(5) 297–306 (2002)
23. BJ Hughes-Formella, K Bohnsack, F Rippke, G Benner, M Rudolph, I Tausch and J Gassmueller, Anti-inflammatory effect of hamamelis lotion in a UVB erythema test, *Dermatology* 196(3) 316–322 (1998)
24. Y Miyachi, S Imamura and Y Niwa, Decreased skin superoxide dismutase activity by a single exposure of ultraviolet radiation is reduced by liposomal superoxide dismutase pretreatment, *J Invest Dermatol* 89(1) 111–112 (1987)
25. BC Pence and MF Naylor, Effects of single-dose ultraviolet radiation on skin superoxide dismutase, catalase and xanthine oxidase in hairless mice, *J Invest Dermatol* 95(2) 213–216 (1990)
26. K Punnonen, P Autio, U Kiistala and M Ahotupa, In-vivo effects of solar-simulated ultraviolet irradiation on antioxidant enzymes and lipid peroxidation in human epidermis, *Br J Dermatol* 125(1) 18–20 (1991)
27. Y Shindo, E Witt and L Packer, Antioxidant defense mechanisms in murine epidermis and dermis and their responses to ultraviolet light, *J Invest Dermatol* 100(3) 260–265 (1993)
28. K Okada, Y Takahashi, K Ohnishi, O Ishikawa and Y Miyachi, Time-dependent effect of chronic UV irradiation on superoxide dismutase and catalase activity in hairless mice skin, *J Dermatol Sci* 8(3) 183–186 (1994)
29. Y Shindo and T Hashimoto, Antioxidant defence mechanism of the skin against UV irradiation: study of the role of catalase using acatalasaemia fibroblasts, *Arch Dermatol Res* 287(8) 747–753 (1995)
30. P Filipe, I Emerit, J Vassy, JP Rigaut, E Martin, J Freitas and A Fernandes, Epidermal localization and protective effects of topically applied superoxide dismutase, *Exp Dermatol* 6(3) 116–121 (1997)
31. L Naderi-Hachtroudi, T Peters, P Brenneisen, C Meewes, C Hommel, Z Razi-Wolf, LA Schneider, J Schuller, M Wlaschek and K Scharffetter-Kochanek, Induction of manganese superoxide dismutase in human dermal fibroblasts: a UV-B mediated paracrine mechanism with the release of epidermal interleukin 1 alpha, interleukin 1 beta and tumor necrosis factor alpha, *Arch Dermatol* 138(11) 1473–1479 (2002)
32. L Hellemans, H Corstjens, A Neven, L Declercq and D Maes, Antioxidant enzyme activity in human stratum corneum shows seasonal variation with an age-dependent recovery, *J Invest Dermatol* 120(3) 434–439 (2003)
33. F Steckel, R Gieseler, D Pollet and U Hoppe, Heat shock protein expression and UV-light induced damage in cultured human skin cells, *Proceedings of the XXth Congress IFSCC, Cannes, Sept 14-18, 1998*, vol 1 (1998) pp 133–140
34. L Declercq, L Hellemans, E Goyarts, D Gan, H Corstjens, I Sente, T Mammone, K Marenus and D Maes, Induction of heat shock proteins to protect against seasonal deficiency in antioxidant defense capacity of human stratum corneum, *Proceedings of the 22nd Congress IFSCC, Edinburgh, Sept 24–27, 2002*, vol 2 (2002) podium 20
35. Council Directive n 76/768/CEE of July 27, 1976. (JOCE L 262 of Sept 1976)

36. C Mas-Chamberlin, F Lamy, P Mondon, S Scocci, L de Givry, F Vissac and K Lintner, Heat- and UV-stable cosmetic enzymes from deep sea bacteria, *Cosmet Toil* 117(4) 22–30 (2002)
37. K Lintner, F Lamy, C Mas-Chamberlin, P Mondon, S Scocci, P Buche and F Girard, *IFSCC Magazine* 5(3) 195–200 (2002)
38. P Lehmann, B Melnik, E Holzle, N Neumann and G Plewig, The effect of UV-A and UV-B irradiation on the skin barrier. Skin physiologic, electron microscopy and lipid biochemistry studies, *Hautarzt* 43(6) 344–351 (1992) (In German)
39. C Magnoni, E Euclidi, L Benassi, G Bertazzoni, A Cossarizza, S Seidenari and A Giannetti, Ultraviolet B radiation induces activation of neutral and acidic sphingomyelinases and ceramide generation in cultured normal human keratinocytes, *Toxicol In Vitro* 16(4) 349–355 (2002)
40. L Coderch, M de Pera, J Fonollosa, A De La Maza and J Parra, Efficacy of stratum corneum lipid supplementation on human skin, *Contact Derm* 47(3) 139–146 (2002)
41. PM Elias and R Ghadially, The aged epidermal permeability barrier: basis for functional abnormalities, *Clin Geriatr Med* 18(1) 103–120 (2002)
42. E Lamaud and W Schalla, Influence of UV irradiation on penetration of hydrocortisone. *In vivo* study in hairless rat skin, *Br J Dermatol* 111(supp 27) 152–157 (1984)
43. AV Rawlings, A Davies, M Carlomusto, S Pillai, K Zhang, R Kosturko, P Verdejo, C Feinberg, L Nguyen and P Chandar, Effect of lactic acid isomers on keratinocyte ceramide synthesis, stratum corneum lipid levels and stratum corneum barrier function, *Arch Dermatol Res* 288 383–390 (1996)
44. K Kaidbey, B Sutherland, P Bennett, WG Warner, C Barton, D Dennis and A Kornhauser, Topical glycolic acid enhances photodamage by ultraviolet light, *Photodermatol Photoimmunol Photomed* 19(1) 21–27 (2003)
45. *Cosmetic Lipids and the Skin Barrier*, T Förster, ed, New York: Marcel Dekker (2002), and references therein
46. PJ Matts, JE Oblong and DL Bissett, A review of the range of effects of niacinamide in human skin, *IFSCC Magazine* 5(4) 285–289 (2002)
47. O Tanno, Y Ota, N Kitamura, T Katsube and S Inoue, Nicotinamide increases biosynthesis of ceramides as well as other stratum corneum lipids to improve the epidermal permeability barrier, *Br J Dermatol* 143(3) 524–531 (2000)
48. AL Almada, New Research on vitamin E, soy & avocado, *Functional Food and Nutraceuticals* (Nov/Dec 2001)
49. LSP Ramdin, J Richardson, CR Harding and M Rosdy, The effect of ascorbic acid (vitamin C) on the ceramide subspecies profile in the SkinEthic epidermal model, *Proceedings of Stratum Corneum III Basel, September 12–14, 2001*, Poster 40
50. PM Elias, P Nau, K Hanley, C Cullander, D Crumrine, G Bench, E Sideras-Haddad, T Mauro, ML Williams and KR Feingold, Formation of the epidermal calcium gradient coincides with key milestones of barrier ontogenesis in the rodent, *J Invest Dermatol* 110 399–404 (1998)
51. A Haratake, K Ikenaga, N Katoh, H Uchiwa, S Hirano and H Yasuno, Topical mevalonic acid stimulates de novo cholesterol synthesis and epidermal permeability barrier homeostasis in aged mice, *J Invest Dermatol* 114(2) 247–252 (2000)
52. DM Both, K Goodtzova, DB Yarosh and DA Brown, Liposome-encapsulated ursolic acid increases ceramides and collagen in human skin cells, *Arch Dermatol Res* 293(11) 560–575 (2002)
53. K Lintner, F Lamy, C Mas-Chamberlin, P Mondon, S Scocci, P Buche and F Girard, Heat-stable enzymes from Deep Sea bacteria: A key tool for skin protection against UV-A induced free radicals, *IFSCC Magazine* 5(3) 195–200 (2002).
54. FX Maquart, A Simeon, S Pasco and JC Monboisse, Regulation of cell activity by the extracellular matrix: the concept of matrikines, *J Soc Biol* 193(45) 423–428 (1999)
55. K Lintner and O Peschard, Biologically active peptides: from a lab bench curiosity to a functional skin care product, *Int J Cosm Sci* 22 207–218 (2000)
56. French Pat FR03/05707, K Lintner
57. French Pat FR 99/00743 and WO/0043417, K Lintner (Jan 2003)

58. K Katayama et al, A pentapeptide from type I procollagen promotes extracellular matrix production, *J Biol Chem* 268(14) 9941–9944 (1993)
59. K Lintner, Promoting production in the extracellular matrix without compromising barrier, *Cutis* 70(6S) (suppl) 13–16 (2002)
60. C Mas-Chamberlin, K Lintner et al, Relevance of antiwrinkle treatment of a peptide: 4 months clinical double blind study vs excipient, *Ann Dermatol Vener* 129 Proceedings 20th World Congress of Dermatology, Book II, Paris: Masson (2002)
61. L Robinson, NC Fitzgerald, DG Doughty, NC Dawes, CA Berge and DL Bissett, Palmitoyl-Pentapeptide offers improvement in human photoaged facial skin, *Ann Dermatol Vener* 129 Proceedings 20th World Congress of Dermatology, Book II, Paris: Masson (2002)

Antiaging Effects of a Skin Repair Active Principle

L. Rigano and C. Andolfatto

Laboratori L. Rigano, Milan, Italy

F. Rastrelli

Kalichem Italia, Botticino Sera, Italy

KEY WORDS: *antiaging, sodium DNA, cell renewal, skin moisturization, skin elasticity*

ABSTRACT: *A particular extract of DNA from the gonadic tissue of male sturgeons is shown to have cell renewal effects with possible antiaging benefits for skin moisture, thickness, elasticity and a reduction in skin wrinkledness.*

Sodium DNA is an ingredient with activity at the cellular level. This fact has led to its incorporation in numerous high-end antiaging skin care products. An explanation of that activity and results of several tests of one sodium DNA material are presented in this chapter.

The Many Levels of Aging

The slow, inevitable skin aging process is characterized by a progressive degeneration of the skin tissue as well as by a variety of attendant visible changes in the skin surface. The skin acquires a new appearance as wrinkles form and become increasingly conspicuous, the epidermal layer thins, and the skin decreases in firmness and elasticity. These changes show the passage from youth to adulthood to old age.

Such visible effects can be seen well before the age of 30 in humans and result from major changes in skin cells and the structures supporting the tissue. They are caused by a variety of genetic, metabolic, hormonal, biological and environmental factors.

The first and most notable sign of aging is a decrease in the skin's water retention capability and the resultant decrease in dermis elasticity. This is visible when facial muscles are contracted and face wrinkles become deeper.

Moreover, as the years pass, biological and environmental damage accumulates and will not be repaired naturally. The most important changes concern collagen and elastin fibers, the basic constituents of the connective tissue. The amount of collagen, which is synthesized by the fibroblasts, tends to decrease as the activity of the fibroblasts is modified; this adds to the effects of the sun radiation and the fall in estrogen during menopause.

During menopause the elastin fibers atrophy as the dermis thins and their production is altered; thus, the dermis loses its viscoelastic properties. In addition, the epidermis becomes less efficient in performing its barrier function because of a loss of keratinocytes and a reduction in the thickness of the hydrolipid film thickness on its surface.

The skin tends to look dull, becomes dry and dehydrated, less firm and not homogeneous, grows thin and slackens. This is caused by a number of factors. On the one hand, the input of biological nutrients becomes poor due to reduced skin vascularization and slow cell renewal; on the other hand, the progressive degeneration of the dermal connective tissue and the structural changes in the epidermis play their role. Environmental stress must also be considered. Skin cells are exposed daily to UV rays, infrared (IR), osmotic stress and poor moisturization; therefore, they become an easy target for thousands of free radicals. Furthermore, psychological stress weakens the body's defense system, making it more vulnerable to their attacks.

In conclusion, it seems clear that action is necessary on different levels to slow down the skin aging process.

Extracted DNA to Stimulate Cell Repair

More than 30 years ago in Russia, experiments were performed with

the aim of developing an effective treatment for diseases related to ionizing radiation. Among the biologically active materials tested was deoxyribonucleic acid (DNA) extracted from the gonadic tissue of male sturgeons. The extract was carefully purified, depolymerized and neutralized with sodium hydroxide according documented procedures.^{1,2} The extract later received the INCI name Sodium DNA.

Positive feedback on sodium DNA was obtained in 1986, when it was employed to treat diseases related to the Chernobyl nuclear disaster, however the results were never precisely quantified. Indeed, in animal studies carried out some years later,³ the same compound was shown to protect and repair γ -radiation-induced lesions. In the following years, many clinical tests proved its efficacy at treating different types of skin lesions and illnesses.^{4,5} Scientists looked at its nucleotides, the basic units of nucleic acids. They found that nucleotide segments of DNA with a molecular mass from 250 to 500 kD were able to control the formation of wrinkles. Moreover, intradermal administration of DNA fragments in aesthetic surgery patients accelerated wound healing.^{6,7} This paved the way to research on sodium DNA as an antiaging active in cosmetics.

The most likely explanation of this ingredient's antiaging effect is based on the fact that some segments of DNA act as donors of purine and pyrimidine bases, which are key molecules for the vitality of all cells. Sodium DNA passes through the cell membrane by pinocytosis (**Figure 1**), an endocytosis method of transport facilitated by sodium ions, which are combined with the poly-deoxyribonucleotides. Therefore, the cells presumably use the acquired amount of sodium DNA both as a structural base to synthesize the nucleic acids and their cofactors and to metabolize their own DNA.

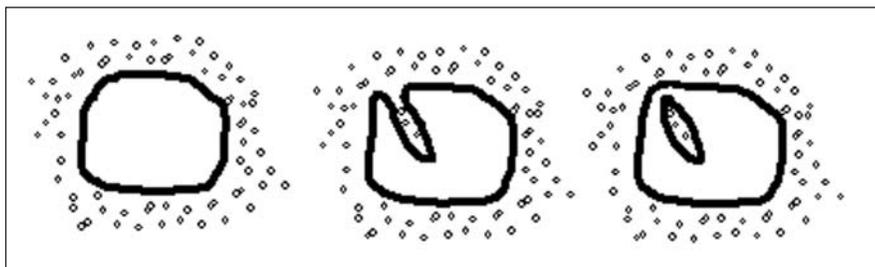


Figure 1. Pinocytosis as a method of transport of exogenous molecules in the cell

These processes occur very easily in cells that are under metabolic conditions or extreme stress. This is the case of altered keratinocytes or fibroblasts, both of which are typical in aged skin. Thanks to the cell integration process, sodium DNA acts to stimulate cell repair activity, regenerate epithelial and granulation tissues and reduce the symptoms of inflammation, thus accelerating the healing of skin microlesions.^{8–11}

***In vitro* Tests**

In vitro tests were used to assess the regenerating and photoprotective property of sodium DNA toward two cell types—keratinocytes and fibroblasts. The particular form of sodium DNA used in these tests was a highly purified commercial product^a with molecular weight in the range 250–500 kD. This product will be called DNA-Na in the following discussion.

Keratinocytes and fibroblasts were obtained from two healthy donors by biopsy. Both specimens were grown in culture and incubated on plates containing DNA-Na at different concentrations.

The skin's cell renewal rate decreases naturally as the years go by. This is the cause of skin aging. As a measure of the regenerating power, the growth rate of the cells treated with DNA-Na was determined at 24, 48 and 72 h after incubation and compared to the untreated cells used as controls.

To assess the property of protecting the cells exposed to radiation, the vitality of the cells treated with DNA-Na was tested 24 h after exposure to a UV source and compared to the untreated cells used as controls. Test results showed that DNA-Na stimulated cell proliferation and proved effective in protecting them.

In detail, it stimulated the growth of keratinocytes. Moreover, increased cellular growth was recorded 72 h after exposure at 1% concentration of DNA-Na (**Figure 2**). DNA-Na also improved the vitality of fibroblasts, whose growth was increased 24 h after exposure (**Figure 3**). Furthermore, phototoxicity tests suggested that DNA-Na had no harmful cytotoxic effects and carried out a protective function from the damage induced by UV rays toward fibroblasts.^{12,13}

^a Kalinat Anti-Wrinkle (INCI: Sodium DNA), Kalichem Italia LC, Botticino Sera, Italy. Kalinat Anti-Wrinkle is a registered trademark of Kalichem Italia.

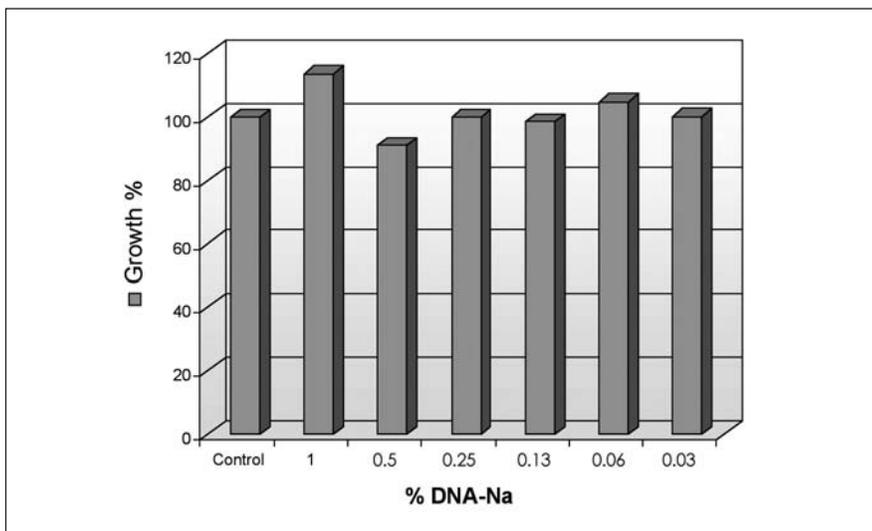


Figure 2. Growth of keratinocytes 72 h after exposure at different concentrations of DNA-Na

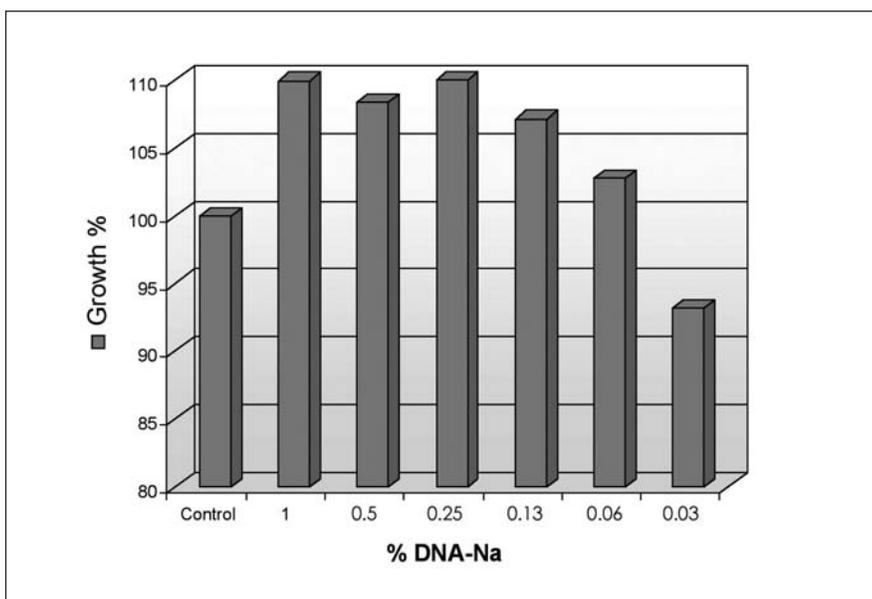


Figure 3. Growth percentage of fibroblasts 24 h after exposure at different concentrations of DNA-Na

In vivo Tests

To assess the efficacy of an emulsion (**Formula 1**) containing DNA-Na aimed at increasing moisturization, elasticity and thickness and

reducing wrinkledness, a test was performed after prolonged use and its results were compared to results from a placebo emulsion.¹⁴ This double-blind study enrolled 20 Caucasian female volunteers aged 30–60, with an average age of 49 years. These volunteers applied the test products on the two periocular zones, twice daily for eight weeks.

Formula 1. Antiaging test emulsion

A. BHT	0.05% wt/wt
Phenoxyethanol (and) methylparaben (and) ethylparaben (and) butylparaben (and) propylparaben	1.10
Olivoyl hydrolyzed wheat protein (and) cetearyl alcohol (and) glyceryl oleate (and) glyceryl stearate (and) potassium hydroxide	9.00
Ethylhexyl palmitate	8.00
Isopropyl myristate	6.50
Dimethicone	1.00
Cetyl alcohol	4.50
B. Water (<i>aqua</i>)	qs to 100.00
Disodium EDTA	0.05
Glycerin	3.20
C. Imidazolidinyl urea	0.25
Sodium DNA (Kalinat Anti-Wrinkle, Kalichem Italia)	0.25

Numerous instrumental measurements of skin properties were taken before the first application and on the day following the final application.

- Moisturization level (in arbitrary corneometric units) was measured¹⁵ by a corneometer^b. It is related to the modification of conductance as measured by the probe applied to the skin surface.
- Elasticity was measured¹⁶ by a cutometer^c. Its probe exerts a cycling suction on the skin surface and the consequent skin deformation is measured by electrical means.

^b Model CM 825 Corneometer, Courage & Khazaka, Germany

^c Cutometer 575, Courage & Khazaka

- Skin wrinkledness was measured¹⁷ on skin imprints that are prepared with a quick-hardening resin^d and image analysis^e of skin replicas.
- Skin thickness measurements¹⁸ used scanning software^f and an ultrasound scanner at high resolution, with high frequency (320 MHz) ultrasound emission. This frequency allows skin scanning to a depth of 3 cm, with an axial resolution of 50 μm and a lateral resolution of 350 μm .¹⁸

During the eight-week product application period, the test subjects were not allowed to use emulsions other than the ones being evaluated and were asked to abstain from prolonged exposure to UV rays.

The resulting instrumental data revealed that the emulsion containing the active principle had induced an increase in the mean basal values of skin moisturization from 60.9 corneometric units before treatment to 63.7 corneometric units after treatment, whereas the placebo values were essentially unchanged. The data also revealed a significant ($P < 0.05$) decrease of 4% in the mean basal values of maximum wrinkledness (**Figure 4**). However, the best results were recorded for biological elasticity and skin thickness. In the site treated with the active emulsion, the mean basal values of elasticity and skin thickness showed a highly significant ($P < 0.01$) increase of 25.6% and 8.7%, respectively.

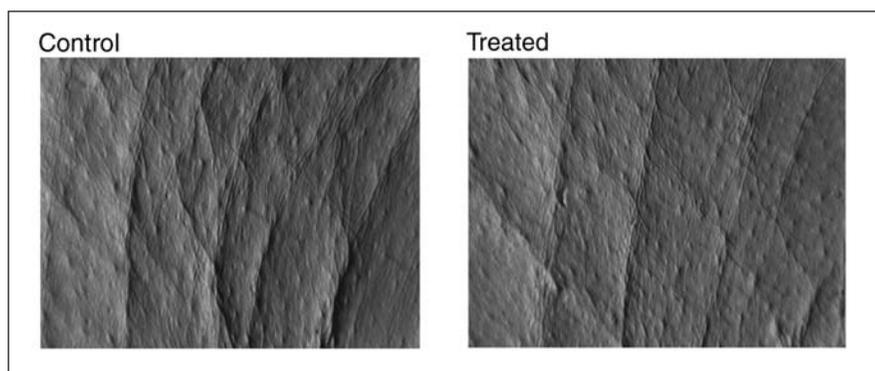


Figure 4. Wrinkles in the skin site before (control area) and after (treated area) the application of DNA-Na

^d Silflo-Flexico Ltd., UK

^e Quantilines, Monaderm

^f Dermascan C Version 3, Cortex Technology, Denmark.

Dermascan C is a registered trademark of Cortex Technology.

Formulas 2–3 are given as guidance for an optimized use of sodium DNA.

Formula 2. Body cream for dry skin

A. Water (<i>aqua</i>)	71.00% wt/wt
Betaine	1.00
Panthenol	0.20
Allantoin	0.10
Disodium EDTA	0.05
Glycerin	4.00
B. PVP/VA copolymer	0.20
C. Ammonium acryloyl-dimethyl taurate/VP copolymer	1.40
D. Olivoyl hydrolyzed wheat protein (and) cetearyl alcohol (and) glyceryl oleate (and) glyceryl stearate (and) potassium hydroxide	2.00
Butylene glycol dicaprylate/dicaprate	6.00
Phenoxyethanol	0.80
Salicylic acid	0.20
Tocopheryl acetate	0.30
E. Potassium azeloyl diglycinate	1.00
Alcohol (and) water (<i>aqua</i>) (and) <i>Plantago lanceolata</i> (and) <i>Berberis aquifolium</i>	2.00
<i>Arctium lappa</i> (and) propylene glycol	1.00
<i>Hedera helix</i> (and) propylene glycol	1.00
<i>Urtica dioica</i> (and) propylene glycol	1.00
Water (<i>aqua</i>)	4.00
Sodium DNA	0.15
Diazolidinyl urea	0.30
Fragrance (<i>parfum</i>)	0.30
Silica	<u>2.00</u>
	100.00

Procedure: Prepare A in the main mixer under vacuum. Add B; after complete dispersion, add C, then mix and homogenize until complete swelling. Heat to 70°C. Melt D at 70–75°C; then add D to the main mixer. Homogenize ABCD for 10 min. Cool to 40°C while mixing; then add E in order. Cool to RT under vacuum.

Characteristics: White-ivory creamy emulsion; pH 5.3; viscosity (Brookfield RVT): 23000 mPa.s 5 rpm, 25°C.

Comment: Gel-cream easy to spread. The synergy between sodium DNA and the epithelizing, soothing, anti-inflammatory activity of the other actives protects and moisturizes, thus making the product especially suitable for dry skin.

Formula 3. Antiaging emulsion

A. Water (<i>aqua</i>)	51.00% wt/wt
Panthenol	0.20
Allantoin	0.20
Disodium EDTA	0.10
B. Xanthan gum	0.25
C. Carbomer	0.60
D. Olivoyl hydrolyzed wheat protein (and) cetearyl alcohol (and) glyceryl oleate (and) glyceryl stearate (and) potassium hydroxide	4.00
PPG-15 stearyl ether	1.00
Phenoxyethanol	0.60
<i>Shorea stenoptera</i> butter	2.00
C12-15 alkyl benzoate	6.00
Cetyl alcohol	0.60
Tocopheryl acetate	0.50
BHT	0.01
E. Water (<i>aqua</i>)	14.44
Sodium DNA	0.50
Quaternium-15	0.10
Water (<i>aqua</i>) (and) phospholipids (and) superoxide dismutase	1.50
Water (<i>aqua</i>) (and) <i>Fagus sylvatica</i>	2.00
Glycerin (and) water (<i>aqua</i>) (and) <i>Buddleja davidii</i> (and) <i>Thymus vulgaris</i>	3.00
Glycerin (and) water (<i>aqua</i>) (and) <i>Plantago lanceolata</i>	1.00
Water (<i>aqua</i>)	4.00
Sodium hyaluronate	0.10
Water (<i>aqua</i>)	2.00
Citric acid	0.20
Sodium hydroxide	3.80
Fragrance (<i>parfum</i>)	<u>0.30</u>
	100.00

Procedure: Prepare A in the main mixer under vacuum. Add B; mix with turbine under vacuum; after complete dispersion of AB, add C. Mix under vacuum until complete swelling. Heat to 70°C. Melt D at 70°C then add to the main mixer. Homogenize 10 min. Cool to 40°C while mixing, then add E in order. Cool to room temperature while mixing with blades under vacuum.

Characteristics: Ivory creamy emulsion; pH 6.8, viscosity (Brookfield RVT): 68,000 mPa.s at 5 rpm, 25°C

Comment: With emollient and rich texture, this emulsion is effective in controlling the age signs at different skin levels. The principles include sodium DNA with antiaging effects, in synergy with actives of vegetable origin, with antioxidant molecules and soothing, moisturizing, protective actives having filming and moisturizing properties.

Conclusions

Epidermal keratinocytes and skin fibroblasts of human origin were tested for cell vitality and phototoxicity. Within the bounds of *in vitro* models, the test results suggested that nucleotide segments of sodium DNA in a proprietary material called DNA-Na in this article had a protective and regenerating effect on the skin. *In vivo* tests of a formulation containing DNA-Na showed a significant effect on increasing the basal values of moisturization, elasticity and skin thickness as well as in reducing skin wrinkledness.

Even if it is well-known that nucleotides, nucleosides, purine bases and pyrimidine bases enhance cell proliferation *in vitro*, the mechanisms involved in these actions are still controversial. These compounds are reported both to synergize with growth factors and to act directly on purinergic receptor A₂ inducing per se a proliferative response. Moreover, they could stimulate the skin's photoprotective mechanisms and an immediate action against free radicals.¹⁹ Indeed, when cultured fibroblasts were incubated with radioactive amino acids in the presence of oligonucleotides, the incorporation of the tracer into secreted proteins increased significantly.⁹

In conclusion, regardless of the protection and repair mechanism involved, cell proliferation after contact with DNA-Na takes place, and the tested active principle proved *in vivo* to have a multipurpose activity in improving the appearance of age signs. This activity is related not only to improved water coordination, but also to increased cohesiveness of superficial skin layers, as demonstrated by the improvement of skin thickness and elasticity.

Published November 2006 *Cosmetics & Toiletries* magazine.

References

1. CA Emanuel and IL Chaikoff, The large scale preparation of sodium deoxyribonucleate from ripe salmon testes, *J Biol Chem* 203 164 (1953)
2. S Zamenhof, Preparation and assay of deoxyribonucleic acid from animal tissue, *Methods Enzymol* 3 696-704 (1957)
3. UG Henning, Q Wang Q, NH Gee and RC von Borstel, Protection and repair of gamma-radiation-induced lesions in mice with DNA or deoxyribonucleoside treatments, *Mutat Res* 350(1) 247-254 (1996)
4. L Zagami and F Lesca, Management of cervico-vaginal dystrophies, *Minerva Ginecol* 43(4) 185-190 (1991)

5. JuP Vainberg, DN Nosik, EN Kaplina, NN Nosik, LB Kalnina and LA Lavrukina, Action of drugs based on native DNA against RNA and DNA containing viruses, *Klin Med (Mosk)* 73(6) 3 (1995)
6. M Cavallini, Biorevitalization and cosmetic surgery of the face: Synergies of Action, *J Appl Cosmetology* 22(7–9) 125–132 (2004)
7. L Valdatta, A Thione, C Mortarino, M Buoro and S Tuinder, Evaluation of the efficacy of polydeoxyribonucleotides in the healing process of autologous skin graft donor sites: A pilot study, *Curr Med Res Opin* 20(3) 403–408 (2004)
8. S Thellung, T Florio, A Maragliano, G Cattarini and G Schettini, Polydeoxyribonucleotides enhance the proliferation of human skin fibroblast: involvement of A2 purinergic receptor subtypes, *Life Sci* 64(18) 1661–1674 (1999)
9. P Sini, A Denti, G Cattarini, M Daglio, ME Tira and C Balduini, Effect of polydeoxyribonucleotides on human fibroblasts in primary culture, *Cell Biochem Funct* 17(2) 107–114 (1999)
10. P Rubegni, G De Aloe, C Mazzatenta, M Figiani and L Cattarini, Clinical evaluation of the trophic effect of polydeoxyribonucleotide (PDRN) in patients undergoing skin explants. A pilot study, *Curr Med Res Opin* 17(2) 128–131 (2001)
11. MP Rathbone, PJ Middlemiss, JW Gysbers, C Andrew, MA Herman, JK Reed, R Ciccarelli, P Di Iorio and F Caciagli, Trophic effects of purines in neurons and glial cells *Prog Neurobiol* 59(6) 663–690 (1999)
12. F Marzatico, Study concerning the in-vitro evaluation of keratinocytes and fibroblasts proliferation in presence and in absence of a substance that works as cell-growth stimulator, *University of Pavia, Department of Physiological-Pharmacological-Cellular & Molecular Sciences. Prot. N° M320503V* (2003)
13. F Marzatico, In-vitro phototoxicity study on modified 3T3 NRU to evaluate the vitality of fibroblasts irradiated with a UVA source in presence and in absence of a cell-protecting substance, *University of Pavia, Department of Physiological-Pharmacological-Cellular & Molecular Sciences. Kalichem Study* (2003)
14. Instrumental evaluation of the efficacy of an anti-wrinkle cosmetic ingredient; Study 176/05/01-02, *ISPE, Milano*
15. E Berardesca, EEMCO guidance for the assessment of stratum corneum hydration: Electrical methods, *Skin Res Technol* 3 126–132 (1997)
16. L Rodriguez, EEMCO guidance for the assessment of tensile functional properties of the skin—Part 2, *Skin Pharmacol Appl Skin Physiol* 14(1) 52–67 (2001)
17. JL Leveque, EEMCO guidance for the assessment of skin topography, *Eur Acad Dermatol Venereol* 14(1) 52–67 (1999)
18. JM Kirsch, J Hanson and G Tikjob, The determination of the skin thickness using non-conventional diagnostic ultrasound equipment, *Clin Experimental Dermatol* 9 280–285 (1984)
19. IM Hadshiew, MS Eller, I Moll and BA Gilchrest, Photoprotective mechanisms of human skin. Modulation by oligonucleotides, *Hautarzt* 53(3) 167–173 (2002)

Wrinkle Reduction by Stimulation of the Skin's Mechanical Resistance

Catherine Lenaers, David Boudier, Christa Chauprade, Delphine Rondeau and
Brigitte Closs

Silab, Brive, France

KEY WORDS: *antiaging, skin mechanical resistance, mechano-receptor, integrin, vinculin, alpha-smooth muscle actin*

ABSTRACT: *A rye extract rich in arabinoxylans is shown to stimulate the synthesis of mechano-receptor proteins and alpha-SMA fibers in the skin, improving the skin's mechanical resistance and reducing the appearance of gravity wrinkles.*

Wrinkles are a symptom of structural failure in the dermis. They indicate that the skin is losing its ability to support its own weight, and that fibroblasts in the dermis are losing their capacity to attach to collagen fibers and transmit mechanical information.

This chapter reports on an arabinoxylans-rich rye extract that stimulates the skin's lifting properties and boosts the mechanical resistance of the dermis.

Skin Resistance Mechanisms

The dermis is a support connective tissue composed primarily of fibroblasts and a vast microfibril network of collagen, elastin and proteoglycans. This fiber network undergoes a constant but very slow renewal, during which the synthesis of macromolecules and their degradation by matrix metalloproteinases (MMPs) are equilibrated until adulthood. This balance depends on such factors as nutritional

supplies, hormonal status, the influence of toxic compounds or the external environment.

In humans at around the age of 30, the skin begins to accumulate a number of changes to cells and their support. In this process called intrinsic aging, the skin acts as if its support structures had lost their intrinsic mechanical properties due to the aging of the structural material or, more likely, due to insufficient maintenance of that material by the cells. In fact, those intrinsic mechanical properties suffer due to the loss of the interaction between fibroblasts and the extracellular matrix (ECM). This interaction normally is ensured by mechano-receptors that are linked to alpha-smooth muscle actin (alpha-SMA) fibers. Mechanical stress is transmitted through the epidermis and then the ECM to reach the mechano-receptors located at the fibroblast plasma membrane. Afterwards, the mechano-receptors transduce the mechanical information into intracellular messages.

The clinical manifestations of these deteriorations involve two major types of surface deformations—expression wrinkles and gravity wrinkles. Mechanical forces in tissues and the phenomenon of gravitation play a fundamental role in their appearance.¹ The formation of gravity wrinkles occurs in the skin wherever it has the capacity to stretch under the influence of its own weight. On the face, for example, the upper eyelids and the lower part of the face are the preferred locations.

The dermis also is altered by UV radiation, especially UVA. It leads to an increase in the production of MMPs that are responsible for the degradation of macromolecules that constitute the ECM. The process of extrinsic aging is also characterized by a reduced fibroblast proliferation capacity and by a reduction of their metabolic activity and migration capacity. This reduction in migration is correlated with a decrease of integrins, which are the proteins involved in the attachment of fibroblasts to collagen fibers and in the transmission of mechanical information.^{2,3}

The ECM is the adhesion substrate of fibroblasts and the mechanical support of the skin. The ECM transmits mechanical stress all the way to fibroblasts. These cells play the role of a strain gauge, detecting mechanical stresses transmitted by receptors

responsible for the junction between the fibroblast and its network of collagen fibers. These mechano-receptors, or shock absorbers, activated by the application of pressure on the surface of the skin, play a central role in the cell mechanics of the skin (**Figure 1**). They integrate the mechanical signal and then act as mechano-effectors by transmitting the shock to adjacent cells by changing their cytoskeleton and activating a variety of intracellular signaling pathways.⁴ Fibroblasts respond to these signals by synthesizing components of the dermal matrix and simultaneously inhibiting the production of MMPs and pro-inflammatory cytokines.⁴⁻⁶ Thus, fibroblasts produce a more resistant support to adapt the resistance of the skin to the mechanical stress, and the translated mechanical information enables the control of biological homeostasis of the dermis.⁷

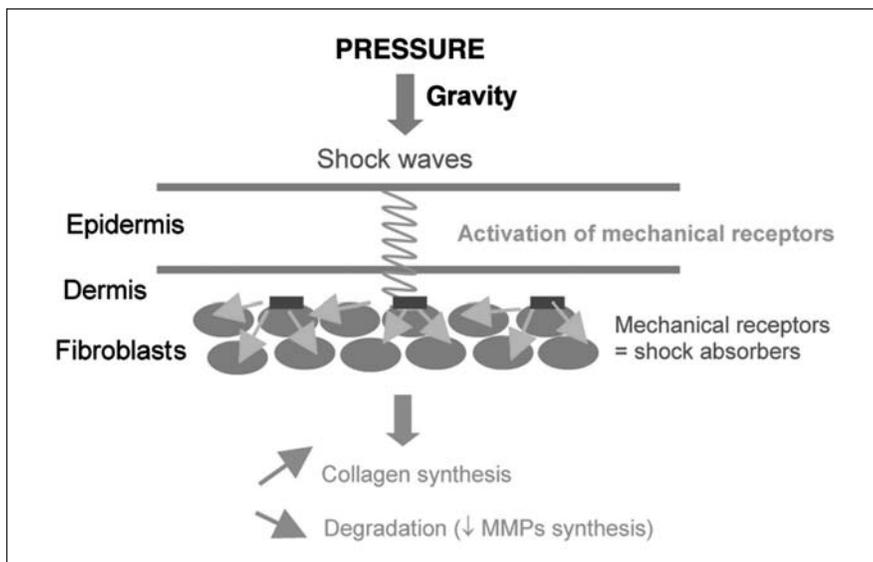


Figure 1. Mechanisms involved in skin resistance

Mechano-receptors and alpha-SMA

The attachment of fibroblasts to collagen fibers, or initial adhesion, is ensured by alpha2beta1 integrins, which are dimeric transmembrane proteins produced in the fibroblasts and specifically involved in activating the cell-signaling pathway.⁸ Faced with a mechanical stress perceived as a signal by the cell, initial adhesion is reinforced

to form a mechano-receptor (**Figure 2**) composed of a set of structural and signaling proteins, among which are talin, vinculin and paxillin.

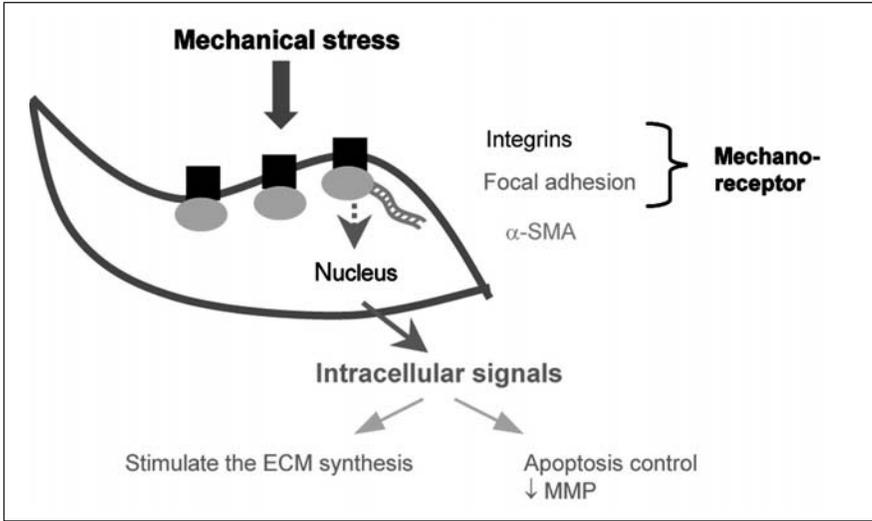


Figure 2. Role of the mechano-receptor and alpha-SMA in cell mechanics

Formation of the mechano-receptor is marked by the incorporation of vinculin in initial adhesion. Vinculin is a relevant marker of the mechano-receptor because it is absent in initial adhesions and its accumulation is correlated with the formation of the mechano-receptor^{9,10} and with the adhesion force of fibroblasts to the matrix.¹¹ Stress fibers attached to this mechano-receptor include alpha-SMA, responsible for the generation of retractile forces of fibroblasts.⁸

The mechano-receptors are activated in conditions of stress and translate the mechanical message into an intracellular signal that organizes the alignment of fibroblast stress fibers. The presence of alpha-SMA in fibroblasts ensures the maturation of mechano-receptors, thereby enabling a more efficient transmission of the mechanical signal. The increased tension of the ECM will stimulate the expression of alpha-SMA by fibroblasts.¹¹ The association of mechano-receptors with alpha-SMA is not only essential for generating tension forces, but also plays a central role in the regulation of dermal mechanical resistance by ensuring a perpetual equilibrium between the states of contraction and relaxation of the skin.

Reinforcement of the initial adhesion into mechano-receptors requires only 10–40 sec. This speed suggests that the formation of mechano-receptors is regulated locally and that it plays a major role in the transmission of forces to the ECM to modulate the mechanical resistance of the skin. If the mechanical stress continues, the mechano-receptor continues to develop into a broad and stable focal adhesion that extends along the periphery of the cell. This process requires about 60 min and enables fibroblasts to induce a longer-term elevated mechanical response, because the focal adhesion can exert higher forces than mechano-receptors.⁹

Age-reduced Interaction Between Fibroblasts and the ECM

As humans age or accumulate exposure to UV, their fibroblasts produce reduced quantities of alpha2beta1 integrins.^{2,3} That reduction leads to a loss of their attachment to the ECM, a contact that is indispensable for maintaining the mechanical properties of the skin.

Aged or photoexposed skin is characterized by a degraded ECM composed of fragmented collagen fibers. This deteriorated support no longer ensures optimal adhesion of fibroblasts, leading to a state of mechanical relaxation. In this state, mechano-receptors are dissolved by the breakdown of vinculin,^{4,10} leading to a loss in the cell-matrix interaction and thus a reduction in tension of the support. In addition, the expression of alpha-SMA by fibroblasts from old skin is reduced⁷ and the organization of these fibers is also altered,² causing a reduction in the retractile forces of fibroblasts and in their response to mechanical stresses. The mechanical resistance of the skin to pressure and gravity shocks is thereby reduced, causing the appearance of wrinkles.³

Favoring the interaction of fibroblasts with their support is thus essential for stimulating the adhesion capacities of fibroblasts and for restoring the mechanical properties of the dermis.

A Rye Extract for Increased Mechanical Resistance of Skin

Researchers at Silab began a search for a new material that would increase the skin's mechanical resistance. Having identified the

biological markers—integrins, vinculin and alpha-SMA—involved in the skin's mechanical resistance, the Silab researchers studied the behavioral differences between normal human fibroblasts and aged human fibroblasts in terms of synthesis of the selected biological markers. Using *in vitro* methods, the researchers screened different plant molecular structures for activity on the three biological markers before finally selecting arabinoxylans. Arabinoxylans, usually extracted from cereals, are polysaccharides composed of a repeated xylan backbone that can be substituted by one or two arabinose.

Published data was searched for a plant that was rich in arabinoxylans and met several additional criteria: low toxicological risk, high potential efficacy, available industrial supply, and free of patent for cosmetic applications. Fifteen plants were screened. They included maritime pine, millet fiber, sugar-apple, cotton, linseed, soy, hemp, nut grass, millet seed, chervil, wheat, apple marc, rye and root of water lily. The results of the screening showed that only the rye (*Secale cereale*) raw material responded to the three biological markers and to the other criteria of raw material selection. Up to now, no other raw material having the same properties as the rye extract has been found. But, now that arabinoxylans has been identified as the active fraction of the rye extract, one can imagine finding another plant rich in arabinoxylans having the same effects as the rye and corresponding to the same concept.

The active ingredient was obtained from the rye seeds after controlled enzymatic hydrolysis. A unique purified rye extract^a was composed of carbohydrates (92%), mineral ashes (4%) and proteins (3%). Quantification of carbohydrates was carried out by the assay of DuBois et al.¹² The carbohydrate analysis by GC/FID led to identification of glucose and arabinoxylan oligomers. The rye extract with this composition and approximately 10% arabinoxylans is the subject of the study reported here.

The phenolic compound ferrulic acid was used as a marker to study the rye extract in formula and to follow its stability after 6, 12 and 18 months at 42°C. The method used for these studies was HPLC (high performance liquid chromatography) analysis. The rye extract was also tested with *in vitro* methods in order to check its activity over time.

^a Coheliss (INCI: Water (aqua) (and) Secale cereale (rye) (seed extract). Coheliss is a registered trademark of Silab.

Material and Methods

The rye extract was tested *in vitro* on normal human fibroblasts and aged human fibroblasts. The syntheses of alpha2beta1 integrins and vinculin were analyzed using quantitative polymerase chain reaction (PCR). The alpha-SMA expression was quantified by spectrofluorimetry and visualized using immunocytology.

Synthesis of alpha2beta1 integrins and vinculin: An *in vitro* study was carried out by quantitative PCR on a pool (4 donors) of normal human fibroblasts, compared to a model of aged human fibroblasts. The aged human fibroblasts were obtained after 25 successive cell transfers. Cell aging was verified by visualizing a senescence marker^b.

Normal and aged human fibroblasts were inoculated in petri dishes 100 mm in diameter and incubated at 37°C for 48 h in an atmosphere of 5% CO₂. The cells were then grown for 24 h in the presence or absence of rye extract at 0.25% (v/v) or TGF-beta1^c at 1 ng/mL used as reference molecule.

At the end of incubation, the cells were recovered and total RNA was extracted. The RNA was reverse-transcribed and the complementary DNA obtained was analyzed by quantitative PCR. Beta-actin mRNA, the internal standard, also was analyzed in parallel to the mRNA of alpha2beta1 integrins and vinculin. Quantification of fluorescence incorporation (SYBR Green) was measured^d continuously. The analysis of cycle threshold (relative quantification) was carried out with software^e.

Expression of alpha-SMA: Two *in vitro* studies were carried out to evaluate the expression of alpha-SMA by normal human fibroblasts and aged human fibroblasts.

In the first study, the expression of alpha-SMA was quantified by spectrofluorimetry. Normal and aged human fibroblasts were inoculated in 100 mm diameter petri dishes and incubated at 37°C for 48 h in an atmosphere of 5% CO₂. Then the cells were grown for 24 h in the presence or absence of rye extract at 0.25% and 0.50% (v/v) or TGF-beta1^c at 1 ng/mL. This treatment was repeated once.

^b beta-Galactosidase, available as C-50030 from Sigma, USA

^c T-7039 from Sigma, USA

^d iCycler thermocycler, model MyiQ, Bio-Rad, USA

^e Genex software, Bio-Rad

After four days of incubation with the products, fibroblasts were immunolabeled. The cells were recovered, permeabilized and incubated for 45 min at 4°C with an alpha-SMA monoclonal antibody^f followed by the addition of a second antibody^g. The level of alpha-SMA was quantified with a plate reader^h by fluorescence excitation at 488 nm and emission at 530 nm.

The number of cells was quantified by the incorporation of a solution of propidium iodide^k at 15 µg/mL. The cell count was estimated with a plate reader of fluorescence excitation at 365 nm and emission at 605 nm.

In the second study, the expression of alpha-SMA was visualized by immuno-cytology. Normal and aged human fibroblasts were inoculated on glass slides^m in complete culture medium for 48 h. Then the cells were treated with the rye extract at 0.10% (v/v) diluted in complete culture medium and incubated at 37°C in an atmosphere of 5% CO₂ for 48 h. This treatment was repeated once.

After four days of treatment with the products, alpha-SMA were labeled by immunocytology. The slides were rinsed in phosphate-buffered saline (PBS) and fixed in a 4% (v/v) solution of paraformaldehydeⁿ for 15 min. Then the slides were rinsed in PBS, permeabilized in a solution of saponin^p at 0.50% (w/v) for 5 min and again rinsed in PBS buffer. Finally, the cells were incubated with a murine alpha-SMA monoclonal antibody^f followed by the addition of a second antibody^q. Visualization was realized with a microscope^r coupled to an image analysis system^s.

Biomechanical properties of the skin: An *in vivo* study of the skin's biomechanical properties was conducted on 20 healthy female volunteers aged 39–70 years (mean age 56 ± 8 years). Measurements of the face were made instrumentally^t before and after 56 days of twice

^f A-2547 from Sigma, USA

^g Murine Alexa Fluor-488-conjugated anti-IgG, A-11017 from Interchim, France

^h Fluorolite 1000, Dynex, USA

^k P-4170, Sigma, USA

^m Labtek, USA

ⁿ F-1635, Sigma, USA

^p S-4521, Sigma, France

^q FITC-coupled murine anti-IgG, Alexa Fluor A488 from Molecular Probes, USA

^r Olympus IX 70 microscope, Japan

^s VisioLab 2000, Biocom, France

^t SEM 575 Cutometer, Courage & Khazaka, Germany. Cutometer is a registered trademark of Courage & Khazaka.

daily treatment with the rye extract formulated (**Formula 1**) at 4% in an emulsion vs. placebo.

Skin tone and the tensor effect were determined in order to monitor the biomechanical properties of the skin under the effect of the treatments. Skin tone was evaluated with the parameter X and tension was quantified with the parameters U_f and U_e . U_f is the visco-elastic component of the skin; if U_f decreases, the skin is less extensible, thus more taut. U_e is the elastic component of the skin; if U_e decreases, the skin is less flexible, thus more taut. In this study the negative form of the parameter was used to obtain a positive measurement of the tone and tension.

Antiwrinkle and smoothing properties: An *in vivo* study was conducted on 20 healthy female volunteers aged 39–70 years (mean age 56 ± 8 years). Silicone polymer replicas were made of the crow's-feet and the nasolabial fold before and after 56 days of twice daily treatment with the rye extract formulated (**Formula 1**) at 4% in an emulsion vs. placebo.

Formula 1. Test emulsion

Isononyl isononanoate (Lanol 99, Seppic)	5.0% wt/wt
Water (<i>aqua</i>) (and) <i>Secale cereale</i> (rye) seed extract (Coheliss, Silab)	4.0
Cetearyl alcohol/cetearyl glucoside (Montanov 68, Seppic)	2.5
Arachidyl alcohol (and) behenyl alcohol (and) arachidyl glucoside (Montanov 202, Seppic)	2.0
Butylene glycol	2.0
Methylparaben (and) ethylparaben (and) butylparaben (and) propylparaben (and) isobutylparaben (and) phenoxyethanol (Phenonip, Clariant)	0.7
Sodium polyacrylate (and) C13-14 isoparaffin (and) <i>Paraffinum liquidum</i> (mineral) oil (and) polyacrylamide (and) polysorbate 85 (Sepigel 501, Seppic)	0.3
Water (<i>aqua</i>)	qs to 100.0

The antiwrinkle effect was analyzed by observing the replicas with a profilometer equipped with an image analyzer¹. Three param-

eters were studied: the number, total surface and total length of wrinkles. The smoothing effect at the crow's-feet was analyzed by observing replicas with a profilometer equipped with an image analyzer^v. Two parameters characteristic of skin surface relief were studied: the index of mean roughness (Ra) and that of maximal roughness (Rz).

Results

Synthesis of alpha2beta1 integrins and vinculin: The expression of mRNA of alpha2beta1 integrins and vinculin by untreated aged fibroblasts was reduced by 16% (**Figure 3**) and 19% (**Figure 4**), respectively, in comparison to untreated normal human fibroblasts. Tested at 0.25%, the rye extract increased the expression of mRNA of alpha2beta1 integrins and vinculin in normal fibroblasts by 20% and 28%, respectively, and also restored their normal levels of expression by aged human fibroblasts. The rye extract boosted the synthesis of alpha2beta1 integrins and vinculin, thereby favoring the formation of mechano-receptors and increasing adhesion between fibroblasts and the ECM, as well as the transmission of mechanical messages.

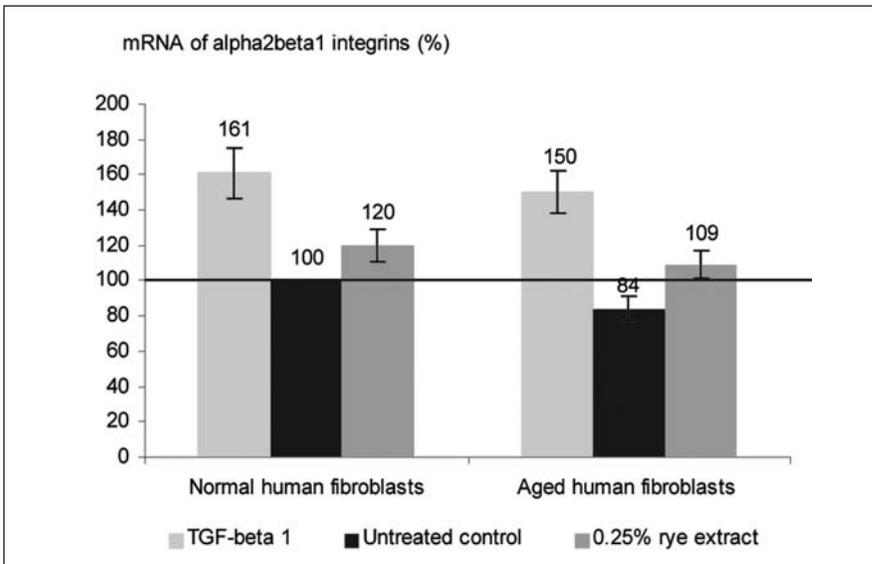


Figure 3. Effect of the rye extract on the expression of alpha2beta1 integrins mRNA by normal and aged fibroblasts

^u Quantirides 99, Monaderm, Monaco. Quantirides is a registered trademark of Monaderm.

^v Quantilines, Monaderm

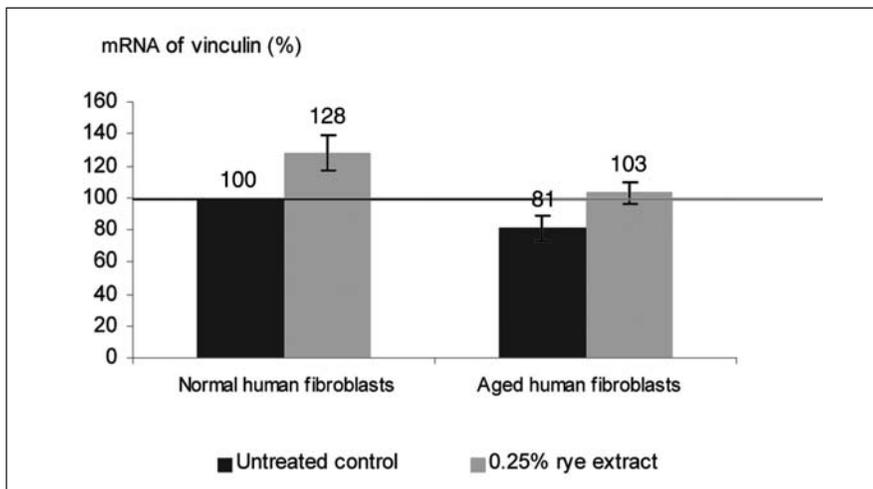


Figure 4. Effect of the rye extract on the expression of vinculin mRNA by normal and aged fibroblasts

Expression of alpha-SMA: In cultures of untreated aged human fibroblasts, alpha-SMA levels were reduced by 60% compared to untreated normal human fibroblasts (**Figure 5**). Tested at 0.50%, the rye extract produced a 62% increase in alpha-SMA expression by normal human fibroblasts and exhibited a dose-dependent tendency to restore alpha-SMA expression by aged human fibroblasts.

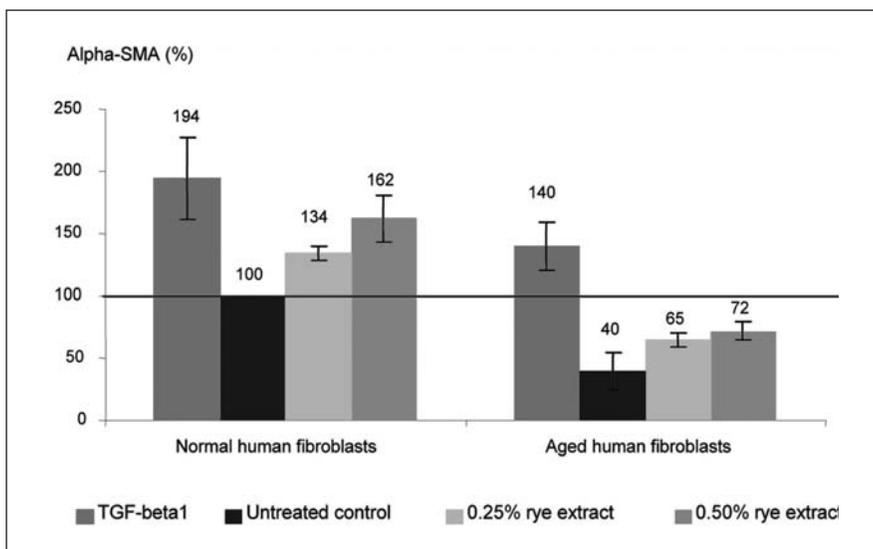


Figure 5. Effect of the rye extract on the expression of alpha-SMA mRNA by normal and aged fibroblasts

The expression of alpha-SMA was visualized by immunocytology. The qualitative results were consistent with those obtained with the spectrofluorimetric assay: the capacity of aged human fibroblasts to express alpha-SMA fibers was reduced, but was stimulated after treatment of cells with the rye extract at 0.10%. The rye extract thus stimulates the contractile properties of fibroblasts that enable the skin to respond to a variety of mechanical stresses to which it is subjected on a daily basis.

Biomechanical properties of the skin: After 56 days of twice daily applications and in comparison to the placebo, the rye extract significantly increased the parameter $-X$ characteristic of skin tone by 19% ($P = 0.0279$). A study of the distribution of the results showed that 72% of the volunteers presented improved skin tone.

In addition, the rye extract also significantly improved parameters $-U_f$ (16%, $P = 0.0136$) and $-U_e$ (19%, $P = 0.0173$), representative of skin tension. This effect was observed in 78% of the volunteers. The rye extract thus renders skin tissue firmer.

Antiwrinkle and smoothing properties: After 56 days of twice daily applications and in comparison to the placebo, the rye extract at 4% in an emulsion presented a significant antiwrinkle effect ($P < 0.05$) at both the crow's-feet and the nasolabial fold, respectively: a 13% and 23% decrease in the number of wrinkles, a 15% and 26% reduction of the total surface of wrinkles, and a 16% and 27% reduction of their length.

The replicas shown in **Figure 6** illustrate the skin surface of the crow's-feet area before and after a volunteer was treated with the rye extract. A study of distribution of the results showed that 67% and 78% of the volunteers presented a decrease in the total wrinkled surface at the crow's-feet and nasolabial fold, respectively. In addition, the rye extract also smoothed skin microrelief by significantly reducing parameters R_a (-7.8% , $P = 0.0071$) and R_z (-5.0% , $P = 0.0247$). The rye extract attenuated roughness of skin microrelief and thereby improved its surface properties.

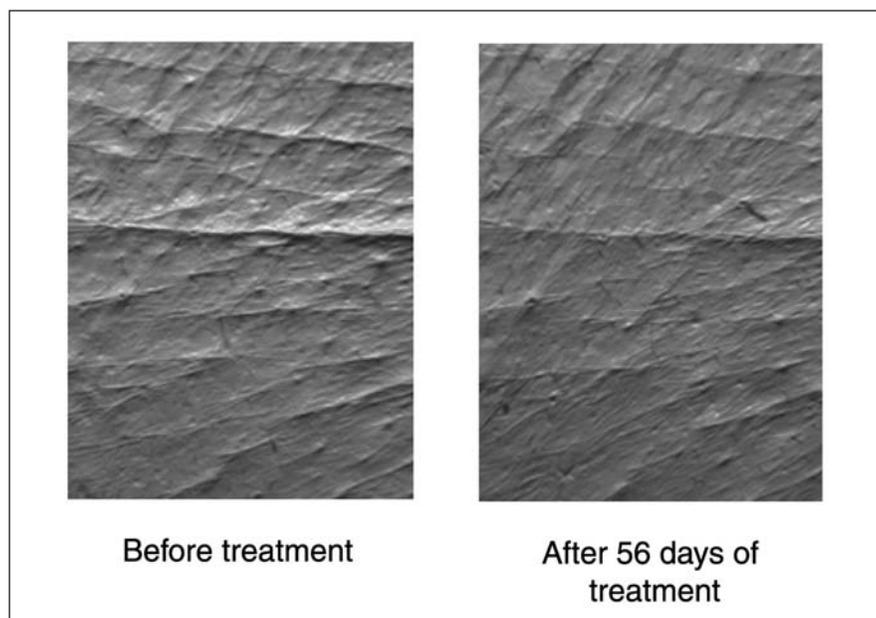


Figure 6. Visualization of the antiwrinkle effect of the rye extract on the crow's-feet of a volunteer

Conclusion

The capacity of the skin to adapt to mechanical stresses, or cell mechanics, can be considered as a new approach to the fight against the signs of aging, especially those of chronobiological aging.

An active substance, rich in arabinoxylans and purified from rye seeds, was shown here in several studies to act as an internal tensor agent. The expression of proteins of the mechanical receptor ($\alpha 2\beta 1$ integrins and vinculin) and of α -SMA fibers by aged human fibroblasts was reduced in comparison to those of normal human fibroblasts. The biological properties of the rye extract restored the expression of $\alpha 2\beta 1$ integrins and vinculin to normal levels, and had a similar effect on the synthesis of α -SMA by aged human fibroblasts.

Tested directly on volunteers, the rye extract formulated at 4% in an emulsion firmed skin tissue as shown by the significant 19% increase in skin tone ($-X$) and increases of 16% and 19% in two skin tension parameters ($-U_f$ and $-U_e$, respectively) after 56 days of twice daily applications.

The rye extract also significantly improved the surface properties of the skin. It attenuated wrinkles at the crow's-feet in 72% of the volunteers and at the nasolabial fold in 78% of the volunteers, and it smoothed skin microrelief by reductions of 7.8% and 5.0% in the indexes of mean roughness (Ra) and maximal roughness (Rz), respectively.

The rye extract boosts the natural skin equipment that participates in its mechanical resistance and restores its natural lifting properties.

Published November 2006 *Cosmetics & Toiletries* magazine.

References

1. CM Lapière, Rides d'expression et plis gravitationnels: des mécanismes physiologiques diamétralement opposés, *Dermatol Pratique* 280(5) 10–11 (2004)
2. MJ Reed, NS Ferara and RB Vernon, Impaired migration, integrin function, and actin cytoskeletal organization in dermal fibroblasts from a subset of aged human donors, *Mec Ageing Dev* 122(11) 1203–1220 (2001)
3. J Varani, L Schuger, MK Dame, C Leonard, SEG Fligel, S Kang, GJ Fisher and JJ Voorhees, Reduced fibroblast interaction with intact collagen as a mechanism for depressed collagen synthesis in photodamaged skin, *J Invest Dermatol* 122(6) 1471–1479 (2004)
4. D Kessler, S Dethlefsen, L Haase, M Plomann, F Hirche, T Krieg and B Eckes, Fibroblasts in mechanically stressed collagen gels assume a "synthetic" phenotype, *J Bio Chem* 276(39) 36575–36585 (2001)
5. M Flück, MN Giraud, V Tunç and M Chiquet, Tensile stress-dependent collagen XII and fibronectin production by fibroblasts requires separate pathways, *Biochim Biophys Acta* 1593(2–3) 239–248 (2003)
6. KM Südel, K Venzke, H Mielke, U Breitenbach, C Mundt, S Jaspers, U Koop, K Sauermann, E Knussmann-Hartig, L Moll, G Gercken, AR Young, F Stäb, H Wenck and S Gallinat, Novel aspects of intrinsic and extrinsic aging of human skin: beneficial effects of soy extract, *Photochem Photobiol* 81(3) 581–587 (2005)
7. CA Lambert, AC Colige, CM Lapière and BV Nusgens, Coordinated regulation of procollagens I and III and their post-translational enzymes by dissipation of mechanical tension in human dermal fibroblasts, *Eur J Cell Biol* 80(7) 479–485 (2001)
8. FG Giancotti, Integrin signaling: specificity and control of cell survival and cell cycle progression, *Curr Opin Cell Biol* 9(5) 961–700 (1997)
9. CG Galbraith, KM Yamada and MP Sheetz, The relationship between force and focal complex development, *J Cell Biol* 159(4) 695–705 (2002)
10. QM Chen, VC Tu, J Catania, M Burton, O Toussaint and T Dille, Involvement of Rb family proteins, focal adhesion proteins and protein synthesis in senescent morphogenesis induced by hydrogen peroxide, *J Cell Sci* 113 4087–4097 (2000)
11. B Hinz, V Dugina, C Ballestrem, B Wehrle-Haller and C Chaponnier, Alpha-smooth muscle actin is crucial for focal adhesion maturation in myofibroblasts, *Mol Bio Cell* 14(6) 2508–2519 (2003)
12. M DuBois et al, *Analytical Chemistry* 28(3) 350–356 (1956)

Peptides in the Pipeline for Antiaging

Bud Brewster

Cosmetics & Toiletries *magazine*

KEY WORDS: *peptides, antiaging, amino acids*

ABSTRACT: *This chapter reviews peptides and their future use.*

Want to give a clear signal about your intentions to add antiaging efficacy to a skin care product? If so, add an ingredient whose whole purpose in life is signaling, i.e., delivering a chemical message that up regulates or down regulates a particular cellular function. That signaling ingredient is the peptide.

A peptide begins with an α -amino acid, which is a molecule that contains amine and carboxyl functional groups attached to the same carbon atom (**Figure 1**). Twenty of these amino acids are used by cells in protein biosynthesis specified by the general genetic code. A peptide is a molecule formed by arranging any of these amino acids in a defined order and linking each acid to the next with an amide bond.

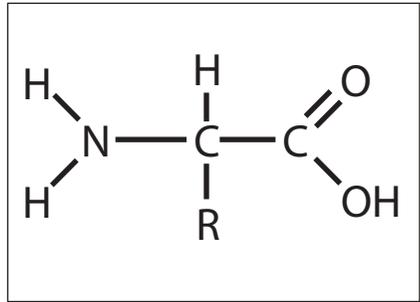


Figure 1. The general structure of an amino acid

The 20 amino acids and their abbreviations are listed in **Table 1**. A peptide will contain at least one of those acids, and may repeat many times with or without occurrences of one or more of the other acids. An example is the sequence glycine-histidine-lycine, or GHK, found bound to copper in human serum. Research by Loren Pickart on wound healing and anti-inflammation in the 1970s established that this GHK-

Table 1. Amino acids used by cells to make proteins

Amino Acid	Abbreviations	
Alanine	A	Ala
Cysteine	C	Cys
Aspartate	D	Asp
Glutamate	E	Glu
Phenylalanine	F	Phe
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Lysine	K	Lys
Leucine	L	Leu
Methionine	M	Met
Asparagine	N	Asn
Proline	P	Pro
Glutamine	Q	Gln
Arginine	R	Arg
Serine	S	Ser
Threonine	T	Thr
Valine	V	Val
Tryptophan	W	Trp
Tyrosine	Y	Tyr

Cu peptide stimulates tissue regeneration and reverses certain aspects of aging.¹ This copper peptide was commercialized in the 1980s and is found today in the Visibly Firm line of antiaging products by Neutrogena and in antiaging products from many other companies.

The GHK in the copper peptide acts as a carrier for the larger-molecular-weight copper and enhances copper's penetration into the cell, where copper is a known co-factor in the production of collagen during wound healing.² But peptides with different amino acid sequences play many different roles in skin antiaging.

"Probably the newest use of peptides is as regulators of cellular functions," Zoe D. Draelos, MD, wrote earlier this year.²

Draelos is a clinical associate professor of dermatology at Wake Forest University School of Medicine in Winston-Salem, N.C.

"Owing to the fact that the body uses peptides for communication between cells, it was theorized that perhaps engineered peptides might be able to up regulate or down regulate cutaneous functions that had decayed with time due to the cumulative effects of aging," Draelos wrote.

"This theory was investigated more thoroughly by chemists under the direction of Karl Lintner at Sederma, France," Draelos continued. "They developed a variety of peptides and tested them in cell culture to determine their biologic effects. The most interesting peptide was found to be a pentapeptide composed of lysine, threonine, threonine, lysine and serine, or the KTTKS peptide."

Because peptides are fragile and have to penetrate into the skin before they can exert their cellular effects, Lintner and co-workers linked the KTTKS peptide to palmitic acid and then commercialized it as a hydroglycolic solution^a containing 100 ppm of palmitoyl pentapeptide-3, or pal-KTTKS. This molecule stimulates the skin fibroblasts in order to reconstitute the extracellular matrix, according to company technical data sheets.³ It reportedly leads to the synthesis of collagen I and IV, fibronectin and glycosaminoglycans.

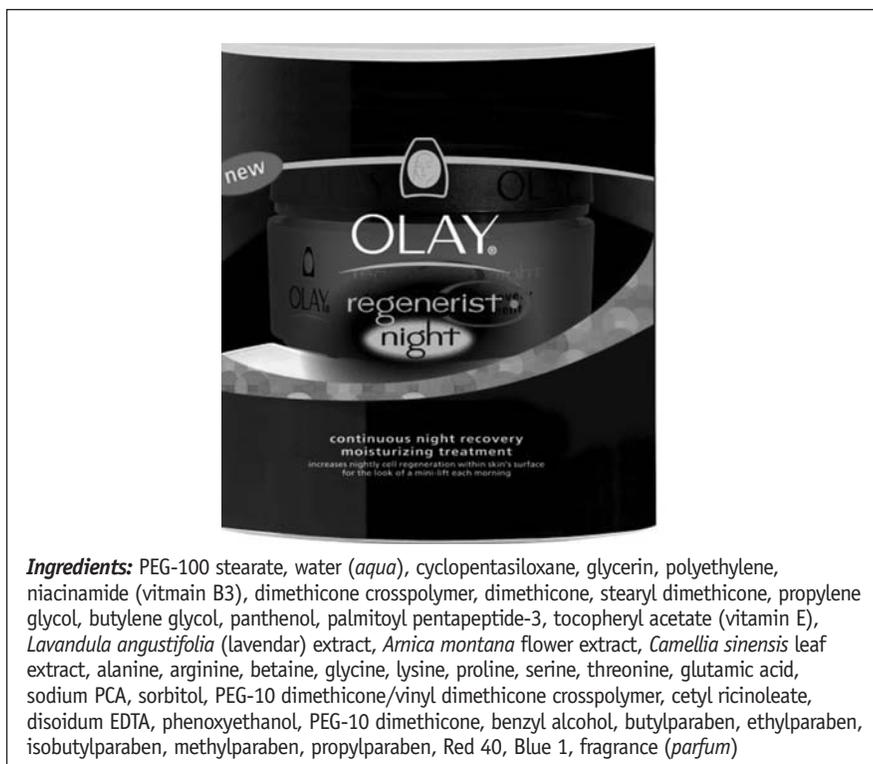


Figure 2. Olay Regenerist Continuous Night Recovery

Meanwhile, skin care scientists at Procter & Gamble, collaborating with Lintner and his colleagues, incorporated the pal-KTTKS peptide into prototype formulations, combining it with other antiaging ingredients to create an amino-peptide complex that is the basis of technology used in Olay Regenerist, Olay's antiaging range that launched in 2003. One of the products in that product line is

^a Matrixyl (INCI: Glycerin (and) water (*aqua*) (and) butylene glycol (and) carbomer (and) polysorbate 20 (and) palmitoyl pentapeptide-3) is a registered trademark of Sederma, France.

“Regenerist Continuous Night Recovery” (**Figure 2**). It recently was named as the winner of the 2006 Cosmetic Executive Women Beauty Awards in the category “Facial Skincare: Antiaging under \$20.”

Draelos is enthusiastic about the future of peptides. “Restoring youthful cellular communications is an enticing model to reverse the cell senescence that results from messages that are either not received or are improperly received,” she wrote. “Certainly, there will be advances made in peptide technology as the ability to measure effects of the new biologics progresses.”²

The Patent Pipeline

A September 2006 search on the terms *peptide* and *aging* in the online database of the US Patent and Trademark Office returned two issued patents and three patent applications disclosing peptides used as active ingredients in antiaging cosmetic compositions. They display a variety of amino acid sequences and peptide functions.

Amino acids from milk: The proteins in milk contain amino acids claimed to alleviate or prevent the effects of aging in skin, according to a patent application⁴ filed in 2004 by John A. Smith of Liverpool, Great Britain.

Casein is a phosphoprotein that is one of the chief constituents of milk and the basis of cheese. The invention calls for treating the skin with a polypeptide, or a derivative of a polypeptide, that is an amino acid sequence present in an alpha-S2 casein precursor. The sequence contains at least three amino acids and does not include the N-terminal end of the full alpha-S2 casein precursor. The preferred sequence is KVIPYVRYL. There may be as few as three and as many as 50 amino acids in the peptide. The most preferred number of amino acids is 9 to 31. Symrise GmbH controls the manufacturing and distribution of these peptides.

Apparently the peptides of this invention function as growth factors in the skin. The inventor notes antiaging claims made by Estée Lauder for a product containing epidermal growth factor and another containing whey proteins.

The patent application describes a procedure by which a typical peptide according to this invention can be prepared from cheese

why. Then *in vitro* tests demonstrate that this typical peptide increases collagen synthesis in fibroblasts and stimulates the growth of keratinocytes.

Curiously, the peptide of this invention, or a derivative of that peptide, also has an effect in alleviating or preventing periodontal disease.

LKKTET polypeptide: Inhibition or reversal of skin aging by actin-sequestering peptides is claimed in a 2004 patent application⁵ from Allan L. Goldstein of Washington, D.C. Actin is a global structural protein that polymerizes to form an actin filament that becomes the three-dimensional network, or cytoskeleton, inside human cells. Goldstein discovered that actin can be sequestered by peptides containing the amino acid sequence LKKTET. One example is thymosin beta4, a polypeptide isolated from the thymus. Thymosin beta4 contains 43 amino acids and is believed to play a role in endothelial cell differentiation and migration, T cell differentiation, actin sequestration and vascularization.

The LKKTET amino acid sequence appears to be involved in mediating actin sequestration or binding. The inventor speculates that its activity may be due, in part, to the ability to polymerize actin. No method is disclosed for preparation of the peptide except for a claim that it can be recombinant, synthetic, or an antibody.

Compositions containing this LKKTET peptide may reduce skin aging by effectuating growth of the connective tissue through extracellular matrix deposition, cellular migration and vascularization of the skin, according to the inventor. Improvements in skin elasticity, size reduction of an area of age-related skin darkening, and lightening of an area of age-related skin darkening are among the benefits claimed following topical or systemic administration.

RGS: The RGS amino acid sequence acts against skin aging by modulating the concentration of adenosine 5'-triphosphate (ATP) in the cell, the concentration of intracellular calcium, and the production and activation of proteins essential to the skin. This discovery was reported this year in a patent application⁶ by three French inventors.

The inventors note that the ATP molecule plays a central role in many cellular mechanisms. It is the principal source of energy for

the cells and it is a marker of cellular activity. For example, cell activity correlates closely with an increase in the synthesis of essential molecules, such as proteins or DNA. So, by increasing the quantity of intracellular ATP, the cell is stimulated and receives the energy needed to synthesize the enzymes that will induce mechanisms of activation and make it possible to increase cellular metabolism, according to the patent application.

The peptide described by these inventors corresponds to the general formula x_nRGSx_n in which x_n is an unspecified amino acid or one of its derivatives and n is an integer ranging between 0 and 3. Thus, the peptide could contain as many as nine amino acid residues, and must contain at least the sequence arginine-glycine-serine or derivatives from them. Peptides of this type can be obtained by chemical synthesis, which is the preferred method, or by fermentation, enzymatic synthesis or protein extraction.

In vitro studies reported in the application demonstrate this peptide's effect on the amount of intracellular ATP, protein expression of the extracellular matrix, keratin expression, cell differentiation, adipocytes and intracellular cAMP. **Formula 1** is among the several formulations included in the application.

Formula 1. O/W emulsion⁶

A. Cetearyl alcohol (and) cetearyl glucoside	5.00% wt/wt
<i>Simmondsia chimensis</i> (jojoba) seed oil	5.00
<i>Paraffinum liquidum</i> (mineral) oil	5.00
Isopropyl palmitate	7.00
B. Glycerin	5.00
Allantoin	0.10
Polyacrylamide (and) C13-14 isoparaffin (and) laureth-7	0.30
Preservative	0.50
Fragrance (<i>parfum</i>)	0.50
Arg-Gly-Ser peptide	1.5 ppm
Water (<i>aqua</i>)	qs

SNAP-25 amino acids: Facial wrinkles can be caused when the muscles in the epidermis are tensed, dragging the skin inwards. As described in a Lipotec patent⁷ issued in March 2006, this muscular tension is the result of hyperactivity of the nerves innervating the facial muscles. Nerve hyperactivity is characterized by the uncontrolled and excessive release of neurotransmitters that excite muscle fibers. Molecules that control neuronal exocytosis contribute to relaxing muscular tension and, consequently, to eliminating wrinkles.

The Lipotec patent discloses peptides from the neuronal protein SNAP-25 that function temporarily like Botox^b to paralyze the muscles that cause wrinkles in the skin. These peptides imitate the paralytic effects of the botulinum toxins in Botox, but the peptide molecules are simpler, more stable, less expensive, and do not cause immune reactions.

The peptide of this invention has a sequence of three to 30 adjacent amino acids from the amino end of SNAP-25 and functions as a neuronal exocytosis inhibitor. Cosmetic compositions containing this peptide optionally may contain one or more peptides from the carboxyl end of SNAP-25.

KTTKS in a fusion peptide: Improved delivery of the KTTKS sequence of amino acids is claimed for a fusion peptide disclosed in a patent⁸ issued in August and assigned to LG Household & Health Care Ltd. in Korea. This is the same amino acid sequence at the core of the Olay Regenerist technology. The Korean invention fuses the KTTKS peptide to another peptide that improves skin absorption and, importantly, enables cellular access.

It is known that peptides of amino sequences 182 to 216 and 197 to 241 of human collagen type I are effective in preventing aging of the skin by using a collagen synthesis process. However, these peptides have weak skin absorbency due to their water solubility, even when the peptides are grafted to palmitic acid, according to this patent. The Korean inventors solved this problem by using a relatively new protein penetration technology to invent a fusion peptide in which a Tat peptide that can penetrate cells is bound to a peptide derived from the C terminal of human collagen I (CHCI).

The Tat peptide is a type of protein of the Human Immunodeficiency Virus (HIV) type-1 that is self-penetrating; i.e., the Tat

^b Botox is a registered trademark of Allergan Inc.

peptide spontaneously passes through a cell membrane for easy penetration such that transportation into the cell is realized. The critical signal for opening the cell's lipid barrier is in the amino acid sequence RKKRRQRRR, which is located in the N terminal region of the Tat peptide. By covalently bonding skin-activating ingredients such as CHCI to a Tat peptide, the inventors produced a fusion peptide that directly and effectively penetrated into cutaneous cells.

The collagen component CHCI is a peptide containing from 5 to 35 amino acids that are continuous within the range of amino acids numbered 182 to 246 in human collagen type I. All fusion peptides prepared according to this invention will contain the KTTKS sequence. The preferred variants are YKTTKSS or EYKTTKSSRL, all from within the range of amino acids numbered 210 to 219.

This fusion peptide is claimed to be highly stable and have superior skin absorption capability. It reportedly provides a skin antiaging agent having superior synthesis of collagen and hyaluronic acid, antiaging effects and improved durability of the effects. The patent discloses a method for preparing one of these peptides. Also disclosed are the results from *in vitro* tests of the peptide's collagen synthesis, antiaging effects, transdermal absorption (penetration into skin cells), skin irritation and cytotoxicity.

Published November 2006 *Cosmetics & Toiletries* magazine.

References

1. L Pickart, *Reverse Skin Aging Using Your Skin's Natural Power*, Bellevue, Washington: Cape San Juan Press p 24 (2005)
2. ZD Draelos, From proteins to peptides—What they mean to the dermatologist, *US Dermatology Review* 2006, pp 92–93; www.touchbriefings.com/pdf/1746/ACF475C.pdf#search=%22%22from%20proteins%20to%20peptides%22%22 (Accessed Sep 9, 2006)
3. Matrixyl, The messenger peptide for dermal matrix repair: An alternative to retinol and vitamin C, a technical data sheet published by Sederma, France. www.evotique.com/matrixyl.pdf#search=%22%22The%20messenger%20peptide%20for%20dermal%20matrix%20repair%22%22 (Accessed Sep 25, 2006)
4. US Pat Applic 20040014653, Peptide composition, JA Smith, Great Britain (Jan 22, 2004)
5. US Pat Applic 20040067227, Inhibition or reversal of skin aging by actin-sequestering peptides, AL Goldstein, USA (Apr 8, 2004)
6. US Pat Applic 20060013794, Cosmetic or pharmaceutical composition comprising peptides with the sequences arg-gly-ser, C Dal Farra, N Domloge and J-M Botto, France (Jan 19, 2006)
7. US Pat 7,015,192, Neuronal exocytosis inhibiting peptides and cosmetic and pharmaceutical compositions containing said peptides, MCB Mira et al, assigned to Lipotec SA, Spain (Mar 21, 2006)
8. US Pat 7,094,407, Fusion peptide containing human type-I collagen derived peptide, preparation thereof, and skin anti-aging cosmetic composition comprising the same, N-G Kang et al, assigned to LG Household & Health Care Ltd, Korea (Aug 22, 2006)

An Herbal Blend for Antiaging Effects: TCM in Personal Care

Daniel Schmid, Esther Belser and Fred Züllli

Mibelle Biochemistry, Buchs, Switzerland

KEY WORDS: *sensitive skin, antiaging, protein oxidation, matrix metalloproteinase (MMP), traditional Chinese medicine (TCM)*

ABSTRACT: *A combination of Chinese herbs is shown here, by gene array analysis, to stimulate the skin's resistance against free radicals, oxidative stress and toxic molecules, while also reducing extracellular matrix degradation. The extract also is shown to help against skin irritation in vivo.*

Traditional Chinese medicine (TCM) is a holistic approach to healing that developed in China about 3,000 years ago and that typically includes therapies such as acupuncture, qigong exercises and herbal medicine. It is highly respected as a means to treat skin disorders and is especially well-perceived by European and Eastern cultures. Believing in the efficacy of TCM, the authors collaborated with expert Severin Bühlmann, PhD, to incorporate this approach into an herbal blend to treat dry, sensitive skin that is prone to psoriasis. The formula also was designed to induce an anti-inflammatory and soothing effect in skin.

Herbal Activity

Anti-inflammation: The anti-inflammatory activity of *Lonicera japonica* is well-documented in the literature. It is an important

Chinese herb that is used to treat inflammatory skin disorders. Ochnaflavone is a biflavonoid and the pharmacologically active compound found in *Lonicera*. It has been shown to block the inflammation pathway at two points: at the transcription factor NF-KB, and at the enzyme cyclooxygenase-2.^{1,2} NF-KB, a regulator of gene activity, is responsible for the formation of gene products that are used to induce an inflammatory response. When NF-KB is blocked, the typical inflammatory reaction to stimuli such as UV light or free radicals is also blocked. Cyclooxygenase-2 synthesizes lipid mediators called prostaglandins that are used to develop inflammation by, for example, increasing local blood circulation. Cyclooxygenase-2 is the target of many pharmaceuticals for relief from pain and inflammation.

Antibacterial activity: *Xanthium sibiricum* contains xanthanolide sesquiterpenes—pharmacologically active compounds that are reported to have antibacterial activity.³ In addition, *Cyperus rotundus* has been shown to exert antibacterial effects as well as strong antioxidant activity.⁴ While the individual activity of each of these Chinese herbs is important, the final activity results from this specific combination. Such combinations or blends are found in traditional recipes of Chinese medicine.

Combining these anti-inflammatory and antibacterial activities, the authors developed an herbal TCM blend^a comprising the aqueous extracts of *Lonicera japonica* flowers, *Xanthium sibiricum* fruits, and *Cyperus rotundus* roots. The efficacy of this TCM blend was tested *in vitro* by gene array analysis and *in vivo* in a clinical trial.

Gene Array Analysis

To determine the efficacy of the TCM blend, gene array analysis based on a DNA microarray technology^b was performed *in vitro* using keratinocytes. This method analyzes the effects of certain compounds on the metabolism of cells. It is a well-established technique that uses chip and robotics technology to measure the activity and expression of hundreds to thousands of different genes in one experiment.

^a EpiCalmin TCM (INCI: *Lonicera Japonica* (Honeysuckle) Flower Extract (and) *Xanthium Sibiricum* Fruit Extract (and) *Cyperus Rotundus* Root Extract (and) Phenoxyethanol (and) Water (Aqua)) is a trademark of Mibelle Biochemistry.

^b The PIQR Skin Microarray analysis used for this study is a product of Miltenyi Biotec, GmbH.

The DNA microarray is a solid chip, about the size of a fingernail, onto which thousands of prepared microscopic spots of DNA oligonucleotides are arrayed from a specific DNA sequence. Each spot contains picomoles of that specified sequence; in this case, a short section of a gene. This particular array contained 1,308 genes that are known markers for disorders in: the skin barrier, the extracellular matrix, DNA repair, signaling, detoxification, inflammation, skin cancer and other skin disorders.

The *in vitro* test was performed according to the following protocol. Normal human keratinocytes were taken from the same donor and incubated for 24 hr either in the TCM blend or in the medium only, which served as a control. Since RNA contains information on the activity of each individual gene, the cells of both cultures were centrifuged and the RNA of the cell pellets was isolated. The RNA of the control culture was labeled with a green fluorescent dye; the RNA from the TCM-treated culture with red fluorescent dye (preparation of the cDNA).

The DNA array was incubated with both labeled RNA preparations. Laser scanning was then performed on the DNA array (see **Figure 1**). A green fluorescent spot indicated the gene was less active and thus

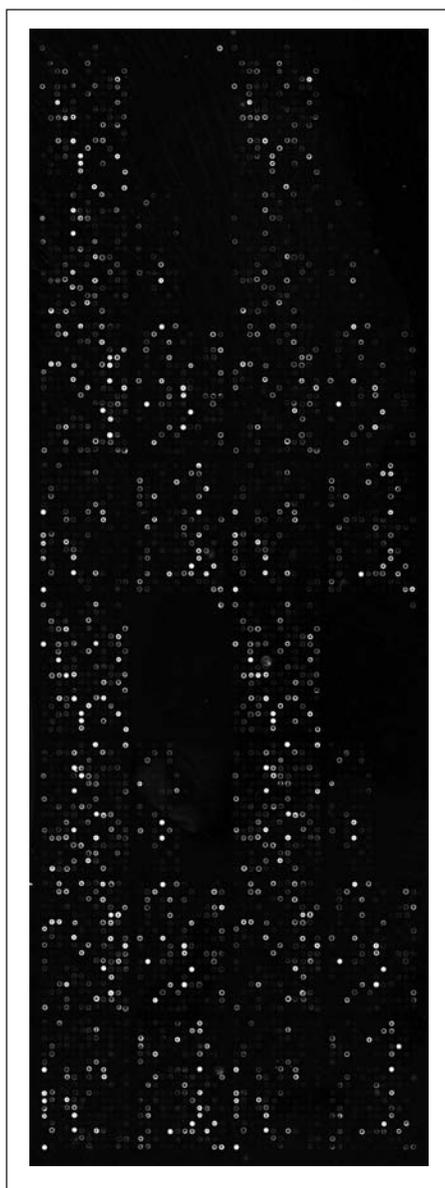


Figure 1. Laser scanning image of the microarray showing the effect of the TCM blend on the expression of 1,308 genes

down-regulated in the presence of the active ingredient. A red fluorescent spot showed that the gene was more active or up-regulated. In the case of no difference in gene activity between the control and the TCM-treated culture, the corresponding spot was fluorescent yellow.

The results of the gene array are summarized in **Table 1**. Only those genes that were highly up-regulated (> 1.9), or highly down-regulated (< 0.4) were taken into account for the efficacy evaluation. The highly up-regulated genes could be grouped by researchers into one main cellular function—protection against free radicals, oxidative stress and toxic molecules, and include:

- Gamma-glutamylcysteine synthetase is the enzyme used for the synthesis of glutathione, an important cellular antioxidant. Glutathione is a tripeptide with the sulfhydryl group of cysteine as its active site. In the reduced state, the sulfhydryl group can provide a reducing equivalent to unstable reactive oxygen species.
- Glutaredoxin and thioredoxin are both proteins that also contain sulfhydryl groups of cysteine residues as active sites. They compose an enzymatic system to deliver reducing equivalents or electrons.
- The heme oxygenase enzyme catalyzes the degradation of heme, an iron-containing molecule that is important for binding oxygen in hemoglobin. Bilirubin, a degradation product of heme, is an efficient scavenger of reactive oxygen species. Thus, the up-regulation of heme oxygenase is therefore a means to protect cells against oxidative stress.
- The enzyme UDP-glucuronosyltransferase helps in the neutralization of fat-soluble toxic molecules such as drugs or environmental toxins. The enzyme increases their solubility in water by adding a glucuronic acid sugar molecule to them. The increased resistance against toxic molecules clearly helps to protect against skin irritation.

The up-regulation of these genes was not surprising because free radicals and oxidative stress are classic triggers for inflammatory reactions in the skin. Again, the most down-regulated genes encode three enzymes of the same family, the matrix metalloproteinase

(MMP) family that comprises enzymes responsible for degradation of the extracellular matrix. MMPs play an important role in the metabolism of matrix components such as collagen and elastin and are essential, for example, in wound healing. However, it is also known that they are predominant in aged skin and lead to an imbalance in the synthesis and degradation of matrix components, causing skin to lose its firmness and elasticity. MMPs also are involved in photoaging of the skin; exposure of skin cells to UV radiation leads to the up-regulation of the expression of MMP genes.⁵

Inhibition of the expression of MMPs or of their enzymatic activity is the mechanism of action of many actives against skin aging, including the TCM blend under investigation. The results in **Table 1** show a drastic down-regulation of MMP genes in keratinocytes after incubation with the TCM blend, suggesting its protective/antiaging efficacy.

Table 1. Genes that were highly up- or down-regulated in keratinocytes after incubation with the TCM blend

Gene	TCM Blend: Control Expression Ratio	Activity of Gene Product	Role/Function in Cells/Tissue
Gamma-Glutamylcysteine Synthetase	3.36	Biosynthesis of glutathione	Glutathione is a free radical scavenger
Glutaredoxin	1.93	Thiol-reducing enzyme	Redox regulation
Thioredoxin	1.95	Thiol-reducing enzyme	Redox regulation
Heme Oxygenase	4.91	Heme degradation; induced by oxidative stress	Defense against oxidative stress
UDP-Glucuronosyltransferase	2.57	Conjugation with glucuronic acid	Removal of toxic lipid-soluble chemicals
MMP 1	0.11	Degrades native collagen	Skin thinning
MMP 3	0.1	Degrades broad range of extracellular matrix components	Skin thinning
MMP 9	0.3	Degrades denatured collagen	Skin thinning

Anti-irritancy Clinical Trial

The soothing and anti-inflammatory efficacies of the TCM blend were tested with 20 subjects using the anionic surfactant sodium dodecyl sulfate (SDS) as the skin irritant. This is a well-known skin irritant that easily penetrates the skin and disrupts the skin barrier. A protocol was developed to test for regenerative as well as protective activities. First, an aqueous solution of 2% SDS was applied under occlusion for 24 hr to a test site on the inner side of the forearm. The TCM blend, a placebo cream, and a 5% TCM blend cream were then applied after the first SDS application, twice daily for nine days (see **Formula 1**).

Formula 1. The composition and percentage of the tested creams

Ingredient	Placebo cream (%)	TCM blend cream (%)
<i>Lonicera japonica</i> (honeysuckle) flower extract (and) <i>Xanthium sibiricum</i> fruit extract (and) <i>Cyperus rotundus</i> root extract (and) phenoxy- ethanol (and) water (<i>aqua</i>) (EpiCalmin TCM, Mibelle)	---	5.00%
Hydrogenated lecithin	30.00	30.00
Sodium carboxymethyl betaglukan	5.00	5.00
<i>Prunus armeniaca</i> kernel oil	4.50	4.50
<i>Sesamum indicum</i> oil	4.50	4.50
Pentylene glycol	4.50	4.50
<i>Butyrospermum parkii</i> butter	3.00	3.00
Phenoxyethanol	0.50	0.50
<i>Cananga odorata</i>	0.20	0.20
Acrylates/C10-30 alkyl acrylate crosspolymer	0.15	0.15
Xanthan gum	0.10	0.10
Sodium hydroxide	0.06	0.06
Water (<i>aqua</i>)	qs to 100.00	qs to 100.00

The resulting inflammatory response and its gradual disappearance during nine days was documented by measuring skin hydration^c and skin redness^d, then the SDS solution was applied again for 24 hr. On day 10, hydration and redness were measured to detect for possible protective effects of the TCM ingredient.

The placebo carrier cream (see **Formula 1**) contained a lamellar-organized lipid fraction comprising hydrogenated phospholipids, and despite the richness of the carrier cream, the cream containing the TCM blend still showed clear advantages over the placebo. For example, the redness of the zone treated with the active ingredient declined significantly faster after SDS application, and also showed less redness and thus better protection against the second SDS application (see **Figure 2**). Compared to the placebo, the effect of the TCM test cream on skin hydration was even more pronounced (see **Figure 3**). After barrier disruption, application of the cream containing the active ingredient resulted in a faster, stronger moisturizing effect compared to the placebo, with highly significant protection against the second SDS application.

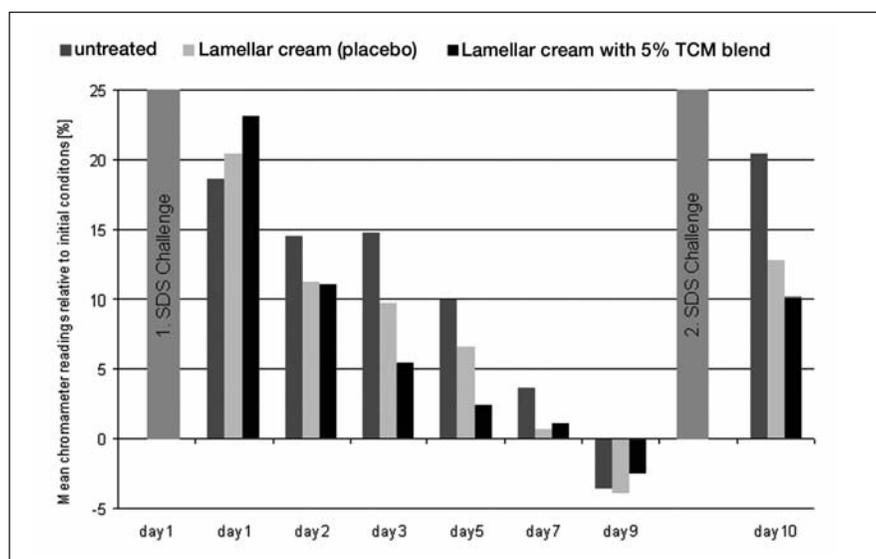


Figure 2. Skin redness after SDS irritation

^c The Corneometer CM 825 PC used for this measurement is from Courage & Khazaka GmbH of Cologne, Germany.

^d The Chromameter CR 300 used for this measurement is from Minolta of Japan.

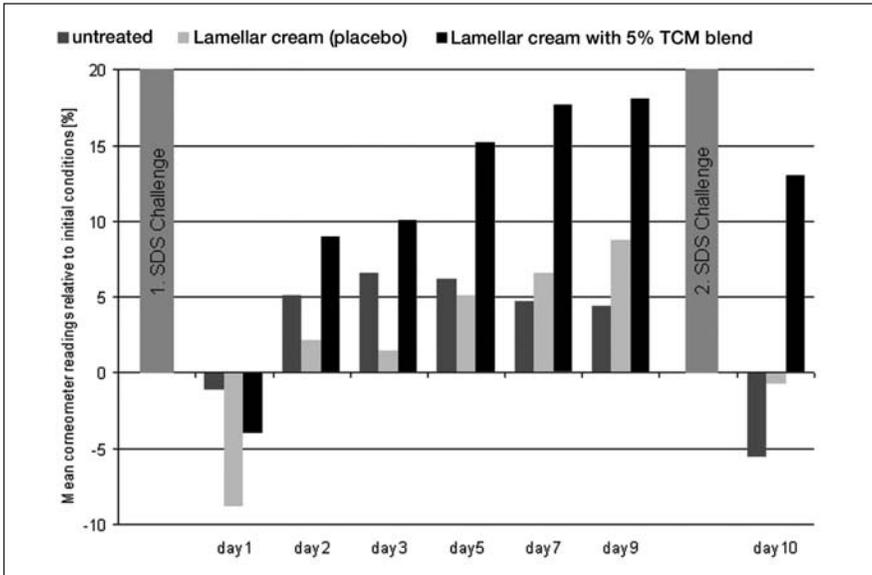


Figure 3. Skin hydration after SDS irritation

Conclusion

A cosmetic blend based on historic TCM ingredients was designed to treat dry, easily irritated skin. Its effects on keratinocytes were tested in a gene array experiment measuring 1,308 markers for skin disorders. Results suggest the TCM blend offers strong antiaging potential.

Free radicals and oxidative stress are triggers for inflammatory reactions in the skin and over time, they become factors in normal and premature skin aging. The oxidation of proteins is especially regarded as a cause of cellular aging because oxidized proteins lose their activity and tend to form aggregates. They also cause the loss of cellular function and lead to tissue impairment.⁶ The sulfur-containing side chains of cysteine and methionine are the principal targets of protein oxidation.

The tested TCM blend was found to stimulate the cell's own defense system by up-regulating the thiol-reducing enzymes glutaredoxin and thioredoxin; it therefore can be concluded that the blend represents a means to prevent protein oxidation and to regenerate existing oxidized proteins.

Aged skin is characterized by an imbalance in the extracellular matrix: the biosynthesis of matrix components is reduced and their degradation is increased, resulting in a loss of elasticity and thinning of the skin. The tested TCM blend counteracted this tendency by inhibiting the formation of extracellular matrix-degrading enzymes. It also was shown to protect against premature skin aging by reducing oxidative damage and the break down of collagen.

TCM treatments are known best by the experts that study and practice them in the East, and oftentimes are lesser-known in the West primarily due to language barriers. As the interest in plant-based ingredients continues to grow, other traditional Chinese recipes to treat skin conditions could open up new potential for the cosmetic industry.

Published January 2009 *Cosmetics & Toiletries* magazine.

References

1. SJ Suh et al, The naturally occurring biflavonoid, ochnaflavone, inhibits LPS-induced iNOS expression, which is mediated by ERK1/2 via NF-kappaB regulation, in RAW264.7 cells, *Arch Biochem Biophys* 447(2) 136–46 (2006)
2. MJ Son et al, Naturally occurring biflavonoid, ochnaflavone, inhibits cyclooxygenases-2 and 5-lipoxygenase in mouse bone marrow-derived cells, *Arch Pharm Res* 29(4) 282–6 (2006)
3. Y Sato et al, A xanthanolide with potent antibacterial activity against methicillin-resistant *Staphylococcus aureus*, *J Pharm Pharmacol* 49(10) 1042–4 (1997)
4. R Yazdanparast and A Ardestani, *In vitro* antioxidant and free radical scavenging activity of *Cyperus rotundus*, *J Med Food* 10(4) 667–74 (2007)
5. J Varani et al, Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin, *J Invest Dermatol* 114 480–6 (2000)
6. VA Vernace, T Schmidt-Glenewinkel and ME Figueiredo-Pereira, Aging and regulated protein degradation: Who has the upper hand? *Aging Cell* 6 599–606 (2007)

An ECM-derived Tetrapeptide to Counterbalance ECM Degeneration

Mike Farwick, Ursula Maczkiewitz and Peter Lersch

Evonik Goldschmidt GmbH, Essen, Germany

Tim Falla

Helix Biomedix Inc., Bothell, Wash. USA

Susanne Grether-Beck and Jean Krutmann

Institut für Umweltmedizinische Forschung, Heinrich-Heine-University

Düsseldorf, Germany

KEY WORDS: *peptides, antiaging, collagen, hyaluronic acid, fibronectin*

ABSTRACT: *Degradation of dermal and epidermal proteins and the reduced proliferation of collagen and hyaluronic acid in the dermis occur during aging. Thus, antiaging technologies must to correct these deficiencies to induce skin regeneration and combat the signs of aging. Data presented here demonstrates that ECM-derived tetrapeptides have the potential to counterbalance ECM degeneration.*

The extracellular matrix (ECM) is the structural backbone of many tissues, especially the skin, and represents a main target for cosmetic applications. ECM proteins are believed to play a pivotal role in cellular migration, proliferation and gene regulation during wound healing. Fragments from ECM constituents have been found capable of stimulating ECM biosynthesis to compensate for tissue destruction.¹ Their mechanisms have been implicated in wound healing, skin aging and skin's response to UV irradiation;^{2,3} from this knowledge, new actives have evolved, as the authors describe here.

Building from the concept that ECM constituents stimulate ECM biosynthesis, bioinformatic methods were employed to identify highly repetitive amino acid motifs with inherent antiaging activities. Several dozens of tetrapeptides were found scattered across sequences of the major ECM macro-molecules.⁴ Ten peptides showed the desired effect of significantly increasing collagen protein in supernatant, thus verifying the underlying assumption that breakdown products of ECM proteins stimulate the ECM neosynthesis.

Of the ten peptides, the five most promising were subjected to further analysis including concomitant collagen determination in the supernatant, as well as gene expression analysis of the ECM marker genes: collagen (COL1A1), fibronectin (FN1) and hyaluronic acid synthetase (HAS1).

Collagen, being a dermal protein responsible for skin strength and elasticity, was examined since its degradation leads to wrinkles that accompany aging.⁵ Hyaluronic acid, one of the main components of the ECM, is a nonsulfated glycosaminoglycan that binds water, also ensuring the elasticity of the skin.⁶ Fibronectin, a glycoprotein that helps to create a cross-linked network within the ECM, was also of interest since it provides binding sites for other ECM components such as hyaluronic acid and collagen.⁷

In the end, one tetrapeptide, glycine-glutamic acid-lysine-glycine (GEKG, or INCI: tetrapeptide-21)^a, was evaluated *in vivo* for effects on these genes.

Material and Methods

Human dermal fibroblasts (HDFs) prepared from neonatal foreskin were cultured for four days in a humidified 5% carbon dioxide atmosphere in Eagle's minimal essential medium^b supplemented with: 5% fetal calf serum^c, 0.1% l-glutamine, 2.5% sodium bicarbonate, and 1% streptomycin/amphotericin B, until they reached confluence. For the studies described here, only early passage fibroblasts (< 12) were used so as to avoid any changes in their original phenotype during subculture. Cells were kept in 6-well plates for culture.

^aTego Pep 4-17 (INCI: Tetrapeptide-21 (and) Glycerin (and) Butylene Glycol (and) Water (aqua)), is a product of Evonik Goldschmidt GmbH.

^bEMEM is a product of Life Technologies GmbH, Eggenstein, Germany.

^cFetal Calf Serum is a product of Greiner, Frickenhausen, Germany.

For isolation of total RNA, kits^d were used according to manufacturer instructions. The RNA concentration was determined via photometric measurement^e at 260/280. To avoid repeated free-thaw cycles for the prepared RNA for multiple experiments, aliquots of total RNA (100 ng) were applied for cDNA synthesis using a synthesis system^f for the reverse transcription step with random heaters. For each gene, a specific primer pair was designed^g based on the cDNA sequence published as indicated. For each gene expression determination, three independent experiments were performed and the mean value of these was calculated.

PCR reactions were carried out using a continuous fluorescence detection device^h and softwareⁱ. Each sample was analyzed twice, employing the universal protocol over 46 cycles. Detailed reaction conditions included: 10 min 94°C of hot-start taq polymerase activation, 20 sec 95°C denaturation, 20 sec 55°C annealing, and 30 sec 72°C extension. For comparison of relative expression in real time PCR control cells and treated cells, the $2^{-\Delta\Delta C(T)}$ method was used.

The tested peptides were applied at concentrations of 1 µg/mL for 24 hr to human dermal fibroblast cell cultures, and RNA was extracted to perform gene expression profiling. Induction of hyaluronic acid-synthase-1 and collagen was analyzed by real time PCR with the primer pairs shown in **Table 1**. Since collagen production depends not only on stimulated gene expression, but also on a complex process of post-translational modification, researchers further quantified the collagen concentration in the fibroblast cell culture supernatants using a collagen assay^{k,8}. All samples were incubated in the presence of β-aminopropionitrile (50 µg/mL) to increase the stability of the collagen.

In addition, an *in vivo* study was conducted with a panel of 60 volunteers divided into four groups. Four variations of a test cream were developed (see **Formula 1**), including a placebo cream without

^dThe RNeasy Total RNA Mini Kit is a product of Qiagen.

^eBioPhotometer is a product of Eppendorf.

^fSuperScript III First-Strand Synthesis System is a product of InVitrogen.

^gPrimer Express 2.0 Software is a product of Applied Bio Systems. ^hThe Opticon 1 machine is a device from MJ Research, Waltham, MA, USA.

ⁱThe SYBR Green PCR Master Mix software is a product of Applied Biosystems.

^kThe Sircol Collagen Assay is a product of Biocolor.

the active, a positive control cream incorporating 10 ppm palmitoyl pentapeptide-4,^{2,3} a cream with 10 ppm GEKG, and a cream with 100 ppm GEKG. The samples were applied to the inner forearms of subjects twice daily for eight weeks. Before and after eight weeks of application, skin volume and roughness were analyzed using a skin surface characterization device^m.

Table 1. Primer pairs

18S rRNA	5'-GCCGCTAGAGGTGAAATCTCTG-3' 5'-CATTCTGGCAAATGCTTTCG'-3'
Collagen 1A1	5'-CCTGCGTGTACCCCACTCA-3' 5'-ACCAGACATGCCTCTTGTCCTT-3'
HAS-1	5'-GCGGGCTTGTCAGAGTACT-3' 5'-AACTGCTGCAAGAGGTTATTCCTATAT-3'
Fibronectin	5'-GAAAGTACACCTGTTGTCATTCAACA-3' 5'-ACCTTCACGCTGTCACTTCCA-3'

Results

Of all those tested, the most active peptide found had the sequence GEKG (see **Figure 1**). At a concentration of 1 µg/mL, the peptide increased the amount of secreted collagen protein in the supernatant approximately 2.5-fold. On the mRNA level, all three tested ECM marker genes were induced, resulting in a 2.5-fold increase of COL1A1 expression. In addition, HAS1 encoding for the hyaluronic acid was 5.7-fold, and the gene encoding for fibronectin was induced by 10.5-fold. The well-balanced induction of these important ECM constituents by the GEKG peptide suggests strong antiaging effects.

Formula 1. Sample cream used for *in vivo* tests

Polyglyceryl-3 methylglucose distearate	3.0%
Glyceryl stearate	2.0%
Stearyl alcohol	1.0%
C12-15 alkyl benzoate	9.5%
PPG-3 myristyl ether	9.5%
Glycerin	2.5%
1,2-Butanediol	0.25%
Polysorbate 20	0.025%
Peptide	varied
Preservative	0.8%
Fragrance (<i>parfum</i>)	0.1%
Water (<i>aqua</i>)	qs to 100.0%

^mThe Visioscan VC 98 is a device of Courage & Khazaka Electronic GmbH.

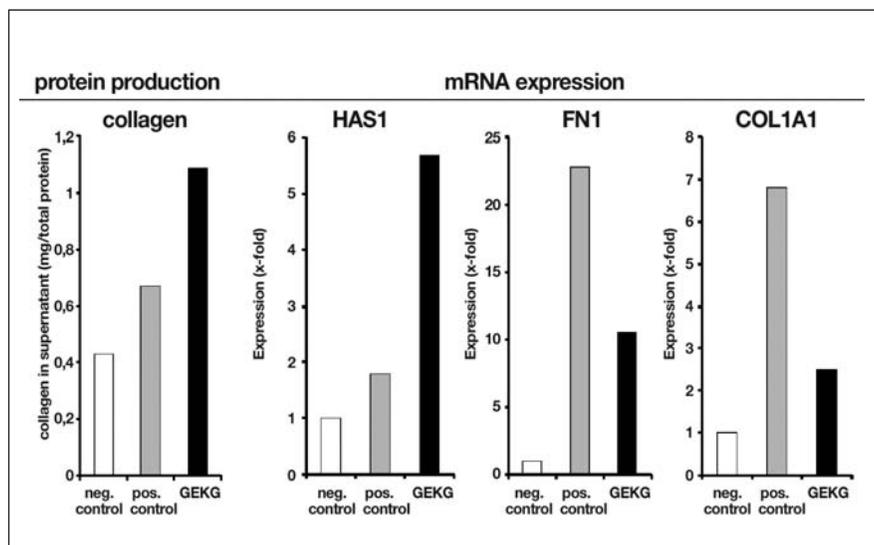


Figure 1. *In vitro* effects of GEKG on secreted collagen levels and ECM marker gene expression in human dermal fibroblasts

Although the palmitoyl pentapeptide-4 positive control showed a stronger induction in COL1A1 gene expression, the effect was not completely translated into collagen protein. This could be due to different gene induction kinetics. For instance, GEKG may cause a faster response on expression levels that is immediately translated into protein production, and that afterward diminishes to minor levels; whereas the positive control peptide could take longer to react to the stimulus, thus delaying the response.

The *in vivo* relevance of GEKG activity was additionally tested by a vehicle-controlled biopsy and elasticity study. After eight weeks' application of a cream formulation containing 50 ppm GEKG, both collagen gene expression and skin elasticity significantly increased, compared with the vehicle and untreated skin (data not shown but available).

Thus, to demonstrate the proposed antiaging effect of GEKG *in vivo*, a parameter "volume" measurement was taken of the previously mentioned 60 volunteers. This software-based method compares the distribution of gray scales in photographs taken of the volunteers' skin before and after the eight-week application period. It calculates the theoretical amount of liquid that would be necessary to fill the wrinkles and generate a plain surface. A reduction of the parameter

volume is interpreted as an overall improvement in skin structure resulting from the reduction of skin wrinkles in number and depth.

With an increasing concentration of GEKG, an increased reduction of the volume was observed. Compared with the positive control, a significant increase was obtained with 100 ppm GEKG (see **Figure 2a**).

Besides parameter volume measurements, parameter roughness was assessed via the same skin texture analysis device^{m,9,10}. These roughness parameters originate from the DIN-parameters Ra–Rz, and describe the depth of fine and coarse wrinkles. R1–R5, for instance, describe the maximum and average amplitude of a surface structure, as well as the mean height level. In the case of GEKG in skin, **Figure 2b** again demonstrates a dose-dependent effect; increasing concentrations of GEKG increased the reduction of skin roughness. Compared with the positive control, 10 ppm GEKG showed comparable efficacy whereas 100 ppm GEKG doubled the effect.

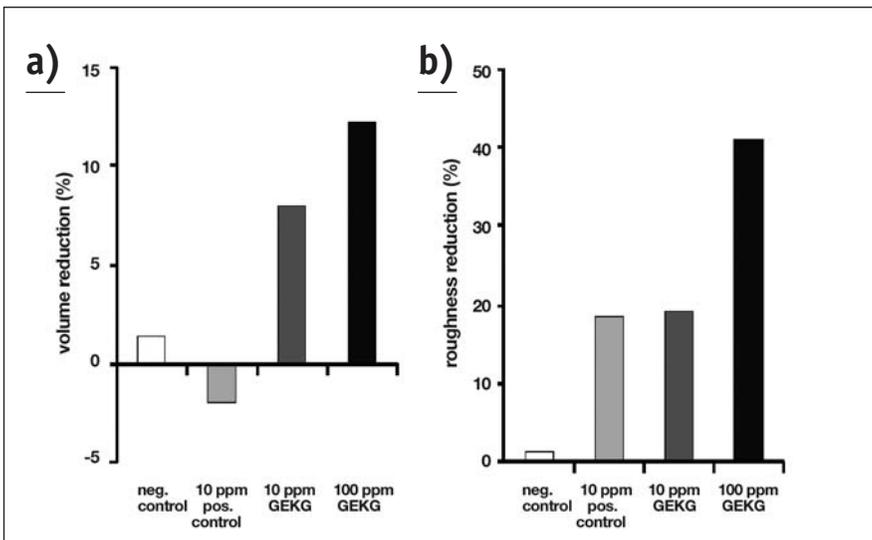


Figure 2. Topical application of GEKG reduced: a) the parameter volume, and b) skin roughness *in vivo*

Only 10 ppm of palmitoyl pentapeptide was tested, which is closer to its maximum suggested use level. GEKG at 10 ppm and

100 ppm translates to approximately 0.5–5.0% of the tetrapeptide-21 use concentration, which contains 2,000 ppm peptide.

Figure 3 shows the skin structure of one volunteer who applied the formulation containing 100 ppm GEKG for eight weeks. The pictures demonstrate an overall improvement of the skin structure; the wrinkles are less deep and less pronounced and the skin roughness is decreased.

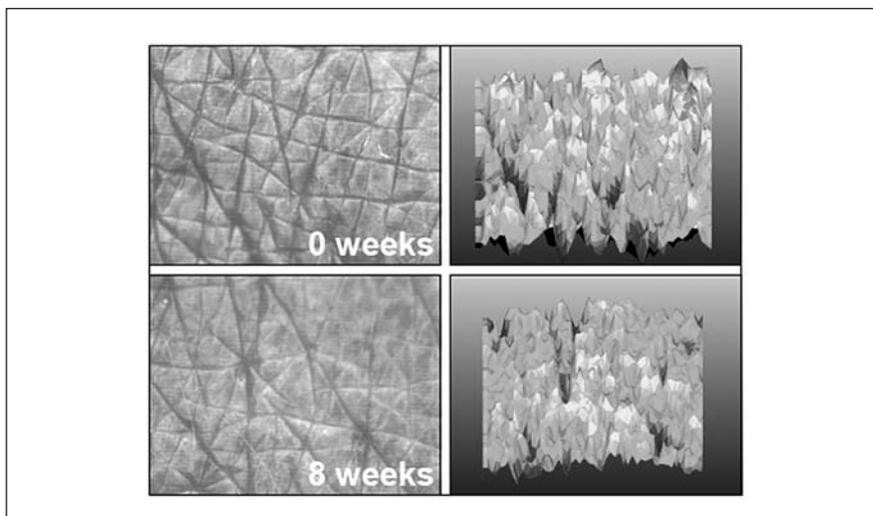


Figure 3. Photographs of one volunteer's skin taken before and after eight weeks of topical application of 100 ppm GEKG; for software-supported analysis, each photograph was digitalized and the differences were calculated

Conclusion

Aging is associated with changes in the skin at all levels. For instance, degradation of key dermal-epidermal and dermal proteins occur, together with reduced epidermal proliferation and collagen and hyaluronic acid synthesis in the papillary dermis. Antiaging technologies must correct these deficiencies in order to induce skin regeneration to combat the resulting signs of aging.

The data presented in this study demonstrates that ECM-derived tetrapeptides have the potential to counterbalance the ECM degeneration observed during skin aging. *In silico* analysis identified approximately 30 abundant tetrapeptide motifs in ECM proteins, and *in vitro* analysis showed that 10 of these motifs can stimulate collagen synthesis.

GEKG was identified as a highly active tetrapeptide that is able to stimulate dermal repair and renewal mechanisms. An increased expression of ECM-synthesizing enzymes like hyaluronic acid synthetase, as well as expression and production of ECM proteins like collagen and fibronectin, was observed. Finally, *in vivo* data confirmed the efficacy of GEKG, leading to an improved skin structure and reduced wrinkles.

Published June 2006 *Cosmetics & Toiletries* magazine.

References

1. LR Robinson, NC Fitzgerald, DG Doughty, NC Dawes, CA Berge and DL Bissett, Topical palmitoyl pentapeptide provides improvement in photoaged human facial skin, *Int J Cosm Sci* 27(3) 155–160 (2005)
2. KT Tran, P Lamb and JS Deng, Matrikines and matricryptins: Implications for cutaneous cancers and skin repair, *J Dermatol Sci Oct* 40(1) 11–20 (2005)
3. KT Tran, L Griffith and A Wells, Extracellular matrix signaling through growth factor receptors during wound healing, *Wound Repair Regen* 12(3) 262–8 (May–Jun 2004)
4. US 20070299105, Peptide fragments for inducing synthesis of extracellular matrix proteins
5. M Yaar and BA Gilchrist, Photoaging: Mechanism, prevention and therapy, *Br J Dermatol*, 157(5) 874–87 (Nov 2007)
6. L Baumann, Skin aging and its treatment, *J Pathol* 211(2) 241–51 (Jan 2007)
7. M Larsen, VV Artym, JA Green and KM Yamada, The matrix reorganized: Extracellular matrix remodeling and integrin signalling, *Curr Opin Cell Biol* 18(5) 463–71 (Oct 2006)
8. VV Yurovsky, Tumor necrosis factor-related apoptosis-inducing ligand enhances collagen production by human lung fibroblasts, *Am J Respir Cell Mol Biol* 28(2) 225–31 (2003)
9. K De Paepe, JM Lagarde, Y Gall, D Roseeuw and V Rogiers, Microrelief of the skin using a light transmission method, *Arch Dermatol Res* 292(10) 500–10 (2000)
10. A Pagoni, Photo-aging and photodocumentation, *Cosmet Toil* 117(1) 39–46 (2002)

Understanding Reactive Oxygen Species

Paolo U. Giacomoni, PhD

Estée Lauder

KEY WORDS: *oxygen, reactive oxygen species, ROS, lipids, free radicals*

ABSTRACT: *This chapter investigates reactive oxygen species (ROS), hydroxyl radical production, lipid and protein damage and the physiological relevance of ROS.*

Life is accompanied by a wealth of chemical reactions. As far as life on earth is concerned, one of the most relevant reactants is molecular oxygen (O_2). While oxygen is required by all animals in order to live, it can also be extremely toxic by itself and under the form of reactive oxygen species (ROS) such as H_2O_2 (hydrogen peroxide), $O_2^{\cdot-}$ (superoxide anion), OH^{\cdot} (hydroxyl radical) or 1O_2 (singlet oxygen). These ROS provoke spontaneous, rapid and nonspecific chemical reactions that can wreak havoc on biological systems. In the human body, ROS can be formed intrinsically as a result of normal cellular metabolism or extrinsically as a result of environmental stressors.

Superoxide anion, which is generated in the course of cellular respiration (oxidative phosphorylation), and nitric oxide (NO), which is involved in a variety of processes, including vasodilation, neurotransmission and immune defense, are among the ROS generated by normal physiological chemical reactions. NO can be associated with pro-oxidant or antioxidant activity depending on existing conditions. NO itself is not a strong oxidant, but becomes so after reacting rapidly with superoxide anion to give $ONOO^{\cdot-}$ (peroxynitrite). Peroxynitrite decomposes to OH^{\cdot} (hydroxyl radical) and

NO_2 (nitrogen dioxide), both of which trigger chain peroxidation via H-abstraction (**Figure 1**).

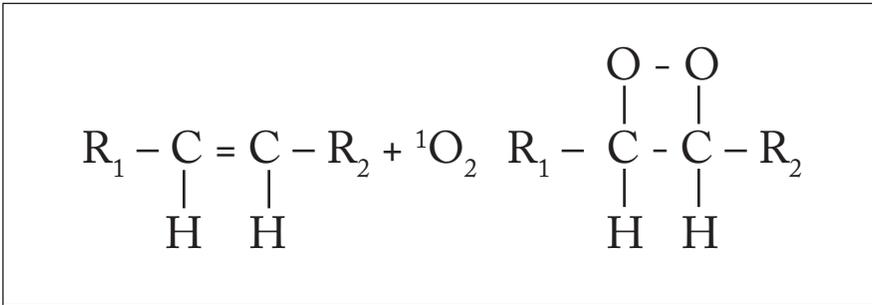


Figure 1. Chain peroxidation via H-abstraction

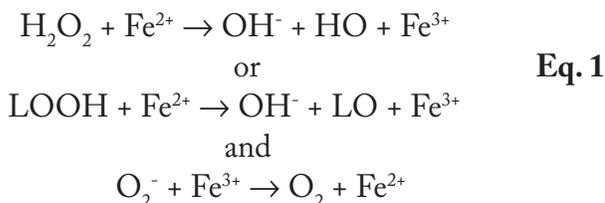
This could occur, for example, at sites of inflammation, where macrophages are recruited and activated. When macrophages are activated, they undergo a respiratory burst that produces and releases large amounts of nitric oxide.

One of the most widespread stressors on the surface of earth is UV radiation from the sun. Significant experimental evidence has been accumulated about the damaging action of solar radiation and its mechanisms. One of the crucial observations was made when growth media was exposed to UVA radiation. The media became toxic to the cultured mammalian and human cells.¹ In attempt to understand the mechanisms of this UVA cytotoxicity, it was observed that when tryptophan is exposed to near UV light (300–400 nm) in aerated water, hydrogen peroxide is generated.²

Hydroxyl Radical Production

Tyrosine, tryptophan, riboflavin and other molecules, known as photosensitizers, can transfer electrons or energy to acceptor molecules. One of the most important acceptors of energy or electron transfer is ground state molecular oxygen (${}^3\text{O}_2$) in which the ground state is triplet oxygen. Ground state molecular oxygen is converted to superoxide anion radical (O_2^-) upon electron transfer or to its singlet state (${}^1\text{O}_2$) upon energy transfer.^{3,4} Singlet oxygen is not a free radical because spin restriction has been eliminated, and it has a completely vacant orbital or no unpaired electrons. However, singlet oxygen does react rapidly to form endoperoxides.

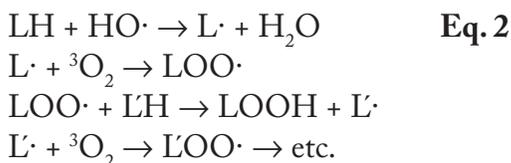
In the presence of physiologically relevant metal ions such as Fe^{2+} or Cu^+ , H_2O_2 can be reduced to OH^- and $\text{HO}\cdot$ via the Fenton reaction:



The Fenton reaction and the production of hydroxyl radicals can be maintained as long as there is superoxide or other reductants to cycle ferric iron into ferrous iron. Superoxide anions can also bind a proton to form the neutral species $\text{HO}_2\cdot$ (protonated superoxide radical), which can penetrate cellular membranes to damage intracellular components.

Lipid and Protein Damage

Lipids and proteins are not directly damaged by solar radiation because they have very low extinction coefficients in the range of wavelengths reaching the surface of the earth. Lipids and proteins are the target of UV-generated ROS. Proteins can undergo the process of metal catalyzed oxidation,⁵ and lipids undergo the peroxidation cascade that results in the multiplication of oxidative damages. This occurs because $\text{HO}\cdot$ can abstract a hydrogen from a lipid, (LH) resulting in a lipid radical ($\text{L}\cdot$), thus initiating free radical-mediated (chain) lipid peroxidation. $^3\text{O}_2$ reacts rapidly with $\text{L}\cdot$ to make peroxy radical ($\text{LOO}\cdot$), which abstracts hydrogen from another lipid to form the lipid hydroperoxide (LOOH) and propagate chains. These reactions are illustrated as follows:



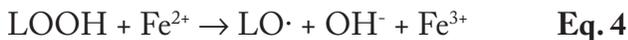
$^1\text{O}_2$ (singlet oxygen), a chemical generated via energy transfer from a sensitizer, can react rapidly with various amino acid residues in

proteins ($k > 10^7 \text{ M}^{-1}\text{s}^{-1}$). Some examples can include tryptophan to give kynurenine, histidine, methionine, tyrosine or cysteine, and this can irritate protein structure and function. It is important to note that for many sensitizers, the rate constant for energy transfer to form $^1\text{O}_2$ is about two orders of magnitude greater than that for electron transfer to $^3\text{O}_2$ with formation of O_2^- . Thus, $^1\text{O}_2$ formation may predominate in skin exposed to solar radiation. By implication, $^1\text{O}_2$ could potentially be the most dangerous reactive species generated in skin during a sunlight challenge. Human cells also possess catalase and superoxide dismutases to eliminate H_2O_2 and superoxide respectively, but do not have enzymes to take care of singlet oxygen.

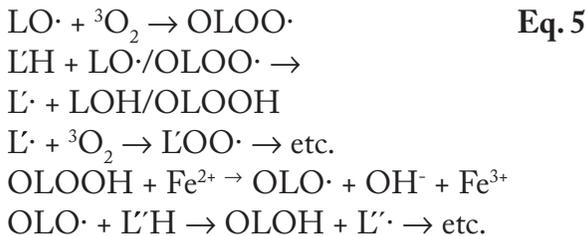
$^1\text{O}_2$ can add directly to an unsaturated lipid to give a conjugated double bond LOOH without free radical intermediacy.



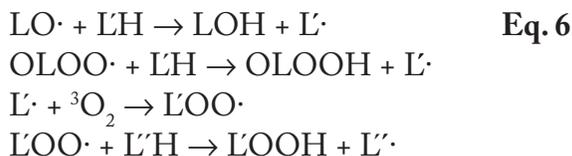
If some iron is nearby, the following equation results.



Of course, oxygen and other lipids are found in living tissues, organs and membranes. If the living tissues, membranes and organs possess oxygen, the following reaction occurs.



If the living tissues, membranes and organs possess oxygen, the following reaction occurs.



If participating lipids become limiting, radicals will react with one another to produce nonradical products, thus terminating chain propagation.



The Physiological Relevance of ROS

Free radicals are reactive because they have an unpaired electron, rather than the normal pair of electrons found in the outer shell of stable molecules. Most free radicals have high chemical reactivity, thus they are usually short-lived. As a result, they seek to “pair” with other molecules, often initiating a cascade of events that may destabilize and possibly destroy cells or other molecules in the body. If these free radicals are not controlled, they can cause significant damage to proteins, lipids, membranes and DNA.

The human body has evolved a number of mechanisms, both enzymatic and nonenzymatic, to deal with these potential damaging radicals.^{6,7} These processes include: enzymes such as superoxide dismutase, glutathione peroxidase and catalase⁸; scavengers such as tocopherol, ascorbic acid, α -lipoic acid and uric acid; quenchers of excited states such as β -carotene, melanin and xanthin; and chelators, to sequester metals such as heme.

Stressful conditions can create an abundance of free radicals that can overwhelm the body's own natural defenses. These ROS threaten the integrity of skin and other tissues, but the skin especially is at risk for at least two reasons: Skin is exposed to O_2 from the inside at the levels provided by the blood and from the outside at the higher levels that occur in the air. Skin that is compromised has heightened sensitivity to environmental stresses. Therefore, external application of free radical scavengers may help prevent formation or remove the free radicals before they can cause damage. The strategy for exogenous help is similar to that provided by the body itself, including sunscreens, chelators, quenchers, scavengers and enzymes.

ROS attack proteins, DNA and membranes indiscriminately. First of all, oxidized enzymes are impaired and without enzymes the

physiology of the cell is severely jeopardized. Secondly, DNA can be damaged by ROS, such as protonated superoxide, which provokes damage such as 8-oxo-guanosine.

Last but not least, cellular membranes, the backbone of which is constituted by phospholipids arranged in bi-layers, can be severely damaged by ROS. Abiding to rigorous polarity, the negatively charged heads of phospholipids line the inner and the outer surfaces of the plasma membranes and make them hydrophilic. The hydrophobic interaction of lipophilic tails of the phospholipids maintain the membrane in a structure which, when not specifically organized in a cation pump or in receptors able to internalize specific ligands, is impermeable to electrolytes. When membrane lipids are peroxidized, the relatively hydrophilic hydroperoxyl groups will disrupt the hydrophobic interactions. This will provoke the displacement of the peroxide-containing segments toward the outer surfaces of the membrane that are in contact with water. Resulting structural changes are reflected in increased membrane permeability and loss of membrane potential, for example, in zeiosis and cellular blebbing, which have serious consequences on overall cell function.

Taming Free Radicals

Exposure to UV radiation leads to:

- accelerated aging,
- increased wrinkling,
- thickening of the epidermis,
- sagging of the skin and
- loss of elasticity.

Indeed, the inflammatory response to any external aggression is performed by immune cells. In order for these cells to get to the site of action, they release H_2O_2 on the endothelium to exit the blood vessels. They then release singlet oxygen and metallo-proteinases to create a path in the dermis to reach the damaged cell that is digested by releasing peroxides. All of these oxidative cascades provoke damages that are only partially removed, and aging is known to be defined as the accumulation of damages.^{9,10}

Destruction of skin lipids by peroxidation contributes to dry skin

and rough appearance. Peroxidized lipids are also skin irritants. They can migrate to distant sites to exert measurable oxidizing stress far from the site of formation.

Optimum protection needs a multifaceted approach to the control of free radicals. One antioxidant does not address all of the different free radicals that may be formed by an oxidative stress. Thus it is best to use a blend. In selecting antioxidants, a chemist must consider concentration and the effect of synergies or antagonists.

Published May 2005 *Cosmetics & Toiletries* magazine.

Symbols used:

Molecular oxygen	$^3\text{O}_2$ (This is the ground state. It is triplet oxygen.)
Hydrogen peroxide	H_2O_2
Superoxide anion	$\text{O}_2\cdot^-$
Hydroxyl radical	$\text{OH}\cdot$
Singlet oxygen	$^1\text{O}_2$ (Singlet oxygen is not a radical. Normally oxygen has one unpaired electron in each of the outer orbitals. In the case of singlet oxygen, the two unpaired electrons pair up in the inner orbital, leaving the outer orbital empty. This not a favorable energy state, therefore, singlet oxygen is very reactive.)
Nitric oxide	NO
<u>Peroxynitrite</u>	ONOO \cdot
Zeiosis and cellular blebbing	Bubble formation on the surface of the membrane. This is the first step in a cascade of events when a cell undergoes apoptosis (programmed cell death).

References

1. JD Stoen and RJ Wang, Effect of near-ultraviolet and visible light on mammalian cells in culture II. Formation of toxic photoproducts in tissue culture medium by blacklight. *Proc Natl Acad Sci U S A* 71 3961–5 (1974)
2. HN Ananthaswamy and A Eisenstark, Near-UV induced breaks in phage DNA: sensitization by hydrogen peroxide (a tryptophan photoproduct) *Photoche Photobiol* 24 439–442 (1976)
3. MJ Davies and JW Truscott, Photo-oxidation of proteins and its consequences in (P.U. Giacomoni, editor) *Sun Protection in Man*, Elsevier, Amsterdam New York 251–275 (2001)
4. IE Kochevar and RW, Redmond Photosensitized production of Singlet Oxygen in (L Packer and H Sies editors) *Singlet Oxygen, UVA and Ozone—Methods in Enzymology* vol 319 Academic Press San Diego London New York 20–28 (2000)

5. K Kim, SG Rhee and ER Stadtman, Nonenzymatic cleavage of proteins by reactive oxygen species generated by dithiothreitol and iron *J Biol Chem* 260 15394–15397 (1985)
6. EE Boh, Free Radicals and aging of skin, *Cosmet Dermatol* 14 37–40. (2001)
7. Y Shindo, E Wit and W Epstein et al., Enzymic and non-enzymic antioxidants in epidermis of human skin., *J Invest Dermatol* 102 122–124. (1994)
8. D Darr and I Fridovich, Free radicals in cutaneous biology, *J Invest Dermatol* 102 671–675 (1994)
9. PU Giacomoni and P D'Alessio, Skin Aging, The relevance of Antioxidants in (S Rattan and O Toussaints, editors) *Molecular Gerontology* Plenum Press New York London 177–192 (1996)
10. VR Winrow, PG Winyard, CJ Morris and DR Blake. Free radicals in inflammation: second messengers and mediators of tissue destruction, *Br Med J* 49 506–52 (1993)

Measuring Reactive Oxygen Species in Skin with Fluorescence Microscopy

Nancy E. Kinkade, PhD

Eastman Chemical Co., Kingsport, Tenn., USA

KEY WORDS: *two-photon fluorescence microscopy, free radicals, rice bran oil, UV irradiation, tocopherols and tocotrienols*

ABSTRACT: *Several signs of skin aging have been attributed to the presence of free radicals. This chapter discusses the application of two-photon fluorescence microscopy to test the effects of sample formulations on the number of reactive oxygen species (ROS) formed in skin models.*

Free radicals are involved in natural physiological processes in the skin but also are responsible for oxidative stress resulting in damage to cellular components—ultimately leading to premature aging and diseases. Oxidative stress can occur when the number of free radicals surpasses the capacity of the body's natural defense mechanisms. This can be due to environmental factors such as exposure to UV radiation and pollutants.

Several *in vitro* tests exist^{1,2} to assess the free radical scavenging ability of ingredients in cosmetics and personal care, and there are even *in vivo* tests available to assess the efficacy of products to prevent lipid oxidation.^{3,4} This paper discusses the application of two-photon fluorescence microscopy to determine the effects of cosmetic formulations on the amount of reactive oxygen species (ROS) formed in skin models.

Skin Aging and ROS

As the largest organ in the human body, the skin is responsible for several important physiological functions including regulating body temperature, protecting internal organs and providing sensation and excretion, to name a few. Many people, however, are more focused on attributes that accompany healthy and functioning skin—attributes associated with beauty. Smooth, supple, evenly colored, wrinkle-free skin generally is sought, but as the skin ages it experiences a variety of changes, some of which lead to wrinkling, uneven pigmentation and thinning of the skin. Several signs of skin aging have been attributed to the presence of free radicals.

Free radicals—molecules containing an odd number of electrons—often are unstable and highly reactive. Reactive oxygen species (ROS) include free radicals that contain oxygen and hydrogen peroxide. Free radicals and ROS are unavoidable since they are natural by-products of biological processes such as cellular respiration and oxidative phosphorylation.

The human body has natural defenses to scavenge free radicals, but if an excessive amount of free radicals are unleashed through exposure to UV radiation or pollution such as cigarette smoke or ozone, the natural antioxidants in the body can be overwhelmed. Evidence exists that suggests free radicals are responsible for many diseases, including skin cancer^{5,6} and photo-aging due to damage done to cellular components.^{7,8}

Many everyday personal care regimens are beginning to incorporate the use of products containing sunscreen filters. While some consumers still strive for that “perfect tan,” many more understand the dangers associated with UV radiation. Unfortunately, evidence is growing that sunscreen filters may not be the panacea once thought. E. Damiani recently reported that many of the common organic sunscreen filters become less effective after UVA irradiation.⁹ Furthermore, KM Hanson reported that while the sunscreen filters reduced the formation of ROS in the short term, more ROS were formed one hour after application of sunscreen than were formed where no sunscreen was used.¹⁰

Protecting Against ROS

Rice bran oil has been used as a beauty ingredient in Asia for centuries and traditionally earned women the reputation of *nuka bijin* or “bran beauties.” Nearly 20 years ago, research scientists in Japan conducted a study of sake factory workers because they were found to have naturally beautiful hands. The results indicated the cause to be regular handling of rice bran hulls by the workers. The mix of components in this natural extract results in a material suited for cosmetic products. Rice bran extract is rich in many beneficial, naturally occurring ingredients including rice bran wax, squalene and phytosterols, as well as tocopherols and tocotrienol isomers. Several of the components in rice bran oil extract are well-known antioxidants.¹¹⁻¹⁶ Tocopherol and tocotrienols are of interest because of their antioxidant and moisturization properties imparted to skin.¹⁷ The tocopherols and tocotrienols present in rice bran oil extract consist of eight isomers commonly known as the vitamin E family (see **Figure 1**).

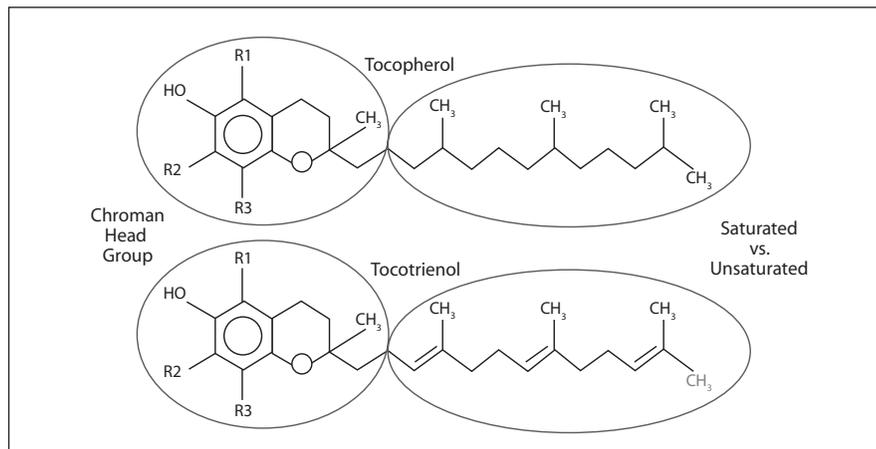


Figure 1. The vitamin E family

Vitamin E is often equated with synthetic alpha-tocopherol. It is important to recognize that natural vitamin E is more than just one isomer, and that while all eight isomers have similar chemical properties due to their similar structures, those slight differences in structure are now known to result in significant differences in their biological functions.¹⁸ The eight tocopherol and tocotrienol isomers

have been reported to have similar antioxidant activities, although in at least one study, gamma-tocopherol was reported to be more easily oxidized than alpha-tocopherol,¹⁹ and in another study, alpha-tocotrienol was found to be a better antioxidant than alpha-tocopherol.²⁰

While the jury may still be out regarding which isomer is best, it is clear that tocopherols and tocotrienols are lipid-soluble ROS scavengers that protect cellular components. To better understand the tangible ROS-scavenging benefits of rice bran extract, two prototype moisturizing creams were formulated with 1% and 5% rice bran extract. *In vitro* test data obtained with these creams had previously shown that rice bran extract scavenges free radicals and protects lipids from oxidation.²¹ In the method described herein, fluorescence microscopy and epidermal skin models were used to explore the ROS-scavenging benefits of rice bran extract.

Two-photon Fluorescence Microscopy

In August 2006, a study was commissioned^a to investigate the effect of 1% and 5% rice bran extract cream formulas^b on the formation of ROS in a skin model^c. The researcher commissioned developed the protocol and carried out the experiments using formulations prepared by the author.

The study performed incorporates two-photon fluorescence imaging microscopy to investigate the affect of personal care ingredients on free radicals in the skin.²² This method uses dihydrorhodamine (DHR)^d as the fluorescent probe. DHR is nonfluorescent until it reacts with ROS such as singlet oxygen or peroxyxynitrite and forms rhodamine-123 (R123). The fluorescence of R123 can be detected by fluorescence microscopy.

The 2002 Hanson and Clegg fluorescence microscopy study used *ex vivo* skin. The protocol for this study uses a living skin model^c—a multilayered, highly differentiated tissue model of the human epidermis. It consists of normal human-derived epidermal keratino-

^a Eastman Chemical commissioned the study performed by Kerry M. Hanson, PhD, at the University of California at Riverside.

^b NutriLayer (INCI: *Oryza sativa* (rice) bran oil extract) is a product of Eastman Chemical, Kingsport, TN, USA.

^c The EpiDerm skin model was provided by MatTek, Ashland, MA, USA.

^d DHR fluorescent probe was obtained from Molecular Probes, Carlsbad, CA, USA.

cytes and contains *in vivo*-like lipid and ceramide profiles. Hanson and Clegg found this skin model to yield more reproducible results than *ex vivo* human skin tissue, which shows variability due to differences in pigmentation, age and body site.^{23,24}

The protocol for the study discussed herein calls for incubating the tissues after applying 100 μl of 15 μM DHR at 37°C and 5% CO_2 for 2 hr, applying 2 mg cm^{-2} of each test formulation to the surface of separate skin tissues with a positive pressure pipet and spreading over the skin with the tip of a glass rod. The formulation was allowed to absorb into the tissue for 2 hr before imaging. Free radical formation can be induced through many means; doing so with UV irradiation is both simple and noninvasive. The rice bran extract used in this study neither absorbed, nor reflected, UVA or UVB radiation. UV irradiation was performed using a solar simulator^e that emits both UVB and UVA radiation. A standard dose of 40 mJ cm^{-2} was used, which is equivalent to approximately 20 min of mid-day summer sun in Bondville, Illinois, USA.²⁵

Two-photon fluorescence microscopy imaging was accomplished using a microscope coupled to a titanium-sapphire laser^f.

Each treated tissue was imaged before and after UV irradiation. Before irradiation, the tissue was imaged one time at four different depths to acquire data on the strata basale, spinosum, granulosum and corneum. These “before” images gave an auto-fluorescence background level. After UV irradiation, each tissue was imaged over two areas at four different depths analogous to the “before” images. Image areas were typically 20x20 μm or greater. A variety of cosmetic formulations were investigated as shown in **Table 1**.

The formulations were prepared in Kingsport, Tennessee, USA, and shipped to California where they were tested.^a The images, as shown in **Figure 2**, were collected using software.^g

The different colors in the images represent the fluorescence caused by differing amounts of free radicals formed in a placebo treated (base cream), and a rice bran extract treated tissue sample imaged at four different depths into the skin tissue. The placebo and

^e The solar simulator was obtained from Solar Light Co., Glenside PA, USA.

^f The Zeiss Axiovert microscope coupled to a Tsunami titanium-sapphire laser is a device of Spectra-Physics, Mountain View, CA, USA.

^g SimFCS software was utilized.

rice bran extract cream formulations, shown in **Table 1**, were identical except the placebo contained water in place of rice bran extract. Post-collection data analysis used methods similar to those used in flow cytometry.

Table 1. Composition of the formulations used in the two-photon fluorescence microscopy study

INCI Name	Base Cream	5% Rice Bran Extract	1% Rice Bran Extract	Trade Name/ Supplier
Water (<i>aqua</i>)	92.85	87.85	91.85	
Glycerin	2.00	2.00	2.00	
Carbomer	0.20	0.20	0.20	Carbomer Ultrez 10, Noveon
Cetearyl alcohol (and) cetareth-20	2.00	2.00	2.00	Promulgen D, Noveon
Glyceryl dilaurate	0.50	0.50	0.50	Lexemul GDL, Inolex Chemical
Cetyl alcohol	1.50	1.50	1.50	
Dimethicone	0.20	0.20	0.20	DC 200 Fluid 350 cSt, Dow Corning
<i>Oryza sativa</i> (rice) bran oil extract	0.00	5.00	1.00	NutriLayer, Eastman Chemical
Triethanolamine	0.25	0.25	0.25	
Propylene glycol (and) diazolidinyl urea (and) methylparaben (and) propylparaben	0.50	0.50	0.50	Germaben II, ISP (Sutton Labs)

The intensity of each cell was recorded for each image at each depth for each tissue sample. A histogram of the data was generated and a statistical analysis completed to determine the mean of each histogram. The mean is the fluorescence intensity for the image at the depth z . A comparison of the mean values between the rice bran extract containing formulations and the control formulation was made and is expressed as the percent quenching of ROS. As shown

in **Figure 3**, both the 1% and 5% rice bran extract creams reduced the concentration of ROS present in the model epidermis after exposure to UVA and UVB, compared with the amount of ROS formed when the base cream formulation alone was used.

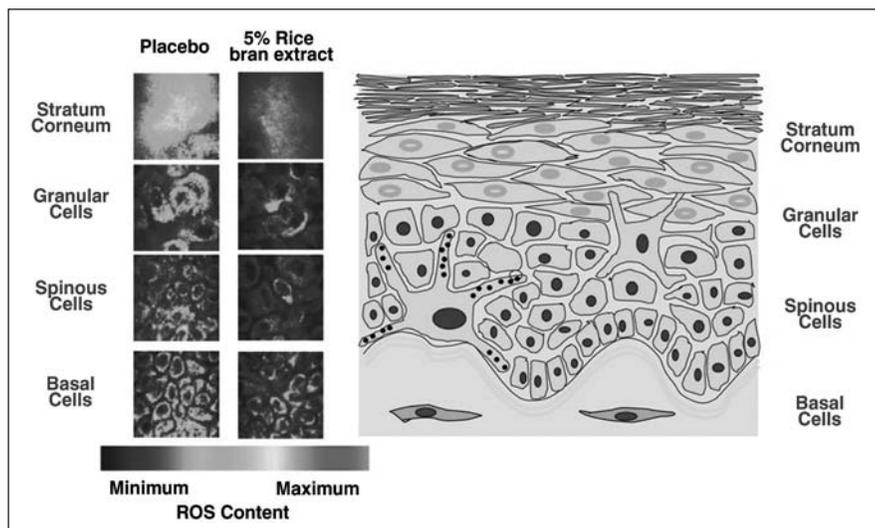


Figure 2. Two-photon fluorescence microscopy comparison of ROS present in placebo versus rice bran extract.

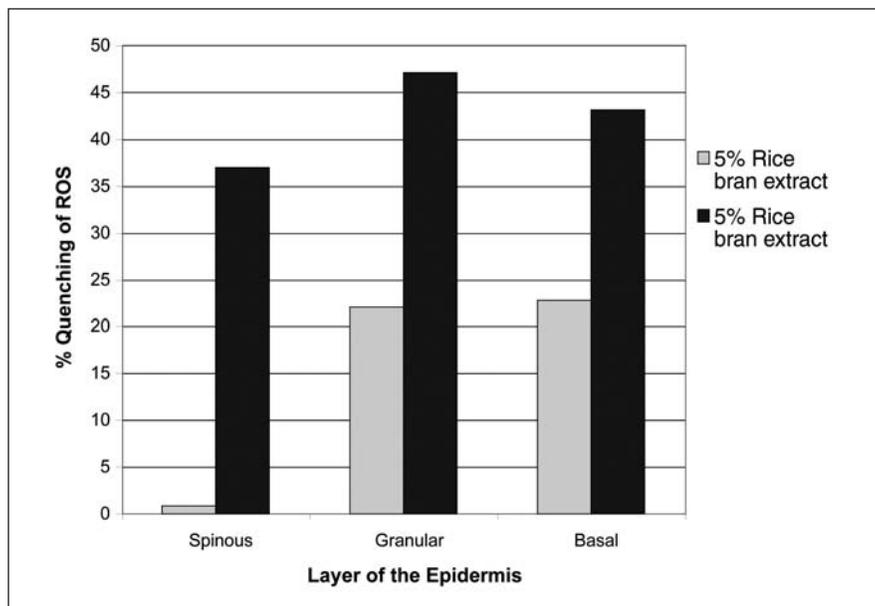


Figure 3. ROS-quenching activity of different rice bran extract concentrations in the granular, spinous and basal layers

Although the 1% rice bran extract cream did not significantly reduce the ROS in the stratum granulosum, there were 20% less ROS found in the strata spinosum and basal than with the base cream. The 5% rice bran extract cream reduced the ROS in the strata granulosum, spinosum and basal by almost 50% more than what was detected using the base cream. No results are shown from the stratum corneum because it was determined that it is a highly scattering, inhomogeneous region that cannot provide statistically significant data without a larger sample population than what was used for this study.

These results clearly indicate that the antioxidants found in the rice bran extract penetrated into the epidermis where they were beneficial at protecting living and regenerating cells. This is not surprising since it is well-documented in the open literature that tocopherols, tocotrienols, phytosterols and some of the other components in rice bran extract readily penetrate the stratum corneum.²⁶ This is crucial if they are to protect the cellular components from free radical damage.

Conclusion

The ROS study performed showed that incorporating rice bran extract into personal care or cosmetic products reduced the amount of ROS formed in model skin tissue. Antioxidants in rice bran extract penetrated through the stratum corneum of the model skin tissue and quenched free radicals in the strata granulosum, spinosum and basal.

The data provided by fluorescence microscopy can be used to support claims such as raw material benefits in personal care products including antiaging and sun care. The research demonstrates a two-photon fluorescence microscopy method to evaluate the effect of personal care ingredients on free radicals at various depths in skin tissue models. It can also be used to investigate the photoprotective effectiveness of sunscreens or sunscreens combined with free radical scavengers, such as rice bran oil extract or other ingredients, on a substrate that closely resembles human skin.

References

1. J Buenger et al, An interlaboratory comparison of methods used to assess antioxidant potentials, *Int J Cosmet Sci*, 28 135–146 (2006)
2. E Pelle et al, An *in vitro* model to test relative antioxidant potential: Ultraviolet-induced lipid peroxidation in liposomes, *Arch Biochem and Biophys* 283 2 234–240 (1990)
3. E Pelle et al, Cigarette smoke-induced lipid peroxidation in human skin and its inhibition by topically applied antioxidants, *Skin Pharma Applied Skin Physiol* 15 63–38 (2002)
4. E Pelle et al, A test for antioxidant activity in cosmetic formulations, *J Cosmet Sci* 53 237–240 (2002)
5. S Jiang, D Quan and H Maibach, Chemoprevention of skin cancer: Role and antioxidants, *Cosmet Toil* 122 1 28–31 (2007)
6. C Nishigori, Y Hattori and S Toyokuni, Role of reactive oxygen species in skin carcinogenesis, *Antioxidants & Redox Signaling* 6 561–571 (2004)
7. Q Chen and BN Ames, Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells, *PNAS* 91 4130–4134 (1994)
8. M Berneburg et al, Induction of the photoaging-associated mitochondrial common deletion *in vivo* in normal human skin, *J Invest Dermatol* 122 1277–1283 (2004)
9. E. Damiani et al, Changes in ultraviolet absorbance and hence in protective efficacy against lipid peroxidation of organic sunscreens after UVA irradiation, *J Photochem Photobiol B: Biol* 82 (2006)
10. KM Hanson et al, Sunscreen enhancement of UV-induced reactive oxygen species in the skin, *Free Radic Biol Medicine* 41 8 1204–1212 (2006)
11. B Idson, Vitamins and the skin, *Cosm & Toil* 102(12)79–89 (1993)
12. B Idson, Dry skin moisturizing and emolliency, *Cosm & Toil* 107(7)69–76 (1992)
13. C Fox, Topical bioactive materials, *Cosm & Toil* 109 (9) 83–104 (1994)
14. W Stone and T Huang, Cosmeceuticals, *NutraCos* 1 9–12 (2003)
15. C Weber et al, Efficacy of topically applied tocopherols and tocotrienols in protection of murine skin from oxidative damage induced by UV-irradiation, *Free Rad Biol & Med* 22 (5) 761–769 (1997)
16. R Wachter, B Salka and A Magnet, Phytosterols, *Cosm & Toil* 110 (7) 72–78 (1995)
17. E Idson, Vitamins and the skin (vitamins in cosmetics), *Cosm & Toil* 108 12 79–89 (1993)
18. A Pappas, The Vitamin E Factor, Harper Perennial (1999)
19. M Potokar, W Holtmann and A Werner-Busse, Effects of topical and oral vitamin E on pigmentation and skin cancer induced by ultraviolet irradiation in SKH:2 hairless mice, *Fat Sci Technol* 38 1 87–97 (Oct 1990)
20. Suzuki et al, Structural and dynamic membrane properties of alpha-tocopherol and alpha-tocotrienol: Implication to the molecular mechanism of their antioxidant potency *Biochem* 32 40 10692–10699 (1993)
21. NE Kinkade, NutriLayer Phytolipids: A new skin care ingredient from rice bran oil, Congresso Brasileiro de Cosmetologia, Sao Paulo, Brazil (Apr 20–21, 2006)
22. KM Hanson and RM Clegg, Observation and quantification of UV-induced ROS generation in *ex vivo* human skin, *Photochem Photobiol* 7 57–63, (2002)
23. KM Hanson and RM Clegg, Two-photon fluorescence imaging and ROS detection within the epidermis, *Methods in molecular biology: Epidermal cells, methods and protocols*, K Turksen, ed., Humana Press, Clifton, NJ, 412–421 (2003)
24. KM Hanson and RM Chappell, Sunscreen and antioxidant effects upon UV-induced ROS in human skin: A two-photon fluorescence microscopy study, *Eur J Biochem*, 217 589–598 (2003)
25. Surface Radiation Research Branch, *Surfrad Network UV Monitoring*, National Oceanic and Atmospheric Administration (2001)
26. M Kanimoura, Percutaneous absorption of alpha-tocopherol acetate, *J Vitaminol* 14 150 (1968)

Using Photochemiluminescence to Quantify the Antioxidative Capacity of Topicals

Hongbo Zhai, M.D. and Howard I. Maibach, M.D.

University of California School of Medicine

KEY WORDS: *grapefruit extract, stability, emulsion, melanin, erythema*

ABSTRACT: *The current work aimed to formulate a stable w/o emulsion containing grapefruit extract by entrapping the extract in the inner aqueous phase. The final formula was found to have skin-whitening, moisturizing, cleansing and antiwrinkle effects, among others.*

Exposure to ultraviolet radiation (UVR), air pollutants, chemical oxidants and aerobic microorganisms may damage the skin.^{1,2} In particular, reactive oxygen species (ROS) are considered a likely contributor to skin aging, cancer and certain skin disorders.^{1,3,4}

Natural defense systems in healthy skin can protect against exogenous oxidative stress. Excessive free radical attack, such as over-exposure to UVR, however, can overwhelm cutaneous antioxidant capacity, leading to oxidative damage and ultimately to skin cancer, immunosuppression and premature skin aging.¹⁻⁴ Therefore, supplying exogenous antioxidants to the endogenous antioxidant system may prevent or minimize ROS-induced photoaging.^{4,5} This is why topical products with antioxidant properties have proliferated worldwide.

Recently, *in vitro* and *in vivo* methods have been utilized for the evaluation of antioxidant capacity.⁴⁻⁶ Most are based on the measurement of the relative abilities of antioxidants to scavenge

radicals in comparison with the antioxidant potency of a standard antioxidant compound. However, some technologies require complicated performance and are time-consuming or expensive. We introduced a rapid, accurate and facile method to quantify the antioxidative capacity with a sensitive photochemiluminescence device.

Photochemiluminescence Device

A photochemiluminescence (PCL) device^a was utilized (**Figure 1**). Its principle is described: Defined free radicals (superoxide anion radicals) are generated in the measuring system by the exposure of a photosensitizer to a UV light source. The free radicals are detected by their reaction with a chemiluminogenic substance and the measurement of the emitted light. The light flashes are detected in the PCL device by a photomultiplier. These generated radicals are partially scavenged by reaction with the sample antioxidants and remaining radicals are quantified by the previously mentioned detection principle.



Figure 1. Lab setup for using photochemiluminescence to quantify the antioxidative capacity of a sample

The results are presented in equivalent concentration units of synthetic vitamin E^b for lipid-soluble substances or ascorbic acid for

^a Photochem is a registered trademark of Analytik Jena AG, Jena, Germany, and Analytik Jena USA, Inc., Texas, USA.

water-soluble substances. Several concentrations of these standard compounds are used to establish a calibration curve and the detector signal of each run is monitored for 180 seconds. For measurements of the integral antioxidative capacity of lipid-soluble (ACL) antioxidants, an ACL kit^c was used.

Versatile Applications for PCL

Photochemiluminescence (PCL) is used to improve the antiradical properties of cosmetics and control the long-term stability of products. It also has applications in numerous fields outside of cosmetics and personal care.

In biology it aids examination of oxidative stress phenomena from increased UV irradiation and other environmental factors, enabling improvements in the resistance to these factors.

In the food industry, it helps improve the shelf life of products and determines the antioxidative efficacy of food supplements. Other applications have been found in medicine, naturopathy, environmental medicine, pharmacy and chemistry.

PCL enables the determination of these biochemical properties:

- Integral antioxidative capacity of most diverse substance mixtures, such as blood plasma, tissue homogenates, oils, foodstuffs, cosmetics and pharmaceutical products.
- Selective antiradical properties of individual, non-enzymatic substances.
- Activity of superoxide dismutase.

Testing Topical Formulations

A vitamin E-containing oil-in-water emulsion^d with 22.5% oil content for facial skin care and its vehicle control were tested. The tested emulsion contains *aqua*, petrolatum, myreth-3 myristate, glycerin, cetearyl alcohol, tocopheryl acetate, cetareth-20, dimethicone, sodium PCA, sodium citrate, sodium carbomer, *parfum*, benzyl alcohol, methylparaben and propylparaben. The active ingredient was 2.3% vitamin E. The vehicle control was identical with the exception of the deletion of vitamin E.

^b Trolox is a registered trademark of Hoffman-LaRoche, Basel, Switzerland.

^c Analytik Jena AG, Jena, Germany, and Analytik Jena USA, Inc., Texas, USA

^d Sebamed cream, Sebamed is a registered trademark of Sebapharma GmbH & Co., Boppard, Germany.

Preparation of sample: Sample was made at a 5% concentration as follows: 37.5 mg of sample was placed into a 2.0 mL flat-top microcentrifuge tube^e to which 750 μ L of butanol^f (HPLC-grade) was added. The mixture was shaken with a vortex^g vigorously for 30 seconds. After centrifugation for 5 minutes at 10,000 rpm, the supernatant was collected and transferred into a new microfuge tube through a 0.45 μ m filter^h.

Procedure for sample analysis: Analysis was performed according to the standard protocol with a modification.⁷ Six equal aliquots of each testing sample were analyzed. Ratio of the assay mixture in microliters was 50:2300:200:25 for the sample, the ACL-diluent, the reaction buffer, and the photo sensitizer and detection reagent work solution, respectively. A light emission curve was recorded during 180 seconds, and inhibition was the evaluation parameter of antioxidant activity. Amounts of antioxidative substances were calculated by synthetic vitamin E used as standard to establish a calibration curve and expressed as nmol equivalents in antioxidant activity of vitamin E. Further details of measuring method and principles of PCL analysis are found elsewhere.⁷⁻¹⁰

Statistical analysis: Statistical analysis was performed using a computer program^k. A paired Student's *t* test was used in comparison of significance difference between the vitamin E-containing formulation and its vehicle control. All tests of comparisons were two-sided at a significance level of 0.05.

Results

The quantity of antioxidant capacity for the vitamin E-containing formulation and its vehicle control were 2.28 ± 0.05 and 0.16 ± 0.03 , respectively. The vitamin E-containing formulation showed a markedly significant ($P < 0.001$) effect of antioxidant over its vehicle control.

^e Fisher Scientific, Pittsburgh, Penn., USA

^f Fisher Scientific, Fair Lawn, N.J., USA

^g Scientific Industries, Inc., Bohemia, N.Y., USA

^h Osmonics, Inc., Minnetonka, Minn., USA

^k SigmaStat is a registered trademark of SPSS Science, Chicago, Ill., USA.

Discussion and Conclusions

Antioxidants often are found in skin care products claiming to decrease photoaging and reverse photodamage. These topical products may contain enzymatic and/or nonenzymatic antioxidants. However, vitamin E is a common component in these formulations. Its antioxidant effect has been documented.^{1,3,4,11-13} From the method tested, the vitamin E formulation has demonstrated its superior antioxidant effect over its vehicle control.

PCL analysis provides advantages over the other methodologies. It is simple, quick, sensitive, economical, convenient and reliable. It may detect the antioxidative capacities of even low concentrations of antioxidant. We suggest it may act as a screening method; keep in mind that the method and data were generated from an *in vitro* experiment. Actual antioxidant effects of antioxidant-based formulation candidates should also be evaluated *in vivo* in man. Additionally, basic requirements for a scientific paper reporting antioxidant testing are recommended.¹⁴

Published January 2006 *Cosmetics & Toiletries* magazine.

References

1. JJ Thiele, F Dreher and L Packer, Antioxidant defense systems in skin, in *Cosmeceuticals, Drugs vs. Cosmetics*, P Elsner and HI Maibach, eds, New York: Marcel Dekker (2000) pp 145–187
2. JJ Thiele, Oxidative targets in the stratum corneum. A new basis for antioxidative strategies, *Skin Pharmacol Appl Skin Physiol* 14 87–91 (2001)
3. DP Steenvoorden and GM van Henegouwen, *The use of endogenous antioxidants to improve photoprotection*, *J Photochem Photobiol B: Biol* 41 1–10 (1997)
4. F Dreher and HI Maibach, Protective effects of topical antioxidants in humans, in *Oxidants and Antioxidants in Cutaneous Biology*, JJ Thiele and P Elsner, eds, Basel: Karger (2001) pp 157–164
5. F Stab, R Wolber, T Blatt, R Keyhani and G Saueremann, Topically applied antioxidants in skin protection, *Methods Enzymol* 319 465–478 (2000)
6. R Kohen, Skin antioxidants: their role in aging and in oxidative stress—new approaches for their evaluation, *Biomed Pharmacother* 53 181–192 (1999)
7. IN Popov and G Lewin, Photochemiluminescent detection of antiradical activity. IV: testing of lipid-soluble antioxidants, *J Biochem Biophys Methods* 11 1–8 (1996)
8. IN Popov and G Lewin, Photochemiluminescent detection of antiradical activity: II. Testing of nonenzymic water-soluble antioxidants, *Free Radic Biol Med* 17 267–271 (1994)
9. G Lewin and IN Popov, Photochemiluminescent detection of antiradical activity. III: A simple assay of ascorbate in blood plasma, *J Biochem Biophys Methods* 28 277–282 (1994)
10. IN Popov and G Lewin, Antioxidative homeostasis: characterization by means of chemiluminescent technique, *Methods Enzymol* 300 437–456 (1999)
11. J Fuchs, *Potentials and limitations of the natural antioxidants RRR-alpha-tocopherol, L-ascorbic acid and beta-carotene in cutaneous photoprotection*, *Free Radic Biol Med* 25 848–873 (1998)

12. F Dreher, B Gabard, DA Schwindt and HI Maibach, *Topical melatonin in combination with vitamins E and C protects skin from ultraviolet-induced erythema: a human study in vivo*, *Br J Dermatol* 139 332–339 (1998)
13. D Darr, S Dunston, H Faust and S Pinnell, *Effectiveness of antioxidants (vitamin C and E) with and without sunscreens as topical photoprotectants*, *Acta Dermatol Venereol* 76 264–268 (1996)
14. H Verhagen, OI Aruoma, JH van Delft, LO Dragsted, LR Ferguson, S Knasmuller, BL Pool-Zobel, HE Poulsen, G Williamson and S Yannai, *The 10 basic requirements for a scientific paper reporting antioxidant, antimutagenic or anticarcinogenic potential of test substances in in vitro experiments and animal studies in vivo*, *Food Chem Toxicol* 41 603–610 (2003)

Protecting the Genome of Skin Cells from Oxidative Stress and Photoaging

Louis Danoux, Christine Jeanmaire, Vincent Bardey, Gilles Périé,
Marie-Danielle Vazquez-Duchêne, Véronique Gillon, Florence Henry,
Philippe Moser and Gilles Pauly

Laboratoires Sérobiologiques, A division of Cognis France, Pulnoy, France

KEY WORDS: *photoaging, mitochondrial DNA, nuclear DNA, DNA damage, UVA, UVB, DNA repair, GADD45 α , kaempferol sophoroside*

ABSTRACT: *According to a concept proposed here, protection of nuclear DNA and mitochondrial DNA against UVB and UVA radiation can break the vicious cycle responsible for skin photoaging.*

A new concept to fight the cycle responsible for photoaging has been proposed. It addresses ultraviolet (UVA and UVB) radiations of nuclear deoxyribonucleic acid (nuDNA) and mitochondrial DNA (mtDNA), and provides global protection by effects that are both preventive and curative. The concept is illustrated using a new phytochemical active aimed at preventing photoaging by protecting and repairing UV-induced DNA damage.

Overview of DNA and the Genome

In human cells, the genome is represented by linear DNA in the nucleus, and also by circular DNA located in cell organelles named mitochondria. DNA constitutes an important cellular component because it is the carrier of genetic information that is read as a blueprint by which cells perform necessary operations in order to grow or survive.

The nucleus is the cell domain that harbors DNA, which constitutes the genetic material or genome. DNA is formed of chained nucleotide bases whose specific sequences, termed *genes*, carry the hereditary information.

The nucleus conceals the initial steps of the central dogma of biology, which states that genes are transcribed into transportable messenger ribonucleic acid (mRNA) carrying the instructions up to cytoplasm where mRNAs are translated into determined proteins.

A second part of the genome is found in mitochondria. Mitochondria generate energy in the form of adenosine triphosphate (ATP) through an oxygen-dependent enzymatic pathway. The circular stranded mtDNA contains genes-encoding proteins essential to producing energy.

Although mtDNA represents a small part of human genome, it plays a crucial role in aging. In fact during energy production, mitochondria release reactive oxygen species (ROS); typically 1–5% of consumed oxygen is released as ROS that causes mtDNA damage leading to the weakening of the cell energetic potential and, at the end, to the cell senescence and death. This vicious cycle now is accepted as an important cause of aging.¹

DNA Damage and Repair

Damage: DNA undergoes both direct and indirect effects, respectively, from UVB at the nuclear level and from UVA, particularly at the mitochondrial level. Concerning the UVB toxicity (see **UV Toxicity**), a recent study demonstrated that the level of thymidin dimers was increased distinctly even at infraerythral dose. UVA radiation induces damage in mitochondria; the most frequent is called *common deletion* (see sidebar on UV toxicity).

In order to maintain the integrity of DNA, the mammalian cells are equipped with a sophisticated system (see sidebar on the DNA repair system) involving many proteins committed first to detect DNA alterations, then to delay the cell cycle allowing the repair of damaged cells. This system is particularly important because it is well established that it decreases during aging and a deficiency in repair of DNA damage results in improper cell functioning.

UV Toxicity

UVB (wavelength 290-320 nm) can cause the dimerization of two adjacent pyrimidine bases into cyclobutane pyrimidine dimers (CPD) or 6-4 pyrimidine-pyrimidone (6-4PP).⁹ This type of lesion distorts the DNA helix. As a consequence, it inhibits transcription and replication, and modifies gene expression. These solar scars might not be visible immediately because the level of thymine dimers (a kind of CPD) was increased distinctly even at infraerythemal dose.¹⁰

UVA (wavelength 320-400 nm) can penetrate deeper into dermis where it triggers the release of supplementary ROS from mitochondria. Therefore, dermal cells' mtDNA—which has no protective proteins and potent DNA repair enzymes—constitutes a privileged target to endogenously and exogenously generated ROS. In particular, repetitive UVA exposure leads to the formation of “common deletion,” which is a large-scale deletion of 4977 bp in mtDNA of dermal cells. It has been shown that the rate of common deletion is increased in photoexposed skin.¹¹

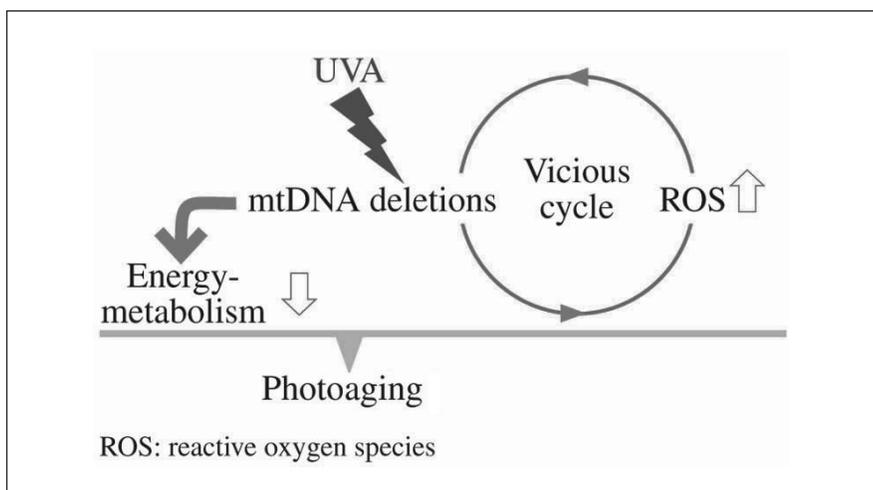


Figure 1. Accelerated skin photo-aging by UVA-induced lesions in mitochondria

The DNA repair system is not exhaustive; some UV-induced dimers can persist in skin up to three weeks after the UV irradiation. Furthermore this system can be saturated, particularly in the case of oxidative stress, when ROS are produced in excess. In mitochondria, the respiratory chain generates ROS that, associated with ROS

produced during UV irradiation, cause considerable damage to macromolecules such as proteins and DNA. This mitochondrial DNA damage starts a vicious cycle through the expression of deleterious proteins that in turn enhance the release of ROS and the oxidative stress. Such overload of ROS production, exceeding the natural potential of DNA repair, explains the phenomenon of skin photoaging. Moreover, nucleotide excision repair (NER) of nuDNA is induced only at low irradiation and even might be lowered by strong UV exposure or aging.²

This data makes it obvious that to preserve the good quality of human skin, it is necessary to protect both the nuDNA of the epidermis from UVB, and the mtDNA of the dermis from UVA. The traditional approaches (see sidebar on preventing and curing DNA damage) have been claimed to protect skin from sunlight exposure, but most of them concern either nuDNA or mtDNA through either protective or curative mechanisms. Our approach was to propose a new active ingredient that covers all the effects of UV in order to efficiently break the vicious cycle responsible for photoaging. This total protection of DNA can be advantageously completed by a stimulating effect on the endogenous systems of DNA repair.

DNA Repair System

The mechanisms of DNA repair imply several pathways grouped in base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and strand breaks repair.

The BER concerns ROS-induced lesions of nucleic bases and employs specific glycolases; it renews aberrant bases in nuDNA and mtDNA.

The NER is the most important DNA repair pathway for UV-induced lesions. In human skin, the 6-4PP and CPD are repaired through NER pathway in a matter of hours.

The MMR repairs errors occurring during DNA replication, whereas the repair of single strand breaks is done by a BER mechanism.

A New Active Against Photoaging

With this goal of global skin preservation, a new active ingredient^a based on an extract of the plant *Senna alata* (L.) ROXB. (Fabaceae) has been developed. In this chapter, this proprietary *Senna alata* extract will be called SAE.

Senna alata (L.) ROXB., also called candle bush, is a fast-growing perennial shrub that grows 1-3 meters tall. Native to South America, *Senna alata* (L.) ROXB. now is distributed throughout the tropics, and occasionally planted for medicinal and ornamental purposes. In different traditional medicine, the crushed leaves are used for numerous skin diseases such as eczema. The leaves are known to contain flavonoids, especially kaempferol-3-O-sophoroside (K3OS) (C₂₇H₃₀O₁₆; molecular weight = 610.52; CAS No. 19895-95-5). Different fractions were isolated from the raw plant extract and K3OS was identified as an active molecule. It was described that this kind of molecule can preserve DNA from oxidative stress.³

Preventing and Curing DNA Damage

Preventive approaches: The most immediate preventive strategy is to reduce the rate of DNA attacks by the regular use of filters. The sun protection factor (SPF) based on erythema often is overestimated and is not guaranteed for each user of sunscreen.¹² Therefore the use of complementary ingredients to protect human skin is meaningful:

- Polyphenols from green tea, vitamin E and vitamin C have been claimed to protect DNA from ROS.¹³
- Concerning mtDNA, β-carotene can protect human fibroblasts from repetitive UVA irradiations.¹⁴

Curative treatments: The topical use of enzymes, such as photolyase and T4 endonuclease V, has been proposed to treat the skin.¹⁵

Another way is to stimulate the DNA repair system. The stimulation of enzymes is relevant because it has been established that the DNA repair potential decreases with aging. A recent method is the use of thymidine dinucleotide (pTpT) or UV-irradiated DNA¹⁶ to enhance the DNA repair. The pTpT activity could be mediated through the activation of p53 protein that controls the expression of gadd45α, a protein that promotes the cell cycle arrest, allowing the DNA repair process to occur.

^a DN-Age [INCI name: Water (and) glycerin (and) Senna alata leaf extract] is a product of Laboratoires Sérobiologiques, a division of Cognis France.

Methods

Protection against UVB: Two methods were used to evaluate SAE's protection against UVB.

- **“Comet” assay on human keratinocytes.** The protection of nuDNA was tested first through the “comet” assay on human keratinocytes. This assay is a very sensitive method for evaluating DNA strand breaks. The human keratinocytes were cultivated within growth medium for three days at 37°C, CO₂=5%; then cells were treated by SAE in a balanced salt solution followed by irradiation (UVB at 50 mJ/cm²). After incubation for one day, cells were harvested and embedded in low-melting-point agarose gel on microscope slides, incubated in alkaline buffer solution for DNA unwinding, and processed by electrophoresis. The more fragmented the DNA is, the more it migrates during electrophoresis to form the “tail” of the comet. Then DNA was stained by a fluorescent probe, ethidium bromide, and the measurement of comet length was obtained by image analysis with a confocal microscope. Vitamin E was the reference because it reduces ROS-induced DNA fragmentation.⁴
- **Immunohistochemistry study of thymine dimers on human keratinocytes.** The protection of thymine dimers was evaluated by immunohistochemistry on primary cultures of human keratinocytes. Human keratinocytes were cultured in standard medium during four days; then SAE or K3OS was added in balanced salt solution and the cells were irradiated (UVB at 20 mJ/cm²). Four hours after irradiation, thymine dimers were detected by a specific monoclonal antibody and revealed by fluorescein isothiocyanate. Images were collected by a confocal microscope and analyzed. Results were expressed as the percentage of the surface occupied by thymine dimers staining.

Protection against UVA: The protection of mtDNA was evaluated on UVA-irradiated human dermal fibroblasts by measuring the rate of “common deletion,” referring to a protocol based on RT-PCR (reverse transcriptase polymerase chain reaction) with vitamin E

as reference.⁵ Human dermal fibroblasts were exposed three times a day and four consecutive days per week to sub-lethal doses of UVA (8 J/cm²) during two weeks. The products of damaged DNA amplification were visualized in agarose gel stained with ethidium bromide and quantified by phosphorimager analysis.⁶

Stimulation of DNA repair: To explore more deeply the potential of SAE, the gene expression of treated primary human keratinocytes was screened by using cDNA (complementary DNA) arrays. Primary cultures of human keratinocytes were treated for three hours by SAE in cell culture medium; then the cells were recovered and total RNA was extracted^b, converted in cyanine 3- or cyanine 5-labeled cDNA by the reverse transcriptase and hybridized on MWG “Pan human 10K” arrays.

The assay was repeated three times and the results were normalized and evaluated. Then, quantitative RT-PCR (qRT-PCR) method was used to confirm the stimulation of gadd45 α gene expression. The protein gadd45 α promotes the cell cycle arrest allowing the DNA repair process to take place, and avoids the amplifying of damaged cells.⁷ It also facilitates the nucleotide excision process that is the most important DNA repair pathway for UV-induced lesions; gadd45 α is UV responsive and contributes to maintenance of genomic stability.

In this case, the qRT-PCR assay requires the culture of human keratinocytes within growth medium for 3-5 days at 37°C, CO₂=5%, followed by the introduction of SAE in a balanced salt solution. After an incubation of 90 or 180 minutes, the cells were harvested and total RNA was extracted, and mRNA was amplified with SYBR green incorporation^c. The results were expressed by the gadd45 α crossing point versus the house keeping gene crossing point and normalized by a stable calibrator tested in each run of qRT-PCR.

Characterization of the active molecule: Different fractions were isolated from the raw plant extract. Then the activity of each fraction to protect human keratinocytes from UV toxicity was screened. The active fraction was thoroughly investigated by HPLC (high performance liquid chromatography) to isolate the main molecule that was tested and structurally determined by ¹H-NMR, ¹³C-NMR and ESI MS.

^b Kit Nacherly nagel “NucleoSpin RNAII”

In vivo study of skin microrelief: protective effect against cutaneous photodamage was evaluated on 12 healthy female volunteers (ages 30–50 years) with skin phototype II or IIIA.

The volunteers were irradiated^d on four consecutive days in the back area by UV(A+B). A cream containing 3% SAE was tested against the placebo cream. An irradiated but untreated skin area was simultaneously appraised as a control site. Products were applied daily for six consecutive days before irradiations (pre-treatment) and just before each of the four irradiations (treatment) with randomization, at the dose of 2 mg/cm².

Skin was irradiated individually by increasing doses of 0.75 MED, 0.9 MED, 1 MED, and 1.2 MED. Negative Silflo replicas were made six days after the last irradiation. Alteration of the microrelief was studied by confocal microscopy of the replica. Then Fast Fourier Transform (FFT) was applied and results were expressed as a ratio of principal moments of inertia, also called second moments of the FFT. After UV(A+B), the ratio increases, corresponding to a higher anisotropy of the cutaneous microrelief.

Results

SAE displayed a convincing potential to protect globally the genome integrity of skin cells.

- It decreased the level of inflammatory mediators produced by UVB-irradiated keratinocytes.
- It reduced the rate of DNA lesions such as fragmentations and thymidine dimer formation induced by the UVB irradiation of keratinocytes.
- It specifically protected mtDNA as demonstrated by a decrease of the rate of common deletions observed in UVA-irradiated fibroblasts.

Following are the results.

DNA photoprotection by the comet assay on human keratinocytes:

During electrophoresis, the UVB-induced DNA fragments migrated from the nucleus to form a tail like a comet. The measurement of the tail length on the horizontal axis allowed the quantification of UVB

^c Kit Roche LC FastStart DNA Master SYBR Green I

^d Oriol Solar simulator equipped with a Schott filter WG 320

effect. The UVB-induced DNA fragmentation was decreased significantly after SAE treatment whereas vitamin E had no effect (**Table 1**).

Table 1. DNA photoprotection evaluated by the “comet” assay on human keratinocytes

Tested products	Comet length in μm
Control without UV	25 \pm 2
UVB at 50 mJ/cm ²	55 \pm 4
UVB + vitamin E at 0.0003%	54 \pm 8
UVB + SAE at 1%	37 \pm 4 (*)
Statistics:	
Mean \pm SEM on 3 assays	
Student's t test	
(*) p<0.05	

Immunohistochemistry study of thymine dimers on primary cultures of human keratinocytes: The results (**Figures 2 and 3**) demonstrate that UVB radiation of 20 mJ/cm² notably induced the formation of thymine dimers in the nucleus of human keratinocytes. SAE as well as K3OS provided a strong protection with a dose-dependent effect.

UVA-induced common deletion in mtDNA of human dermal fibroblasts: Repetitive exposures to UVA led to common deletion in mtDNA, visualized in **Figure 4** by the 772 bp DNA fragment in B lane. SAE provided a good protection against the UVA-induced mtDNA mutations in human dermal fibroblasts as did vitamin E.

Stimulation of gene expression: The gene expression profiling by cDNA arrays provided interesting targets involved in the DNA gene repair process. This biologic mechanism is based on the booster effect of gadd45 α (growth arrest and DNA damage) gene expression allowing an increase in skin cell DNA repair. Profiling revealed that the expression of gadd45 α gene was stimulated in human keratinocytes treated by SAE compared to nontreated keratinocytes. qRT-PCR study confirmed that SAE and K3OS significantly enhanced by approximately 30% the expression of gadd45 α gene after 90 and 180 minutes of keratinocyte treatment (**Table 2**).

Table 2. Stimulating effect on gadd45 α expression in human keratinocytes

Ratio of gene expression referring to house-keeping gene	Incubation for 90 minutes	Incubation for 180 minutes
Control medium	0.37 \pm 0.03	0.39 \pm 0.05
K3OS at 0.1%	0.48 \pm 0.05 (*)	0.50 \pm 0.14
SAE at 1%	0.51 \pm 0.10 (*)	0.50 \pm 0.09 (*)
Statistics: Mean \pm SD on 3 or 4 assays Student's t test (*) p<0.05		

In vivo protective effect: The protective effect against cutaneous photodamage was established after repetitive UV irradiations. Cutaneous microrelief was significantly protected by SAE after four consecutive daily UV(A+B) radiations (**Figures 5 and 6**). Skin microrelief aspect was preserved totally. The skin anisotropy was reduced by 29% in comparison with the control site that was irradiated but not treated. In the same experimental conditions, placebo cream had no effect.

Conclusion

The potential of a proprietary *Senna alata* extract—called *SAE* in this chapter—to preserve DNA from UVB attack was established by the comet assay and the immunohistochemistry study of thymine dimers in primary human keratinocytes. The K3OS also reduced the rate of UV-induced thymine dimers. Therefore K3OS can explain, at least in part, the protective effects of SAE. The SAE protection against UVA was demonstrated by measuring the rate of common deletion in human dermal fibroblasts. It is essential to preserve mitochondria from UVA because mtDNA lacks protective proteins and furthermore mitochondria are not equipped with an efficient system of DNA repair.

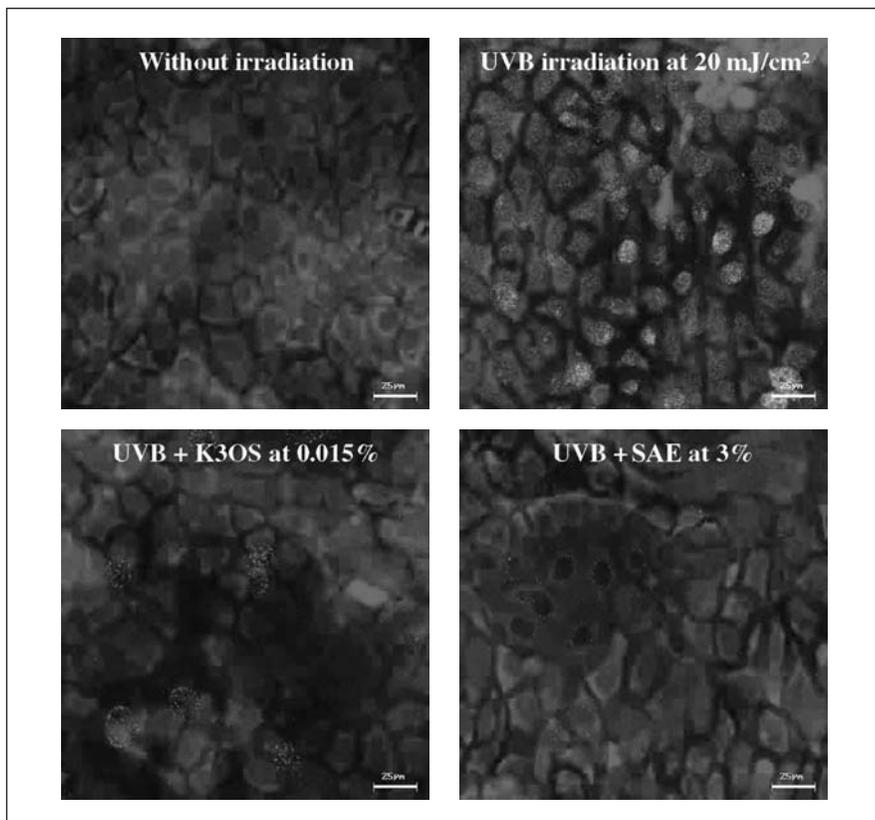


Figure 2. Visualization by immunocytochemistry of thymine dimers formation in irradiated keratinocytes

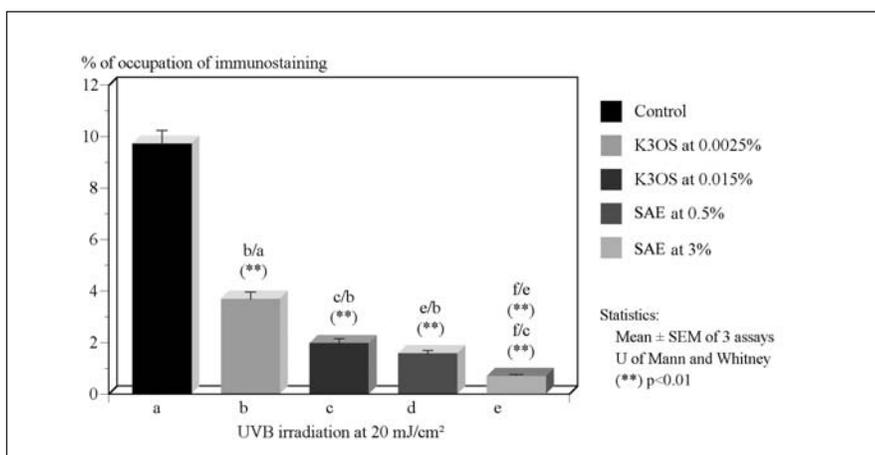


Figure 3. Quantification of thymine dimers formation in irradiated human keratinocytes

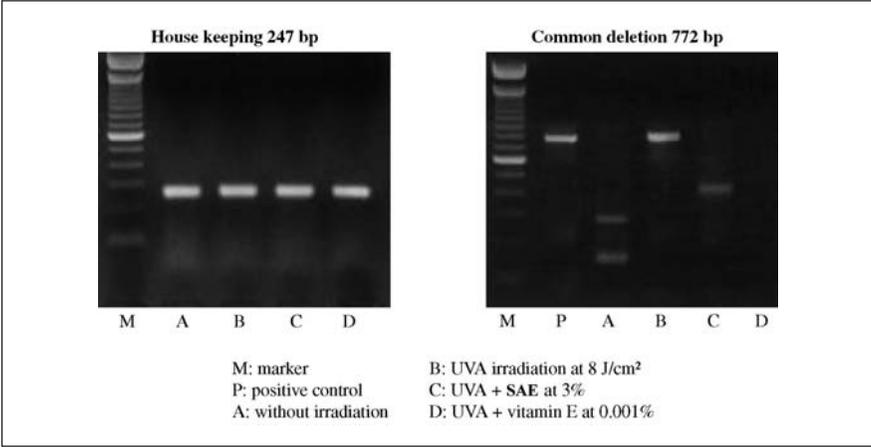


Figure 4. Image of gel electrophoresis results of house keeping gene and common deletion

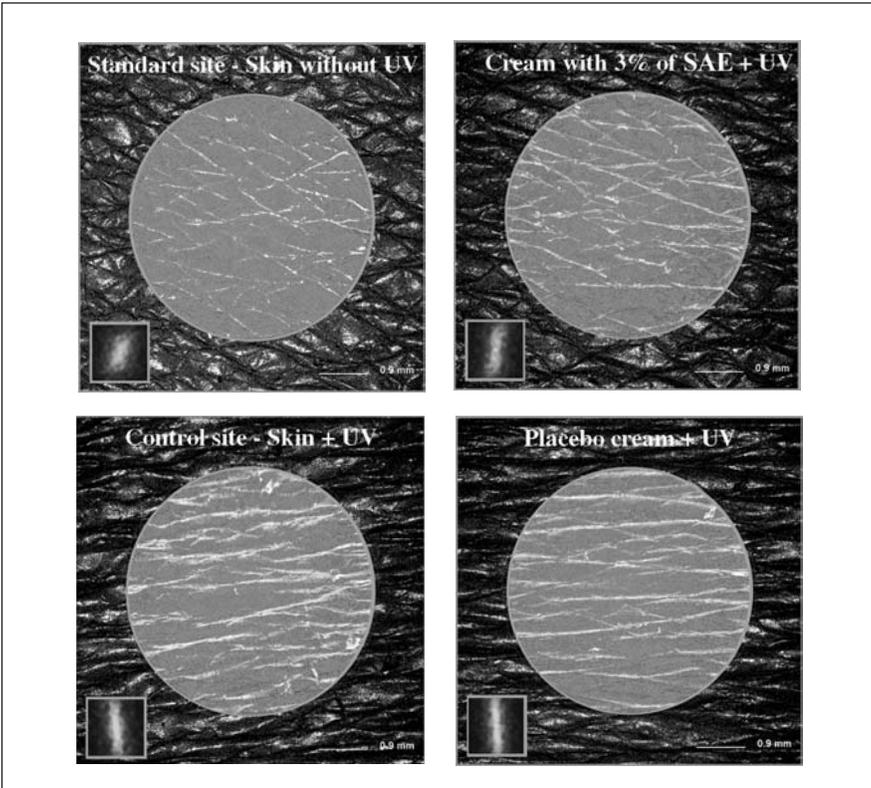


Figure 5. Visualization of skin microrelief alteration on negative replica by confocal microscopy six days after repetitive UV(A+B) irradiation

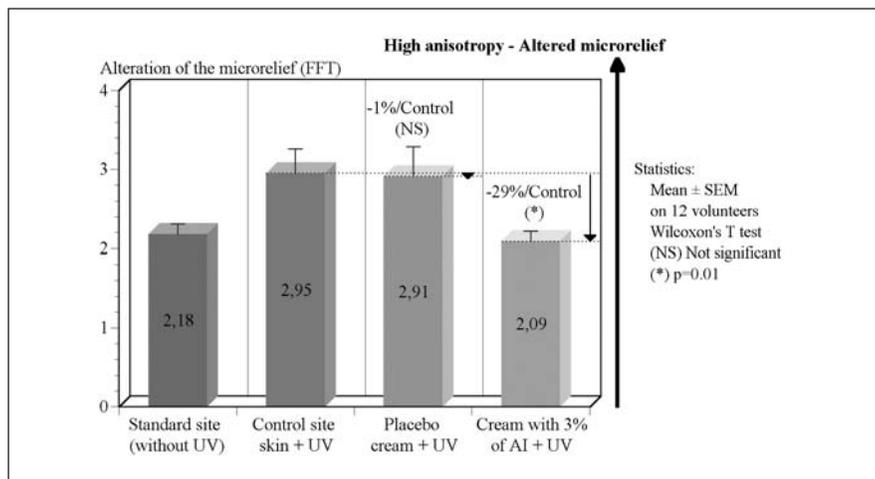


Figure 6. Quantitative study of skin microrelief on negative replica six days after last UV irradiation

Terms and Abbreviations

ATP	adenosine triphosphate
cDNA	complementary DNA
DNA	deoxyribonucleic acid
K3OS	kaempferol-3-O-sophoroside
mRNA	messenger RNA
mtDNA	mitochondrial DNA
NER	nucleotide excision repair
nuDNA	nuclear DNA
qRT-PCR	quantitative RT-PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SAE	proprietary <i>Senna alata</i> extract

The presence of K3OS in *Senna alata* leaves has been shown in the upper layers of the epidermis especially in the nucleus of vegetal cells.⁸ From the association of K3OS to vegetal cell nucleus, a strong hypothesis of its effectiveness in protecting DNA of plant cells from UVB damage can be driven. This protective mechanism may be, by analogy, transferred from the vegetal cells to the skin cells.

The profiling of gene expression enabled a more in-depth explanation of the mechanism of action: both SAE and K3OS have distinctly stimulated the gene expression of gadd45 α and can help skin cells recondition their genomic material.

The use of sophisticated techniques such as cDNA arrays and NMR afforded a better comprehension of the SAE action mechanism to preserve the skin cells from solar irradiation. By both its curative and preventive effects, this new active ingredient, based on a concept of full DNA protection involving both mtDNA and nuDNA, will be able to break the vicious cycle responsible for the human skin photoaging.

The effect of this active ingredient was shown on the skin microrelief and thus has demonstrated that it can be used to fight against detrimental effects of UVA and UVB radiations on human skin.

Published December 2005 *Cosmetics & Toiletries* magazine.

References

1. YH Wei, CY Lu, CY Wei, YS Ma and HC Lee, *Chinese J Physiology* 1–11 (2001)
2. B Becker, T Vogt, M Landthaler and W Stolz, *J Invest Dermatol* 116(6) 983–988 (2001)
3. MC Lazze, R Pizzala, M Savio, LA Stivala, E Proserpi and L Bianci, *Mutation Research* 535 103–115 (2003)
4. S Maalouf, M El Sabban, N Darwiche and H Gali Muhtasib, *Molecular Carcinogenesis* 121–130 (2002)
5. M Berneburg, S Grether Beck, V Kurten, T Ruzicka, K Briviba, H Sies and J Krutmann, *J Biological Chem* 15345–15349 (1999)
6. M Berneburg, N Gattermann, H Stege, M Grewe, K Vogelsang, T Ruzicka and J Krutmann, *Photochem Photobiol* 66(2) 271–275 (1997)
7. Q Zhan, KA Lord, I Alamo Jr, MC Hollander, F Carrier, D Ron, KW Kohn, B Hoffman, DA Liebermann and AJ Fornace Jr, *Mol Cell Biol* 14(4) 2361–2371 (1994)
8. L Danoux, C Jeanmaire, V Bardey, F Henry, P Moser, O Freis, D Gauché and G Pauly, Proceedings of the 23rd IFSCC Congress, Orlando, Oct 24–27, 2004 (2004) pp 11–15
9. M Ichihashi, in 1st Asian Dermatology Symposium, *Cosmetic Dermatology & Photoaging* 68–76 (1997)
10. N Chouinard, JP Thierrien, DL Mitchell, M Robert, R Drouin and M Rouabhia, *Biochem Cell Biol* 79 507–515 (2001)
11. M Berneburg, J Krutmann, MA Birch Machin and JL Rees, *J Invest Dermatol* 111(4) 709–710 (1998)
12. K Hemminki, VJ Bykov and JA Marcusson, *J National Cancer Institute* 91(23) 2046–2047 (1999)
13. H Mireles-Rocha, I Galindo, M Huerta, B Trujillo-Hernandez, A Elizalde and R Cortes-Franco, *Acta Derm Venereol* 82(1) 21–24 (2002)
14. L Eicker, V Kurten, S Wild, G Riss, R Goralczyk, J Krutmann and M Berneburg, *Photochem Photobiol* 2(6) 655–659 (2003)
15. KH Kraemer and JJ DiGiovanna, *J Am Acad Dermatol* 46(3) 463–466 (2002)
16. T Mammone, M Ingrassia, D Gan, K Marenus and D Maes, *Skin Pharmacol Appl Skin Physiol* 15(1) 26–34 (2002)

From the Sea: Algal Extracts for Skin Homeostasis

Diane Bilodeau and Isabelle Lacasse

Atrium Innovations Inc., Quebec City, Quebec, Canada

KEY WORDS: *algal extracts, skin barrier improvement, free radical protection, antiaging benefits, anti-inflammatory action*

ABSTRACT: *The authors discuss the abilities of marine-derived active ingredients to protect the skin from barrier disruption, aging, free radicals and inflammation. Four marine species are examined that provide such benefits via various sulfated polysaccharides and polyphenols.*

Homeostasis refers to the ability that all living organisms possess to achieve and maintain physiological stability while facing constant environmental changes. Being at the frontier of the internal and external milieus, the skin certainly is one of the most challenged organs. Desiccants, free radicals, inflammatory mediators or even the simple passage of time are major insults capable of disrupting skin homeostasis.

Supporting Homeostasis with Marine Extracts

A global strategy for skin care must protect the skin on multiple fronts. Therein, the marine biomass has various solutions to offer. For example, the brown seaweed, *Ascophyllum nodosum*, which typically is found in the cold marine waters of the Northern Hemisphere, can easily tolerate desiccation, temperature variations and high saline water. Desiccation tolerance in algae has been linked to

its capacity to increase cell wall thickness with emersion to air. As cell wall thickness increases, more polysaccharides become available for retaining water, thus decreasing the rate of desiccation.¹

Also of interest is the robust brown seaweed *Fucus serratus* that grows well on slow-draining shores where it has to protect itself from heavy wave action and sand abrasion. Not to be forgotten is the red seaweed *Palmaria palmata* found on moderately exposed rocky shores where it grows under the shade of other types of algae. *P. palmata* has developed pigments with antioxidant activity to better absorb light.²

Yet another promising biomass is *Enteromorpha compressa*, the marine green alga that grows abundantly in rock pools and sandy rocks. Being exposed to a high oxygen concentration in rock pools, *E. compressa* also has developed potent antioxidant mechanisms that may be linked to polycaccharides and polyphenol fractions.³

Each of these algae is rich in various and specific sulphated polysaccharides such as fucoidans and alginates (*A. nodosum* and *F. serratus*), carrageenans and galactans (*P. palmata*), and ulvans (*E. compressa*), that have found applications in the cosmetic industry. They also contain various polyphenols that may add to their benefits. To better characterize the cosmetic potential of these algal sources, a profile of their modulation of gene expression in skin fibroblasts and keratinocytes was established using cDNA microarray technology, as this paper describes, and the results were validated with corresponding functional assays performed on skin cells. The concurrent benefits of these ingredients were then assessed through consumer testing. Results shown here reveal that alone or in combination, these marine actives could be an answer for a global strategy to preserve skin homeostasis.

DNA Microarray: The Technique

DNA (gene) arrays are commonly used for monitoring expression levels of numerous genes simultaneously. The technique is particularly useful to decipher how cells react to a particular treatment and may give important clues as to the molecular mechanism of action of active ingredients in the cosmetic field. The protocol is fairly complex and is based on the specificity of DNA-RNA interactions. In cells,

messenger RNAs (mRNA) are molecules produced in the nucleus through transcription, as a reflection of gene activation. These mRNAs are then exported into the cytoplasm where they serve as blueprints to guide protein synthesis. The level of mRNA for a given protein varies with cellular activity.

To measure the effect of an active on the gene expression of skin cells, the mRNA content of treated cells is extracted and tagged with red fluorescence, then mixed with the mRNA content of control cells previously labeled with green fluorescence. Samples of this mixture of RNA transcripts are gently placed over an array of pre-identified microscopic DNA spots attached to a solid surface such as glass. These various DNA fragments are complementary (cDNAs) and can therefore bind, as Velcro bands^a, to their unique corresponding messenger mRNA. The end result fluorescence for each cDNA spot will obviously depend upon the ratio between red and green mRNA.

If a gene has been more activated in treated cells, the corresponding mRNA will be more abundant than in control cells, resulting in red fluorescence of the complementary cDNA spot on the array. On the opposite, gene silencing following treatment will result in green fluorescence. Should treatment have no impact on a specific gene, the corresponding spot on the array would appear as yellow, reflecting an equal amount of red and green mRNA transcripts.

***In vitro* Tests**

To characterize the skin's response to selected marine water extracts, a fibroblast suspension was prepared and integrated into a reconstructed epidermis model. Following incubation of the co-cultures for 24 hr at 37°C, in medium containing the extract in water only or without the test compound as the control, total RNA was extracted for each culture, purified and fluorescently labeled. Microarray hybridization was assayed using a confocal laser scanner to measure fluorescence intensities, allowing simultaneous determination of the relative expression levels of all the genes represented in the array. A selection of up to 164 genes pertinent to skin physiology was grouped by clusters of physiological interest on the mini chips.

^a Velcro is a product of Velcro Industries B.V.

F. serratus: The DNA array-based profiling of *F. serratus* extract points toward its role as a modulator of keratinocyte differentiation. In the presence of the extract^b alone, gene expression of cell cytokeratins 14 and 16 was up-regulated while that of cytokeratins 1 and 10 was down-regulated (see **Table 1**). This activity is in line with the stimulation of a pro-differentiation pathway.⁴

Table 1. Results obtained with *F. serratus* extract in the reconstructed epidermis cDNA microarray experiment

Gene	Effect of <i>F. serratus</i> extract		
	Up-regulation	Down-regulation	No effect
Antigen KI-67			X
Aquaporin 3	X		
β -defensin 4	X		
Corneodesmosin	X		
Cornulin	X		
Cytokeratin 1		X	
Cytokeratin 10		X	
Cytokeratin 14	X		
Cytokeratin 16	X		
Involucrin	X		
NICE-1	X		
RNAse 7	X		
Transglutaminase K	X		

Other markers of differentiation, including corneodesmosin, cornulin, involucrin, NICE-1 and especially transglutaminase K (TGK/TGase1), were also up-regulated. TGK is an enzyme that plays an important role in the formation of the cornified layer during the differentiation of epithelial keratinocytes.⁵ The lack of influence of the extract on the expression of the Ki-67 antigen, a proliferation marker, supports a rather selective effect in modulating epidermal differentiation for improved barrier function.⁶ In addition, β -defensin-4 and RNAase 7 expressions were also up-regulated, indicative of *F. serratus*' potential to provide antimicrobial resistance for additional skin protection.⁷

^b Homeo-Shield (INCI: *F. serratus* extract (and) glycerol) is a registered trademark of Atrium Innovations, Quebec City, Quebec, Canada.

The up-regulating effect of *F. serratus* extract on transglutaminase (TGK) gene expression has been validated at the protein level through a measure of TGK activity. Normal Human Epidermal Keratinocytes (NHEK) were incubated at 37°C for 72 hr with or without (control) the extract alone, at a concentration of 0.33%. The enzyme TGK was then extracted from cells and assayed by measuring the covalent addition of 3H-putrescin to casein. The casein was precipitated with trichloroacetic acid (TCA), the precipitates collected on filters, and the radioactivity measured by liquid scintillation.

Following *F. serratus* extract treatment, transglutaminase activity was significantly induced—5 x over control cells, $p < 0.01$ (see **Figure 1**). This result supports the cDNA microarray observations and demonstrates that *F. serratus* extract modulates the epithelial differentiation process.

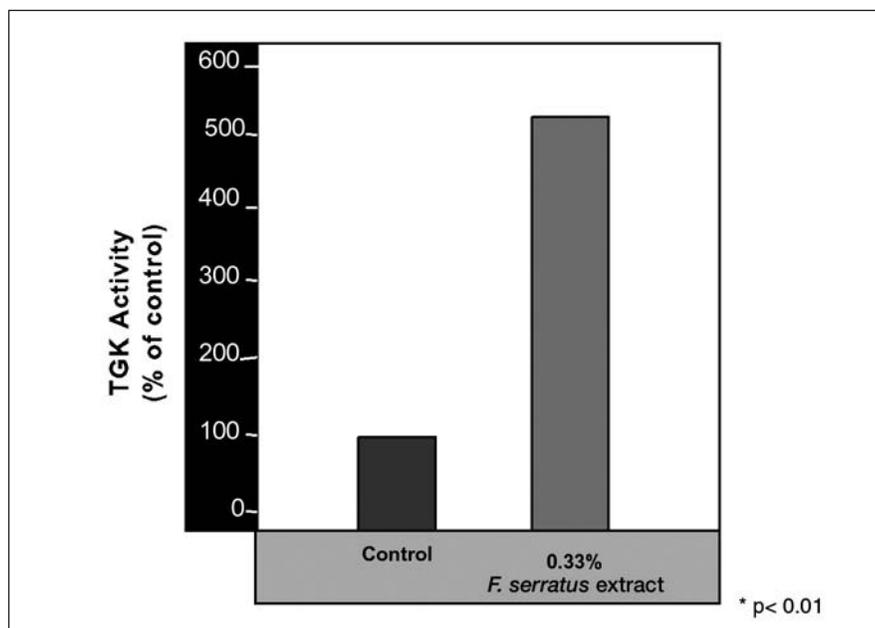


Figure 1. Effect of **F. serratus** extract on the enzymatic activity of transglutaminase K

An increase in transglutaminase activity by promoting maturation of the cornified envelope should improve skin barrier function.^{5,8} This suggests *F. serratus* extract would be beneficial in skin care formulas aimed at protecting from adverse environmental influences and transepidermal water loss (TEWL).

***E. compressa* and *P. palmata*:** A DNA array-based profile obtained on combined *E. compressa* and *P. palmate* water extracts^c demonstrated that this combination synergizes to switch on gene coding for various antioxidant enzymes in aged fibroblasts and reconstructed epidermis (see **Table 2**).

Both the green *E. compressa* and the red *P. palmata* are known to develop antioxidant strategies when challenged by higher level of stress, an activity that has been associated with the presence of polysaccharides and some phenolic compounds that may also be responsible for their remarkable color.^{2,3}

The gene expression of catalase, SOD 1 (cytosolic form), SOD 2 (mitochondrial form) and glutathione peroxidase was augmented in the presence of the combination of water extracts from *E. compressa* and *P. palmata*. Such a stimulation of the cellular antioxidant machinery would be expected to limit the destructive effects of reactive oxygen species (ROS).⁹ The reduced expression of oxidative stress-responsive 1 (OXSR1), a stress marker whose expression normally correlates with a high concentration of ROS, further validates the free radical scavenging ability of the ingredient.

The combination of *E. compressa* and *P. palmata* extracts acts at other levels of antioxidant protection as well by increasing the gene expression of heme-oxygenase 1 (HO-1) without affecting the constitutively expressed heme-oxygenase 2. HO-1 is part of an inducible protection system that primes skin defenses against UV-induced oxidative stress.¹⁰ Finally, the mixture's ability to induce thioredoxin could assist in protecting skin cells from UV-induced lipid peroxidation.¹¹ This effect on lipid peroxidation was further documented using fluorescence cytometry analysis.

Young and aged fibroblasts were both studied. It is known that cellular aging causes an increase in the endogenous formation of metabolically generated ROS, leading to significant lipid peroxidation.¹² Accordingly, the mixture^c was tested for its efficacy in reducing the level of lipid peroxidation occurring in aged fibroblasts. Aged cells were incubated at 37°C for 24 hr with or without (control) the test product at a concentration of 1% in water only. A probe specific for lipid peroxides was added to the culture media for 45 min and washed away. The test mixture was then reintroduced or not (control) in the culture media.

^c Homeoxy (INCI: Sorbitol (and) *E. compressa* extract (and) *P. palmata* extract (and) water (aqua)) is a registered trademark of Atrium Innovations, Quebec City, Quebec, Canada.

Table 2. Results obtained with *E. compressa* and *P. palmata* extracts on the expression of antioxidant enzymes in different skin cell systems analyzed in the described cDNA microarray experiment

Gene	Aged fibroblasts		Reconstructed epidermis*	
	Up-regulation	Down-regulation	No effect	Up-regulation
Catalase	X			
Glutaredoxin	X			
Glutathione peroxidase	X			
Glutathione reductase	X			
Heme oxygenase 1	X			X
Heme oxygenase 2			X	
Metal-regulatory transcription factor		X		
Oxidative stress response 1		X		
Peroxiredoxin 1	X			
SOD 1 (cytosolic)			X	X
SOD 2 (mitochondrial)	X			X
Thioredoxin		X		X
Thioredoxin reductase 1			X	

*Note: On reconstituted epidermis, up-regulation was the only observed effect

Following an additional incubation, cells were trypsinized and fluorescence measured by flow cytometry. The presence of oxidation is reflected by a decrease in the fluorescence of the probe. Results showed that the presence of the extracts significantly reduced lipid peroxidation in aged cells to a level similar to that of young cells; 41%, $p < 0.01$.

UV exposure also is known to trigger lipid peroxidation. Thus, using a similar protocol, researchers also investigated the action of the ingredient on aged fibroblasts exposed to UVA and UVB irradiation at 180 mJ/cm^2 . While UV exposure caused a further increase in the level of lipid peroxidation in aged fibroblasts, a protective effect was observed in the presence of the extracts; 32%, $p < 0.01$ (see **Figure 2**).

The *E. compressa* and *P. palmata* blend allowed aged skin cells to restore their innate free radical scavenging capacity to that of younger cells, therefore limiting membrane lipid peroxidation. These activities could reduce and prevent the signs of chronological as well as actinic aging in the skin.

A. nodosum on aging: cDNA array-based profiles differ between young and aged skin fibroblasts and this fact provides information on the effects of aging in skin cell biology. Not surprisingly, genes responsible for the synthesis of structural proteins such as α -smooth muscle actin, collagen 1 and 3, cytoplasmic β -actin, decorin and lamin A, as well as genes involved in cell adhesion such as integrin beta-1 and laminin beta-1, displayed reduced expression in aged cells in the microarray experiment.

On the other hand, expression of MMP3 gene was up-regulated in these cells. MMP3 activation has been associated with the breakdown of extracellular matrix components such as collagen fibers and fibronectin during the processes of chronological and actinic aging.¹³ Interestingly, incubating these aged cells in the presence of a depolymerized fucan fraction derived from *A. nodosum*^d reverses these deleterious effects of aging, re-establishing a younger pattern of gene expression (see **Table 3**).

The rejuvenating potential of the *A. nodosum* fraction was confirmed when aged cells were found to recover their lost responsiveness to growth factors such as epidermal growth factor (EGF)

while grown in the presence of the extract. EGF is naturally produced in the body where, among other things, it stimulates skin cell production, an effect that is diminished with aging.¹⁴ In a proliferation assay, EGF was used to stimulate cell turnover in young and aged fibroblasts.

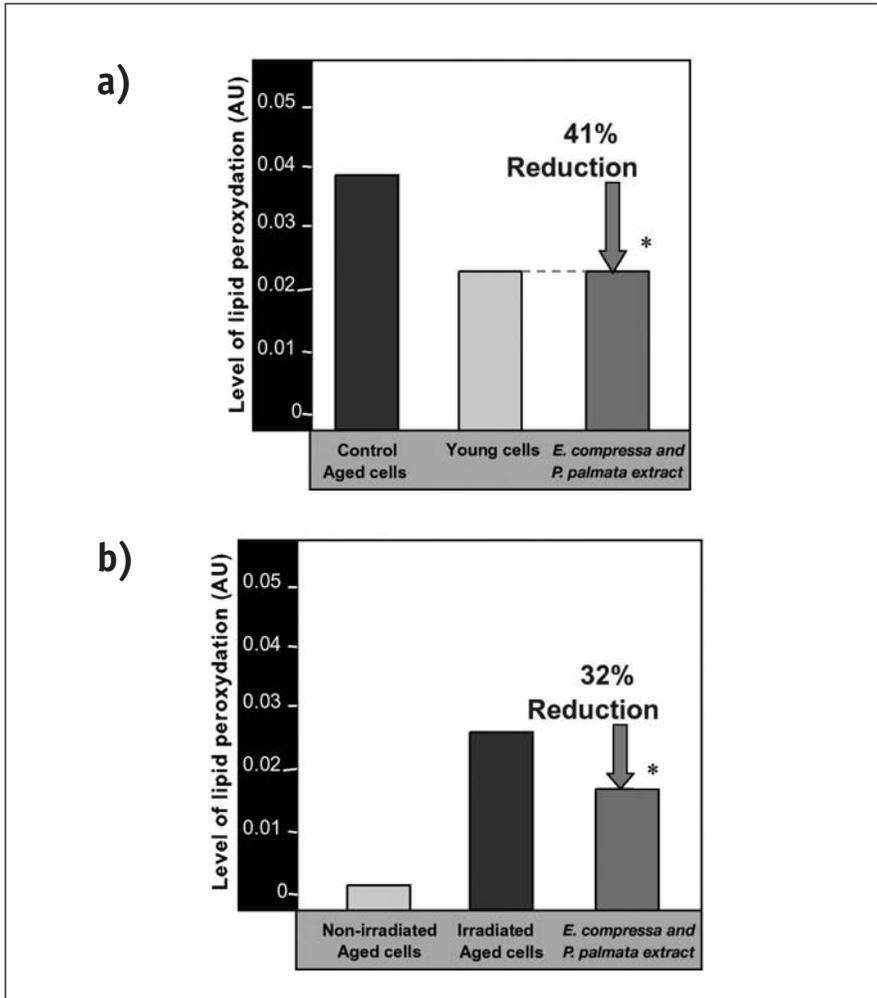


Figure 2. Effect of a combination of *E. compressa* and *P. palmata* extract on a) metabolically- and b) UV-induced lipid peroxidation

Aged and young cells were seeded at low density and incubated for 72 hr in the presence or not (control) of EGF, with and without (control) the tested 0.1% in water extract. Supernatants were removed, cells were rinsed and cell nuclei were stained by incubating

Table 3. Results obtained with *A. nodosum* fraction, enriched in depolymerized fucans, in an aged-cell cDNA microarray experiment

Gene	Effect of cellular aging ¹			Reversal effect of extract ²	
	Up-regulation	Down-regulation	No Effect	Up-regulation	Down-regulation
α -smooth muscle actin		X		X	
Collagen 1 α -1		X		X	
Collagen 3 α -1		X		X	
Cytoplasmic β -actin		X		X	
Decorin		X		X	
Glutathione reductase		X		X	
HSP 27		X		X	
Integrin β -1		X		X	
Lamin A		X		X	
Laminin β -1		X		X	
MMP3	X				X
Plasminogen activator inhibitor 1			X	X	

¹ Effect of aging on gene expression: Aging mainly translates into a down-regulation of mRNA transcript levels of genes coding for important functional and structural proteins

² Effect of *A. nodosum* fraction, enriched in depolymerized fucans, on the reversion of aging-induced modulation of gene expression

with Hoechst dye solution. After extensive washing, analysis of the labeling by nuclei count was performed with a cell analyzer.

As expected, and contrary to young cells, aged cells initially did not respond to the presence of the growth factor; however, addition of an extract derived from *A. nodosum*^d restored the responsiveness of aged cells to EGF, as revealed by an increase in proliferation capacity (see **Figure 3**).

The proliferation assay results obtained with *A. nodosum* are in line with the cDNA profiling and support a rejuvenating potential for this active; it may help to restore the integrity of the extracellular matrix, especially at the dermal-epidermal junction, and it may also promote optimal cell turnover and increase the rate of skin renewal to a level normally associated with younger age.

A. nodosum on inflammation: A different fraction^e of *A. nodosum*, this time enriched in nondepolymerized fucans, was shown to reduce inflammatory activity.

To reproduce inflammatory conditions, keratinocytes were stimulated with phorbol 12-myristate 13-acetate (PMA). In response to such stress, cells normally release prostaglandin E2 (PGE₂), a lipid involved in inflammatory reactions.¹¹ However, when keratinocytes were stressed with PMA (0.1 µg/mL) in the presence of the active water extract at 0.6%, PGE₂ production was reduced by 60%, $p < 0.01$ (see **Figure 4**). PGE₂ production was measured from the culture supernatant using an ELISA detection system.

In accordance with this result, the active derived from a fraction of *A. nodosum* enriched in nondepolymerized fucans could be used to protect the skin from harmful inflammatory responses that may result from environmental insults such as chemical or UV exposure.

In vivo Tests

The compatibility and complementarity of the described ingredients in combination^f were evaluated using consumer assessments. Such

^d Homeo-Age (INCI: *A. nodosum* extract (and) sorbitol (and) water (aqua)) is a registered trademark of Atrium Innovations, Quebec City, Quebec, Canada.

^e Homeo-Soothe (INCI: *A. nodosum* extract (and) sorbitol (and) water (aqua)) is a registered trademark of Atrium Innovations, Quebec City, Quebec, Canada.

^f The Homeosta-SEA line of ingredients is a registered trademark of Atrium Innovations, Quebec City, Quebec, Canada.

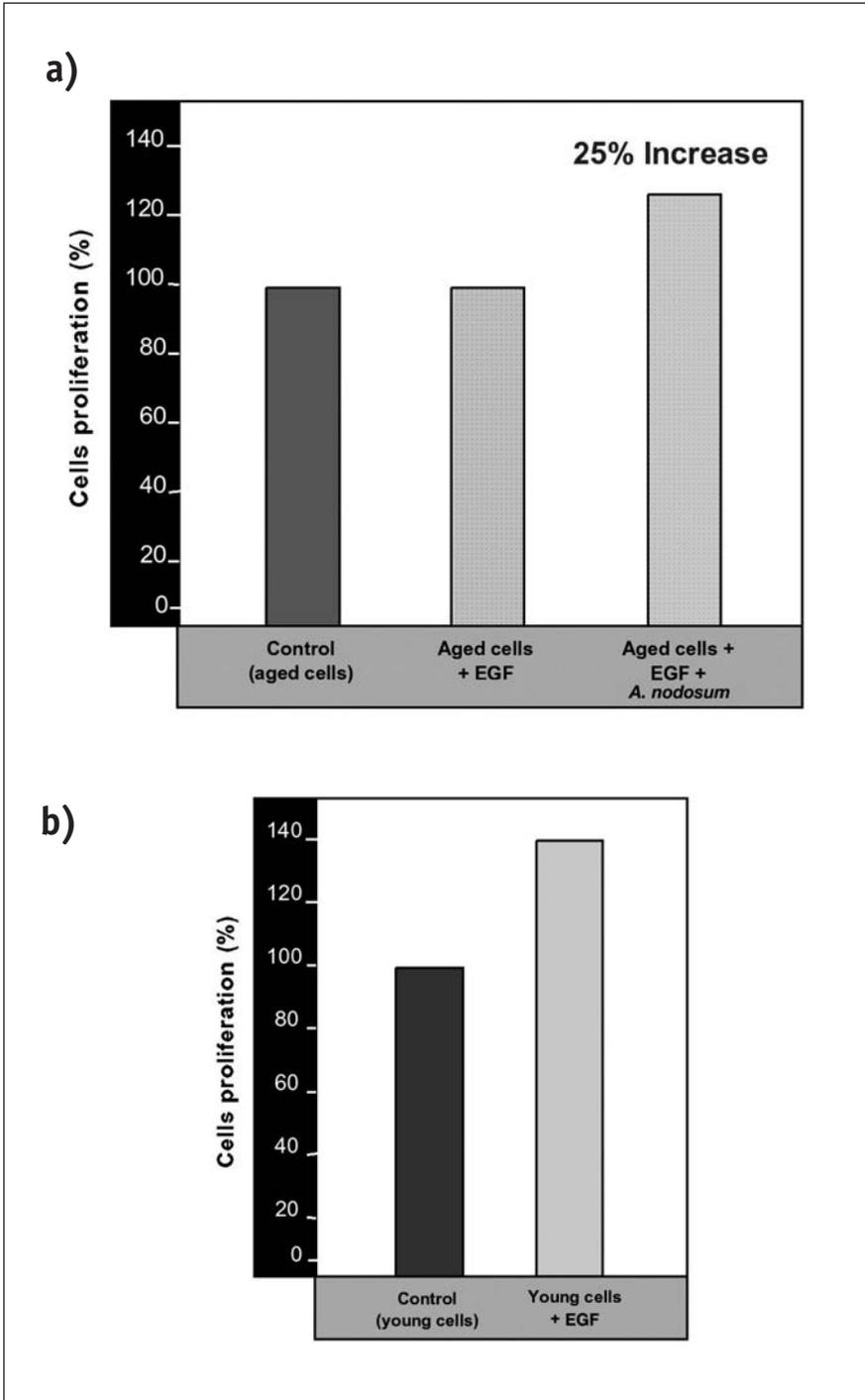


Figure 3. Effects of *A. nodosum* extract on cellular growth in a) aged cells, compared with b) a young cell control and young cells treated with only EGF

tests make it possible to directly identify and evaluate the benefits generated to the end users.

For the first consumer test, a panel of 105 women aged 40–75 years was asked to apply a daily regimen consisting of two different cosmetic formulations for a period of 28 days. The tested day cream contained 0.5% nondepolymerized *A. nodosum* plus 0.5% of the combined *E. compressa* and *P. palmata* extract (**Formula 1**), while the night cream contained 0.5% depolymerized *A. nodosum* plus 1% *F. serratus* extract (**Formula 2**).

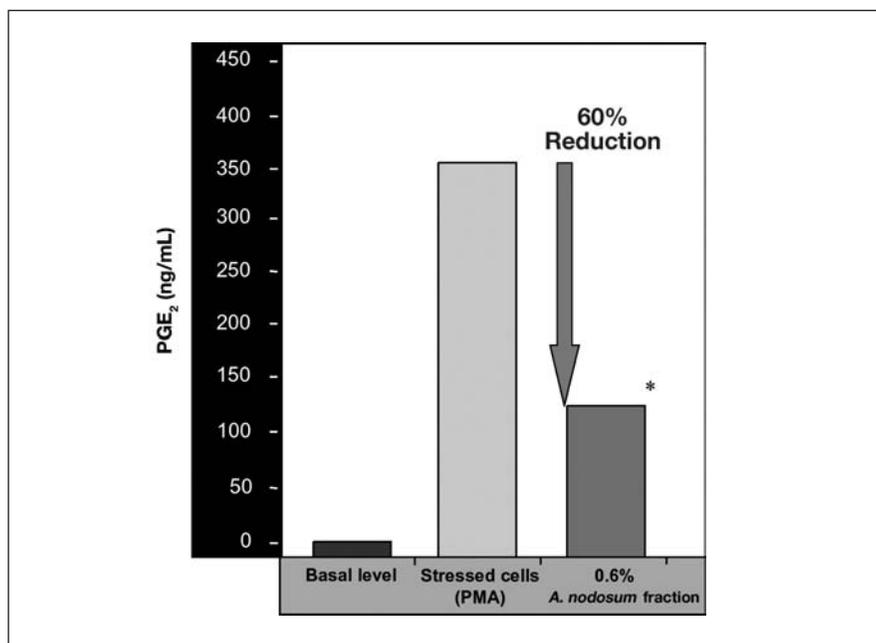


Figure 4. Effect of *A. nodosum* fraction, enriched in nondepolymerized fucans, on stress-induced production of PGE₂.

Subjects were asked to evaluate the benefits of the cream formulations with respect to defined criteria linked to the appearance of signs of aging such as wrinkles, skin tone and skin firmness. The rationale supporting potential antiaging effects for these specific cream formulations relied on the presence of ingredients with anti-irritant and antioxidant activities—i.e., *F. serratus* extract plus *E. compressa* and *P. palmata* extract, respectively—in the day cream, and ingredients with metabolic-enhancing and skin barrier replenishing activities—depolymerized *A. nodosum* and *F. serratus* extract,

respectively—in the night cream. The regimen performance is summarized in **Figure 5**. An overall satisfaction of 84% was reported after 28 days and this particular regimen was reported to reduce the appearance of wrinkles, improve skin texture and complexion, and to protect the skin from dehydration.

Formula 1. Antiaging day cream

A. Cetearyl glucoside (and) cetearyl alcohol	1.5% w/w
Glyceryl stearate	1.0
Sodium cetearyl sulfate	0.3
Squalene	1.0
Dicaprylyl carbonate	2.0
Dicaprylyl ether	2.0
<i>Paraffinum liquidum</i> (mineral) oil	5.0
Dimethicone	1.0
Sodium polyacrylate	1.0
B. Glycerin	3.0
Water (<i>aqua</i>)	qs to 100.00
Preservative	0.7
C. <i>A. nodosum</i> extract (and) sorbitol (and) water (<i>aqua</i>) (Homeo-Soothe, Atrium)	0.5
Sorbitol (and) <i>E. compressa</i> extract (and) <i>P. palmata</i> extract (and) water (<i>aqua</i>) (Homeoxy, Atrium)	0.5

Procedure: Heat A and B to 80°C. Add B to A and slowly cool to 60°C. Homogenize until sodium polyacrylate is dispersed and begin cooling to ambient temperature. Add C and mix until homogeneous. Viscosity: 41,000cps; Brookfield RVF#6–10 rpm 25°C.

In a second consumer test, the soothing effect of a different regimen was self-assessed by subjects presenting a general sensitive skin as evaluated by a dermatologist. This test cream incorporated 0.5% nondepolymerized *A. nodosum* extract, and 0.5% of the combined *E. compressa* and *P. palmata* extract plus 0.5% *F. serratus* extract (**Formula 3**).

The rationale behind testing this formulation was that sensitive skin expresses pro-irritant, pro-oxidant and defective barrier

components.¹⁶ A panel of 102 women with sensitive skin, aged 18–50 years, used the test cream formulation twice daily for a period of 28 days. Results were reported as a self-assessment of the formulation's efficacy toward signs of skin sensitivity such as tingling sensations being reduced, soothing and calming effects (see **Figure 6**). Results showed that 85% of the consumers were satisfied with the soothing cream after 28 days of use, with reports that skin better resisted aggressions and protected sensitive skin from inflammatory reactions.

Formula 2. Antiaging night cream

A. Cetearyl glucoside (and) cetearyl alcohol	1.5% w/w
Glyceryl stearate	1.0
Sodium cetearyl sulfate	0.3
Squalene	1.0
Dicaprylyl carbonate	2.0
Dicaprylyl ether	2.0
<i>Paraffinum liquidum</i> (mineral) oil	5.0
Dimethicone	1.0
Sodium polyacrylate	1.0
B. Glycerin	3.0
Water (<i>aqua</i>)	qs to 100.00
Preservative	0.7
C. <i>A. nodosum</i> extract (and) sorbitol (and) water (<i>aqua</i>) (Homeo-Age, Atrium)	0.5
<i>F. serratus</i> extract (and) glycerol (Homeo-Shield, Atrium)	0.5

Procedure: Heat A and B to 80°C. Add B to A and slowly cool to 60°C. Homogenize until sodium polyacrylate is dispersed and begin cooling to ambient temperature. Add C and mix until homogeneous. Viscosity: 44,500cps; Brookfield RVF#6–10 rpm 25°C.

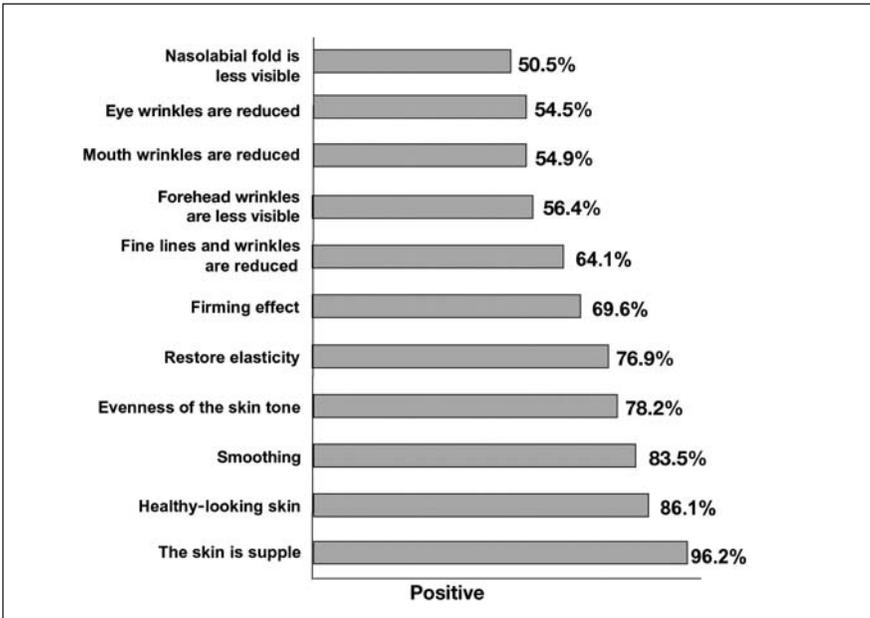


Figure 5. Self-appreciation of a twice daily application of the described algae extract regimen cream formulations on the appearance of signs of aging

Formula 3. Soothing cream

A. Cetearyl glucoside (and) cetearyl alcohol	1.5% w/w
Glyceryl stearate	1.0
Sodium cetearyl sulfate	0.3
Squalene	1.0
Dicaprylyl carbonate	2.0
Dicaprylyl ether	2.0
<i>Paraffinum liquidum</i> (mineral) oil	5.0
Dimethicone	1.0
Sodium polyacrylate	1.0
B. Glycerin	3.0
Water (<i>aqua</i>)	qs to 100.00
Preservative	0.7
C. <i>A. nodosum</i> extract (and) sorbitol (and) water (<i>aqua</i>) (Homeo-Sooth, Atrium)	0.5
<i>F. serratus</i> extract (and) glycerol (Homeo-Shield, Atrium)	0.25
Sorbitol (and) <i>E. compressa</i> extract (and) <i>P. palmata</i> extract (and) water (<i>aqua</i>) (Homeoxy, Atrium)	0.5

Procedure: Heat A and B to 80°C. Add B to A and slowly cool to 60° C. Homogenize until sodium polyacrylate is dispersed and begin cooling to ambient temperature. Add C and mix until homogeneous. Viscosity: 36,500cps; Brookfield RVF#6–10 rpm 25°C.

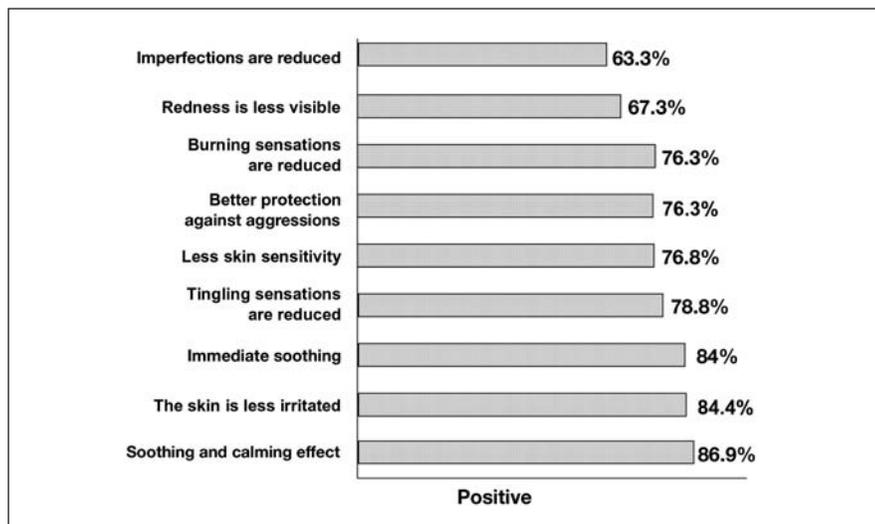


Figure 6. Self-appreciation of a twice daily application of an algae extract regimen cream formulation on sensitive skin parameters

Conclusion

Each ingredient from the discussed line of algal extracts shows a biological activity profile addressing at least one major threat to skin homeostasis. *F. serratus* extract, for example, could be used to protect the epidermal barrier; *E. compressa* and *P. palmata* extracts exhibited free radical scavenging affects; and the profiles of a depolymerized fucan fraction of *A. nodosum*, as well as a different fraction of *A. nodosum*, are indicative of reversing the signs of aging and controlling pro-inflammatory mediators in the skin, respectively. The specificity and complementarity of these ingredients suggest their use alone or in combination to aid skin health and appearance. The proposed line of algal extracts represents an armamentarium for the formulation chemist wishing to develop finished products endowed with defined biological mechanisms of action while respecting the delicate skin homeostasis.

Published April 2008 *Cosmetics & Toiletries* magazine.

References

1. AD Boney, *Biology of marine algae*, Hutchinson Educational Ltd, London (1969)
2. YV Yuan, MF Carrington and NA Walsh, Extracts from dulse (*P. palmata*) are effective antioxidants and inhibitors of cell proliferation *in vitro*, *Food Chem Toxicol* 43(7) 1073–81 (2005)

3. AD Ansell, RN Gibson and M Barnes, Oceanography and marine biology, an annual review, *UCL Press*, London (1998)
4. C Jacques, AM de Aquino and M Ramos-e-Silva, Cytokeratins and dermatology, *Skinmed* 4(6) 354–60 (2005)
5. K Hitomi, Transglutaminases in skin epidermis, *Eur J Dermatol* 15(5) 313–9 (2005)
6. T Scholzen and J Gerdes, The Ki-67 protein: From the known and the unknown, *J Cell Physiol* 182(3) 311–22 (2000)
7. F Niyonsaba and H Ogawa, Protective roles of the skin against infection: Implication of naturally occurring human antimicrobial agents beta-defensins, cathelicidin LL-37 and lysozyme, *J Dermatol Sci* 40(3) 157–68 (2005)
8. T Hirao, A novel non-invasive evaluation method of cornified envelope maturation in the stratum corneum provides a new insight for skin care cosmetics, *IFSCC Magazine* 6(2) 103–109 (2003)
9. DR Bickers and M Athar, Oxidative stress in the pathogenesis of skin disease, *J Invest Dermatol* 126(12) 2565–75 (2006)
10. M Allanson and VE Reeve, Immunoprotective UVA (320–400 nm) irradiation up-regulates heme oxygenase-1 in the dermis and epidermis of hairless mouse skin, *J Invest Dermatol* 122 1030–1036 (2004)
11. Y Nishinaka, H Nakamura, H Masutani and J Yodoi, Redox control of cellular function by thioredoxin: A new therapeutic direction in host defense, *Arch Immunol Ther Exp (Warsz)* 49(4) 285–92 (2001)
12. J Vinson and S Anamandla, Comparative topical absorption and antioxidant effectiveness of two forms of coenzyme q10 after a single dose and after long-term supplementation in the skin of young and middle-aged subjects, *IFSCC Magazine* 8(4) 287–92 (2005)
13. P Brenneisen, H Sies and K Scharffetter-Kochanek, UVB irradiation and matrix metalloproteinases: From induction via signaling to initial events, *Ann N Y Acad Sci* 973:31–43 (2002)
14. KT Tran, SD Rusu, L Satish and A Wells, Aging-related attenuation of EGF receptor signaling is mediated in part by increased protein tyrosine phosphatase activity, *Exp Cell Res* 289(2) 359–67 (2003)
15. JN Lawrence, JJP Dally and DJ Benford, Measurement of eicosanoid release in keratinocyte cultures to investigate skin irritation and tumour promoting activity, *Toxicol in vitro* 9(3) 285–290 (1995)
16. M Leibold and LG Herrmann, Impaired skin barrier function in dermatologic disease and repair with moisturization, *Cutis* 76(6 Suppl) 7–12 (2005)

Measuring the Antioxidant Potential of an Açai Extract

Karina Coyado Bispo

Beraca, São Paulo, Brazil

KEY WORDS: *açai, total antioxidant status, antiaging, free radicals, testing*

ABSTRACT: *The antioxidant potential of cosmetic materials can be evaluated by several methodologies, including a commercial kit that measures total antioxidant status, as illustrated here with a commercial extract from the fruit of the açai, a Brazilian palm tree. Applications in antiaging products are suggested.*

One of the challenges in developing antiaging products is to inhibit the oxidative damages caused by free radicals. Free radicals can damage cellular genetic material, causing changes in the cell's proliferative and biosynthetic capacity and resulting in aging of the organism.¹⁻³ Free radicals can be inhibited by molecules that present antioxidant activity. These molecules have reducing activity; they are able to donate electrons to the free radical in a way that neutralizes its unpaired electron, eliminating its reactivity.

The development of new cosmetic products presenting antioxidant activity will be essential for the fight against aging. Today, the most frequently used antioxidants are ascorbic acid (vitamin C) and α -tocopherol (vitamin E). However, exploration of Earth's vegetal biodiversity is uncovering new plant materials. It is of great impor-

tance not only to find new materials with anti-oxidant potential but also to use a standard methodology to determine how much potential each of those materials has for use in the fight against aging.

This chapter discusses one of those methodologies—called Total Antioxidant Status or TAS—and illustrates its use in evaluating the antioxidant potential of an extract from the fruit of a Brazilian palm tree—the açai.

Açai's Antioxidant Phytochemicals

The açai palm tree, one of several species of the genus *Euterpe*, grows in northern Brazil, where it is known by the native people as *içá-çai*, which means “fruit that cries.” The name is pronounced ah-SAH-ee. The vernacular name is also sometimes spelled *assai palm* in English. The berries are harvested as a food that has been enjoyed for decades by the people of Brazil. A bowl of açai is usually eaten in the morning with breakfast.

The dark purple or green pigmentation of açai has led to several experimental studies of its anthocyanins, a group of polyphenols that give the deep red or purple color to berries, other fruits and vegetables that are high in antioxidant value. Some studies have shown that açai has 10 times more antioxidants than red grapes, whose red pigment gives red wine its antioxidant value.

Antioxidant Activity Evaluation

Method: Different methods exist to measure the antioxidant activity of a product, which is also sometimes referred to as the product's total antioxidant potential or total antioxidant capacity. For this study the chosen method was Total Antioxidant Status (TAS).

Free radicals form in the human body during normal cellular metabolism and following exposure to UV light, gamma radiation, cigarette smoke and other environmental pollutants. However, the body has an antioxidant system capable of removing free radicals and protecting the body from destructive molecules such as H_2O_2 , $RO\cdot$, $ROO\cdot$ and $O_2\cdot$ radicals. This system has three main groups of antioxidants—the primary, secondary and tertiary defenses. The TAS test measures the total antioxidant effect of these three defense systems: ⁴

- Primary antioxidants include superoxide dismutase (SOD), glutathione peroxidase, and metal-binding proteins such as ferritin or ceruloplasmin. These antioxidants work by preventing the formation of new free radical species.
- Secondary antioxidants include vitamins E and C, β -carotene, uric acid, bilirubin and albumin, among others. Secondary antioxidants trap radicals, thereby preventing chain reactions.
- Tertiary antioxidants, such as DNA repair enzymes, work by repairing biomolecules damaged by free radicals.

For this study, TAS was measured using a commercial kit^a that uses an assay based on the radical cation 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and the reaction between a peroxidase (metmyoglobin) and hydrogen peroxide. The reaction produces free radicals that normally oxidize the ABTS ions; i.e., the free radical takes an electron from the ion. However, if an antioxidant is present, the antioxidant will supply the electrons, thereby inhibiting the oxidation of ABTS. This TAS assay measures the ability of a sample to inhibit ABTS oxidation.

The ABTS oxidation is monitored through a spectrophotometer using a wavelength of 600 nm. A significant decrease in the production of oxidized ABTS in the presence of the sample indicates the presence of antioxidant substances.

The test has an internal standard that is run together with each sample TAS determination. The standard is 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a molecule that has antioxidant activity and that is a positive control to the test.

The reaction mixture for TAS determination is placed in tubes. Each experimental unit (controls, samples, blanks and its replicates) is run in different tubes. A control without the sample is used to determine the the maximum ABTS oxidation. The sample is added to other tubes also determining the quantity of oxidated ABTS.

Material: The objective of this study was to determine the TAS of a particular commercial açai extract^b, referred to here simply as

^a Randox Total Antioxidant Status Kit, a product of Randox Laboratories Ltd., Crumlin, County Antrim, UK

^b Biofunctional Açai Extract Organic—Açai Extract Organic (INCI: Glycerin (and) water (aqua) (and) Euterpe oleracea pulp oil) is a product of Beraca.

the tested açai extract. This extract is produced using the açai dried pulp, vegetable glycerin and water. The active ingredient—anthocyanins—is standardized.

Procedure: The kit uses only 20 μL of sample for TAS determination. In the present test, the sample was tested diluted in the following concentrations: 1, 2, 5 and 10% by volume. It was tested diluted because interferences were noted when the pure sample was tested.

Calculating TAS: The kit gives absorbance that results from ABTS oxidation after the reaction. This absorbance is determined using a spectrophotometer. The presence of antioxidant molecules results in a decrease in the resulting absorbance. The TAS value can be calculated after first calculating a factor based on the absorbances of the reaction mixture before (A_2), and after (A_1) the reaction, as indicated in the following equations:

$$\text{Factor} = C_s / (\Delta A_{\text{blank}} - \Delta A_{\text{standard}}),$$

where:

C_s = Concentration of the standard

$\Delta A = A_2 - A_1$ (for blank, sample and standard).

Then the TAS value can be calculated as follows:

$\text{TAS} = \text{Factor} \times (\Delta A_{\text{blank}} - \Delta A_{\text{sample}})$ TAS is expressed in units of mmol/L of antioxidant activity. This unit is an equivalent unit of the antioxidant activity of the 6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid standard of the test.

The TAS value was plotted as a function of different sample concentrations, and the value for the pure sample was calculated using the equation obtained from the linear equation fitted to the plotted curve. The equation was fitted using statistical software^c. The equation was as follows:

$$\text{TAS} = a \times \text{Sample Concentration} + b$$

where TAS is in units of mM, a is the slope of the curve, and b is the intercept on the Y axis.

After a and b were determined, the TAS of an undiluted sample was calculated using the same equation and a sample concentration of 100% by volume. Because of interferences obtained when the pure sample was tested, this extrapolation method was used to estimate the pure sample's TAS value.

^c Sigma Plot for Windows, version 9.01, is a product of Systat Inc.

A statistic test was applied only to determine if the slope of the fitted linear equation of TAS versus sample concentration was different from zero. A p value less than 0.05 suggests a confidence level of 95%. In this case, the slope of the fitted curve differed from zero with a confidence level of 95%, thus confirming that the sample has antioxidant activity. In other words, one can be relatively certain that the sample has some antioxidant activity because the slope is not zero, meaning that TAS increased when the sample concentration was increased.

Results and discussion: The açai extract was tested to determine its antioxidant activity at several concentrations. The results were graphed (**Figure 1**). From the plotted curve it was possible to determine the following first-degree equation:

$$\text{TAS} = a \times \text{Sample Concentration} + b,$$

where a is (0.279 ± 0.006) and b is (0.15 ± 0.03) .

The correlation coefficient (r) was 0.9995, showing a strong correlation between TAS and the sample concentration.

Using this equation it was possible to determine the TAS in the undiluted sample was 26.8 ± 0.6 mM/L. It would be interesting to compare this value to TAS values for more widely used antioxidants, but the test reported here was not performed with a comparative purpose. It was performed only to determine the absolute TAS for this açai sample.

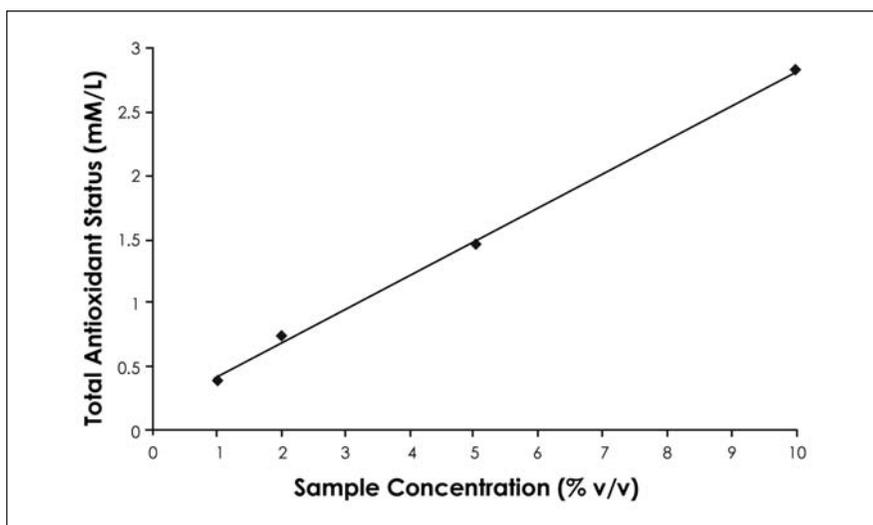


Figure 1. Total antioxidant status of the tested açai extract at selected concentrations

It is important to remember that TAS has a unit expressed as an equivalent antioxidant activity of the molecule 6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid. This antioxidant is not as common as vitamin C or α -tocopherol. At present, the author does not have information about the correlation of the antioxidant activity of 6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid and other antioxidants. For this correlation, further tests or bibliographic research will be necessary.

Conclusion

This commercial açai extract^a, tested with a particular assay kit^b, showed a total antioxidant status of 26.8 ± 0.6 mM/L.

Published August 2008 *Cosmetics & Toiletries* magazine.

References

1. APM Loureiro, PD Mascio and MHG Medeiros, Formação de adutos exocíclicos com bases de dna: implicações em mutagênese e carcinogênese, *Quim Nova* 25(5) 777–793 (2002)
2. T Lu, Y Pan, SY Kao, C Li, I Kohane, J Chan and BA Yankner, Gene regulation and DNA damage in the ageing human brain, *Nature* 429 883–891 (2004)
3. T Finkel and NJ Holbrook, Oxidants, oxidative stress and the biology of ageing, *Nature* 408 239–247 (2000)
4. King James Medical Laboratory Web site, available at: www.kingjamesomegatech-lab.com/total.htm (Accessed Jun 25, 2008)

Protecting Skin from UV Oxidative Stress with a New (Cys-Gly)₂ Dimer Peptide

A. Plantivaux

Sophia Antipolis University, Nice, France

E. Bauza, T. Marchand, C. Dal Farra and N. Domloge

Vincience Research Center, Sophia Antipolis, France

KEY WORDS: *UV, antioxidant, SOD, catalase, protein carbonylation, lipid peroxidation*

ABSTRACT: *In the presented studies, researchers investigated the effect of the (Cys-Gly)₂ dimer peptide against UV-induced oxidative stress on cultured human fibroblasts. The dimer peptide was found to act on a range of mechanisms involved in cell defense against such stress.*

UVA exposure is a main cause of oxidative stress in skin. It damages proteins via cross-linking, carbonyl formation and denaturation; lipids through peroxidation; and DNA via reactive oxygen species, which in turn may cause promutagenic DNA lesions and DNA single-strand breaks. In skin, protection from oxidative stress is ensured by enzymic and nonenzymic antioxidants.^{1,2} Superoxide dismutase (SOD) enzymes are known to destroy superoxide radicals. SOD exists in humans in two forms: CuZn SOD and Mn SOD.³

Following the SOD defense mechanism is the action of catalase—a ubiquitous protein found primarily in cellular peroxisomes. Catalase destroys the hydrogen peroxide formed by SOD and by

other processes. Catalase increasingly has become recognized as an enzyme centrally involved in protecting the skin from UV oxidative stress.⁴ Recent data has demonstrated a significant decline of SOD and catalase activity in photoaged skin.⁵

After a large screening study, the dimeric cysteYL-glycin dipeptide, or (Cys-Gly)₂ dimer peptide, which can be obtained either by synthesis or extracted from plants, was chosen to conduct studies because it provided the best protection against oxidative stress. The presented studies investigated the effect of the (Cys-Gly)₂ dimer peptide against UV-induced oxidative stress on cultured human fibroblasts. For this purpose, the expression of SOD and catalase after UVA or UVB stress was studied, and the outcome on protein carbonylation and lipid peroxidation was evaluated.

Materials and Methods

Cell culture: NHDF human neonatal skin fibroblasts^a were cultured in Dulbecco's Modified Eagle Medium (DMEM) at 1 g/L glucose and supplemented with 10% fetal calf serum (FCS), 1% glutamine^a and a 0.2% antibiotic^b at 37°C, 5% CO₂.

Cell treatment, UV irradiation: At 70% of confluence, cells were treated with 1% of the dimer peptide 24 h prior to and during UV irradiation^c.

After irradiation, another application of the dimer peptide at 1% was administered to cells in three sets of samples. These were placed in an incubator for 30 min, 4 h and 24 h, respectively. For sample preparation for assays of SOD activity, catalase activity and protein carbonylation, fibroblasts were rinsed and lysed with 200 µL of the following buffer: 0.5M phosphate buffer, 0.1M aprotinin, 0.1M alpha-toluenesulfonyl fluoride (phenylmethylsulphonyl fluoride or PMSF), and 0.2% surfactant^d at pH 7. Three times, the samples were frozen in liquid nitrogen and thawed at 37°C to extract cell proteins. Protein concentration was determined by the bicinchoninic acid assay method (BCA) method^e (see **BCA Assay**).

^a Testing materials were obtained from Cambrex, Verviers, Belgium.

^b Primocin is a product of Invivogen, San Diego, CA, USA.

^c Vilber Lourmat VL-215M lamp is a product of Fischer Bioblock, Illkirch, France.

^d Triton X-100 is a product of Sigma, Saint Louis, MO, USA.

^e The BCA method was adopted from Pierce, Brebriere, France.

BCA Assay

The bicinchoninic acid assay (BCA assay) is a biochemical assay for determining the total level of protein in a solution. The total protein concentration is exhibited by a color change of the sample solution from green to purple in proportion to protein concentration, which then can be measured using colorimetric techniques. The BCA assay relies on two reactions: First, the peptide bonds in protein reduce Cu²⁺ ions from the cupric sulfate to Cu⁺. The amount of Cu²⁺ reduced is proportional to the amount of protein present in the solution. Next, two molecules of bicinchoninic acid chelate with each Cu⁺ ion, forming a purple-colored product that absorbs light at a wavelength of 562 nm. The amount of protein present in a solution can be quantified by measuring the absorption spectra and comparing it with protein solutions of known concentrations.

—Source: Wikipedia website; Available at: http://en.wikipedia.org/wiki/Bicinchoninic_acid_assay.
(Accessed Nov 10, 2006.)

SOD assays: 15 μg of cytosolic proteins were separated by electrophoresis at 80 V and 4°C on an 8% nondenaturing polyacrylamide gel in standard tris-glycine buffer. A modified photochemical method of Beauchamp and Fridovich (1971) using nitro-blue tetrazolium was used to locate SOD activity on the gels.⁶ The gel was quantified using a quantification program^f.

Catalase studies: Catalase activity was assayed according to a method adapted from Beers and Sizer⁷ by monitoring the initial disappearance of H₂O₂ at 240 nm on a recording spectrophotometer. Catalase activity was calculated in μmol/min/g as follows:

$$\text{Catalase Activity} = \frac{(A_{240}/\text{min} \times 107)}{(43.6 \times \text{mg of protein/mL})} \quad \text{Eq.1}$$

Protein carbonylation studies: Protein carbonylation was assessed using the Enzyme-Linked ImmunoSorbent Assay (ELISA) technique.⁸ Proteic samples were derivatized with 2,4-dinitrophenylhydrazine (DNP). DNP then was revealed with anti-DNP antibodies diluted at 1:5,000 and streptavidin-HRP complex at

^f Alpha-imager V-5.5 program was applied from Alpha Innotech.

1:3,000. Tetramethylbenzidine peroxidase substrate was added, and absorbance was read at 490 nm using a microplate reader. In order to convert the absorbances in carbonyl concentrations, a standard curve was constructed using varying proportions (0-100%) of oxidized BSA with fully reduced BSA. The oxidized BSA solution at 4 g/L was prepared using the BCA method.

Lipid peroxidation: After irradiation, cells were rinsed and lysed with 50 μ L of the following buffer: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 0.5M EDTA, 0.2% surfactant^d, 1 mg/mL aprotinin and 0.1 M PMSF. Proteins were extracted using liquid nitrogen, and samples were centrifuged at 10,000 rpm. The supernatant was harvested for protein determination by the BCA method and for lipid peroxidation detection. Lipid peroxidation was determined by the measurement of thiobarbituric acid reactive substances (TBARS), according to Janero et al.⁹ The resulting pink supernatant was measured in a spectrophotometer at wavelength 532 nm.

Comet assay protocol (single-cell gel electrophoresis): In brief, slides were prepared with a thin layer of agarose and coverslips were quickly pressed. Cells were irradiated, harvested, added to low-melting-point agarose and exposed to alkaline lysis solution for 1 h. Following this, cell lysate was subjected to a DNA-unwinding solution for approximately 20 min and then to electrophoresis for 10 min. After electrophoresis, the slides were stained with propidium iodide^g for 1 min.

A total of 50 randomly captured comets from each slide were examined blindly at 400X magnification using a microscope connected through a camera to an image analysis system. Undamaged cells have an intact nucleus without a tail and damaged cells have the appearance of a comet. To quantify the DNA damage, tail moment was evaluated. Tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail. The comet tail moment is positively correlated with the level of DNA breakage in a cell. The mean value of the tail moment in a particular sample was taken as an index of DNA damage in this sample.

⁹ Testing materials were obtained from Molecular Probes, Eugene, OR, USA.

Results and Discussion

SOD gels showed that UVA stress decreased Mn SOD activity compared with the control. Interestingly, dimer peptide administration to the cells, under UVA stress conditions, enhanced and restored Mn SOD activity up to that of the control (see **Figure 1**).

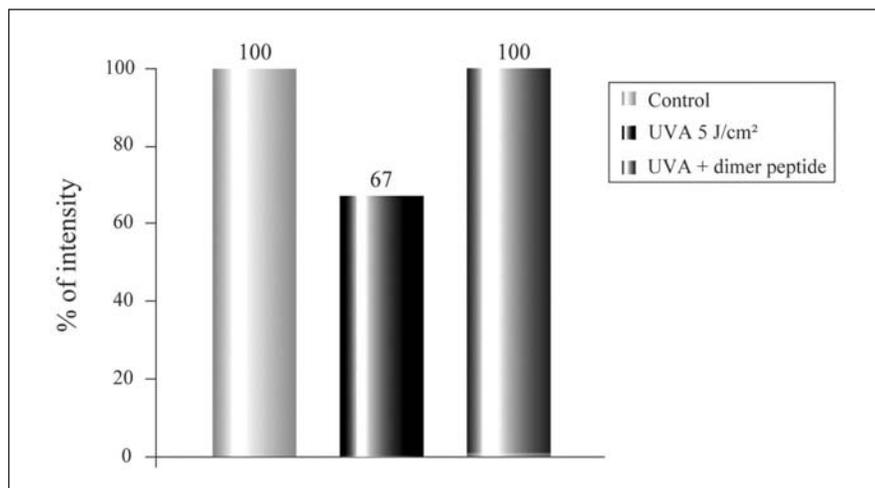


Figure 1. Quantitative evaluation of Mn SOD activity, 4 h after UVA irradiation

In the absence of UVA stress, the application of the dimer peptide at 1% for 24 h did not substantially modulate cell SOD level. In the same manner, the level of CuZn SOD expression exhibited similar but more moderate enhancement. The application of dimer peptide 24 h prior to and during UVB exposure also was found to enhance Cu/Zn SOD expression in UVB-stressed cells, compared with the untreated control cells (data not shown).

In parallel studies, catalase activity was assessed by spectrophotometer measurements, which showed that the addition of dimer peptide to cells enhanced catalase activity in the treated cells (data not shown). Catalase assessment 4 h after exposure to three doses of UVA (1, 5 and 9 J/cm²) showed that when cells were treated with dimer peptide 24 h prior to UVA, the treated cells exhibited a higher level of catalase activity in response to UVA stress (see **Figure 2**).

When cells are irradiated with UVA, the resulting oxidative stress attacks the cells and increases protein carbonylation, which damages cell structure and causes cell functions to deteriorate. Interestingly,

a significant decrease in protein carbonylation was observed in cells treated with dimer peptide 24 h prior to UVA irradiation (see **Figure 3**).

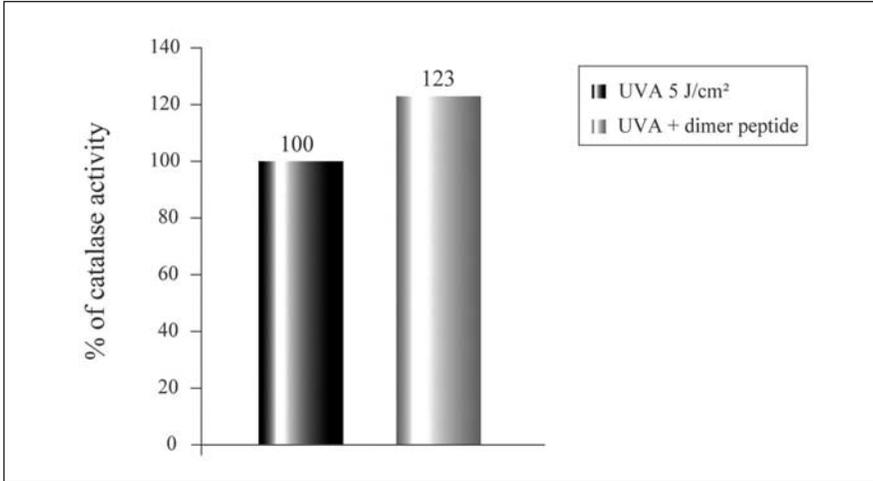


Figure 2. Catalase activity in control and dimer peptide-treated cells, 4 h after UVA irradiation

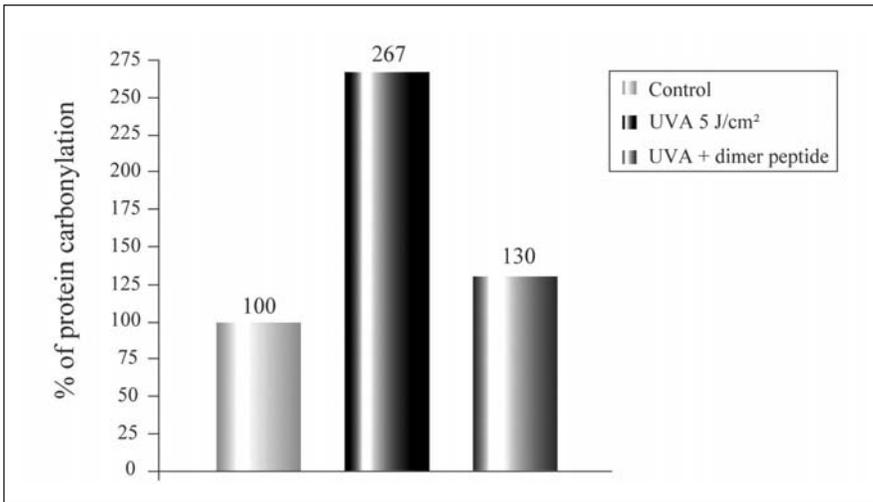


Figure 3. Protein carbonylation in control and dimer peptide-treated cells, 4 h after UVA irradiation

Further studies showed that the addition of dimer peptide to cells reduced their level of protein carbonylation. Parallel studies with UVB stress confirmed dimer peptide’s effect on reducing carbonylation of proteins (data not shown). These findings provide further confirmation of the protective effect of the peptide.

Studies of lipid peroxidation showed that dimer peptide exhibited a protective effect on cell structure and function by decreasing lipid peroxidation. Cell irradiation with 5 J/cm² of UVA increased oxidative stress-induced lipid peroxidation and caused cell damage. Interestingly, irradiated and treated cells with dimer peptide were protected significantly from oxidative stress and exhibited a level of lipid peroxidation that was much lower and close to their nonirradiated, peptide-treated level (see **Figure 4**).

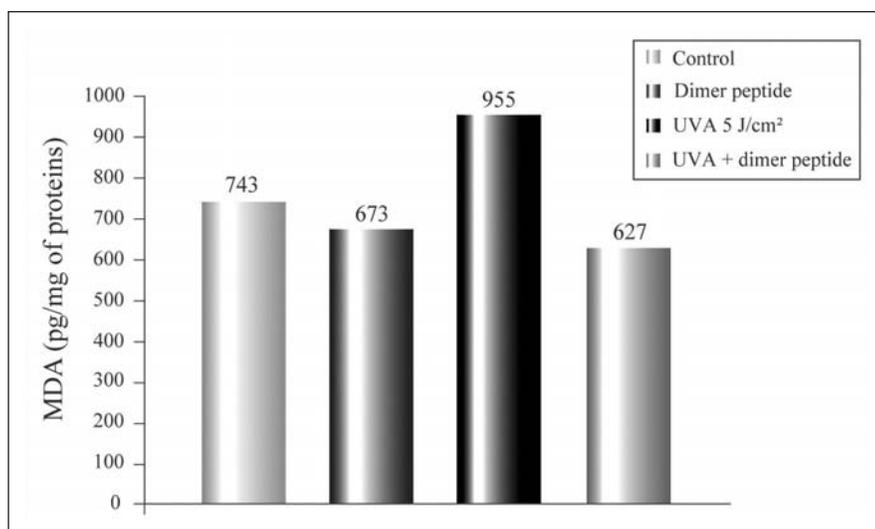


Figure 4. Lipid peroxidation in control and dimer peptide-treated cells, 24 h after UVA irradiation

The comet assay demonstrated the protective effect of the dimer peptide on DNA and revealed a remarkable decrease (73%) of DNA damage in dimer peptide-treated irradiated cells.

Conclusion

Taken together, the results of these studies demonstrate that the synthetic dimer peptide possesses a significant antioxidant effect that appears to operate on the succession of the cell's antioxidant defense mechanisms, thereby offering a genuine enhancement in physiological protection. Consequently, these studies strongly suggest that dimer peptide could be of great use in skin care and antiaging products.

References

1. Y Shindo et al, Enzymic and non-enzymic antioxidants in epidermis and dermis of human skin, *J Invest Dermatol* 102 122–124 (1994)
2. MT Leccia et al, Solar simulated irradiation modulates gene expression and activity of antioxidant enzymes in cultured human dermal fibroblasts, *Exp Dermatol* 10 272–279 (2001)
3. C Carraro et al, Characterization of superoxide dismutase from mammalian skin epidermis, *J Invest Dermatol* 90(1) 31–36 (1988)
4. DP Steenvoorden and GM Van Henegouwen, The use of endogenous antioxidants to improve photoprotection, *J Photochem Photobiol B* 41 1–10 (1997)
5. F Afaq and H Mukhtar, Effects of solar radiation on cutaneous detoxification pathways, *J Photochem Photobiol B-Biol* 63 61–69 (2001)
6. C Beauchamp and I Fridovich, Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels, *Anal Biochem* 44 276–287 (1971)
7. RF Beers and IW Sizer, A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase, *J Biol Chem* 195 133–140 (1952)
8. H Buss et al, Protein carbonyl measurement by a sensitive ELISA method, *Free Radic Biol Med* 23(3) 361–366 (1997). *Erratum in: Free Radic Biol Med* 24(7–8) 1352 (1998)
9. DR Janero, B Burghardt, Thiobarbituric acid-reactive malondialdehyde formation during superoxide-dependent, iron-catalyzed lipid peroxidation: Influence of peroxidation conditions, *Lipids* 24(2)125–131 (1989)

An Aquaporin-inspired Lipid Concentrate for Mature Skin

Mike Farwick, Betty Santonnat and Peter Lersch, Kees Korevaar, Anthony V. Rawlings, Susanne Grether-Beck, Kathrin Medve-Koenigs and Jean Krutmann

EVONIK Goldschmidt GmbH, Essen, Germany, EVONIK Cosmoferm B.V., Delft, Netherlands, AVR Consulting Ltd., Cheshire, England, University Duesseldorf GmbH, Duesseldorf, Germany

KEY WORDS: *stratum corneum, ceramides, SAXD, RT-PCR, mature skin*

ABSTRACT: *The authors describe a novel multi-lamellar concentrate based on ceramide technology and newly identified cell-signalling molecules. This skin-identical blend provides skin protection benefits and is shown to increase molecular markers for water management and barrier components that decline during aging, thus improving skin barrier function, moisturization and elasticity.*

Dry, thin and sagging skin is among the most common complaints of women over the age of 50. This is due to reduced protective, preventative and regenerative aspects of aged skin. Aged skin is manifested by reduced stratum corneum (SC) moisturization, and although transepidermal water loss (TEWL) is known to be normal or improved with age, the epidermal barrier repair capacity after removing the superficial layers of the barrier by tape stripping is significantly impaired.¹

Electron microscopy studies have shown a decreased size and number of keratohyalin granules (KHGs), the repository of pro-flaggrin in the keratinocytes, in aged skin. In this respect, reduced

SC natural moisturizing factors (NMF) have been observed due to reduced amounts of profilaggrin-rich KHGs.² Equally, abnormal intercellular lipid lamellae occur in aged skin¹, accompanied by a reduction in the levels of SC ceramides and especially ceramide EOS-linoleate.³ This is a result of the reduced lipid synthetic capacity of the epidermis that occurs during aging.

Further epidermal changes that occur with aging include the premature expression of involucrin⁴ and the decline in transglutaminase-1 and filaggrin levels.⁵ These changes can impact SC formation and maturation.

An age-related decline in the activity of the rate-limiting enzymes for ceramide, cholesterol and fatty acid synthesis has been reported; namely serine palmitoyltransferase, hydroxy-methyl-glutarycoenzyme-A reductase, and acetylcoenzyme-A carboxylase.⁶ All of these proteins participate in the production of a fully functional SC, but one family of proteins, the aquaporins, that forms channels to facilitate the transport of water across membranes has become of significant interest for its role in epidermal water maintenance.

Aquaporin-3 (AQP3) has been a particular focus because it is an aquaglyceroporin—i.e., it can co-transport glycerol, and its absence results in skin dryness, reduced SC hydration and elasticity, and delayed barrier recovery.⁷ Dumas et al.⁸ have reported an age-related

decline in AQP3 expression that further manifests itself in photo-damaged skin. Thus, a defective osmotic equilibrium could occur in the epidermis and account for the skin dryness observed in older subjects.

Brandner et al.⁹ also have discussed the importance of claudin-1 for the paracellular permeability of epidermal tight junctions.

Clearly, significant epidermal changes occur in aging skin that are responsible for its reduced

Ceramide Key

Significant epidermal changes occur throughout skin aging.

To build an accurate model accounting for these variances, researchers evaluated a lipid mixture containing ceramides in combinations, as described here.

S = sphingosine

P = phytosphingosine

E = esterified fatty acid

O = omega hydroxyfatty acid

N = normal fatty acid

A = alphahydroxy fatty acid

protective, repair and regeneration capacity. The purpose of the presented work was to evaluate a lipid mixture containing the ceramides EOS, EOP, NP, NS and AP cholesterol (see **Ceramide Key**) and behenic acid, together with caprooyl-phytosphingosine and caprooyl-sphingosine.

The first group of lipids were chosen for known SC lipid lamellar-forming and skin protective characteristics¹⁰, whereas the latter were chosen for their effects as epidermal cell signalling molecules^{11,12} that aid epidermal repair and regeneration.

Materials and Methods

Human SC lipids were isolated, as previously described.¹³ An equimolar mixture of synthetic, nonanimal derived cholesterol, behenic acid and a unique combination of well-defined, synthetic, human skin-identical ceramides produced using biotechnology—namely sphingosine-derived CER EOS and CER NS, and phytosphingosine-containing CER EOP, CER NP and CER AP, was prepared. This lipid mixture was then used in small angle X-ray diffraction studies.

A combination of caprooyl-phytosphingosine and caprooyl-sphingosine was added to the described mixture and all lipids were finally preformulated in a multi-lamellar system designed to prevent crystallization to maximize efficacy. This blend^a was then tested in clinical studies at a 5% use level.

In Vitro Small Angle X-ray Diffraction Studies

Measurements were taken at the European Synchrotron Radiation Facility (ESRF, Genoble) as described.¹³ Small angle X-ray diffraction (SAXD) provides information about the supramolecular organization of the barrier lipid molecules in multiple lamellae, consisting of a broad-narrow-broad sequence of electron lucent bands. The long periodicity phase (LPP) with a repeat distance of 12–13 nm is unique for the SC. It consists of two bilayers with a crystalline

^a Skinmimics (INCI: cetareth-25 (and) glycerin (and) cetyl alcohol (and) behenic acid (and) cholesterol (and) ceramide EOP (and) ceramide EOS (and) ceramide NP (and) ceramide NS (and) ceramide AP (and) caprooyl-phytosphingosine (and) caprooyl-sphingosine) is a product of Evonik Goldschmidt GmbH, Essen, Germany.

structure of 5–6 nm each called the short periodicity phase (SPP), separated by a narrow central lipid layer with fluid domains.¹⁵

Skin Bioengineering and Skin Biopsy Clinical Design

The described studies comply with the World Medical Association's Declaration of Helsinki (2000) concerning biomedical research involving human subjects. The efficacy study was performed *in vivo* on 10 healthy volunteers with dry skin, five male and five females, in the age range of 35–65 years. All measurements were performed by the same investigator in an air-conditioned room (room temperature 18–22°C; air humidity ~30–50%). The material^a under evaluation was topically applied daily, 2 mg/cm² of the skin surface, to both the volar forearm and buttock over a period of four weeks by a dermatologist; the contra-lateral areas were left untreated. Skin bioengineering parameters described below were measured on the volar forearm skin whereas at the end of the study, 4-mm punch biopsies were taken for the epidermal molecular analysis.

Determination of Molecular Markers by Real Time PCR

Total RNA was isolated and analyzed as described previously.¹³ Briefly, after reverse transcription with randomw hexamers, the PCR reactions were carried out on a monitor^b using the SYBR Green method. Each sample was analyzed in duplicate and 18S rRNA was used as internal standard. For comparison of relative expression in real time PCR control cells and treated cells, the $2^{-\Delta\Delta C(T)}$ method was used.¹⁴

Skin Bioengineering Tests

TEWL was measured^c, SC moisturization was determined^d quantitated as changes in electrical capacitance in arbitrary units (AU), and skin viscoelasticity was evaluated^e over the four-week study.

^b The Opticon 1 monitor is a device from MJ Research, Waltham, MA, USA.

^c The Tewameter TM300 is a device from Courage and Khazaka, Cologne, Germany.

^d The Corneometer CM825 is a device of Courage and Khazaka.

^e The Cutometer MPA 580 is a device of Courage and Khazaka.

Statistical Analysis

A paired Student's t-test was performed to determine statistical differences between the data obtained from treated and untreated areas; the significance was set at the $p < 0.05$.

Results

SAXD provides information on the periodicity of the SC lamellar lipid phase. The long periodicity phase of approximately 12 nm and the short periodicity phase at approximately 6 nm can be observed in both the natural human SC lipid mixture and the synthetic SC lipid mixture (see **Figures 1a** and **1b**), indicating that this lipid mixture can mimic the lamellar ordering effects of SC lipids.

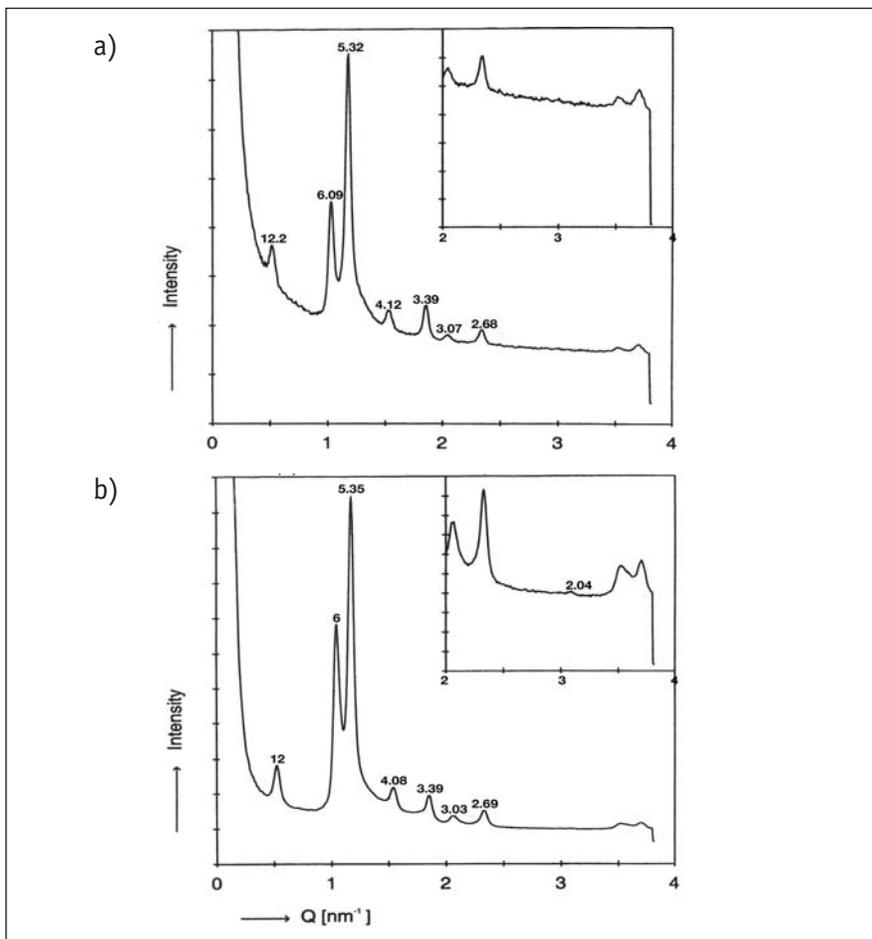


Figure 1. SAXD of a) human stratum corneum lipids and b) the synthetic lipid mixture used in this study

The molecular ratios of the ceramides, nonanimal cholesterol and fatty acid were optimized in such a way that this mixture, even with the limited number of ceramides, closely resembles lamellar and lateral SC lipid organization, delivering the components necessary for formation of a skin-identical lipid barrier.

The effects of topical application of the product were assessed in an *in vivo* study covering both molecular and classical skin parameter readouts. mRNA for protein markers of epidermal differentiation—i.e., involucrin, transglutaminase-1, filaggrin and loricrin; rate-limiting steps of ceramide biosynthesis including serine palmitoyl transferase subunits 1 and 2, and ceramide glucose transferase-1; and epidermal water maintenance involving aquaporin-3 and claudin-1 were assessed on treated versus untreated buttock skin. All keratinocyte markers were found to be increased in the treated buttock versus the untreated control. These effects were specifically pronounced in the volunteer subgroup over the age of 50 (see **Figure 2**).

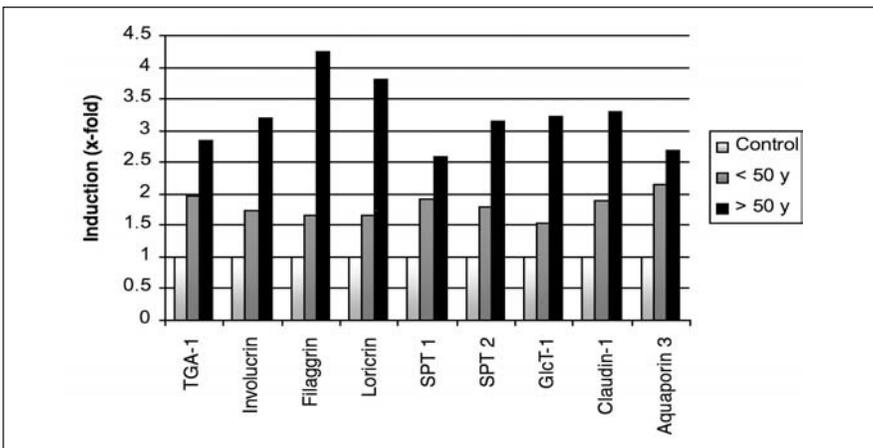


Figure 2. Effect of the topical application of the product on various keratinocyte markers

In the bioengineering study, skin barrier function (**Figure 3a**), skin hydration (**Figure 3b**) and skin elasticity (**Figure 3c**) were significantly improved after four weeks of topical treatment with the o/w cream containing the tested mixture.

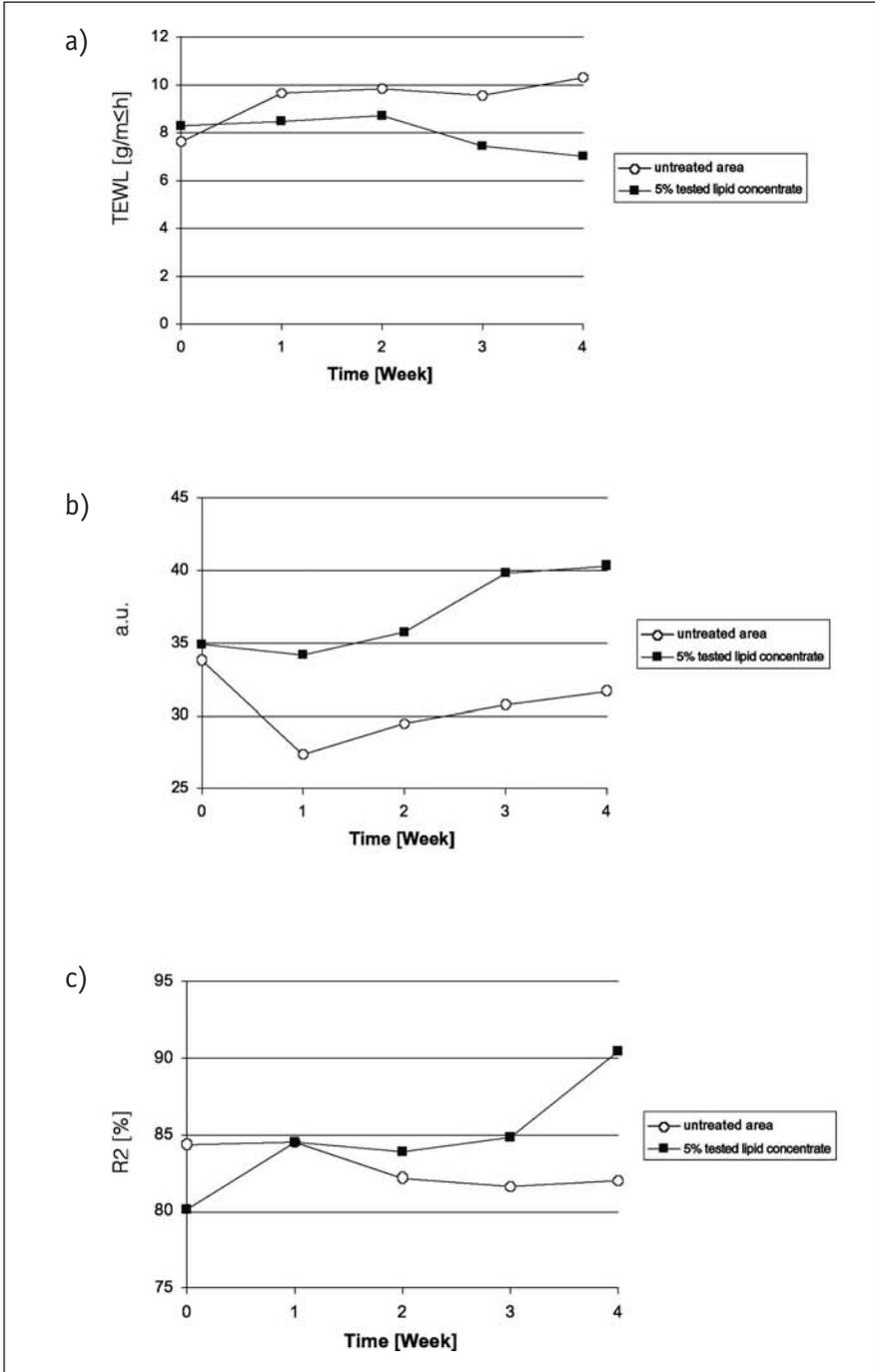


Figure 3. In the bioengineering study, a) skin barrier function, b) skin hydration and c) skin elasticity were significantly improved after four weeks of topical treatment with the o/w cream containing the tested mixture.

Conclusions

The SAXD diffraction work described in this study has demonstrated that a mixture of CER EOS, EOP, NS, NP and AP, together with nonanimal-derived cholesterol and behenic acid, can mimic the lamellar ordering of human SC lipids known to be the most favorable conformation for skin protection. As the number of ceramides decline with aging, this lipid mixture could complement naturally occurring ceramides in skin. Non-natural ceramides have previously been shown to disrupt the lipid matrix.¹¹

Many epidermal proteins are reduced or aberrantly expressed in skin aging. The lipid mixtures used in this study have been shown to increase mRNA for structural epidermal proteins, epidermal synthesis enzymes and proteins involved in epidermal water maintenance—i.e., filaggrin claudin-1 and AQP3. This is due to the cell signalling molecules used in the lipid mixture. These molecules have previously been shown to improve keratinocyte differentiation,^{12,13} however, this is the first study demonstrating not only improvements in gene-induction of these proteins *in vivo* but also the gene-induction of AQP3 in humans *in vivo*. The results point out also that all markers observed were significantly upregulated, and in addition, a higher response for volunteers over age 50 was obtained for all of them.

Over the four-week study period, topical application of the lipid-containing o/w cream resulted in significant improvement of skin physiological parameters such as TEWL, SC hydration and skin elasticity. This is a result of the improvements in epidermal differentiation induced by the cell-signalling molecules and the skin barrier enhancing effects of the long chain ceramides varieties included, normally found in the SC.

This combination of lipids in a multi-lamellar system provides skin protection and improves preventative and regenerative aspects of mature skin, allowing formulators to produce products that target different aspects of the management of water in aged skin; i.e., rebuilding the SC barrier, increasing the presence of epidermal tight junction molecules, improving water flux into the keratinocytes through the aquaporin system and stimulating the synthesis of the NMF precursor protein profilaggrin.

References

1. JT Reed, PM Elias and R Ghadially, Integrity and permeability barrier function of photoaged human epidermis, *Arch Dermatol* 133(3) 395–6 (1997)
2. E Proksch, Dryness in chronologically and photoaged skin, ch 11 in *Dry Skin and Moisturizers—Chemistry and Function*, M Loden and HI Maibach, eds, CRC Press: Boca Raton, FL 117–126 (2006)
3. J Rogers et al, Stratum corneum lipids: The effect of aging and the seasons, *Arch Dermatol Res* 288(12) 765–70 (1996)
4. M Engelke et al, Effects of xerosis and aging on epidermal proliferation and differentiation, *Br J Dermatol* 137(2) 219–25 (1997).
5. JI Contet-Audonneau, J Jeanmaire and G Pauly, A histological study of human wrinkle structure: Comparison between sun-exposed areas of the face with or without wrinkles and sun protected areas, *Br J Dermatol* 140(6) 1038–47 (1999)
6. R Ghadially et al, Decreased epidermal lipid synthesis accounts for altered barrier function in aged mice, *J Invest Dermatol* 106(5) 1064–9 (1996)
7. M Hara, T Ma and AS Verkman, Selectively reduced glycerol in skin of aquaporin-3 deficient mice may account for impaired skin hydration, elasticity and barrier recovery, *J Biol Chem* 277(48) 46616–21 (2002)
8. M Dumas et al, Hydrating skin by stimulating biosynthesis of aquaporins, *J Drugs Dermatol* 6 (6suppl) s20–24 (2007)
9. J Brandner et al, Tight junction proteins in the skin, *Skin Pharmacol Physiol* 19(2) 71–77 (2006)
10. U Wollenweber et al, Application of a skin identical lipid concentrate for enhanced skin moisturization and protection, *SOFW* 130(9) 2–7 (2004)
11. S Pillai et al, Synergy between vitamin D precursor 25-hydroxyvitamin D and short chain ceramides on keratinocyte proliferation and differentiation, *J Invest Dermatol Symp Proc* 1(1) 39–43 (1996)
12. P Lersch et al, Topical application of a sphingokine-lipid-mixture: Improvement of skin performance in humans addressed at a physiological and molecular level, *IFSCC Congress* (2006)
13. M de Jager et al, Acylceramide headgroup architecture affects lipid organization in synthetic ceramide mixtures, *J Invest Dermatol* 123(5) 911–916 (2004)
14. KJ Livak and TD Schmittgen, Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method, *Methods* 25 402–408 (2001)
15. JA Bouwstra, FER Dubbelaar, GS Gooris and M Poncet, The lipid organization in the skin barrier, *Acta Derm Venereol* suppl. 208 23–30 (2000)

Watering Holes in the Stratum Corneum

Bud Brewster

Cosmetics & Toiletries *magazine*

KEY WORDS: *stratum corneum, water, cells, keratin, lipids*

ABSTRACT: *This chapter investigates the connection between desmosomes and moisturization of the stratum corneum (SC). Can this be done by adding water?*

The skin has its own efficient mechanisms and locations for storing water in the stratum corneum (SC). A well-hydrated SC is essential for shedding cells and preventing thickened, dry, rough, scaly skin. But what if the hydration system breaks down? How can the cosmetic chemist help?

One way is to encourage the storage of water in the SC. Can you find four places to store water in **Figure 1**? The figure illustrates the cells of the SC and the lipid bilayers that surround them. The SC is the outermost layer of the epidermis. Its function is to provide a physical barrier between the interior body and the exterior environment.

The cells in this barrier are called corneocytes. They were living cells, with a nucleus and DNA, when they were formed lower in the epidermis, but they mature and lose their vitality and experience other changes as they move up from the epidermis and through the SC on their way to being shed at the skin surface. In the SC they are flat, hexagonal-shaped cells filled with water-retaining keratin proteins surrounded by a protein envelope and lipids. The corneocytes are stacked 10–30 layers high and connected to each other by protein bridges called desmosomes. Stacked bilayers of lipids surround the cells in the extracellular space. The resulting structure is the natural

physical and water-retaining barrier of the skin. One writer called it “a wall of hydrophilic protein bricks (the corneal cells) sealed by a hydrophobic ceramide lipidic cement.”²

The lipids include cholesterol, free fatty acids and sphingolipids. One of the sphingolipids is ceramide, which plays a major role in generating the stacked bilayers of lipids. These lipid structures trap water molecules in their hydrophilic region.

The NMF shown in **Figure 1** is a complex mixture of low-molecular weight, water-soluble compounds. This mixture results from the changes that occur as corneocytes move upward through the stack and enzymes break down the link between two proteins called keratin and filaggrin. If the SC has insufficient water content, proteolytic enzymes are activated and they degrade the filaggrin into individual amino acids. These free amino acids, along with other physiological chemicals such as lactic acid, urea and salts, are called a *natural moisturizing factor* (NMF). This breakdown of filaggrin to amino acids happens only when the skin is dry. Its purpose is to control the osmotic pressure of the skin and the amount of water it holds. NMF components also are found outside of the corneocyte; among these components are sugars, minerals, and amino acids from desmosome breakdown. The components of the NMF are hygroscopic; they hold water to rehydrate the SC and keep it flexible.

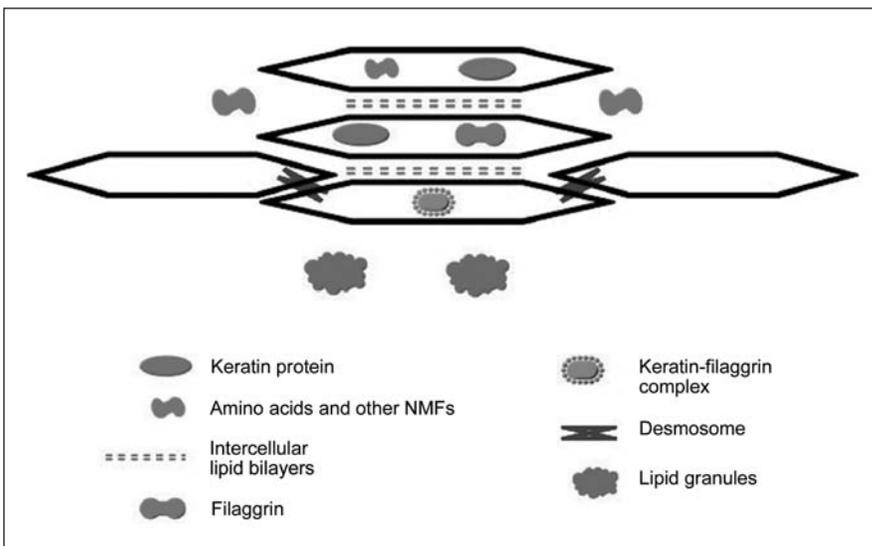


Figure 1. The corneocyte layer of the stratum corneum (Adapted from Reference 1)

The Multifunctional Stratum Corneum

The SC has long been known for its protective function: to retard the diffusion of substances into and out of the skin. Writing in *Dry Skin and Moisturizers: Chemistry and Function*, Albert Kligman notes these complementary functions revealed in recent research:⁴

- Regulation of homeostatic mechanisms
- Mechanisms of repair after disruption of the barrier by disease or exogenous insults
- A depot for antibacterial substances to protect against disease
- Pro-inflammatory cytokines to aid in adaptive reactions against external threats
- A semiotic signaling device for sensing changes that are then transmitted to the tissue below
- A depot for storage of lipophilic drugs such as corticosteroids.

Finally, for enzyme activity to occur there must be water in the intercellular spaces of the SC. According to one writer, this water usually can be seen around degrading desmosomes called lacunae.³

This brief description of water-holding activity in the SC is drawn from the 2001 Marino report, which itself is drawn largely from *Dry Skin and Moisturizers: Chemistry and Function*, edited by Howard I. Maibach and Marie Lodén, and published a year earlier. In the introduction, Albert Kligman writes that recent research and new technologies show that mechanisms other than moisturization are at work in the SC, and some are probably more influential (see **The Multifunctional Stratum Corneum**).⁴ But this column will focus on moisturization mechanisms and efforts by cosmetic chemists to support them.

Writing in *Dermatologic Therapy* in 2004, Anthony V. Rawlings and Clive R. Harding assert that maintenance of an optimal level of hydration by the SC is dependent largely on several factors: the intercellular lamellar lipids that provide an effective barrier to the passage of water through the tissue; the diffusion path length, a tortuous path water must travel among the SC layers; and the water-binding effect of the NMF. “Each maturation step leading to the formation of an effective moisture barrier—including corneocyte

strengthening, lipid processing, and NMF generation—is influenced by the level of SC hydration,” according to Rawlings and Harding.⁵

Lipid Bilayers

The lipid bilayers of the SC hold water (**Figure 2**) and surround corneocytes to provide the permeability barrier. Among the lipids available in the skin, the ceramides are particularly well-suited to generating the stacked lipid structures. Ceramides and other lipids are produced naturally in the granular layer of the epidermis below the SC, but cosmetic chemists have found ways to supply additional ceramides when necessary.

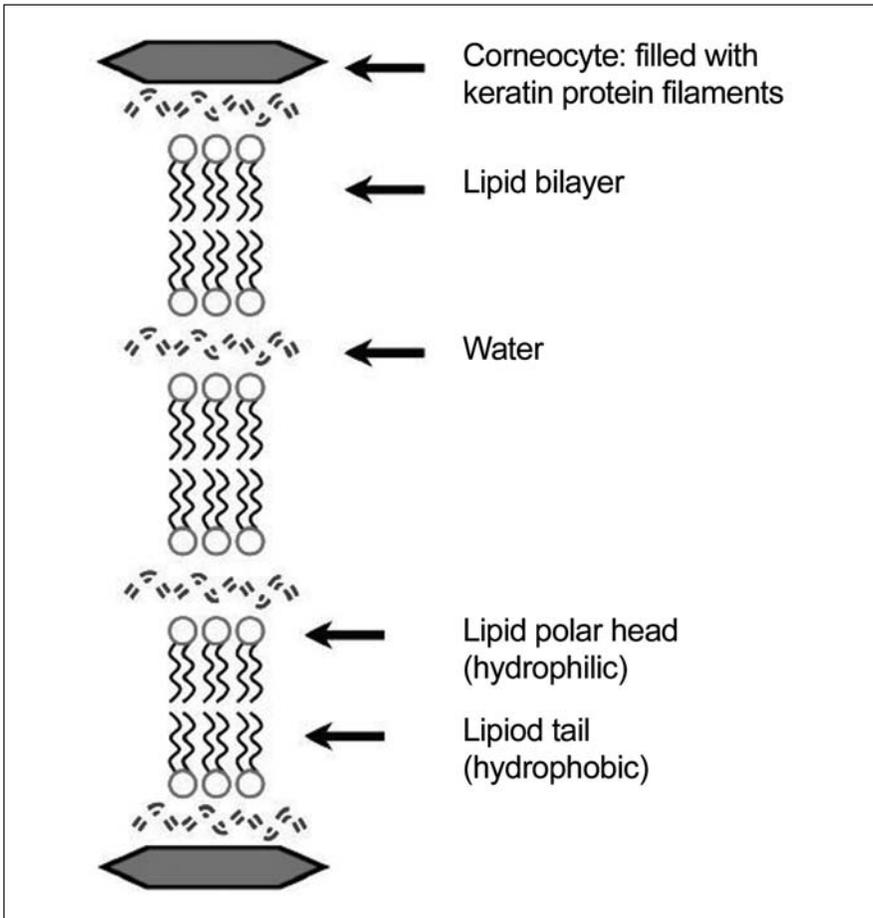


Figure 2. The lipid bilayers of the stratum corneum (From Reference 1)

A 1997 patent² by Odile Enjolras assigned to The Boots Company plc discloses a dermatological or cosmetic composition for skin care containing 0.01%–30% by weight of at least one ceramide and 1%–15% by weight of linoleic acid useful for restoring the barrier function of the SC of dry or very dry skin. The inventor notes that the barrier has to allow some water to flow out through the SC, because that flow is the means by which oxygen and nutrients are delivered to the nonvascularized epidermis. But if the flow is excessive, the skin becomes rough and dry.

A different approach was taken later in Harbhajan S. Paul's patent⁶ assigned to Biomed Research & Technologies, Inc. (Wexford, Pa., USA). His patent reveals the use of topically applied branched-chain amino acids (BCAAs) capable of being catabolized in epidermal cells to form lipid precursors for epidermal lipid synthesis. In addition to strengthening the lipid barrier, these BCAAs also may enhance the NMF by providing metabolites to increase the NMF's constituent chemical pool and by reducing the loss of water-soluble NMF through the skin during washing or other forms of water exposure.

This last point was emphasized in a personal communication⁷ from a researcher at Unilever Research (Trumbull, Conn., USA). "Humectants, including NMF, are still only half of the moisturization story. In skin, the natural lipid bilayers form a continuous protective barrier around the corneocytes. Because NMF and humectants in general are water-soluble, when exposed to water they can be washed away easily. Skin lipids help to protect corneocytes from the effects of water washing by keeping NMF and moisture sealed within the cells. However, surface lipids are lost due to soaps and harsh cleaners, which then exposes the cells to NMF loss.

"Thus in cleansing products, the cosmetic formulator must understand the need for mild cleansing that minimizes any skin-damaging effects. For lotions and care products, the cosmetic formulator must understand the balance of not only replacing humectant function within the surface skin cells, but also the function of the lipids layer to seal moisture and humectants in the surface cells," according to Unilever Research.

Natural Moisturizing Factor

The NMF consists primarily of amino acids or their derivatives such as pyrrolidone carboxylic acid (PCA) and urocanic acid together with lactic acid, urea, citrate and sugars (see **Table 1**). Together they may represent 20%–30% of the dry weight of the SC. In their chapter on NMF in the Lodén and Maibach book, Harding, Bartolone and Rawlings write that “the importance of the NMF lies in the fact that its constituent chemicals, particularly its PCA and lactic acid salts, are intensely hygroscopic. These salts absorb atmospheric water and dissolve in their own water of hydration, thereby acting as very efficient humectants. In essence the amount of NMF in the SC determines how much water it can hold for any given relative humidity.”⁸

As shown in **Figure 1**, corneocytes in the lower layers of the SC contain a keratin–filaggrin complex. This complex of two proteins was formed below the SC in the lower levels of the epidermis. As the degenerating corneocytes move toward the outer layer of the skin, enzymes break down the link between filaggrin and the water-retaining keratin. When the moisture level of the skin is sufficiently decreased, specific proteolytic enzymes in the SC are triggered to break down filaggrin into free amino acids, which together with other chemicals constitute the NMF.

Table 1. The chemical composition of NMF¹¹

Free amino acids	40.0%
Pyrrolidone carboxylic acid	12.0
Lactate	12.0
Sugars, organic acids, peptides, unidentified materials	8.5
Urea	7.0
Chloride	6.0
Sodium	5.0
Potassium	4.0
Ammonia, uric acid, glucosamine creatine	1.5
Calcium	1.5
Magnesium	1.5
Phosphate	0.5
Citrate, formate	0.5

Unilever Research continues the story from there. “As skin moisture is lost, the formation of NMF increases in an attempt to maintain proper moisture balance. NMF remains with the corneocyte to keep it hydrated and flexible. Eventually the corneocyte reaches the surface and the NMF is either washed away (depleting the cell) or lost with the cells in natural desquamation. If NMF is fully depleted from cells, the cells will become dry and stiff and will eventually be perceived as dry skin.”⁷

Cosmetic chemists have created ingredients and topical compositions to deliver NMF components topically to dry skin where they diffuse according to a concentration gradient. For example, Arch Personal Care Products (Plainfield, N.J., USA) has developed BioPlex NMF, comprised of sodium PCA, lactic acid, sodium lactate, urea and collagen amino acids, and claims each of these ingredients acts individually as a moisturizer.

Engelhard Corp. (Stony Brook, N.Y., USA) offers a product called Advanced Moisture Complex and a related highly cationic blend called Advanced Moisture Complex-CAT for better adhesion to the skin surface. Both blends contain sodium PCA, urea and trehalose, which is a naturally occurring, nonreducing disaccharide composed of two glucose molecules that reportedly helps prevent damage from dehydration by maintaining the liquid crystalline lamellar structure of the SC's lipid barrier.

Amino acids and SC moisturization play a limited role in a recent Beiersdorf patent⁹ that addresses numerous formulation issues, including moisturizing, skin smoothing, care action, improved vehicles for cosmetic and dermatological active substances, improved emulsion stability and better biocompatibility. The invention relates to the use of cosmetic and dermatological emulsions, in particular o/w emulsions having at least one aqueous phase, at least one of which contains electrolytes in dissolved form. The electrolytes can include amino acids, especially essential amino acids, which are “advantageous since moisture can be bound into the skin by hydration processes,” according to the patent. In addition to the one or more aqueous phase(s) and the dissolved amino acids, the preparations according to this patent contain the following: one or more partially neutralized esters of monoglycerides or diglycerides of saturated

fatty acids with citric acid; one or more sorbitan monoesters, which are characterized by a specified structural formula; and one or more fatty alcohols, chosen from the group of branched and unbranched alkyl alcohols having 12–40 carbon atoms.

Water at the Desmosomes

A connection between desmosomes and moisturization of the SC is tenuous, but consider this 2004 L'Oréal patent titled "Isolated peptide of the horny layer and uses thereof."¹⁰ The invention concerns an isolated polypeptide from the family of calcium-fixing proteins and its use in compositions for the cosmetic treatment of dry skin. The invention also concerns a DNA sequence coding for the polypeptide and the uses of that DNA sequence.

For example, in cosmetics, this polypeptide can be used to regulate the impairments of epidermal, normal or pathological proliferation or differentiation, as in conditions such as dry skin, hyperkeratosis, parakeratosis, psoriasis, ichthyosis and neoplasia. The polypeptide thus can be introduced into a composition that is intended for moisturizing or making up the skin, mucous membranes or keratinic fibers. The polypeptide also can be used in the treatment of aging, particularly aging of the skin, and in the treatment of skin damage linked to exposure to ultraviolet radiation.

The mechanism enabling all these regulatory activities is apparently the role of calcium flows in the cellular functions. According to the patent, the calcium is an intracellular messenger of outstanding importance. Numerous hormones and extracellular messengers exert their effect by an increase of the intracellular ratio of calcium. Most of the effects of the calcium were determined by a large, relatively homogeneous family of calcium-binding proteins. Among this family, the calmodulins are the most ubiquitous. The role of the calmodulin is to recognize the changes in the concentration of cytosolic calcium ions and to transmit the information to the intracellular proteins. The calmodulin is known for interacting with numerous proteins or enzymes, among which is desmocalmin, a protein of the desmosome that interacts with the keratins.

Summary

Brief patent searches did not identify any inventions claiming to moisturize the SC by directly adding water to the corneocytes. Nor is this apparently an approach used in the industry. But interest has been shown in the other SC water reservoirs: the lipid bilayers, the NMF, and the area around the desmosomes.

The lipid bilayers store water by entrapment. Anthony Rawlings explains that NMF and keratin in the corneocyte probably retain water initially by hydrogen bonding, “but as the keratin unfolds, there are more water bonds. In fact, you usually observe an exponential increase in the water content. Unfreezable water, bound probably to keratin, is the primary bound water. Secondary bound water then forms a water shell around this and binds to NMF.”³

What can the cosmetic chemist do to increase the amount of water stored in each of these places? “Ingredients of the NMF will help to bind water. So will other humectants such as glycerol. But simple occlusive agents also will increase water content,” Rawlings says. “In this respect the combination of bilayer-forming lipids such as ceramides together with humectants give the best benefit in terms of dry skin relief.”

Published May 2006 *Cosmetics & Toiletries* magazine.

References

1. C Marino, Skin Physiology, Irritants, Dry Skin and Moisturizers, Report No. 56-2-2001, Washington State Department of Labor and Industries (Aug 2001) www.lni.wa.gov/Safety/Research/Dermatitis/files/skin_phys.pdf. (Accessed May 17, 2006)
2. US Pat 5,656,278, Dermatological and cosmetic compositions, O Enjolras, assigned to The Boots Company plc, Great Britain (Aug 12, 1997)
3. Personal e-mail correspondence to Bud Brewster from Anthony Rawlings, director, AVR Consulting Ltd., Northwich, Cheshire, UK, dated May 19, 2006
4. A Kligman, Introduction, In *Dry Skin and Moisturizers: Chemistry and Function*, M Lodén and HI Maibach, eds, Boca Raton: CRC Press (2000) p 7
5. AV Rawlings and CR Harding, Moisturization and skin barrier function, *Dermatologic Therapy* 17 43 (2004)
6. US Pat 6,149,924, Composition for enhancing lipid production barrier function, hydrogen peroxide neutralization, and moisturization of the skin, HS Paul, assigned to Biomed Research & Technologies, Inc, USA (Nov 21, 2000)
7. Personal e-mail correspondence from Unilever Research to Bud Brewster, dated May 12, 2006
8. CR Harding, J Bartolone and AV Rawlings, Effects of Natural Moisturizing factor and lactic acid isomers on skin function, In *Dry Skin and Moisturizers: Chemistry and Function*, M Lodén and HI Maibach, eds, Boca Raton: CRC Press (2000) p 230

9. US Pat 6,962,711, Cosmetic and dermatological preparations comprising increased electrolyte concentrations, G Schneider et al, assigned to Beiersdorf AG, Germany (Nov 8, 2005)
10. US Pat 6,800,609, Isolated peptide of the horny layer and uses thereof, B Mehul, D Bernard and L Simonetti, assigned to L'Oréal, France (Oct 5, 2004)
11. Ibid Ref 8, p 231

Treating Wrinkles with Dimethylaminoethanol, Retinol and Mineral Salts

C. Bertin, C. Robert, M. Jousselin, N. Issachar and E. Camel

Johnson & Johnson Consumer, France, Issy les Moulineaux, France, Institut d'Expertise Clinique, Lyon, France

KEY WORDS: *dimethylaminoethanol, retinol, mineral salts, wrinkles, aging*

ABSTRACT: *A placebo-controlled study was conducted to determine if the appearance of wrinkles on facial skin could be improved by a combination of three cosmetic ingredients: dimethylaminoethanol, retinol and mineral salts. Both clinical assessment and video analysis of cutaneous replicas showed a significant improvement of the wrinkle appearance.*

Age-related changes in skin appearance including dryness, wrinkling and laxity, are influenced by the behavior of keratinocytes and fibroblasts. As individuals age, skin cells replace themselves more slowly and the lower layer of the skin, the dermis, decreases in thickness. The network of elastin and collagen fibers that allows human skin to stretch and retract begins to unravel.¹⁻³

On the face, thinner and less elastic skin tends to sag and fold. Permanent crease lines and crow's-feet form on the brow and around the eyes. Tiny wrinkles form around the lips. The tissues of the jaw and neck droop into jowls and double chins. Skin aging is affected by genetic factors such as skin color, environmental factors such as prolonged exposure to the sun,^{4,5} and the consequences of a lifestyle involving smoking, rapid weight loss and stress.^{6,7} The aging process cannot be stopped or reversed but good daily skin care can slow the process.

This article describes a placebo-controlled study conducted to determine if the combination of dimethylamino-ethanol, retinol and mineral salts has a positive effect on one aspect of skin aging, the appearance of wrinkles in facial skin.

Three Cosmetic Ingredients

Different groups of molecules are now recognized and used to slow skin aging. Retinoid and α -hydroxy acid (AHA) families and polypeptides are the most frequently used, alone or in combination.

Retinol: Retinol is one of the most effective molecules now on the market for counteracting the effect of cutaneous aging.^{8,9} Easily absorbed by the epidermis,¹⁰ it increases cellular renewal in the basal layer of the epidermis, normalizes cellular differentiation and regulates the keratinization process.¹¹⁻¹³ Retinol also has an effect on the dermis. Studies conducted on mice¹⁴ and humans^{15,16} have shown that retinol increases the quantity of collagen in the dermis. It also has been shown to increase skin elasticity and to reduce the depth of wrinkles on women.¹⁷

DMAE: Prescribed for many years as a treatment for cognitive disorders due to its remarkable brain-enhancement effects,¹⁸⁻²¹ dimethylaminoethanol (DMAE) has recently become a popular skin-firming cosmetic ingredient (see **DMAE's Mechanism of Action**). Clinical trials have demonstrated the safety and efficacy of DMAE in antiaging formulations.^{22,23}

Mineral salts: The use and development of mineral salts in dermatology are based on the discovery of several interaction sites in the skin. Zinc, copper and magnesium appear to play a role in skin aging.

Zinc is one of the most abundant trace elements in the human body.²⁴ Zinc is the cofactor of more than 90 different enzymes. It protects them from free radicals and UV radiation attacks and regulates keratinization and fibroblast proliferation. Zinc is also involved in melanogenesis and in metabolism of fatty acids, and vitamins A and E.²⁵ Topically applied zinc speeds wound healing, regulates sebum secretion, and exhibits antiseptic and antibacterial activity.

During aging, the natural level of zinc in the skin diminishes.²⁶ Low concentrations of zinc in the epidermis have been observed in older people and may be the result of aging-reduced activity of epi-

dermal enzymes. Conversely, zinc deficiency can retard the activity of other enzymes, leading to intensification of the aging processes.²⁴

DMAE's Mechanism of Action

The skin-firming and antiaging benefits of DMAE are becoming well-documented.^{22,30}

DMAE is a naturally occurring nutrient found in seafood such as anchovies, sardines and salmon. It is also present in small amounts in the human brain. DMAE is a simple amine base ((CH₃)₂NCH₂CH₂OH) with structural similarity to choline. Initially used in cosmetic formulations as a skin permeation aid,³¹ its skin-firming and antiwrinkle properties were rapidly recognized.

Multiple studies have been conducted supporting the efficacy and safety of this ingredient in topical formulations of skin care products for the improvement of the appearance of aging skin.²³ This property may be explained through different mechanisms of action.²² DMAE may increase the supply of acetylcholine that, in turn, may function as an ubiquitous cytokine-like molecule, regulating basic cellular phenomena such as proliferation and differentiation. Indeed, it has been demonstrated that human epidermal keratinocytes possess cholinergic enzymes that synthesize and degrade acetylcholine and express both nicotinic and muscarinic classes of cholinergic receptors on their cell surfaces.³²

Furthermore, it has been demonstrated that DMAE has the ability to contract keratinocytes both *in vitro* and *in vivo*.²⁷ This non-neuronal communication system may be responsible for the immediate short-term firming effect of topically applied DMAE.³³

Other potential cutaneous benefits concern scavenging of free radicals, inhibition of age-related protein cross-linking, stabilization of skin-cell membranes, and modulation of inflammation.

Copper also plays a role in skin via keratin synthesis and has a scavenging effect through the activation of the Cu-Zn superoxydismutase. It induces collagen production by fibroblasts thanks to the lysyl oxidase activation. In melanocytes, copper stimulates tyrosinase, therefore promoting melanin precursor synthesis and photoprotection.²⁵

Magnesium is a mineral of primary importance in the body because it aids in the activation of adenosine triphosphate (ATP), the main energy source for cell functioning. Magnesium also activates several enzyme systems and is important for the synthesis of RNA

and DNA. It is important for the synthesis of several amino acids.

DRMC: In the present study, authors investigate the effects of a cosmetic product containing DMAE, retinol and mineral salts on wrinkles. The mineral salts were a mixture of magnesium aspartate, zinc gluconate and copper acetate. These ingredients have been formulated into a proprietary combination that will be called DRMC in this article. The components of DRMC were chosen because, as already stated, the association of DMAE to mineral salts¹¹ or to retinol¹² increases the antiaging efficacy of the final product.

Materials and Methods

A double-blind, randomized, placebo-controlled study was conducted to test the efficacy of an antiaging product containing DRMC.

Subjects: In the present study, 124 female subjects were divided into two groups: one treated with the DRMC-containing antiaging product (n = 62) and one treated with a placebo (n = 62). Each subject was Caucasian, between the ages of 45 and 66, and with wrinkles and fine lines on her face.

The participants were accepted into the study only after receiving a medical examination and giving their free and informed consent. Women with serious illness or under medical treatment that would likely interfere with the efficacy evaluation were excluded. Also excluded were women who had used a cosmetic containing AHA for two weeks preceding the start of the study or who had followed a treatment containing retinol, retinoic acid or vitamin A for the two months preceding the start of the study. A final exclusion was for women who had recently suffered from sunstroke or who had followed heliotherapy during the month preceding the study.

Treatments: Both the test cream containing the DRMC and the placebo cream were white o/w creams, differing primarily by the presence of DRMC as the active ingredient. The placebo had a formula very similar to that of the test cream, but it was not exactly the vehicle of the test cream. Confidentiality prevents disclosure of the complete formulations of the two creams, but it can be said that all the ingredients, except DRMC, are well-known ingredients without any cutaneous rejuvenation efficacy. The concentrations of DRMC were chosen so they provided an efficacy but remained in the range accept-

able for a cosmetic product. The two creams were randomly assigned to the subjects.

Schedule: At baseline (T0), a trained assessor performed a clinical assessment of the wrinkles present on the complete face. Photographs of the face were taken. Skin replicas were created outlining the right or left eye crow's-feet wrinkles and upper part of the cheek, the forehead, and the area between the eyebrows. The choice of right or left side was determined randomly.

Each subject completed a controlled application of the cream under normal conditions of use and in the presence of laboratory staff. A sufficient amount of the cream was spread with gentle taps from the external corner of the eye to the internal corner, favoring particularly the outline of the eye and the cheekbone, avoiding application too close to the eyelashes. The cream was then applied on the rest of the face, with special attention to the forehead, between the eyebrows, and the area under the chin. Three hours after application, clinical evaluations were performed again and another set of skin replicas was obtained. The purpose of repeating the clinical evaluation and the skin replicas after a 3-hr interval was to take into account the possible immediate short-term firming/tightening effect of topically applied DMAE,²⁷ i.e., the ability of DMAE to induce contraction or shrinkage of keratinocytes both and .

After the initial applications, the cream was applied once daily in the morning, seven days a week for 12 consecutive weeks, by the subject at home on cleansed skin and under normal conditions of use.

After 4, 8 and 12 weeks (T4, T8, T12), each subject returned to the laboratory for a controlled application of the cream. Three hours later, clinical assessment, photographs and skin replicas were taken again on the zones previously defined at T0. This comparison was used to take into account both the short- and long-term firming effects of the DRMC.

Assessing parameters and analyzing data: Facial wrinkles were evaluated by a trained assessor on a 12-cm visual analogical scale. For each subject, seven areas were scored on the face: the forehead, crow's-feet, wrinkles between eyebrows, nasal troughs, cheeks, the periauricular area and the area under the chin.

Replicas of the wrinkles present on the forehead, at crow's-feet and between the eyebrows were obtained with a silicone rubber^a material applied each visit at the same location identified by the particular subject's beauty marks or a mask in translucent plastic with anatomic marks specific to the subject.

Skin imprints were analyzed using a skin analyzer^b with software^c, enabling the researchers to obtain a set of standardized parameters characterizing the cutaneous relief including the total number of wrinkles, total wrinkled surface and total length of the wrinkled surface.

Statistical analysis: The arithmetic mean values and standard errors on the mean of each parameter were determined at each time point of the study and for each treatment group for the skin relief parameters. The arithmetic mean values and the standard deviations for the scores of individual values collected from each subject were also calculated. For both the DRMC group and the placebo group, an intragroup comparison was performed between baseline values and the values obtained 3 hr after the application of the cream at T0, T4, T8 and T12 weeks. Also performed at those time points were intergroup comparisons of the DRMC group versus the placebo group.

Results

Clinical assessment: For both the active- and placebo-treated groups, a progressive and statistically significant reduction in the appearance of facial wrinkles was observed on all areas of the face considered ($P < 0.05$; Wilcoxon test) (data not shown). At the end of the study and for DRMC-treated group, the grading of wrinkles on the forehead and between the eyebrows was reduced respectively by 19% and 17%. On cheeks and nasal troughs, the wrinkle grades decreased by 22% and 17%, respectively. After four weeks of using the DRMC cream, a statistically significant difference in the appearance of crow's-feet wrinkles was demonstrated in comparison to the placebo treatment (see **Table 1**).

^a Silflo silicone rubber is a product of Flexico Corp.

^b Skin Image Analyser (SIA) is a device from Monaderm, Monaco.

^c Quantirides is a program from Monaderm, Monaco.

Table 1. Percentage change in clinical assessment of wrinkles in crow's-foot area after product application at Tx, compared with initial evaluation before any treatments

Time points	Placebo (n = 62)	Antiaging product with DRMC (n = 62)
T0	-2% **	-1% **
T 4 weeks	-8% ** †	-10% ** †
T 8 weeks	-11% ** †	-13% ** †
T 12 weeks	-14% ** †	-17% ** †

** $p < 0.005$, Intragroup comparison with regard to baseline value (Wilcoxon test, "two-tail")
† $p < 0.05$, Intergroup comparison: statistically significant difference between the two products (U test of Mann-Whitney, "two-tail")

The effects of the DRMC treatment lasted until the end of the study: reductions in wrinkle grades were recorded at 17% in the DRMC-treated group after 12 weeks, versus 14% in the placebo-treated group ($P < 0.05$; U test of Mann-Whitney). Photographs of one subject before and after the 12 weeks of DRMC treatment are shown in **Figure 1**.

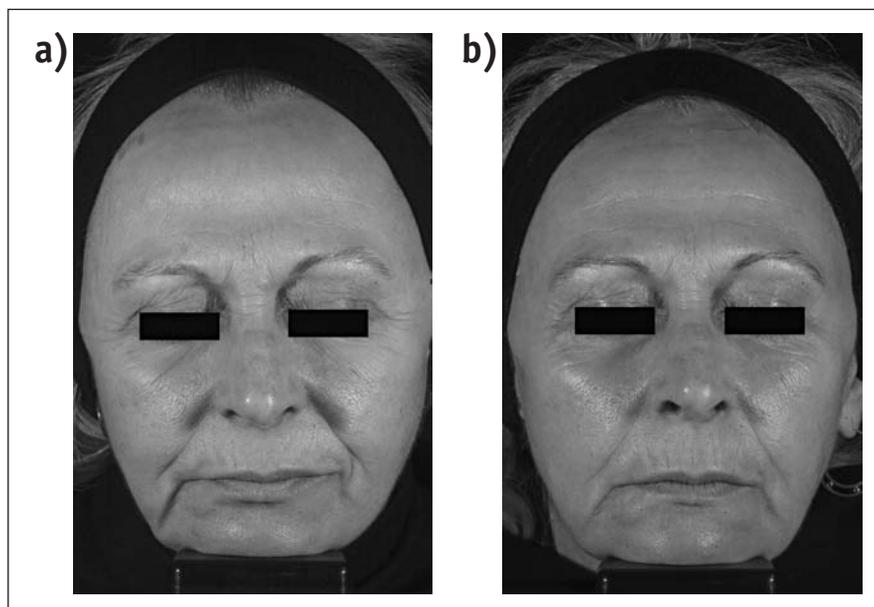


Figure 1. Example of results obtained on one volunteer treated with the antiaging product a) before treatment (T0) and b) at the end of the treatment (T12 weeks)

Cutaneous relief analysis on skin replica: In both groups, depending on the localization, some replicas were not assessable because of problems removing them from the skin. Therefore, the results are given on fewer than 62 replicas.

- *Crow's-foot wrinkles.* No significant variation in crow's-feet wrinkle parameters was measured (see **Table 2**) in the placebo-treated group, except after 12 weeks of treatment. At that time, the total length of the wrinkled surface was reduced by 6%. On the other hand, compared to initial evaluation, DRMC treatment induced a significant reduction in the three parameters analyzed starting from the fourth week of use. The maximum effect was obtained after 8 weeks of application: a 19% reduction in the total number of wrinkles was observed, as well as 16% decrease in the total wrinkled surface. The total length of the wrinkled surface diminished by 25%. These effects remained stable after 12 weeks of treatment. The overall efficacy showed statistically significant improvement obtained faster with the DRMC treatment than with the placebo treatment.
- *Forehead wrinkles.* A statistically significant effect was observed with both the DRMC cream and the placebo cream (data not shown). Twelve weeks after the beginning of the study, treatment with either cream led to a comparable and significant reduction in forehead wrinkles.
- *Wrinkles between the eyebrows.* Compared to the placebo treatment, the DRMC treatment showed a statistically significant effect (see **Table 3**) on the total length of the wrinkled surface as soon as after the first application, but also after 4 and 12 weeks of use. The wrinkles between the eyebrows were reduced 21% with the DRMC cream versus 8% with the placebo at T12 ($P < 0.05$ with the Student t test). At the end of the treatment, the total number of wrinkles as well as the total wrinkled surface between the eyebrows was significantly less in the DRMC-treated group than in the placebo-treated group.

Even with only a placebo treatment, some improvement was noticed, as in the case of forehead wrinkles. The placebo did not

contain moisturizing ingredients, for example, but there is always a moisturizing effect when applying a cosmetic product onto the skin. A real placebo with no physiological effect is nearly impossible to design.

Table 2. Percentage change in SIA analysis of wrinkles in crow's-foot area after product application at Tx, compared to initial evaluation before any treatments

Parameter	Time points	Placebo (n = 57)	Antiaging product with DRMC (n = 59)
<u>Total number of wrinkles</u>			
	T0	N.S.	-5% ($p = 0.064$)
	T 4 weeks	N.S. †	-10% ** †
	T 8 weeks	N.S. †	-19% ** †
	T 12 weeks	N.S. †	-19% ** †
<u>Total wrinkled surface</u>			
	T0	N.S. †	-10% ** †
	T 4 weeks	N.S. †	-16% ** †
	T 8 weeks	N.S. †	-16% ** †
	T 12 weeks	N.S. †	-14% ** †
<u>Total length of the wrinkled surface</u>			
	T0	N.S. †	-12% ** †
	T 4 weeks	N.S. †	-19% ** †
	T 8 weeks	-5% ($p = 0.062$) †	-25% ** †
	T 12 weeks	-6% * †	-24% ** †

* $p < 0.05$ and ** $p < 0.005$, Intragroup comparison with regard to baseline value (Student t test, "two-tail")
† $p < 0.05$, Intergroup comparison: statistically significant difference between the two products (Student t test or U test of Mann-Whitney, "two-tail")
N.S., no significant change

Discussion

The aim of the present double-blind, randomized, placebo-controlled study was to test the efficacy of a combination of retinol, DMAE and mineral salts on wrinkle severity. The 12-week study demonstrated the efficacy of this specific combination.

An obvious improvement of the appearance of facial wrinkles was correlated with a significant reduction of the wrinkles from the crow's-foot area and between the eyebrows. This improvement was

not simply an effect of moisturization, although both creams may have had a moisturizing effect. However, in the DRMC group, a significant improvement versus placebo was observed after four weeks of use on crow's-feet area (**Table 1**). This significant difference between the two groups lasted until the end of the study, proving the results were not simply a moisturizing effect but that real anti-wrinkle activity had occurred in that area. This activity was confirmed by a crow's-foot wrinkle analysis with SIA (**Table 2**). A significant improvement was observed only in the DRMC-treated group and the difference between the products was statistically significant.

Table 3. Percentage change in SIA analysis of wrinkles between the eyebrows after product application at Tx, compared to initial evaluation before any treatments

Parameter	Time Points	Placebo (n = 59)	Antiaging product with DRMC (n = 59)
<u>Total number of wrinkles</u>			
	T0	-7% *	-9% **
	T 4 weeks	-5% *	-11% **
	T 8 weeks	-13% **	-13% **
	T 12 weeks	-6% ($p = 0.052$) †	-16% ** †
<u>Total wrinkled surface</u>			
	T0	N.S.	-8% **
	T 4 weeks	-8% *	-7% *
	T 8 weeks	-11% **	-6% *
	T 12 weeks	N.S. †	-9% ** †
<u>Total length of the wrinkled surface</u>			
	T0	-5% ($p = 0.077$) †	-14% ** †
	T 4 weeks	-7% *	-19% ** †
	T 8 weeks	-15% ** †	-21% **
	T 12 weeks	-8% * †	-21% ** †

* $p < 0.05$ and ** $p < 0.005$, Intragroup comparison with regard to baseline value (Student t test, "two-tail")
† $p < 0.05$, Intergroup comparison: statistically significant difference between the two products (Student t test or U test of Mann-Whitney, "two-tail")
N.S., no significant change

The fact that DRMC's antiaging effect occurred more quickly around the eyes than on the forehead might at first seem inconsistent. In the first

case a significant difference was observed between the DRMC cream and the placebo, whereas in the second case no difference was observed. This could be explained by the fact that the forehead wrinkles are deeper and more marked than in the crow's-feet area. The antiwrinkle effect would be observed first in the crow's-feet area and later on the forehead.

DMAE has well-documented positive effects for skin firming, skin permeation, antiaging and wrinkle reduction (as discussed in **DMAE's Mechanism of Action**). The combination of DMAE with mineral salts (mixture of magnesium aspartate, zinc gluconate and copper acetate) was previously demonstrated to synergistically stimulate fibroblasts in the synthesis of collagen and glycosaminoglycan and to stimulate cell proliferation.²⁸

On the other hand, a study performed on human skin explants has shown that retinol combined with DMAE significantly stimulates collagen synthesis, raises the number of dividing cells in the basal skin layer and increases epidermis thickness.²⁹

The present study confirmed the efficacy on human skin of this particular combination of cosmetic ingredients—DMAE, retinol and mineral salts—as a new skin antiaging treatment having both short-term and long-term firming properties.

Published April 2008 *Cosmetics & Toiletries* magazine.

References

1. NA Fenske and CW Lober, Structural and functional changes of normal aging skin, *J Am Acad Dermatol* 15 571–585 (1986)
2. C Escoffier et al, Age related mechanical properties of human skin, *J Invest Dermatol* 93 353–357 (1989)
3. Y Takema, Y Yorimoto, M Kawai and G Imokawa, Age related changes in the elastic properties and thickness of human facial skin, *Br J Dermatol* 131 641–648 (1994)
4. EF Bernstein et al, Long term sun exposure alters the collagen of the papillary dermis. Comparison of sun protected and photo-damaged skin by northern analysis, immunohistochemical staining and confocal laser scanning microscopy, *J Am Acad Dermatol* 34 209–218 (1996)
5. GJ Fischer, The pathophysiology of photoaging of the skin, *Cutis* 75 5–9 (2005)
6. MF Demierre, D Brooks, HK Koh and AC Geller, Public knowledge, awareness and perceptions of the combination between skin aging and smoking, *J Am Acad Dermatol* 41 27–30 (1999)
7. JS Koh, H Kang, SW Choi and HO Kim, Cigarette smoking associated with premature facial wrinkling: image analysis of facial skin relics, *Int J Dermatol* 41 21–27 (2002)
8. CEM Griffiths, The role of retinoids in the prevention and repair of aged and photoaged skin, *Clin Exp Dermatol* 26 613–618 (2001)
9. REB Watson et al, Retinoic acid receptor alpha expression and cutaneous ageing, *Mech Ageing Dev* 125 465–473 (2004)

10. C Caldera, L Kersaudy, J Badoual and G Olive, The cutaneous absorption of vitamin A, *Dev Pharmacol Ther* 7 213–217 (1984)
11. S Kang et al, Application of retinol to human skin *in vivo* induces epidermal hyperplasia and cellular retinoid binding proteins characteristic of retinoid acid but without measurable retinoic acid levels or irritation, *J Invest Dermatol* 105 549–556 (1995)
12. R Eichner et al, Effects of long-term retinoic acid treatment on epidermal differentiation *in vivo*: specific modifications in the programme of terminal differentiation, *Br J Dermatol* 135 687–695 (1996)
13. BA Gilchrist, Treatment of photodamage with topical tretinoin: an overview, *J Am Dermatol* 36 S27–S36 (1997)
14. E Schwartz, FA Cruesshank, JA Mezick and AM Kligman, Topical all-trans retinoic acid stimulates collagen synthesis *in vivo*, *J Invest Dermatol* 96 975–978 (1991)
15. CE Griffiths et al, Two concentrations of topical tretinoin (retinoic acid) cause similar improvement of photoaging but different degrees of irritation. A double-blind, vehicle-controlled comparison of 0.1% and 0.025% tretinoin creams, *Arch Dermatol* 131 1037–1044 (1995)
16. J Varani et al, Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin, *J Invest Dermatol* 114 480–486 (2000)
17. C Bertin et al, Retinol + Melibiose: An innovative anti-aging combination, 20th IFSCC Congress (1998)
18. RE Osvaldo, 2-Dimethylaminoethanol (deanol): a brief review of its clinical efficacy and postulated mechanism of action, *Curr Ther Res Clin Exp* 6 1238–1242 (1974)
19. JA Lewis and R Young, Deanol and methylphenidate in minimal brain dysfunction, *Clin Pharmacol Ther* 17 534–540 (1975)
20. CL Saccar, Drug therapy in the treatment of minimal brain dysfunction, *Am J Hosp Pharm* 35 544–552 (1978)
21. JF Flood, GE Smith and A Cherkin, Memory retention: potentiation of cholinergic drug combinations in mice, *Neurobiol Aging* 4 37–43 (1983)
22. R Grossman, The role of dimethylaminoethanol in cosmetic dermatology, *Am J Clin Dermatol* 6 39–47 (2005)
23. CA Cole and C Bertin, Dimethylaminoethanol: a new skin-care ingredient for aging skin, In *Textbook of Cosmetic Dermatology*, 3rd edn, R Baran and HI Maibach, eds, Boca Raton: CRC Press (2004)
24. A Frydrych, J Arct and K Kasiua, Zinc: a critical important element in cosmetology, *J Appl Cosmetol* 22 1–13 (2004)
25. B Dreno, Oligoéléments et peau, *Cosmétique* 8 44–47 (1995)
26. G Brooks, The importance of zinc in the ageing process and the cosmetics effects of a novel zinc yeast derivative, *Proceed IFSCC Conference "Biocosmetics–Skin aging,"* vol II (1993) p 89
27. US Pat Applic 20060193777, Method of screening compounds for potential efficacy for the treatment of signs of aging, MD Southall et al (Aug 31, 2006) www.freepatentsonline.com/20060193777.html
28. C Bertin et al, Anti-aging efficacy of the combination of dimethylaminoethanol (DMAE) and mineral salts, *Proceedings of the 23rd IFSCC Congress, Orlando, 2004*, IFSCC (2004)
29. A Robert, J Serrano, T Oddos and G Ries, Use of a skin explant model for evaluating a new combination anti-aging technologies: retinol and dimethylaminoethanol (DMAE), *Proceedings of the 23rd IFSCC Congress, Orlando, 2004*, IFSCC (2004)
30. I Nagy and K Nagy, On the role of cross-linking of cellular proteins in aging, *Mech Ageing Dev* 14 245–251 (1980)
31. BB Michniak, Amines and amides as penetration enhancers, In *Percutaneous Penetration Enhancers*, E Smith and H Maibach, eds, Boca Raton: CRC Press (1995) p 80
32. SA Grando, Biological functions of keratinocyte cholinergic receptors, *J Invest Dermatol Proc* 2 41–48 (1997)
33. I Uhoda et al, Split face study on the cutaneous tensile effect of 2-dimethylaminoethanol (deanol) gel, *Skin Res Technol* 8 164–167 (2002)

Watermelon Survival Strategies for Skin DNA Protection

Cornelia Huber and Thomas Schreier

Pentapharm Ltd., Aesch, Switzerland

KEY WORDS: *DNA damage, p53, 8-oxo-guanine, compatible solute, watermelon, Citrullus lanatus, Kalahari Desert*

ABSTRACT: *Plants living under extreme conditions have developed a strategy to protect their DNA and other important cell structures from environmental stress factors. In particular, watermelon (Citrullus lanatus) has adapted well to extreme conditions. Its survival strategy can be used in the field of skin care, as shown by several tests, including a direct DNA protection assay, the comet assay, and 8-oxo-guanine and p53 detection.*

Ozone depletion and more active outdoor lifestyles have led to increased UV exposure and major health concerns. The ozone layer, an efficient UV shield in the upper stratosphere, has been severely reduced throughout the past decade because of increased environmental pollution.¹

Its depletion has led to increased UV radiation—a serious problem for human skin.

UV radiation may cause skin cancer through the induction of pyrimidine dimers and reactive oxygen species (ROS), resulting in oxidative DNA damage. Guanine is the preferential target for ROS, leading to the formation of 7,8-dihydro-8-oxo-guanine (8-oxo-G) in the skin (**Figure 1**).^{2,3} A marker of oxidative stress, 8-oxo-dG is highly mutagenic due to incorrect base pairing with deoxyadenosine.

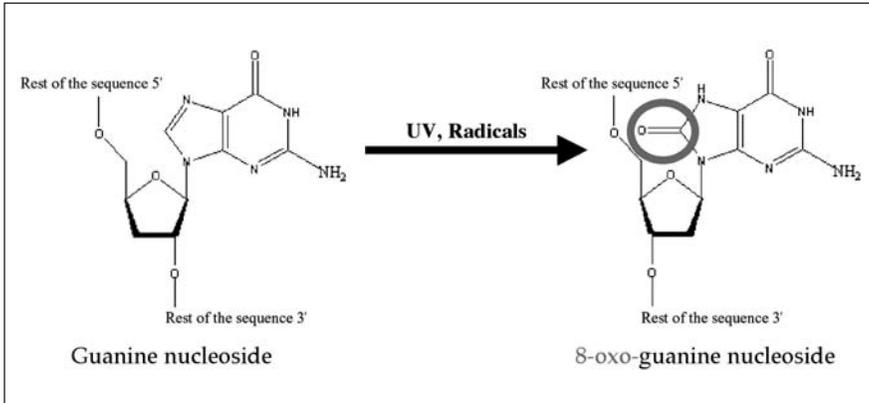


Figure 1. Formation of 8-oxo-guanine as a result of UV radiation or free radical exposure.

DNA damage leads to an up-regulation of p53 protein through increased protein stability.⁴ In normal, healthy keratinocytes, the p53 level is potentially suppressed by a number of regulators such as MDM2. p53 protein has a short half-life of about 5–20 min, hence it is the up-regulation through increased protein stability that prevents its degradation. After DNA damage, p53, or “the guardian of genome,” is activated by phosphorylation and protected from degradation by preventing MDM2 binding. Phosphorylated p53 acts as a transcription factor and promotes expression of further proteins such as p21, gadd45 and BAX by transcriptional activation.

These proteins are involved in controlling the cell cycle (p21), organizing DNA repair (gadd45) and eliminating nonrepairable cells by inducing apoptosis (BAX) (Figure 2).^{5,6} p53 is an marker for damaged DNA; the higher the amount of damaged DNA, the higher the p53 level. In comparison to unprotected cells, cells treated with a DNA-protecting molecule show a lower level of p53.

Defense Development

Organisms living under extreme conditions such as high heat or salt concentration, drought and UV radiation, develop their own defense systems to protect important cell structures such as DNA, enzymes and cell membranes. Plants, yeasts and bacteria growing in deserts synthesize small, water-soluble molecules called *compatible solutes* that protect their important cell structure from damage.

One of the Kalahari Desert’s native plants is watermelon (*Citrullus*

lanatus). Growing under such extreme conditions, the plant has developed its own defense system against heat, drought and UV-induced free radicals by accumulating citrulline—a compatible solute.

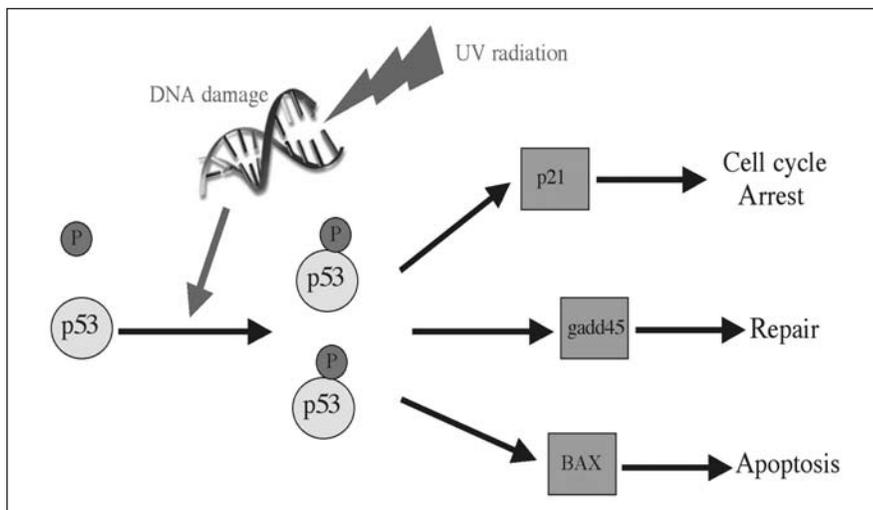


Figure 2. DNA damage results in phosphorylation and thus stabilization of p53. As a consequence of p53 activation, cell cycle arrest, apoptosis or DNA repair can be initiated in the cell.

Search for a Link

The goal of the present study was to incorporate the defense system found in watermelon into a usable form for human skin protection against UV-induced ROS. Therefore, a hydrophilic watermelon extract was chosen and several purification steps such as filtration were applied to the sample to increase the activity on DNA protection. The improvement of skin protection during the purification procedure was demonstrated in the plasmid assay. The plasmid assay is an *in vitro* assay on isolated bacterial plasmid DNA. Induction of single-strand breaks to the supercoiled (intact) plasmid DNA leads to the formation of open circular DNA. In the presence of Cu(II), H₂O₂ causes strand breaks on isolated plasmid DNA.² Through electrophoresis, the open circular plasmid DNA can be distinguished from the supercoiled plasmid DNA (intact DNA) because of the different migration behavior.

The DNA protective effect of the watermelon fraction also has been demonstrated on a skin model based on human keratinocytes

performing the comet assay (single cell electrophoresis assay). The comet assay is a rapid and sensitive fluorescent microscopic method used to examine DNA damage and repair at the individual cell level. Once embedded in a thin agarose gel on a microscope slide, the cells are lysed to remove all cellular proteins, and the DNA subsequently unwinding under alkaline/neutral conditions.

Following unwinding, the DNA is electrophoresed and stained with a fluorescent dye. During electrophoresis, broken DNA fragments (damaged DNA), or relaxed chromatin, migrates away from the nucleus. The extent of DNA liberated from the head of the comet is directly proportional to the DNA damage.

In the next step, the DNA protection activity of the watermelon's natural defense system was shown in a 3-D skin model. The extent of DNA damage and DNA protection after incubation with benzopyrene (BaP) could be directly demonstrated by determining the amount of 7,8-dihydro-8-oxo-guanine (8-oxo-dG) formation. Damaged DNA leads to an increased level of phosphorylated p53. Therefore, phosphorylated p53 should be suitable as a marker for the extent of DNA damage.

Plasmid Assay

DNA strand breaks were measured by the conversion of supercoiled double-stranded DNA^a to open circular form. Then 0.2 µg of DNA was incubated briefly with H₂O₂, Cu(II) and several watermelon extracts after different purification steps in PBS at 37°C at a final volume of 25 µl.

Following incubation, the samples were loaded in 1% agarose gel containing 40 mM Tris, 20 mM sodium acetate and 2 mM EDTA and electrophoresed in a horizontal lab gel apparatus in Tris/acetate/EDTA gel buffer. The DNA was stained with a proprietary unsymmetrical cyanine dye^{b,7}

^a PhiX-174 RF1 is a plasmid DNA manufactured by New England Biolabs.

^b SYBR Gold is a nucleic acid gel stain manufactured by Invitrogen.

^c Nikon E600 is a fluorescence microscope manufactured by Nikon.

Comet Assay

Human keratinocytes were cultivated under standard protocol conditions for keratinocyte cell cultures. To induce oxidative stress, the cells were treated with H_2O_2 . As a positive control, quercetin, known for its DNA protective effect, was chosen. DNA damage and protection appeared immediately after treatment with H_2O_2 and with several concentrations of purified watermelon extract. Therefore the cells were harvested by trypsin-EDTA-treatment, centrifuged for 5 min at 1000 g and resuspended in KFSM-10% FBS.

Then the cells were mixed with low-melting point agarose and extended on slides. After alkaline lysis, the electrophoresis was performed. A fluorescence microscope^c was used for image analysis. The expansion of the comet is expressed as the tail moment, which is calculated by a computer program.

A commercially available *in vitro* 3-D skin model was cultured under appropriate conditions in accordance with the supplier's protocol. After treatment with watermelon extract, the skin model was stressed with benzopyrene (BaP). In order to demonstrate DNA damage, 8-oxo-dG and p53 were detected by using specific antibodies.

Results

Plasmid assay: The DNA sample^a was incubated with 25 μM H_2O_2 and 10 μM Cu(II) in the presence or absence of watermelon extract. The crude watermelon extract showed no protective effect on plasmid DNA (**Figure 3:** lanes 12, 13 and 14), whereas after the first purification step, the watermelon extract seemed to have little protection activity (**Figure 3:** lanes 7 and 8). However, the highly pure watermelon extract showed good protection of the plasmid DNA against damage caused by H_2O_2 /Cu(II) stress.

The plasmid assay proved to be a good tool in increasing protection activity of the watermelon extract during the purification procedure. DNA protection activity was shown to increase after several purification steps, in comparison to the crude extract that did not exhibit protection activity.

Comet assay: High DNA damage, characterized by a long comet, was induced by 100 μM H_2O_2 . Cells were untreated (control and H_2O_2) and treated with quercetin or watermelon extract. After treat-

ment, 100 μ M H₂O₂ were added for 4 hrs and DNA damage was evaluated by comet assay. The purified watermelon extract showed very good DNA protection.

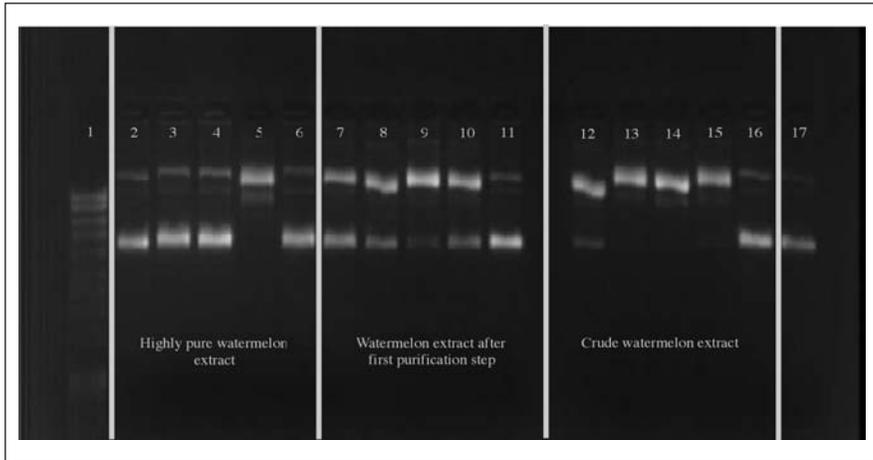


Figure 3. Effects of different watermelon extracts on the H₂O₂/Cu(II)-mediated DNA strand breaks.

Quercetin also showed successful DNA protection and; therefore, was found to be a suitable positive control. comets were reduced after treatment with the watermelon's natural defense system (Figure 4).

Detection of 8-oxo-dG: Human reconstituted epidermis was treated either with four different concentrations of the pure watermelon extract or untreated extract, as a control. The skin models were incubated with BaP and the 8-oxo-dG levels were determined using monoclonal anti-8-oxo-dG antibodies. BaP significantly induced the formation of 8-oxo-dG in the 3-D skin model, whereas the pure watermelon extract provided good DNA protection.

Application of the active fraction of *C. lanatus*' natural defense system substantially inhibited the formation of 8-oxo-dG in the reconstituted epidermis. The protective effect was shown with all tested concentrations (Figure 5).

Quantification analysis indicated a significant reduction of damaged DNA compared to BaP (data not shown).

Detection of phosphorylated p53: An untreated human reconstituted skin model was used as a control and compared to an epidermis treated with four different concentrations of watermelon extract.

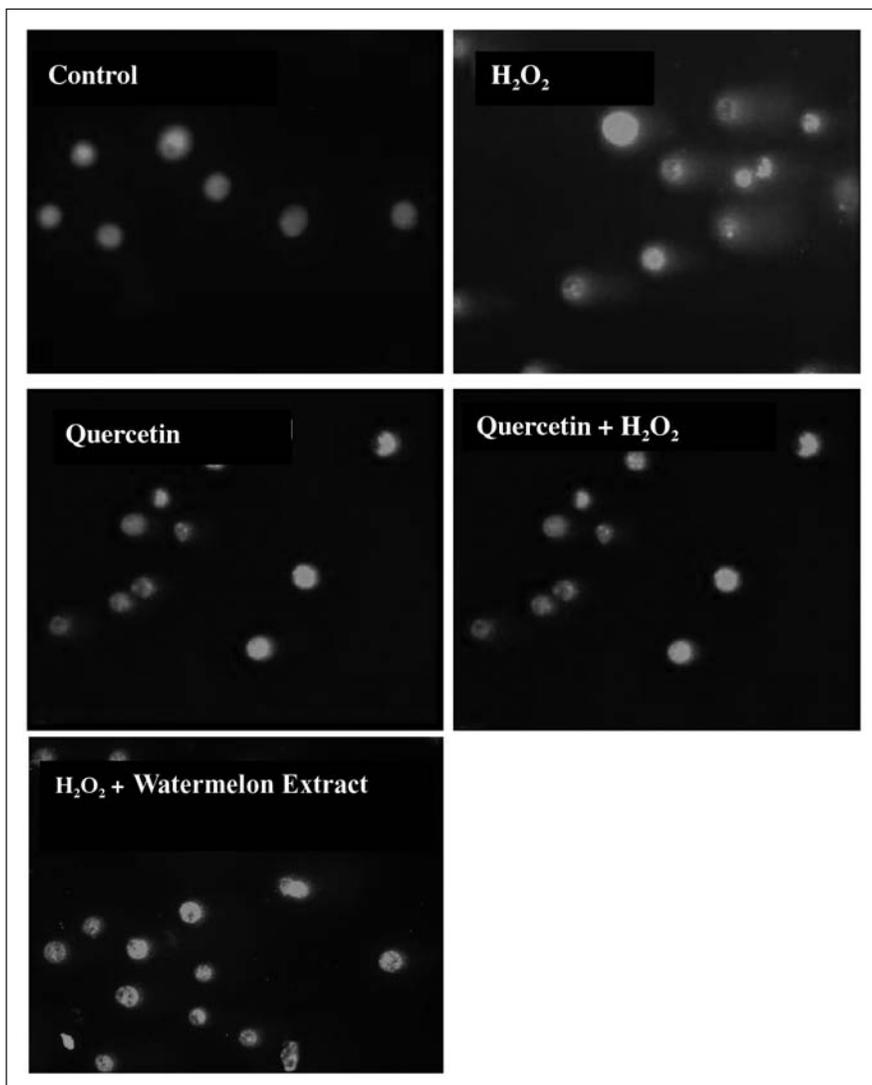


Figure 4. Watermelon extract showed high DNA protection in the comet assay. DNA damage observed by comet assay in keratinocytes.

The amount of p53 in the intact cells was low. BaP addition resulted in DNA damage by formation of 8-oxo-dG. This led to an increase of p53 as shown in this experiment. Purified watermelon extract protected skin DNA in the nuclei from damage, which resulted in a low level of phospho-p53 in the cells (**Figure 6**). Quantification analysis showed a significant reduction of damaged DNA and hence of phospho-p53 compared to BaP (data not shown).

Therefore *C. lanatus*' natural defense system also protects human epidermis from DNA damage caused by extreme stress conditions such as treatment with BaP.

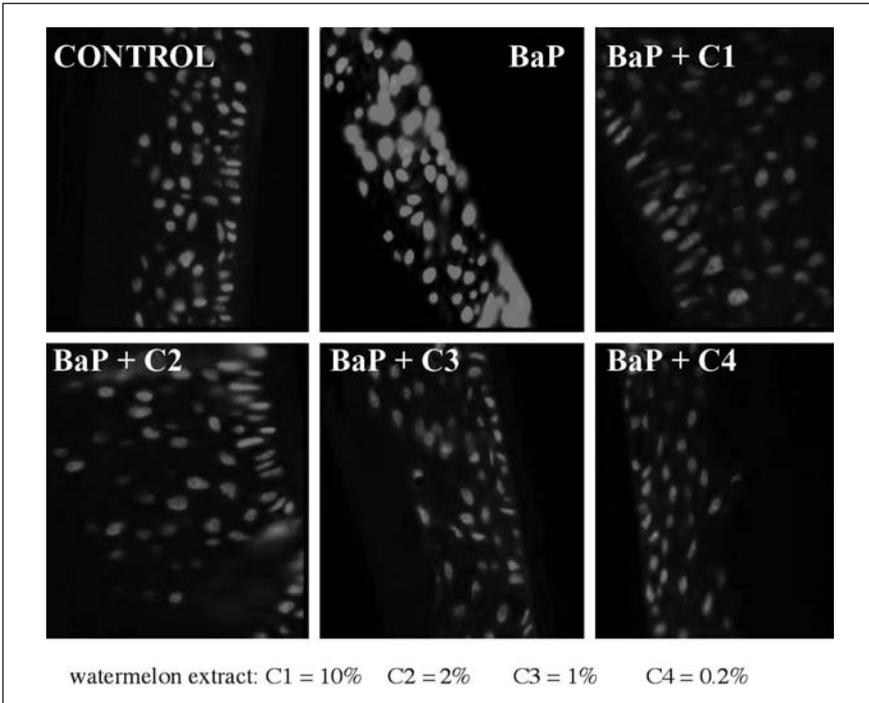


Figure 5. Watermelon extract decreased the formation of 8-oxo-dG in keratinocytes.

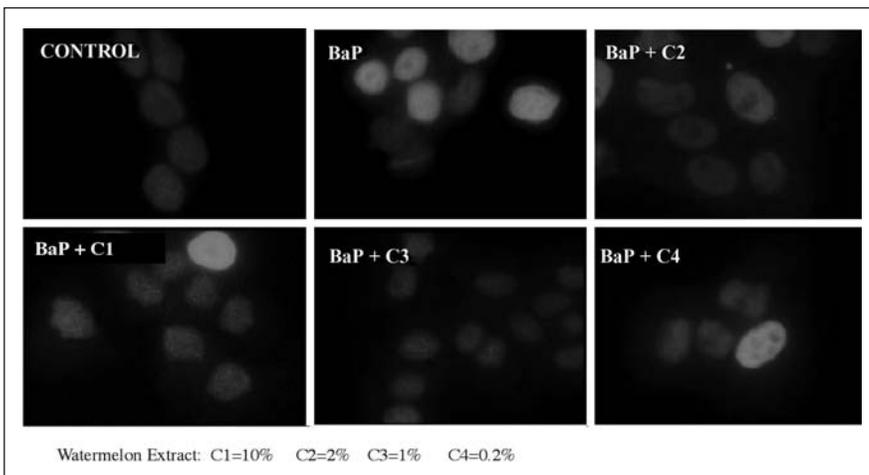


Figure 6. Staining revealed that the percentage of phospho-p53 positive cells labelling is higher in BaP treated cells. As a result of high DNA protection, only low levels of p53 could be detected after treatment with all tested concentrations of the watermelon extract.

Conclusion

Plants and other organisms living under extreme environmental conditions like drought, heat, high UV radiation, pollution and cold have developed their own defense systems to protect important cell structures such as DNA. In this study, *C. lanatus* (watermelon), which synthesizes compatible solutes to protect its essential cell structure from drought and high UV radiation, was shown to have potential applications in skin care to protect skin from environmental stress such as UV radiation and its resulting free radicals.

The DNA protection capabilities of a highly purified *C. lanatus* extract were demonstrated *in vitro* with several cell-based tests including the comet assay, and the efficacy of the active watermelon fraction was further demonstrated in a 3-D skin model *in vitro*. In this *in vitro* model, treatment with radicals caused an increase of 8-oxo-dG and phospho-p53 level in keratinocytes. While 8-oxo-dG is a direct marker for oxidized guanine base, p53 formation is the cell's reply to this damaged DNA. The correlation between 8-oxo-dG and phospho-p53 after cell stress indicates that p53 is an excellent marker to estimate DNA condition and the extent of DNA damage.

Its impressive efficacy in DNA protection has been demonstrated on a human skin model. The purified watermelon extract, containing the plants active principle, shows a natural way of protecting cellular DNA against damage caused by environmental factors. This highly active plant extract is suitable for a range of skin care applications that support the skin's natural defense system and prevent aging.

Published April 2007 *Cosmetics & Toiletries* magazine

References

1. RP Sinha and DP Häder, UV-induces DNA damage and repair: a review, *Photochem Photobiol Sci*, 1(4) 225–236 (2002)
2. H Wei, X Zhang, Y Wang and M Lebowitz, Inhibition of ultraviolet light-induced oxidative events in the skin and internal organs of hairless mice by isoflavone genistein, *Cancer Letters* 185(1) 21–29 (2003)
3. Trautinger, Mechanisms of photodamage of the skin and its functional consequences for skin aging, *Clin Exper Dermatol* 26(7) 573–577 (2001)
4. LVerschooten, L Declercq and M Garmyn, Adaptive response of the skin to UVB damage: role of the p53 protein, *Int J Cos Sci* 28 (1) 1–7 (2006)

5. J Johnson, J Lagowski, A Sundberg and M Kulesz-Martin, P53 family activities in development and cancer: relationship to melanocyte and keratinocyte carcinogenesis, *J Invest Dermatol*, 125(5) 857–864 (2005)
6. D Raj, DE Brash and D Grossman, Keratinocyte apoptosis in epidermal development and disease, *J Invest Dermatol*, 126(2) 243–257 (2006)
7. CS Hsu and Y Li. Aspirin potently inhibits oxidative DNA strand breaks: implications for cancer chemoprevention, *Biochem Biophys Res Comm*, 293(3) 705–709 (2002)

γ -Poly Glutamic Acid: A Novel Peptide for Skin Care

Natalie Ben-Zur and Daniel M. Goldman

Natto Biosciences, Quebec, Canada, Supreme Business Services, Inc., Edison, N.J., USA

KEY WORDS: *polypeptide, exfoliant, humectant, cosmetic application, tensile strength*

ABSTRACT: *Personal care formulators continue to pursue skin care materials that are capable of both moisturization and exfoliation. γ -Poly glutamic acid (INCI: natto gum) meets these multifaceted demands. A review of the literature and research presented here examines the science of natto gum and explores its applications in personal care.*

γ -Poly glutamic acid (γ -PGA) is a novel molecule that is a component of the mucilage of the fermented soybean food product commonly found in Japan called *natto*. The International Nomenclature Cosmetic Ingredient (INCI) name of γ -PGA is *natto gum* and it is classified as a film-forming agent. The sodium salt of γ -PGA, which is more commonly used and thus served as the test sample in this paper, displays the visual appearance of a sticky paste as illustrated in **Figure 1**.

The fermented soybean mucilage consists of a mixture of γ -PGA and fructan produced by *Bacillus natto*.^{1,2} Further research by Bovarnick revealed γ -PGA to be a fermentation by-product freely secreted into growth medium outside of the cell walls of *Bacillus subtilis*.³ This discovery advanced collateral investigations to determine whether this bacteriological process could be reproduced in other species of *Bacillus*.⁴⁻¹¹

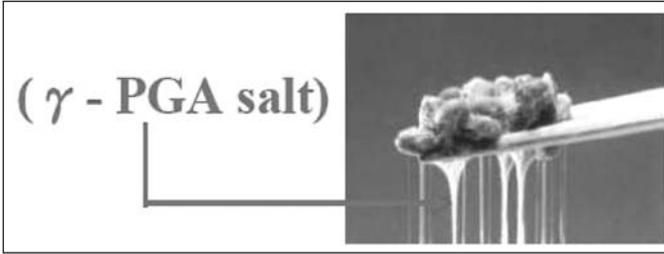


Figure 1. The sodium salt of γ -PGA displays the visual appearance of a sticky paste.

Characteristics of γ -PGA

Recent work by Lung and Da-Yeh further illustrates the applications and production of γ -PGA from microorganisms by gamma irradiating to promote chemical cross-linking between monomeric units to form a γ -PGA hydrogel.¹² The finished gel was lyophilized to assure its physical chemical stability.

The lyophilized product was characterized by Lung and Da-yeh by nuclear magnetic resonance (C^{13} and H^1), Fourier Transform Infrared Spectroscopy and thermal gravimetric analysis in its protonated form, as well as the salts of Na^+ , K^+ , NH_4^+ , Ca^{2+} and Mg^{2+} .

The lyophilized product is a linear homopolypeptide consisting of only glutamic acid residuals composed of both dextro and levo optical isomers. The γ -peptide bond linkages exhibit a range of polymerization from 1000–12000 units. **Figure 2** depicts the repeating units of γ -PGA.

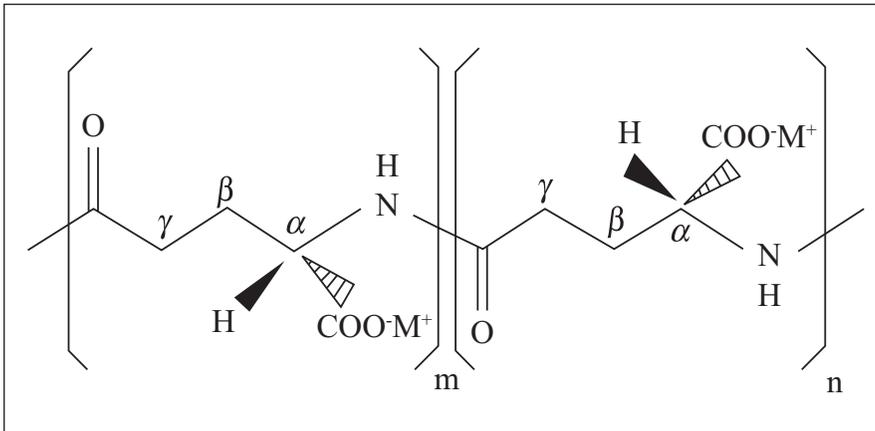


Figure 2. Repeating unit of γ -PGA structure

The molecular weight ranged from 100,000 to 4,000,000 Daltons with an assay value above 95.0%. Physical-chemical properties such as the pH buffering capacity are exhibited in a range of 4.0 to 5.0.

Viscosity can vary as a function of temperature, pH and concentration to influence performance characteristics. The protonated form of γ -PGA is completely soluble in trifluoroacetate, dimethyl sulfoxide, hydrazidine and a high concentration of sulfuric acid. The sodium form, which is soluble in water, can assume different molecular conformations depending on pH conditions and binding to other counter ions. **Figure 3** illustrates the elucidated structure of the protonated form of γ -PGA and **Figure 4** depicts the elucidated structure of the sodium salt of γ -PGA.

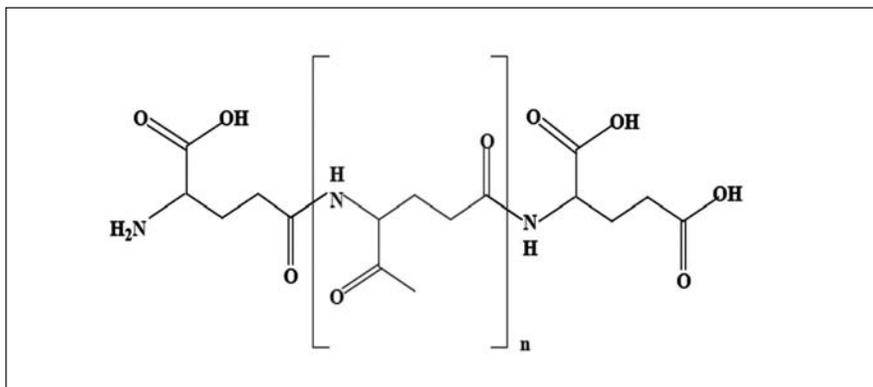


Figure 3. The elucidated structure of the protonated γ -PGA, where $n=H, Na^+, Ca^{++}, Mg^{++}, K^+$.

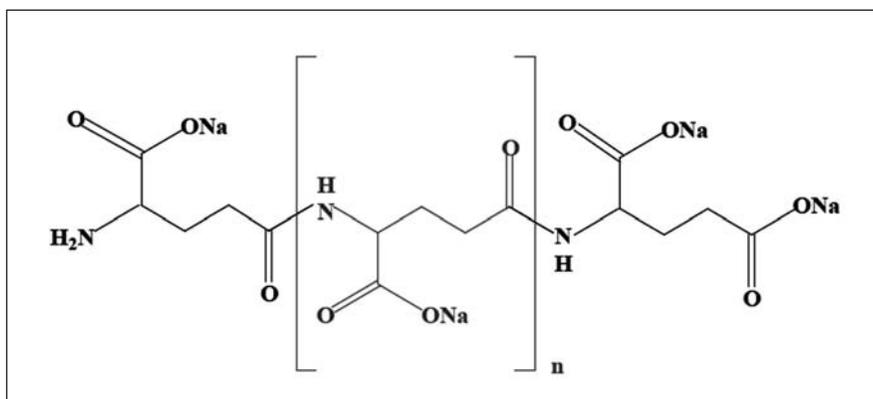


Figure 4. The elucidated structure of the sodium salt of γ -PGA, where $n=H, Na^+, Ca^{++}, Mg^{++}, K^+$.

The chemistry of γ -PGA allows it to be homogeneously miscible as well as chemically stable in a matrix of ingredients typically used in facial creams, such as: glycerin, butylene glycol, alcohol, beta-glucan, hydrogenated lecithin, PEG-50, triethanolamine, disodium EDTA, methylparaben, ethylparaben, propylparaben, fragrance (*parfum*), vitamin E, carbomer, beta carotene, cetyl alcohol and disodium EDTA.

Applications in Cosmetics

Additional qualities of γ -PGA include the ability to form a smooth, elastic, self-moisturizing and soft film on the skin, resulting in improved sensory perception and protection of the outer layer of skin.¹³ It is an excellent hydrophilic humectant and is capable of increasing the production of such natural moisturizing factors as pyrrolidone carboxylic acid (PCA), lactic acid and urocanic acid, as compared to hyaluronic acid and soluble collagen.¹³ Additional work by Ichimara further supports these findings as illustrated in **Figure 5**.¹⁴

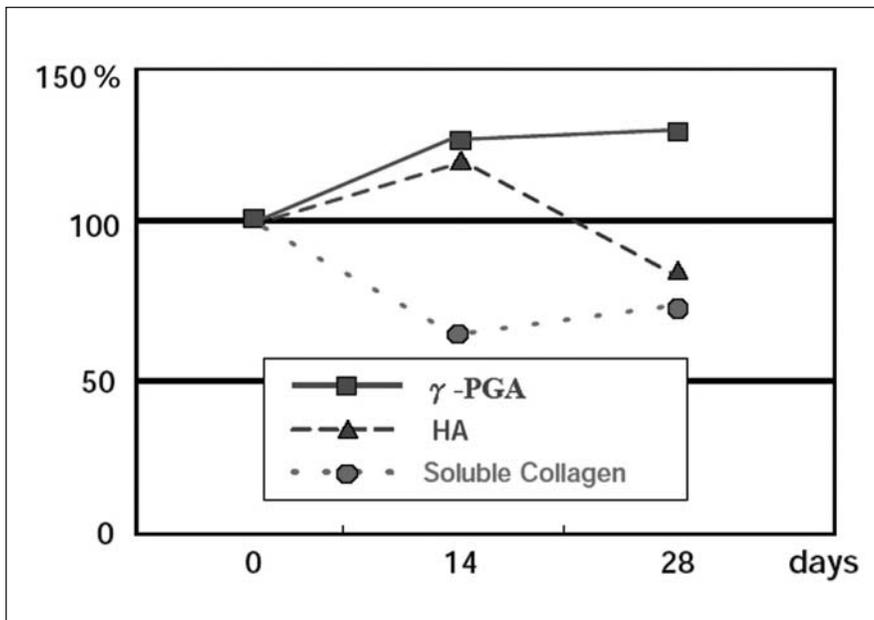


Figure 5. Comparison of γ -PGA, hyaluronic acid (HA) and soluble collagen in the increase of NMF in the stratum corneum

γ -PGA also offers antiaging benefits. A study conducted by Meiji Seika examined the ingredient's potential as a natural replacement for collagen or hyaluronic acid.¹⁵ The origin of γ -PGA from fermented soybeans and its composition of enzymes, oligosaccharides and vitamins was shown in cosmetic formulations to favorably augment the natural moisturizing factor and elasticity of the skin more than collagen and hyaluronic acid during a 28-day period, as detailed in **Figure 6**. The study suggests that γ -PGA could successfully replace collagen and hyaluronic acid in skin care products.

As **Figure 6** demonstrates, products containing γ -PGA reinforce the skin's support structure to energize cells, stimulating the production of lipids and renewal of the epidermis. It has been shown to replenish and nourish the skin, leaving it smoother and plumper; it also is nontoxic to humans, natural and environmentally friendly. The general range of use level is from 0.3–2.5%, depending on the product form being deployed for end product use. The acute oral toxicity test for γ -PGA in the form of its sodium salt gave an $LD_{50} > 5,000$ mg/kg/day. Biodegradability tests of the sodium salt of γ -PGA revealed that 90% degraded in 180 days. **Table 1** compares characteristics of γ -PGA to other peptides.

γ -PGA contains a robust amount of vitamins, oligosaccharides and beneficial enzymes currently not found in the nonfermented soybean product.¹² It can be described as a white to off-white, granulated, free-flowing powder. Purity by HPLC is a minimum of 70%, and the minimum assay value determined by gravimetric analysis of the salt of the γ -PGA is no less than 90% wt/wt. The loss on drying is no more than 10% wt/wt γ -PGA. The heavy metals composition is no more than 0.002% and the total bacterial count test for microorganisms is not greater than 500 CFU/g for a cosmetic-grade material. *Salmonella* and *Escherichia coli* are absent in γ -PGA. Its particle size is 100% through 100 mesh screen.

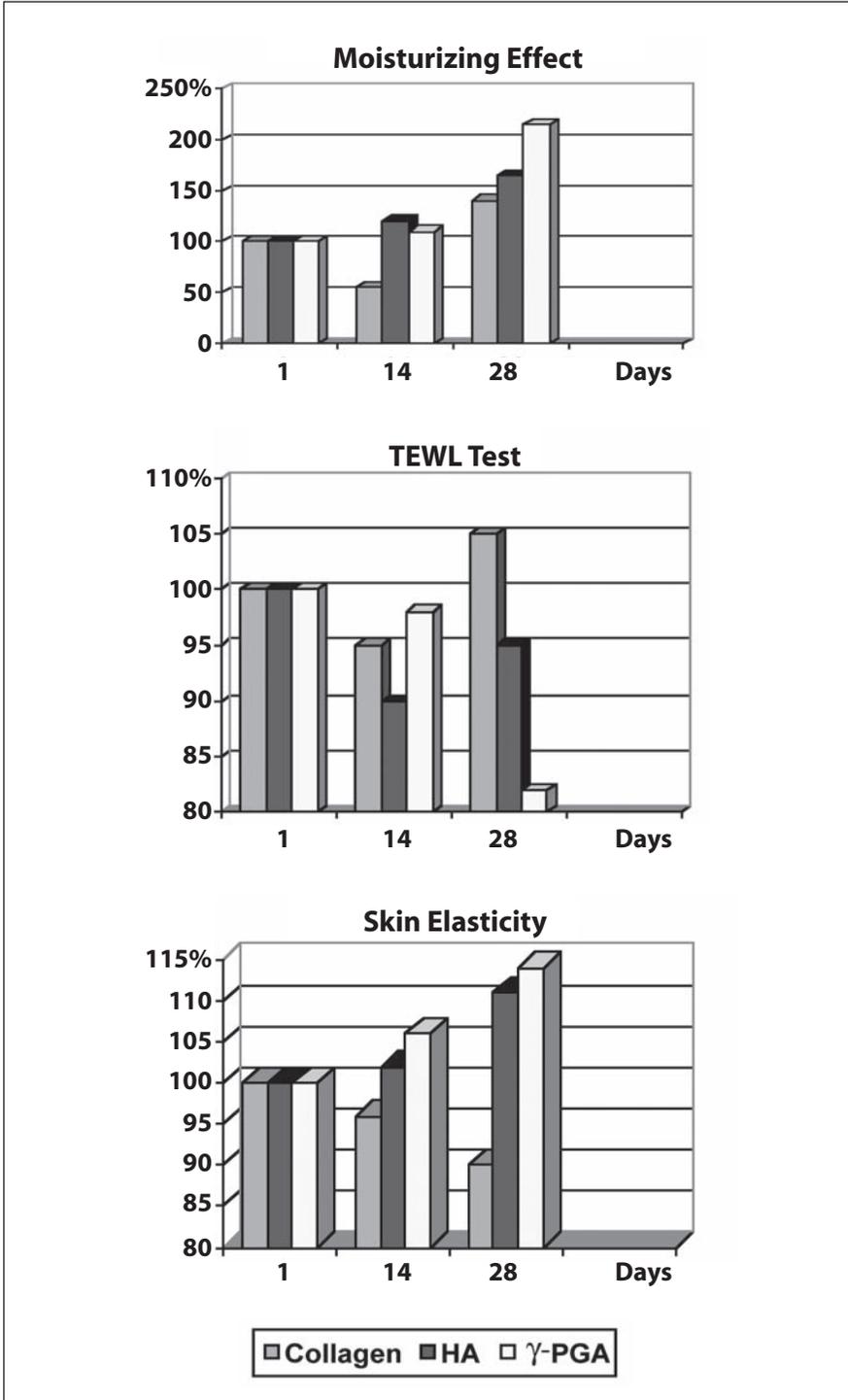


Figure 6. Comparison of collagen, HA and γ -PGA in various parameters over a 28-day period

Table 1.

	γ -PGA	Common Peptide
Building block	Glutamic acid	All amino acids
Optical activity	D-,L-	L-
Type of linkage	γ -peptide	α -peptide
R group	Hydrophilic	Hydrophilic, hydrophobic
Molecular size	10,000 residuals	Less than 10,000 residuals
Gene messages	Nonstandard	Genetic code
Digestible in human body	Slowly	Quickly
Biosynthesized by microorganisms	Rarely	Commonly
Essential for life	No	Yes

γ -PGA Hydrogel

γ -PGA hydrogel is another form of γ -PGA. It is produced by gamma irradiating PGA to promote chemical cross-linking between monomeric units. Microscopic examination of the γ -PGA hydrogel reveals a multi-baglike structure that is capable of enhanced absorption of moisture because of the cross-linking of the peptides (see **Figure 7**).^{16–18}

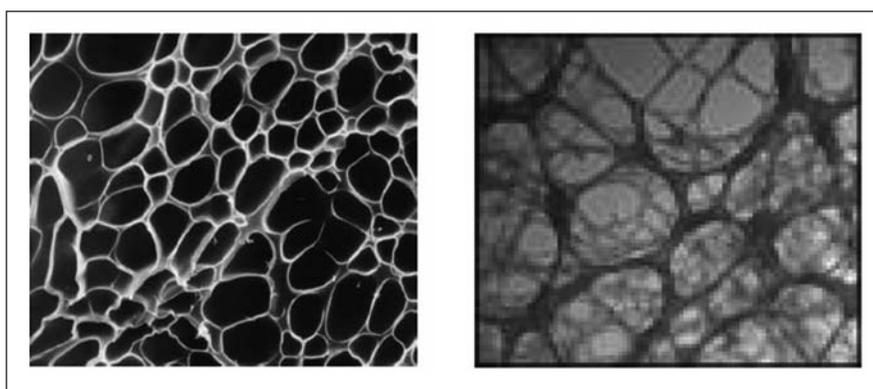


Figure 7. Microscopic examination of the γ -PGA hydrogel reveals a multi-baglike structure.

The physical-chemical properties of the hydrogel enable it to absorb moisture 5,000 times its own weight.^{16,17} The amount of water contained within the γ -PGA hydrogel is influenced by pH

and salt content. The specific water contents and degree of swelling markedly decrease with the addition of electrolytes and pH adjustment to an acidic environment because of the conformational changes that the molecule undergoes in acidic pH. This allows cosmetic formulators to enhance the moisturizing ability of hair or skin formulations with the addition of the proper amount of electrolytes and appropriate pH adjustments. The release of moisture forms a film of protection, which is important in applications such as facial masks that are used to hydrate the skin as well as reduce the appearance of wrinkles. The conceptual before-and-after effects of the γ -PGA hydrogel on the natural moisturizing factor of the skin are depicted in **Figure 8**.

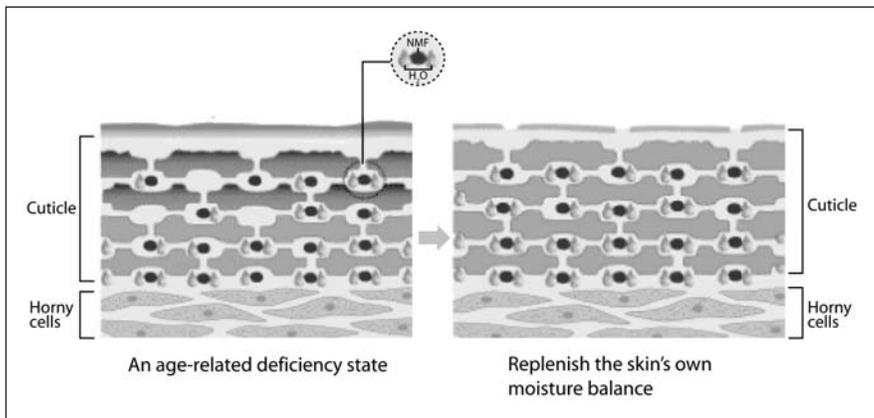


Figure 8. The before-and-after effects of the γ -PGA hydrogel on the natural moisturizing factor of the skin

The moisture loss of the skin was significantly reduced with the use of γ -PGA, compared to its absence. **Figure 9** shows the effects of γ -PGA on transepidermal water loss over a 3 hr time period.¹⁹

Equivalency studies²⁰ were performed against glycerol to examine moisturizing efficiency and water retention on the IMS membrane. The results shown in **Figure 10** reveal that lower concentrations of PGA hydrogel yielded approximately the same results of water retention as higher concentrations of glycerol. This observation reveals that the hydrogel has potential as an alternative to glycerol in cosmetic formulations.

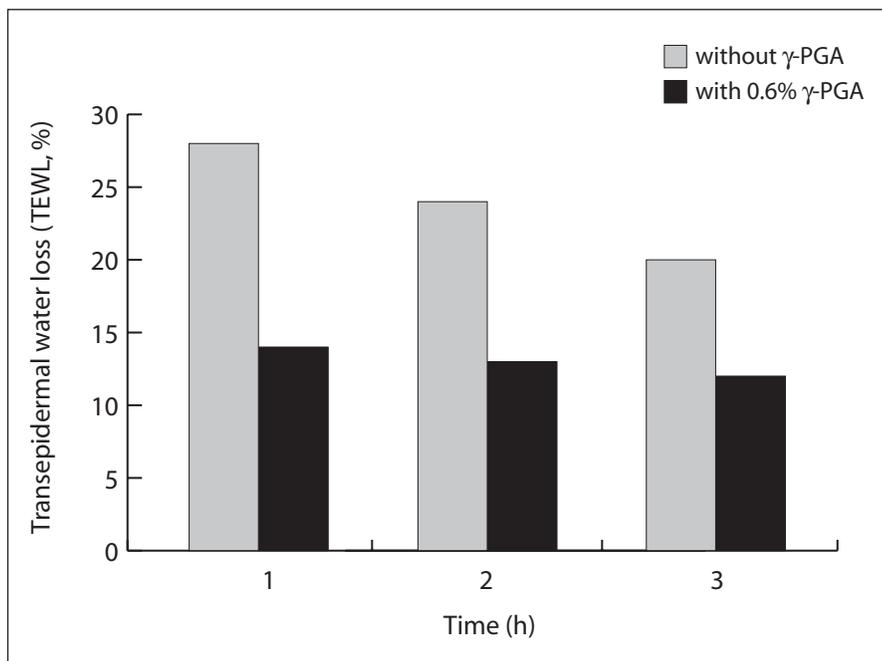


Figure 9. The effects of γ -PGA on TEWL

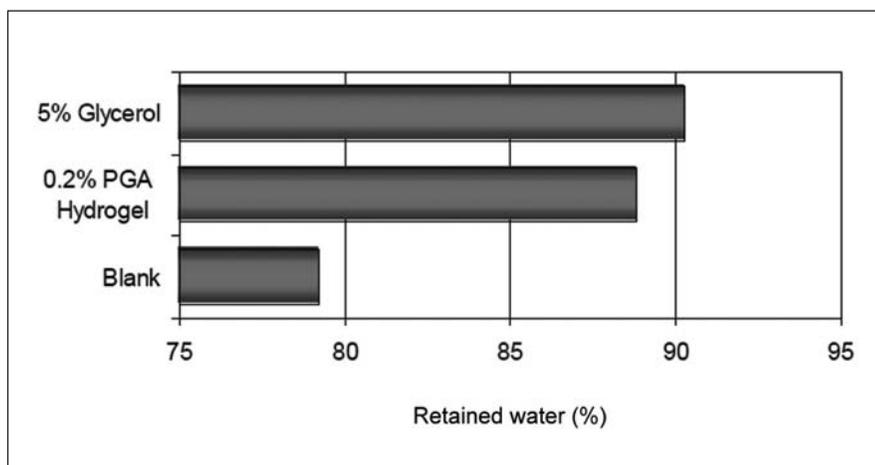


Figure 10. Lower concentrations of PGA hydrogel yielded approximately the same water retention results as higher concentrations of glycerol.

Hair Applications

In addition to skin care, research²⁰ has shown that γ -PGA can be used as an additive to increase hair strength to better withstand the

bleaching process. The material maintains hair strength by increasing its natural moisture retention ability and forming a protective layer on the hair.

It also exhibits the ability to release moisture within the hair formulation to form a barrier capable of diluting the chemical interactions of the applied colorings with the protein makeup of the hair. This diluting action helps to maintain the strength of hair.

The layer formed by γ -PGA was also found to protect hair from damage incurred by bleaching. Investigations were continued to study the effects of the material on maintaining hair strength and preventing hair damage due to bleaching processes (see **Figure 11**).²⁰

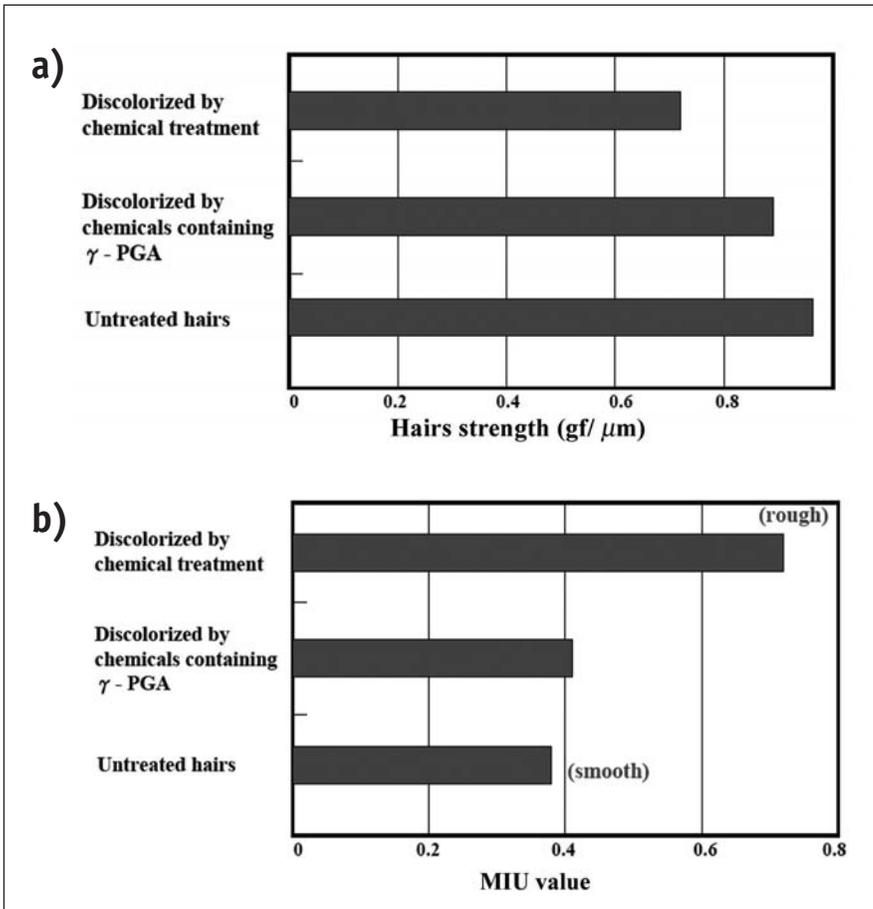


Figure 11. The effects of γ -PGA on a) maintaining hair strength and b) preventing hair damage during chemical processing; comparison of hairs MIU value by different treatments.

Electron microscopy images were taken²⁰ (see **Figure 12**) and detail the differences in physical appearance of hair treated with and without γ -PGA.

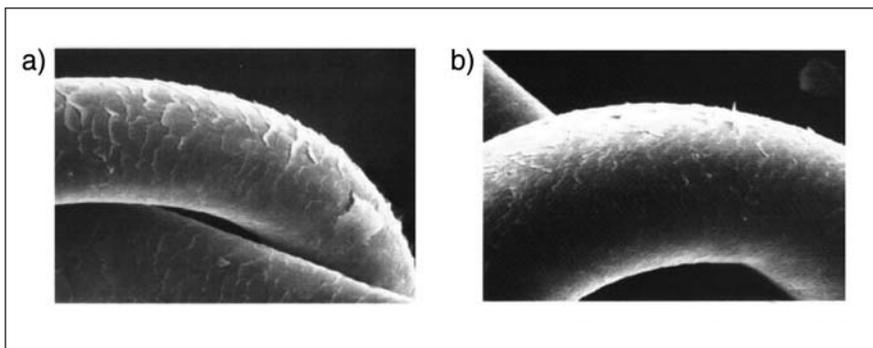


Figure 12. Electron microscopy images of the differences in physical appearance of hair treated a) with and b) without γ -PGA; comparison of hairs MIU value by different treatments.

Treatments exposed the hair to 20 min of bleaching for coloring purposes. A 1.5% sample incorporating γ -PGA was added in the bleaching products to help diminish the damage caused to hair.²⁰

Conclusion

The use of γ -PGA as a value-added component to enhance the properties of existing or novel personal care products as a moisturizer, exfoliant and wrinkle-remover should be investigated through laboratory studies to optimize product development.

By understanding the physicochemical properties of γ -PGA, formulators can develop cosmetic products capable of being both a moisturizer and an exfoliant. The insight gained from the application of γ -PGA could enable formulators to reinvigorate products lacking these attributes. The industry as a whole can benefit from the use of γ -PGA as a low cost, environmentally friendly component with wide ranging applications.

Published April 2007 *Cosmetics & Toiletries* magazine.

References

1. S Sawamura, *Bacillus natto*, J Coll Agri Tokyo 5 189–191 (1913)
2. H Fujii, The formation of mucilage by bacillus natto, part III: Chemical constitutions of mucilage in natto (1), *Nippon Nogeikagaku Kaishi* 37 407–411 (1963)
3. M Bovarnick, The formation of extracellular D(-)glutamic acid polypeptide by bacillus subtilis, *J Biol Chem* 145 415–424 (1942)

4. C Cheng, Y Asada and T Aida, Production of γ -polyglutamic acid by bacillus subtilus A35 under denitrifying conditions, *Agric Biol Chem* 53 2369–2375 (1989)
5. A Goto and M Kunioka, Biosynthesis and hydrolysis of poly γ -glutamic acid from bacillus subtilus IFO3335, *Biosc Biotechnol Biochem* 56 1031–1035 (1992)
6. T Hara and S Ueda, Regulation of polyglutamate production in bacillus subtilus (natto): Transformation of high PGA productivity, *Agric Biol Chem* 46 2275–2281 (1982)
7. RD Housewright, The biosynthesis of homopolymeric peptides in The bacteria: A treatise on structure and function, vol III, eds IC Gunsalus and RY Stanier, Academic Press, New York, 389–412 (1962)
8. H Kubota, Y Nambu and T Endo, Convenient and quantitative esterification of poly (γ -glutamic acid) produced by microorganism, *J Polym Sci Part A: Polym Chem* 31 2877–2878 (1993)
9. S Muraao, The polyglutamic acid fermentation, *Koubunshi* 16 1204–1212 (1969)
10. CB Thorne, CG Gomez, HE Noyes and RD Housewright, Transamination of D-amino acids by bacillus subtilus, *J Bacteriol* 68 307–315 (1954)
11. FA Troy, Chemistry and biosynthesis of the poly (γ -D glutamyl) capsule in bacillus licheniformis. 1. Properties of the membrane mediated biosynthetic reaction, *J Biol Chem* 248, 305–316 (1973)
12. IL Lung and YT Van, Da-Yeh University, Taiwan, *Bioresource Technol* 79 207–225 (April 2001)
13. JP Patent 11240827, Preparation composition for external use containing gamma polyglutamic acid and vegetable extract in combination, issued to K Hasebe and M Inagaki (1999)
14. Increase of NMF in the stratum corneum by γ -PGA, HA and soluble collagen, data provided by Ichimaru, Japan (2002)
15. Comparison table of the effectiveness of γ -PGA, HA and Soluble collagen in cosmetic formulations, data provided by Meiji Seika, Japan (2003)
16. M Kunioka, Properties of hydrogels prepared by irradiation in microbial poly (γ -glutamic acid) aqueous solutions, *Kobunshi Ronbunshu* 50 755–760 (1993)
17. HJ Choi and M Kunioka, Preparation conditions and swelling equilibria of hydrogel prepared by γ -irradiation from microbial poly γ -glutamic acid, *Radiat Phys Chem* 46 175–179 (1995)
18. HJ Choi and M Kunioka, Synthesis and characterization of pH sensitive and biodegradable hydrogels prepared by γ -irradiation using microbial poly-glutamic acid and poly lysine, *J Appl Polym Sci* 58 807–814 (1995)
19. Data collected from Providence University, Taiwan (2001)
20. Data collected by Ichimaru, Japan (2003)

Nutricosmetics: Feeding the Skin

Bud Brewster

Cosmetics & Toiletries *magazine*

KEY WORDS: *nutricosmetics, skin, vitamins, minerals, nutrition*

ABSTRACT: *Do nutricosmetic products actually feed the skin, or are they merely feeding the youth appetite of an aging consumer?*

Everybody agrees that fruits, vegetables, vitamins and minerals are beneficial to human health. Humans have been eating them since the days of Adam and Eve. The question is: Do fruits, vegetables, vitamins and minerals added to cosmetics have a measurable effect on the health and appearance of skin, hair and nails? To put it another way, do nutricosmetic products—which the Kline Group values as a \$1.5 billion global market with 12% growth per annum¹—actually feed the skin, or are they merely feeding the youth appetite of an aging consumer?

The search for the science behind nutricosmetics often leads to a blind alley. For example, as this is written in December 2008, the term *nutricosmetic* yielded not a single “hit” anywhere in all the patents and patent applications on file at the Web site of the US Patent and Trademark Office. Similarly, the Wikipedia page for the term *nutricosmetic* had been deleted in November. The deletion log contained two explanations. One said there was “no indication that the article may meet the guidelines for inclusion.” The other called the deleted article “blatant advertising.”

Advertising has certainly contributed to the growth of the nutricosmetics market. So has the muscle of big companies. In 2002, cosmetics giant L’Oréal and food products leader Nestlé cooperated to create the Inneov range of nutricosmetic products, attracting

follow-up activity at Estée Lauder, Amore Pacific and Shiseido. Many companies, seeing nutricosmetics as a way to add value to their formulations, are competing with their own product lines. For example, the Inneov range, which is used for antiaging, is a skin care product that not only prepares the skin for solar exposure, but also intensifies the tanning process.²

This Bench & Beyond column will look at a few of the nutritional ingredients being added to cosmetics to promote the concept of beauty through a healthy body. Both the claims and the claims support (if any) will be discussed. The column will end with a suggestion about where to find the science behind nutricosmetics.

Skin Nutrition

Inside out or outside in: Nestlé is the world's largest food conglomerate. Its nutrition research occurs at the Nestlé Research Center (NRC) in Lausanne, Switzerland. In this decade, the NRC has extended its expertise in nutrition research beyond foods and beverages, to nutritional supplements for skin and hair health and beauty. The premise is that the skin is not only nourished on the outside with moisturizing creams and topical solutions, but also with nutrients to nourish from the inside.

In collaboration with L'Oréal Dermatological Research, NRC scientists are actively researching nutrients that promote antiaging benefits. Research in this area focuses on the selection of ingredients and the understanding and control of nutrient bioavailability in plasma, skin or hair, and the demonstration of the bioefficacy of these nutrients taken orally:³

- Vitamin A (carotenoids) to maintain and repair skin tissue.
- Vitamin C to reduce the damage caused by free radicals and UV exposure. Over time, free radicals can damage collagen and elastin, the fibers that support skin structure.
- Vitamin E to lessen the skin effects of free radicals and UV exposure.
- Probiotics to improve recovery of the skin and cellular defenses after UV exposure.
- PUFAs (polyunsaturated fatty acids) to reduce dry, scaly

skin. PUFAs play an important role in cell structure, barrier function, lipid synthesis, inflammation and immunity.³

Israeli Biotechnology Research Ltd. (IBR) develops innovative and proprietary natural active ingredients for the cosmetics, therapeutic and foods industries. Its vice president of business development and marketing is Liki von Oppen-Bezalel, who agrees that skin nourishment and protection could be delivered either via supplementation (oral intake) or topically (cosmetics). Each has its pluses and minuses.

“When beauty aids are delivered from within, they face several barriers—bioavailability, bio-distribution and delivery to the skin as the target organ, metabolism and degradation during the long journey from the mouth to the skin,” Oppen-Bezalel tells *C&T* magazine. “However, when these barriers are crossed and the active molecules are delivered to the skin, they enter from the inside and move toward the outer layer. They are still found in the deeper layers of the skin where they can do their work effectively.”

The other route of skin nourishment is from the outside, applying the ingredients topically. “Topical application of actives and ‘skin foods’ has the advantage of easy delivery and achieves effective levels of the materials even when they are applied in small quantities,” Oppen-Bezalel says. “However, topical application involves fast metabolism and degradation on the skin before getting to the place of action by either oxidation or enzymes localized in the skin that destabilize and degrade the actives. And in principle, any penetration to the deeper layers of the skin is an ambivalent desire, based on the definition of the term *cosmetic*.”

Oppen-Bezalel notes that discussion continues about the contribution to skin health and appearance made by various common food ingredients, such as coenzyme Q10, various fatty acids, flavonoids, polyphenols, vitamins A, E and C, minerals, some carotenoids and more. Furthermore, each has “pros and cons for either topical or oral application including stability, formulation issues, color, bioavailability and proven benefits,” she adds.

Drinkable or oral: A 2007 Frost & Sullivan market report² identifies two main categories of nutricosmetics:

- Drinkable nutricosmetics that are taken in liquid form or yogurts, fortified with minerals and vitamins, for better skin care and body health. Examples of drinkable nutricosmetics marketed for skin care properties include Borba's skin balancing water, Groupe Danone's Essensis beauty yogurt and Coca-Cola's Lumae tea.
- Oral nutricosmetics that are ingested as pills for purposes such as antiaging and skin care. Some popular brands include
- Inneov by L'Oréal and Nestlé, Imedeen by Ferrosan, and the Olay Vitamins line from Procter & Gamble.

Both of these types of nutricosmetics enhance skin and hair condition and also protect the skin from UV damage, according to Frost & Sullivan.

Ingredients and Tests

Frost & Sullivan's list of the major ingredients used currently in nutricosmetics includes soy isoflavone proteins, lutein, lycopene, vitamins (A, B6, E), omega-3 fatty acids, beta-carotene probiotics, sterol esters, chondroitin and coenzyme Q10. They are antioxidants promoted for their skin care properties and antiaging effects on free radicals. They also claim anti-inflammatory action to protect the skin against UV radiation.

Discussion of any comprehensive list of nutricosmetic ingredients is beyond the scope of this column, so here is only a sample of those ingredients (**Figure 1**), followed by available research supporting their claims.

Phytoene and phytofluene from tomatoes: IBR Ltd. offers the colorless carotenoids phytoene and phytofluene, which are obtained from tomatoes and the edible unicellular algae of the *Dunaliella* species.

There are more than 700 different carotenoids, many of which differ in the health benefits they offer and the body sites where they are found. Unfortunately, most carotenoids are sensitive to light, a property that considerably limits their use and shortens the shelf-life of products that contain them. In addition, almost all carotenoids have a distinctive visible color, which limits their utility for cosmetic applications. However, phytoene and phytofluene are colorless

(i.e., they absorb light only in the UV range) and dietary phytoene and phytofluene have been shown to accumulate in human skin.⁴ According to IBR Ltd., this accumulation potentially can protect the skin by several mechanisms: as UV absorbers, as antioxidants, and as anti-inflammatory agents. Therefore, IBR has developed both topical^a and dietary^b tomato-based products to bring phytoene and phytofluene to the skin.



Figure 1. Fruits (tomato, kiwi, lychee) for feeding the skin

Lycopene from tomatoes: Scientists at the NRC developed a food ingredient, Lactolycopene, to enhance the bioavailability of the carotenoid lycopene, a nutrient found commonly in tomatoes and tomato products. This unique preparation contains the antioxidant lycopene dispersed among milk proteins for increased nutrient bioavailability.

Lactolycopene was later combined with vitamin C and soy extract in a nutricosmetic supplement called Innéov Fermeté. This antiaging supplement reportedly helps to improve skin firmness and density.

^a IBR-TCLC (INCI: Squalane (and) Solanum Lycopersicum (Tomato) Fruit Extract) is a product and registered trademark of IBR Ltd., Ramat-Gan, Israel.

^b PhytoFLORAL TP, a dietary supplement made by IBR Ltd., is a tomato powder rich in phytoene, phytofluene and beta-carotene. PhytoFLORAL is a registered trademark of IBR Ltd.

The effects of active ingredients in Fermeté in menopausal women have been studied. A clinical test on women aged 45 and over confirmed that after three months of daily consumption, Fermeté efficiently affected the metabolic processes involved in skin aging, modifying skin physiology, and providing antiaging benefits.³

Kiwi seed extract: In 2007, online sources⁵ reported that a company in New Zealand sought to make a nutricosmetic ingredient from the seeds of the kiwi fruit. Kiwifruit Extract Venture Ltd. was already supplying the cosmetics industry with kiwi oil, which contains omega-3 alpha-linolenic acid, and the fruit contains vitamins C, A and E, potassium and flavonoid antioxidants, but the company found that the seeds, which are typically not digested by humans, are a source of two antioxidant flavonoid glycosides: quercitrin (a precursor to quercetin) and kaempferol.

Initial research had centered around topical application of the seed extract, but the company discovered that both the kiwi oil and the seed extract can be taken orally in supplement form. Soon came preliminary evidence that 50 mg of the antioxidant extract taken in oral supplement form can have an improving effect on skin, notably in reducing fine lines and wrinkles, the company reported.

Separately, a 2006 Japanese study conducted on guinea pigs⁶ examined the effects of an aqueous ethanol extract prepared from defatted kiwi seed and its constituents quercitrin and kaempferol (KSE) 3-O-rhamnoside on acne and melanin formation. The authors concluded that the extract “inhibits the enzyme activities involved in acne and melanin formation, and oral administration of KSE is effective in eliminating skin pigmentation.”

Lychee extract: Laboratoires Sérobiologiques (LS) describes its lychee extract^c as “a holistic active ingredient, multifunctional, fruity and attractive, bringing essential ‘well-being’ elements to the skin.” The ingredient is extracted from the pericarp of lychee (*Litchi chinensis* Sonn.), a fruit native to southern China and valued for thousands of years as an astringent, analgesic, stomachic and fortifier. Tea made from the pericarp of the fruit is said to cure skin rashes. LS discovered that extracts from the pericarp also strengthen the skin’s natural defense mechanisms in a number of ways.

^c Litchiderm LS 9704 (INCI: Butylene Glycol (and) Litchi Chinensis Pericarp Extract) is a product of Laboratoires Sérobiologiques, Division of Cognis France.

LS used several tests to support claims for the efficacy of its lychee extract.⁷ A clinical study of skin complexion on 20 women found that the group using a cream containing the lychee extract at 5% for eight weeks scored an improvement in various skin complexion parameters (such as color of the cheekbones and facial tonicity) evaluated on a semiquantitative scale by trained specialists, and this improvement was 39% greater than for a group using a placebo cream. An *in vivo* test demonstrated that the ingredient's scavenging effect was similar to tocopheryl acetate in protecting against oxidative stress. An *in vitro* test indicated good protective activity against UVB- and UVA-irradiated stress. *In vitro* and *ex vivo* tests showed the ingredient's ability to reduce the release of MMP-1 after UVA and UVB irradiation, demonstrating protection against photoaging. Also, a Tagami test showed the ingredient's moisturizing activity lasts up to 24 hr.

Beauty foods: An article⁸ appearing in the November 2006 issue of *Nutraceuticals World*, titled "Beauty from the Inside Out: Claims Versus Science in Cosmeceuticals and Beauty Foods," authored by Joerg Gruenwald, president of Analyze & Realize AG, a Berlin-based specialized business consulting company for herbal medicine, dietary supplements and functional foods, defines *beauty foods* as "common food ingredients that are now entering consumers' awareness as not only being healthy, but also as having a beneficial impact on overall appearance." In contrast to cosmetics and topically used cosmeceuticals, beauty foods claim to improve appearance from within the body. Among the claimed effects are wrinkle reduction and general improvement of facial skin structure, cellulite reduction and body shaping through slimming, and improved structure of nails and hair, but Gruenwald admitted that it is difficult for the consumer to know whether these claims have any basis in fact.

Gruenwald analyzed a variety of available natural or herbal beauty foods, their claims, and the existing clinical evidence supporting those claims. His conclusion was that in general, most cosmeceuticals and beauty foods contain low levels of active components but high levels of claims "without backing those claims with proof of any kind.

"Nevertheless," he concluded, "there is encouraging scientific evidence for the effectiveness of some products, even if the methodology often leaves room for improvement."

Where's the Science?

Where is the science on nutricosmetics that encourages Gruenwald but leaves others like Allen Burke⁹ of QVC and Ralph Bronner¹⁰ of Dr. Bronner's Magic Soaps unconvinced?

The foods industry has the science. For example, BASF is already using food science technology in skin care, according to Serge Rogasik, global marketing director for BASF Beauty Care Solutions. "Several of our most successful topical products have roots in the nutrition world. Retinol and caffeine are obvious examples, but the dill extract behind the Lys'lastine^d concept or Smartvector UV CE^e are all based on food technologies," Rogasik told *C&T* magazine.

The pharmaceuticals industry has the science. The only academic department known to this columnist with a scientific focus on nutricosmetics is the Department of Pharmaceutical Technology, Biopharmaceutics & Nutricosmetics, at the Free University of Berlin. At least one member of that department, R.H. Müller, has published in cosmetics and personal care technical journals, but his subject was nanotechnology, not nutricosmetics. One might even argue that Berlin has the science, because both Free University and Gruenwald are located in that city.

Europe has reason to obtain the science. It is the largest nutricosmetics market, representing 55% of the \$1.5 billion world market, with 41% going to Japan and 3% to the United States, according to Kline & Company in 2008.¹ With the publication of the EU Health Claims Directive (EU 1924/2006), standardized scientific evaluation and evidence of product-related health claims became a legal requirement across Europe in 2006. Gruenwald argues that more proven scientific background on product usage required by that directive will improve the image of the nutricosmetics segment.

Corporate research labs may have the science. After all, the nutricosmetic antiaging boom started in the research labs of L'Oréal and Nestlé. Jay Tiesman, principal scientist/genomics group leader at Procter & Gamble, also was quoted in *FastTalk* magazine: "Our labs can measure not only what's going on at the top of the skin, but also how it responds from the inside. We're gauging its response to exterior damage as well as nutrition. What nutrition triggers a response

on the skin's surface? 'There's still a lot of snake oil out there, but we actually have a much better understanding of beauty products, with much more soon to come.'⁹

Published February 2009 *Cosmetics & Toiletries* magazine.

References

1. www.klinegroup.com/news/speeches/Nutricosmetics-apr08.pdf
2. *Nutricosmetics—Health and beauty within and without*, Frost & Sullivan Market Insight (May 25, 2007)
3. www.foodpacific.com/insight/insight.cfm?object_id=28335&item_name=analysis&displayEditorial=no&List=1
4. F Khachik et al, Chemistry, distribution, and metabolism of tomato carotenoids and their impact on human health, *Exp Biol Med* 227 845–851 (2002)
5. www.nutraingredients.com/Industry/NZ-company-explores-kiwi-nutricosmetic
6. J Tanaka, S-J Shan and H Shimoda, Effect of kiwi seed extract and its flavonoid glycosides on skin functions, *Fragr J* 34(10) 69–74 (2006)
7. www.laboratoires-serobiologiques.com/portals/0/pdf/MP_Litchiderm_EN.pdf
8. http://findarticles.com/p/articles/mi_hb223/is_ai_n29309654?tag=artBody;col18.
9. www.fastcompany.com/magazine/131/fast-talk-beauty-on-the-inside.html
10. www.drbronner.com/drb_press_story10.html

Macroalgae in Nutricosmetics

J.H. Fitton, PhD, and M. Irhimeh, PhD

Marinova, Hobart, Tasmania

KEY WORDS: *nutricosmetic, macro- and microalgae, anti-inflammatory, anti-glycation, tyrosinase inhibition*

ABSTRACT: *Ingested macroalgae and its extracts can provide antiaging benefits such as inhibition of matrix enzymes, glycation, inflammatory activity and elastin calcification. Here, the authors investigated macroalgal extracts on insulin levels of test subjects to determine whether they could control glucose levels.*

Nutricosmetics are specific macro- and micronutrients that are orally delivered to help provide positive changes in the quality of the skin and general appearance. Dietary intake has clear connections with skin health and can even limit UV-induced damage.¹

Macroalgae contains a number of components including micronutrients, carotenoids, fucoidan, vitamin K and omega-3 lipids that are known to provide inhibitory effects on skin damage.²

There is a well-known disparity between skin aging in Japanese women who include macroalgae in their diet compared with Caucasian women of the same age who generally do not ingest macroalgae.³ Environmental factors responsible for these differences include sun exposure and diet.

In general, fucoidan and fucoxanthin products derived from seaweed are appearing more often on the market. As whole macroalgae and its extracts gain an increased presence in the nutraceutical sector, its untapped potential for the antiaging market as a detoxifying

ingredient and micronutrient provider is being realized, in addition to its use in topical cosmetic preparations.

In a previous article, the authors described the historical use and current applications of macroalgae and a bioactive extract, fucoidan, as ingredients in skin care and spa products.⁴ In addition to its cosmetic role, macroalgae has long provided mankind with sources of food and medicine and offered certain specific benefits as nutricosmetics. This article outlines the nutricosmetic benefits of macroalgae and discusses the potential for these natural ingredients to be utilized in the development of novel formulations.

Iodine: Essential Bioactive for Health, Beauty

Macroalgae and its extracts are commonly found in food products. The Asian diet is especially rich in macroalgae based products such as the red macroalga *Porphyra* or nori, the brown alga *Undaria* or wakame, and another brown macroalga *Laminaria sp.* or kombu. All of these macroalgae make excellent food supplements, being nutrient and micronutrient dense, with protein yields exceeding 30% in some cases.

Iodine and other trace elements tend to be more concentrated in perennial kelps,⁵ for example, and protein contents are highest in edible species such as *Porphyra* and *Ulva*.² Macroalgae also contain all the essential amino acids, omega-3 lipids and soluble fiber,² as well as a wide variety of bioactives including lipid-modulating, blood pressure-lowering and glucose metabolism-modifying compounds.⁴ For instance, small peptides found in the brown macroalga *Undaria pinnatifida* have angiotensin-converting enzyme (ACE)-inhibitory activity, which lowers blood pressure.⁶

Micronutrient supplementation is essential to supporting normal healthy skin. Key micronutrients in this area include zinc, selenium and iodine. Macroalgae is a particularly rich source of iodine, and also contributes to selenium and zinc intake. Iodine is a trace element essential for synthesis of the thyroid hormones triiodothyronine and thyroxine, which affect processes and pathways mediating the intermediary metabolism of carbohydrates, lipids and proteins in almost all tissues, including skin.⁷

Selenium is also necessary for the formation of these important hormones. Individuals suffering from thyroid hormone deficiencies also often experience dry skin and thinning hair. Recent research has demonstrated a marked effect of topically applied thyroid hormone on hair density and epidermal proliferation in a mouse model.⁸

Most studies conducted on thyroid hormone tissue effects tend to concentrate on lipid metabolism. The use of iodine supplements for weight loss has been popular in both Western and Asian cultures for centuries. Eating seaweed was a popular Victorian remedy for weight loss, according to the popular Mrs. Grieves.⁹ In addition, there are several traditional Chinese medicines that include macroalgae, such as “concoction of the Jade flask,” which includes the brown macroalgae species *Ecklonia* and *Sargassum* and is used to treat goiter, a condition in which the thyroid gland is enlarged due to iodine deficiency.¹⁰

Today in the Western market, generally the most common use of whole macroalgae in nutraceuticals is as iodine supplements. It is important for manufacturers to consider the iodine levels in products since concentrations vary widely in the different species, from relatively low levels in *Porphyra* and *Undaria* (up to 50 micrograms/g), to extremely high levels (thousands of micrograms/g) in some kelps.⁵

The upper limit for intake is 300 micrograms/day and the upper limit for toxicity is 1,000 micrograms/day.¹¹ In some countries such as Australia, iodine intake is low or marginal due to its paucity in the soil, and supplementation is probably wise.¹² It is important to note that iodine-containing supplements should not be taken by persons already diagnosed with thyroid deficiencies except on the advice of their medical practitioner.

Vitamin K for Youthful Skin

Macroalgae also contains vitamins and other bioactive components. Brown, red and green macroalgae can provide vitamins A, B and C in modest dietary amounts.² These vitamins are essential for healthy skin—for example, vitamin C is necessary for collagen production¹ while the vitamin B group controls homocysteine levels.¹³

Vitamin K or phylloquinone is often overlooked as an essential vitamin for maintenance of elasticity and capillary integrity in skin, and recent studies have emphasized its critical role for the inhibition of elastin degradation. Vitamin K is a required coenzyme for the carboxylation of the amino acid glutamic acid in a small protein called matrix Gla protein or MGP.¹⁴

This carboxylated protein is essential in inhibiting the calcification and subsequent degradation of elastin fibers. Elastin is an elastic matrix component of vascular tissues and skin. The integrity of elastin is an important feature of youthful and healthy tissues. Degradation of elastin by proteases may also lead to the induction of inflammatory processes or so called *inflamm-aging*.¹⁵

Dried seaweeds have very high vitamin K content; for example, dried wakame contains 1,293 $\mu\text{g}/100\text{ g}$, as compared with broccoli, at 305 $\mu\text{g}/100\text{ g}$.¹⁶ This fat-soluble vitamin is best taken orally where the entire benefit can be realized. Thus, orally delivered dried macroalgae could make good nutricosmetic elastin support ingredients.

Carotenoids to Inhibit UV-induced Damage

The active components of macro-algae include pigments such as polyphloroglucinols and carotenoids such as fucoxanthin. Many of these have antioxidant qualities and contribute to the antioxidant nature of aqueous and nonaqueous extracts. Additionally, they are antimutagenic and protect against environmental damage.¹⁷

It has been found that UV-induced skin damage can be ameliorated by dietary means. Components that confer some protection include carotenoids, tocopherols, ascorbate, flavanoids and omega-3 fatty acids.¹ Beta-carotene, lycopene, phytotene, phytofluene and lutein have all been identified in epidermal and dermal layers. They provide some protection from UV-induced skin damage, especially in combination with other antioxidants and immunoprotectives.¹ Carotene, phytotene, phytofluene and lutein are all found in edible macroalgae such as *Porphyra*, wakame and kombu. Indeed, excess intake of beta-carotene derived from the red alga *Porphyra* is expressed as carotenoderma, an orange color in the skin.¹⁶

However, the main antioxidant found in brown macroalgae is fucoxanthin.¹⁸ Fucoxanthin and its derivative, fucoxanthinol, have marked protective effects against inflammation *in vitro*, and also a profound antiobesity effect in animal models and *in vitro*.^{19,20} This component offers significant promise as a nutricosmetic ingredient.

Glutathione, an antioxidant that is sometimes used as an orally delivered skin whitening agent, is a constituent of all macroalgae, with some species containing as much as 3,082 $\mu\text{g}/100\text{g}$.²¹ Additionally, omega-3 fatty acids such as stearidonic acid and hexadecatetraenoic acid are found in edible marine algae such as *Undaria pinnatifida* and *Ulva*, contributing up to 40% of the plants' total fatty acid content.²²

Enzyme Inhibition to Reduce Skin Aging

One of the major effects of UV-induced damage is increased elastase activity in the skin. Thus the inhibition of this enzyme could increase the overall elasticity of the skin when combined with the elastin-sparing activity of vitamin K. Macroalgae can provide ample activity in both these areas.

Fucoidan fractions, alginates and phloroglucinols isolated from marine algae have enzyme inhibitory properties against hyaluronidase, heparanases, phospholipase A2, tyrosine kinase and collagenase expression.²³⁻²⁷ Profound antiviral activity against coated viruses such as herpes and HIV is exhibited by the sulphated polysaccharides found in macroalgae.^{28,29}

Fucoidans are known to have substantial activity on dermal elements within *in vitro* models. For example, fucoidan acts to enhance dermal fibroblast proliferation and deposition of collagen and other matrix factors.³⁰ Matrix metalloproteases that modulate connective tissue breakdown are inhibited by low molecular weight fucoidan due to increased association with its inhibitors. In addition, the serine protease leukocyte elastase was inhibited, thus protecting the elastic fiber network in human skin cultures.³¹ Tyrosinase is also inhibited by fucoidan fractions, indicating its potential for inclusion as whitening agents,³² an effect additional to that of glutathione.

Current Fucoïdan Research

Marinova has developed a solvent-free coldwater process to extract fucoïdan and is currently developing purified, organically certified extracts from a range of brown macroalgae species sourced from Tasmania, Nova Scotia, Tonga and Patagonia.

Over the last six years, the company has funded research into fucoïdan, including work by Irhimeh et al., that has demonstrated a low but significant serum uptake of a high molecular weight *Undaria* derived fucoïdan using a novel antibody technique.³³ This finding was important since it confirmed that systemic effects can be attributed to serum uptake, even from a high molecular weight fucoïdan.

Irhimeh et al. also demonstrated a profound increase in the sticky receptor CXCR4 on blood stem cells, attributed to the fucoïdan extract. This increase was accompanied by healthy changes in cholesterol levels and an increase in the immune regulating cytokine interferon gamma.³⁴ The significant effects of orally ingested fucoïdan suggest potential for rejuvenation nutraceuticals, designed to give the body a boost of immune function.

The company is now carrying out a long term project to determine the effects of macroalgal fractions on matrix enzyme (elastase) activity and glycation, with a particular focus on cosmetics and nutricosmetics.

Glycation is one of the determinants of skin aging. High or irregular blood glucose levels and high insulin levels all correlate with higher levels of glycation. Matrix and serum protein components are gradually glycated in a nonenzymatic process—similar to the browning process that takes place during cooking. The end products of the glycation process are known as advanced glycation end products or AGEs.

In a previous report,⁴ data was discussed on fucoïdan extracts with alpha-glucosidase activity. The action of alpha-glucosidase inhibitors is to delay carbohydrate absorption, reducing the post-prandial increase in blood glucose.³⁵ *Undaria* and *Fucus vesiculosus* fucoïdans were found to be particularly efficacious since they inhibited the activity of alpha-glucosidase by 50% at a concentration of less than 20 µg/mL, in comparison with luteolin that inhibited alpha-glucosidase activity at approximately 18 µg/mL.

In the previous report,⁴ researchers hypothesized that this inhibitory action on alpha-glucosidase would be reflected in reduced insulin levels in serum. In data reported here, the authors investigated insulin levels as a determinant of glucose control and thus potential for glycation.

Methodology

Insulin levels in volunteer blood samples were quantitatively measured using insulin kits and an insulin analyzer^a. The sole restriction was that volunteers were not to have ingested seafood.

This method is a solid-phase, two-site chemiluminescent immunometric assay. The reference fasting range used for the study is 6–27 $\mu\text{IU/L}$. The insulin levels were tested in a group of volunteers ($n = 10$) after receiving the active treatment of 75% *Undaria fucoidan*, 3 g/day, divided into three one-gram doses, for a total of 12 days. The insulin was tested only at two points: baseline and 12 days from receiving the active treatment. Another group of volunteers ($n = 3$) who received the placebo-control treatment was also tested for insulin levels at the same test points (see **Figure 1**).

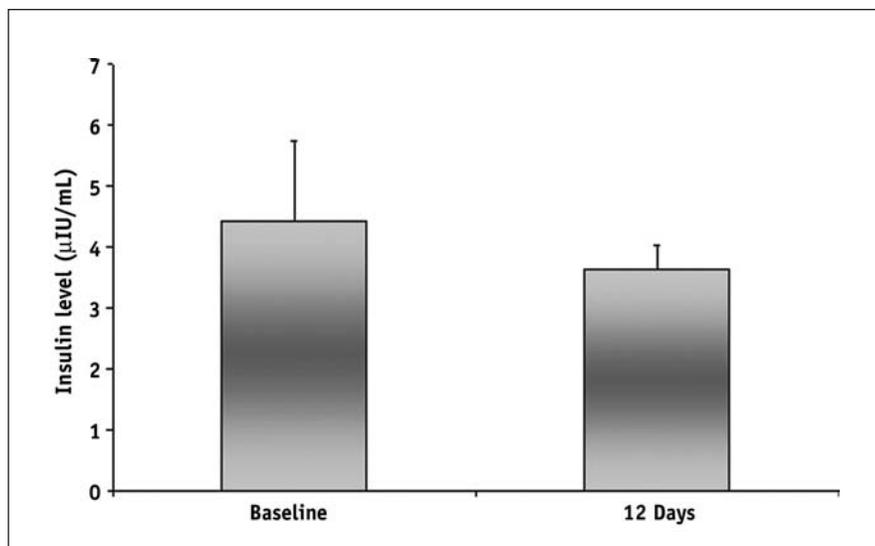


Figure 1. Insulin concentration before and after treatment with 75% fucoidan active; each point represents the average of 10 different individuals (5 male and 5 female) \pm MSE

^a Immulite 2500 insulin kits and the Immulite 2500 analyzer are products of Diagnostic Products Corp., A Siemens Company, Calif. USA.

Results

No change in the average insulin level was found in the placebo group and only a slight decrease was found in the average insulin level of the active treatment group. The slight decrease in insulin levels, however, does suggest that fucoidan extract could act as an inhibitor of glycosidase and reduced glucose loads; yet, this trial was not sufficiently powered to achieve a statistically significant result in healthy adults.

The nutricosmetic area is still modest and relatively underdeveloped. While various fish collagen-based preparations including some vitamin blends have been around for a while, overall there is a much smaller market presence. Further research is definitely warranted.

Anti-inflammatory Fucoidans

Fucoidan is a well-known anti-inflammatory agent with potential for inclusion in nutricosmetics with an antiaging or sunscreen focus. Marinova is currently in clinical trials in this area to investigate the immunomodulatory and anti-inflammatory properties of fucoidans of interest for nutraceutical and nutricosmetic developers. These trials aim to underline the relevance of fucoidans as anti-inflammatory and antiaging ingredients. The trials are being managed and designed by professor Stephen Myers, founding director of the Australian Centre for Complimentary Medicine Education and Research and one of Australia's leading alternative medicine researchers and practitioners.

As explained in the previous article,⁶ fucoidans can be considered to be a dietary fiber and are nontoxic in cell culture. No toxicological changes were observed in rats administered up to 300 mg/kg of fucoidan from *Laminaria japonica* orally. Anticoagulant effects were observed at doses of 900 to 2,500 mg/kg, but no other signs of toxicity were observed.³⁶ Marinova has also undertaken a safety trial in cancer patients in Australia and demonstrated that ingestion of up to 6 g per day of fucoidan has no observable side effects. This study is currently unpublished.

Conclusion

Macroalgae and its extracts are novel nutricosmetic ingredients that can provide cosmetic antiaging benefits such as inhibition of matrix enzymes, glycation inhibition and anti-inflammatory activity, in addition to inhibition of elastin calcification. While macroalgae remain a staple of the spa industry, fucoidan-rich macroalgal extracts have strong potential for commercial success in cosmetics. Key components include fucoidan, vitamin K, minerals and trace elements and carotenoids.

From a consumer's point of view, it tends to take longer to generate beneficial effects on skin through oral supplements, although the effects may be longer-lasting. It is more difficult to market a product that requires persistence and this may account for limited research currently in the nutricosmetics area. Combination products offering two levels of effect—topical, more immediate effects, plus orally administered, longer-term effects—may make more of an impact on consumers.

The nutraceutical world generally does not focus on taking skin measurements as part of clinical studies on supplements, although some supplements likely generate benefits for the skin. As the market matures, the industry may see more of a focus on the health of skin via nutrition.

Published May 2008 *Cosmetics & Toiletries* magazine.

References

1. H Sies and W Stahl, Nutritional protection against skin damage from sunlight, *Annu Rev Nutr* 173–200 (2004)
2. S Aaronson, Algae, in *The Cambridge World History of Food*, K Kiple and KC Ornelas, eds, Cambridge University Press, Cambridge, UK, Vol I, Ch. II.C.I 231–249 (2000)
3. K Tsukahara et al, Comparison of age-related changes in wrinkling and sagging of the skin in Caucasian females and in Japanese females, *J Cosmet Sci* 55(4) 351–71 (Jul/Aug 2004)
4. JH Fitton, M Irhimeh and N Falk, Macroalgal fucoidan extracts: A new opportunity for marine cosmetics, *Cosm & Toil* 122, 55–64 (2007)
5. J Teas, S Pino, A Critchley and LE Braverman, Variability of iodine content in common commercially available edible seaweeds, *Thyroid* 14, 836 (2004)
6. K Suetsuna and T Nakano, Identification of an antihypertensive peptide from peptic digest of wakame (*Undaria pinnatifida*), *J Nutr Biochem* 11(9) 450–4 (Sep 2000)
7. M Moreno, P de Lange, A Lombardi, E Silvestri, A Lanni and F Goglia, Metabolic effects of thyroid hormone derivatives, *Thyroid* 18(2) 239–53 (Feb 2008)
8. JD Safer et al, Thyroid hormone action on skin: Diverging effects of topical versus intraperitoneal administration, *Thyroid* 13(2) 159–65 (Feb 2003)

9. M Grieve, *A Modern Herbal*, Dover Publications Inc., New York (1971)
10. C Tseng, Algal biotechnology industries and research activities in China, *J Appl Phycol* 13, 375 (2001)
11. European Commission Web site, Safe upper intake levels for vitamins and minerals, available at: http://ec.europa.eu/food/food/labellingnutrition/supplements/documents/denmark_annex2.pdf (Accessed Mar 28, 2008)
12. M Li et al, Are Australian children iodine deficient? Results of the Australian National Iodine Nutrition Study, *Med J Aust* 184(4) 165–9 (Feb 20, 2006)
13. P Gisondi, F Fantuzzi, M Malerba and G Girolomoni, Folic acid in general medicine and dermatology, *J Dermatolog Treat* 18(3) 138–46 (2007)
14. D Gheduzzi et al, Matrix Gla protein is involved in elastic fiber calcification in the dermis of *Pseudoxanthoma elasticum* patients, *Lab Invest* 87(10) 998–1008 (Oct 2007)
15. F Antonicelli, G Bellon, L DeBelle and W Hornebeck, Elastin-elasases and inflamm-aging, *Curr Top Dev Biol* 79 99–155 (2007)
16. M Kamao et al, Vitamin K content of foods and dietary vitamin K intake in Japanese young women, *J Nutr Sci Vitaminol* (Tokyo) 53(6) 464–70 (Dec 2008)
17. Y Okai, K Higashi-Okai, Y Yano and S Otani, Identification of antimutagenic substances in an extract of edible red alga, *Porphyra tenera* (Asakusa-nori), *Cancer Lett* 100(1-2) 235–40 (Feb 27, 1996)
18. Y Nishimura, N Ishii, Y Sugita and H Nakajima, A case of carotenoderma caused by a diet of the dried seaweed called Nori, *J Dermatol* 25(10) 685–7 (Oct 1998)
19. X Yan et al, Fucoxanthin as the major antioxidant in *Hijikia fusiformis*, a common edible seaweed, *Biosci Biotechnol Biochem* 63(3) 605–607 (1999)
20. H Maeda et al, Fucoxanthin from edible seaweed, *Undaria pinnatifida*, shows antiobesity effect through UCP1 expression in white adipose tissues, *Biochemical and Biophysical Research Communications* 332, 392–397 (2005)
21. H Maeda, M Hosokawa, T Sashima, N Takahashi, T Kawada and K Miyashita, Fucoxanthin and its metabolite, fucoxanthinol, suppress adipocyte differentiation in 3T3-L1 cells, *Int J Mol Med* 18(1)147–52 (Jul 2006)
22. M Kakinuma, CS Park and H Amano, Distribution of free L cysteine and glutathione in seaweeds, *Fisheries Science* 67, 194 (2001)
23. T Katsube, Y Yamasaki, M Iwamoto and S Oka, Hyaluronidase-inhibiting polysaccharide isolated and purified from hot water extract of *Sporophyll* of *Undaria pinnatifida*, *Food Sci Technol Res* 9, 25 (2003)
24. CR Parish, DR Coombe, KB Jakobsen, FA Bennett and PA Underwood, Evidence that sulphated polysaccharides inhibit tumour metastasis by blocking tumour cell derived heparanases, *Int J Cancer* 40, 511 (1987)
25. T Shibata, K Nagayama, R Tanaka, K Yamaguchi and T Nakamura, Inhibitory effects of brown algal phlorotannins on secretory phospholipase A₂s, lipoxygenases and cyclooxygenases, *J Appl Phycol* 15, 61 (2003)
26. M Wessels, G Konig and A Wright, A new tyrosine kinase inhibitor from the marine brown alga *Styopodim zonale*, *J Nat Prod* 62, 927 (1999)
27. MJ Joe et al, The inhibitory effects of eckol and dieckol from *Ecklonia stolonifera* on the expression of matrix metalloproteinase-1 in human dermal fibroblasts, *Biol Pharm Bull* 29(8) 1735–9 (Aug 2006)
28. DJ Schaeffer and VS Krylov, Anti-HIV activity of extracts and compounds from algae and cyanobacteria, *Ecotoxicology and Environmental Safety* 45, 208 (2000)
29. KD Thompson and C Dragar, Antiviral activity of *Undaria pinnatifida* against herpes simplex virus, *Phytother Res* 18, 551 (2004)
30. R O'Leary et al, Fucoic acid modulates the effect of transforming growth factor (TGF) - β 1 on fibroblast proliferation and wound repopulation in in vitro models of dermal wound repair, *Biol Pharm Bull* 27(2) 266–270 (2004)
31. K Senni et al, Fucoic acid, a sulfated polysaccharide from brown algae, is a potent modulator of connective tissue proteolysis, *Arch Biochem Biophys* 1 445(1) 56–64 (Jan 2006)

32. XJ Kang, FX Wang, CM Sheng and Y Zhu, *Undaria pinnatifida* stem fucoidan biological activity of the composition and bioactivity, *Chinese Pharmaceutical Journal* 41(22) 1748-1750 (2006)
33. MR Irhimeh, JH Fitton, RM Lowenthal and P Kongtawelert, A quantitative method to detect fucoidan in human plasma using a novel antibody, *Methods Find Exp Clin Pharmacol* 27(10) 705–710 (Dec 2005)
34. MR Irhimeh, JH Fitton and RM Lowenthal, Fucoidan ingestion increases the expression of CXCR4 on human CD34+ cells, *Exp Hematol* (2007) (in press)
35. HP Rang, MM Dale and JM Ritter, *Pharmacology*, 3rd edn. Churchill Livingstone, New York (1995)
36. N Li, Q Zhang and J Song, Toxicological evaluation of fucoidan extracted from *Laminaria japonica* in Wistar rats, *Food Chem Toxicol*, 43 421 (2005)

Recent Advances in Slimming Treatments

Karen A. Costa-Strachan, PhD

Prestige Brands Holdings Inc., New York

KEY WORDS: *microcirculation, connective tissue, adipose tissue, xanthines, mesotherapy*

ABSTRACT: *Slimming treatments address a number of targets in skin physiology, including: improving microcirculation, strengthening and protecting the connective tissue, and decreasing adipocyte contents. Traditional and newer approaches to slimming treatments employ a number of ingredients to achieve such ends.*

Popular sites for body contouring include the hips, thighs, buttocks and abdomen. One reason for this is that the hips, thighs and buttocks are areas particularly prone to cellulite. *Cellulite* is a term used to define the puckered, dimpled or “orange-peel” like appearance of the skin often seen in these areas of the body. Cellulite typically begins to develop during or after puberty, so it is thought that estrogen plays a role in its development.¹

Cellulite can manifest in women of all ages, races and body weight. Although weight gain can worsen the appearance of cellulite, it is noteworthy that even slender women can suffer from the appearance of cellulite.^{2,3} Thus, it is not surprising that the hips, thighs and buttocks are sites that are also popular targets for slimming treatment products.

Skin Structure Considerations

The outer layer of the skin reflects activity beneath its surface, especially when there are structural changes occurring. Therefore, to best understand how to target slimming formulations to these areas, it is worthwhile to understand a bit more about the underlying anatomy.

The skin is composed of the epidermis and the dermis. Underlying the dermis is the hypodermis or subcutaneous fat layer. The subcutaneous fat layer can be thought of as being composed of two sub-layers. These two sub-layers include the deeper localized fat deposits and the more superficial compartmentalized layer of fat.

Localized fat deposits typically are the targets of liposuction. Cellulite appears to originate in the fat layer where structural compartmentalization of adipose or fat tissue is predominant.⁴⁻⁶ Small, upright “chambers” are encircled and separated by vertical bands of connective tissue (CT) referred to as *septa*. The compartments are anchored to the dermis above and the fascia below. Overall, the appearance of the compartments can be described as somewhat “honeycomb.”

When the fat cells in these chambers become enlarged and/or fluid accumulates, the chambers begin to bulge, pressing against the septa. This “bulging” phenomenon of the septa contributes toward the appearance of dimples and bumps on the surface of the skin in cellulite.⁶ With age and sun damage, the septa, composed principally of collagen, can become further weakened and thickened, resulting in shrinkage.⁶⁻⁸ As the septa shrink and “pull” on the underlying anchors of the chambers, this can also contribute to the indentations seen on the surface of the skin in cellulite.⁶⁻⁸

Another hypothesis regarding cellulite notes that an inflammatory process caused by decreased circulation may also be involved. It is believed that the early morphological changes leading to cellulite formation are the result of decreased circulation, which itself is the direct result of deterioration of the dermal vasculature—especially a loss of the capillary networks.⁸ The loss of the capillary networks has been hypothesized to be caused by engorged adipocytes coalescing and inhibiting venous return. For more comprehensive information on the etiology of cellulite, including the stages of cellulite formation,

readers are encouraged to read fully the excellent review articles excerpted above and cited in the references.¹⁻⁸

Slimming Treatments

Clearly, there are a number of targets that slimming treatments can address. These areas include: enhancing the removal of fluid buildup by improving microcirculation to the areas in question; strengthening the CT while protecting it from further degradation caused by inflammation and the subsequent release of matrix metalloproteinases (MMPs); and targeting the adipose tissue via stimulation of adipocyte cell metabolism/thermogenesis or some other mechanism to decrease adipocyte contents—lipolysis. Traditional and newer approaches to slimming treatments use a number of ingredients to achieve such ends, which are described here.⁹⁻¹⁴

Enhancing fluid build-up, improving microcirculation: A number of ingredients in slimming treatments may help to enhance fluid buildup and improve microcirculation. Rutin is a citrus flavonoid glycoside found in buckwheat, the leaves and petioles of the *Rheum* species, and the fruit of the Brazilian Fava D'Anta tree. Rutin possesses both anti-adipogenic as well as capillary-strengthening properties which can be beneficial to cellulite.⁹⁻¹⁰

Bilberry extract has been noted to have effects on new capillary formation as well as enhancing microcirculation.¹¹⁻¹² Citrus bioflavonoids from lemons, grapefruits and oranges and fruit bioflavonoids such as hesperidin, eriocitrin and flavonones from grapes, plums, apricots and cherries possess the ability to both strengthen the capillaries and regulate their permeability.¹³

Ginkgo biloba contains flavanoids that have been shown to have vasokinetic effects as well as lipolytic effects.¹⁴

Several reviews¹⁵⁻¹⁹ describe botanical extracts used in cellulite treatments, some of which are briefly mentioned here.

Uva ursi—an herb derived from bearberry, juniper berries, *Elymus repens* (couch grass) and *Agathosma betulina* or *A. crenulata* (buchu) have been associated with loss of excess fluids in tissues, leading to improvements in water retention.

Horsechestnut is claimed to strengthen venous walls, improve circulation and target edema. Aescin, the active constituent of

horsechestnut extract, has the added ability to block elastase and hence help to preserve elastin, an important structural CT responsible for blood vessel integrity and tone.

Hawthorne berry has been shown to improve blood flow while horsetail extract has been associated with strengthening the walls of arteries and veins. Ginseng and angelica extract are known microcirculation boosters. Butcher's broom extract (*Ruscus aculeatus*) contains *ruscogenin* and *neoruscogenin*, both known to strengthen veins. Finally, xanthine derivatives, such as gotu kola as noted later in this article, can also find a presence in slimming treatment formulations for their diuretic effects.

Protecting, strengthening connective tissue: The integrity of the CT in capillaries and veins is vital for their proper functioning. Likewise, the integrity of the CT of the septa in the hypodermis is important. Breakdown of the CT in the vessels leads to capillary permeability and edema while breakdown of connective tissue in the septa causes the compartments of adipose to become more fragile.²⁰ Both the vasculature and the septa in the hypodermis play a role in the development of cellulite.²⁰ All connective tissue, including collagen, elastin, fibronectin and various glycosaminoglycans (GAGs), can be adversely affected by matrix metalloproteinases (MMPs).

MMPs are often released by cells in response to inflammation. Examples of MMPs include various collagenases and elastases. One goal of cellulite-specific slimming formulations is to provide ingredients that target both inflammation and MMP deactivation, as well as ingredients to directly strengthen the connective tissue. Ingredients such as retinoic acid, retinols and vitamin A derivatives and vitamin C have shown strengthening effects on the connective tissue either by direct stimulation of cells to produce healthy collagen or elastin or by playing a supporting role in the biosynthesis of the connective tissue.^{17,20}

Antioxidants in a formulation can help to alleviate the inflammation and resultant release of MMPs that target the destruction of CT.²¹ A formulator's choice of antioxidants is extensive and includes ingredients such as: grape seed extract, green and white tea extracts, vitamin C, vitamin E, coenzyme Q10 (co-Q10), genistein, lycopene, carotenoids such as beta-carotene and lutein, pomegranate extract,

alpha lipoic acid, resveratrol and pine bark extract. Other potential sources of antioxidants include spices such as garlic, onion, turmeric, rosemary, sage, thyme and oregano.

Impacting adipose tissue: One class of ingredients commonly seen in slimming treatment products includes xanthines and xanthine derivatives. Xanthines are a class of pharmacological actives called beta-adrenergic agonists. Although xanthines have a number of well-known pharmacological effects, the main reason they are included in slimming product formulations is because of their ability to stimulate lipolysis, the break down of fat stored as triglycerides. More specifically, xanthine and xanthine derivatives work by inhibiting phosphodiesterase, which ultimately leads to the conversion of triglycerides into free fatty acids and glycerol. Examples of xanthine derivatives include caffeine, theobromine, theophylline and aminophylline.

Xanthine and xanthine derivatives can be found in natural sources such as coffee beans, kola nut extract, guarana seeds, Chinese black tea, chocolate and plants such as yerba mate. Xanthines are also known to possess mild diuretic effects, so they can be included in slimming product formulations for this reason, as well. Xanthine derivatives are not the only materials used to target lipolysis. Milk thistle is an herb that has been used medicinally for its lipotropic ability to help remove fatty substances from the liver.²² Cayenne extract has been associated with increased thermogenesis.²³ A new use for cinnamon may gain more popularity as recent research in adipocytes notes its lipotropic ability as well its insulin-mimetic effects that are due to a water-soluble chemical constituent called methylhydroxychalcone polymer (MHCP).²⁴⁻²⁵

Other popular ingredients in the supplement arena that may have potential in targeting lipolysis in slimming treatments include *Citrus aurantium* (bitter orange) and green tea extracts. Franchi et al. describe how advances in adipocyte physiology and metabolism have led to greater ability to demonstrate active ingredient efficacy in slimming formulations.²⁶ It is noteworthy that at least one contract laboratory with a facility in France offers a full range of screening for the efficacy of actives targeting lipolysis and lipogenesis through the implementation of human adipocytes/preadipocytes.

Recent Advances: Mesotherapy

Slimming formulations can and have drawn from a vast repertoire of active ingredients to produce desired effects. However, the newest method of body contouring to reach the United States may begin to influence not only the ingredients contained in slimming treatments, but also the delivery system of these ingredients.

Mesotherapy was first pioneered by the French physician Michel Pistor in 1958 and was employed traditionally for pain relief.²⁷⁻²⁹ It has since evolved as a means of weight reduction without liposuction. The theory behind mesotherapy or any micro-injection is that the fat-dissolving ingredients are administered directly to the body areas that require treatment. The procedure is touted as risk-free, painless and clinically targeted at areas to slim such as the abdomen, thighs and hips. It is believed that the “mesoactive” substances that are directly administered stimulate the metabolism of fat cells and accelerate their elimination.

Mesotherapy is described as a minimally invasive procedure to target problem areas of the body using microinjections of homeopathic medicines, vitamins, minerals and amino acids. Specifically, mesotherapy has been used to target cellulite, weight reduction and to help eliminate localized fat deposits.

A similar treatment for localized fat reduction was popularized in Brazil. The Brazilian treatment utilized injections of a combination of phosphatidylcholine and deoxycholate as an emulsifier to “dissolve” fat and remove small pockets of adipose tissue.³⁰ Such injections have since become synonymous with mesotherapy, although their history and technique are distinct.

A recent article by Rotunda and Avram notes that experimental studies using individual mesotherapy ingredients for other conditions suggest a number of mechanisms, including lipolysis, disruption of CT and augmentation of circulation, which theoretically may improve cellulite; however, according to the article, peer-reviewed studies have not evaluated whether these effects translate clinically.³¹

Whether mesotherapy is effective or not, the concept of targeted slimming may find its way into cosmetic formulations—just as the cosmetic industry has creatively and elegantly “borrowed” technology from other invasive dermatological procedures, resulting in products

such as Botox^a in a bottle, or laser treatments without a laser. Perhaps a creative combination can be made from both mesotherapy and Botox? This is not such a far-fetched a concept. Lim and Seet note methods whereby the denervation of adipose tissue results in lipotrophy, leading to the postulation that chemodenervations using botulinum toxin may achieve the same result—fat loss.³²

One compelling reason for the development of a cosmetic equivalent to mesotherapy is given in a recent report by the Centers for Disease Control that describes an outbreak of mesotherapy-associated skin reactions.³³ An effective noninvasive cosmetic substitute for any invasive treatment, slimming included, might provide a safer, more marketable, alternative.

Published April 2004 *Cosmetics & Toiletries* magazine.

References

1. ZD Draelos and KD Marenus, Cellulite. Etiology and purported treatment, *Dermatol Surg* 23 12 1177–1181 (1997)
2. B Querleux et al, Anatomy and physiology of subcutaneous adipose tissue by *in vivo* magnetic resonance imaging and spectroscopy: Relationship with sex and presence of cellulite, *Skin Res Technol* 8 2 118–124 (2002)
3. MM Avram , Cellulite: A review of its physiology and treatment, *J Cosmet Laser Ther* 6 4 181–185 (2004)
4. F Mirrashed et al, Pilot study of dermal and subcutaneous fat structures by MRI in individuals who differ in gender, BMI and cellulite grading, *Skin Res Technol* 10 3 161–168 (2004)
5. Rosenbaum M et al, An exploratory investigation of the morphology and biochemistry of cellulite, *Plast Reconstr Surg* 101 7 1934–1939 (1998)
6. GE Pierard et al, Cellulite: From standing fat herniation to hypodermal stretch marks, *Am J Dermatopathol* 22 1 34–37 (2000)
7. AB Rossi and AL Vergnanini, Cellulite: A review, *J Eur Acad Dermatol Venereol* 14 4 251–262 (2000)
8. LK Smalls et al, Quantitative model of cellulite: Three-dimensional skin surface topography, biophysical characterization and relationship to human perception, *J Cosmet Sci* 56 2 105–120 (2005)
9. I Choi et al, Anti-adipogenic activity of rutin in 3T3-L1 cells and mice fed with high fat diet, *Biofactors* 26 4 273–81 (2006)
10. ML Xia et al, Rutin-induced endothelium-dependent vasorelaxation in rat aortic rings and underlying mechanism, *Conf Proc IEEE Eng Med Biol Soc* 6 1 5595–7 (2005)
11. D Bagchi et al, Anti-angiogenic, antioxidant, and anti-carcinogenic properties of a novel anthocyanin-rich berry extract formula, *Biochemistry* 69 1 75-80 (2004)
12. S Roy et al, Anti-angiogenic property of edible berries, *Free Radic Res.* 36 9 1023–31 (2002).
13. DR Bell et al, Direct vasoactive and vasoprotective properties of anthocyanin-rich extracts, *J Appl Physiol*, 100 4 1164–70 (2006)

^a Botox is a registered trade name of Allergan, Inc., Irvine, CA.

14. M Lis-Balchin, Parallel placebo-controlled clinical study of a mixture of herbs sold as a remedy for cellulite, *Phytother Res* 13 7 627–629 (1999)
15. D Hexsel et al, Botanical extracts used in the treatment of cellulite, *Dermatol Surg* 31 866–872 (2005)
16. BI Dickinson and ML Gora-Harper, Aminophylline for cellulite removal, *Ann Pharmacother* 30 3 292–293 (1996)
17. C Bertin et al, A double-blind evaluation of the activity of an anti-cellulite product containing retinol, caffeine and ruscogenine by a combination of several non-invasive methods, *J Cosmet Sci* 52 4 199–210 (2001)
18. J Rao et al, A double-blinded randomized trial testing the tolerability and efficacy of a novel topical agent with and without occlusion for the treatment of cellulite: A study and review of the literature, *J Drugs Dermatol* 3 4 417–25 (2004)
19. EL Sainio et al, Ingredients and safety of cellulite creams, *Eur J Dermatol* 10 8 596–603 (2000)
20. C Pierard-Franchimont et al, A randomized, placebo-controlled trial of topical retinol in the treatment of cellulite, *Am J Clin Dermatol* 1 6 369–374 (2000)
21. Andreassi M et al, Antioxidants in dermocosmetology: From the laboratory to clinical application, *J Cosmet Dermatol* 2 3-4 153–60 (Jul 2003)
22. R Gazak, D Walterova and V Kren, Silybin and silymarin—New and emerging applications in medicine, *Curr Med Chem* 14 3 315–38 (2007)
23. Ohnuki Ket al, CH-19 sweet, a non-pungent cultivar of red pepper, increased body temperature and oxygen consumption in humans, *Biosci Biotechnol Biochem* 65 9 2033–6 (2001)
24. H Cao et al., Cinnamon extract and polyphenols affect the expression of tristetraprolin, insulin receptor, and glucose transporter 4 in mouse 3T3-L1 adipocytes, *Arch Biochem Biophys* (Jan 25, 2007)
25. B Roffey et al., Cinnamon water extracts increase glucose uptake but inhibit adiponectin secretion in 3T3-L1 adipose cells, *Mol Nutr Food Res* 50 8 739–45 (2006)
26. J Franchi et al, The adipocyte in the history of slimming agents, *Pathol Biol (Paris)* 51 5 244–247 (2003)
27. PT Rose and M Morgan, Histological changes associated with mesotherapy for fat dissolution, *J Cosmet Laser Ther* 7 1 17–19 (2005)
28. A Matarasso et al, Mesotherapy for body contouring, *Plast Reconstr Surg* 115 5 1420–1424 (2005)
29. RJ Rohrich, Mesotherapy: What is it? Does it work? *Plast Reconstr Surg* 115 5 1425 (2005)
30. AM Rotunda and MS Kolodney, Mesotherapy and phosphatidylcholine injections: Historical clarification and review, *Dermatol Surg* 32 4 465–480 (2006)
31. AM Rotunda et al, Cellulite: Is there a role for injectables? *J Cosmet Laser Ther* 7 3–4 147–154 (2005)
32. EC Lim and RC Seet, Botulinum toxin injections to reduce adiposity: Possibility or fat chance? *Med Hypotheses* 67 5 1086–1089 (2006)
33. *Morbidity and Mortality Weekly Report (MMWR)*, Center for Disease Control (CDC) 54 44 1127–1130 (Nov 11, 2005)

Macroalgal Fucoidan Extracts: A New Opportunity for Marine Cosmetics

J. Helen Fitton, PhD, Mohammad Irhimeh MSc, and Nick Falk

Marinova Pty. Ltd., Tasmania, Australia

KEY WORDS: *macroalgae, bioactivity, fucoidans, nutraceuticals, antiaging*

ABSTRACT: *This chapter explores the bioactivity and historical applications of macroalgae with a particular focus on fucoidans, as well as outlines the research underlining their growing use in nutraceuticals and discusses their potential in novel cosmetic formulations.*

Macroalgae and macroalgal blends have been included in cosmetic formulations for decades. Used as emollients, skin conditioning agents and viscosity-controlling ingredients, macroalgal products have benefited from their inherent stability, physical and bioactive properties, and natural marine source. In the development of spa products particularly, macroalgae have played a major role, whether as seaweed wraps, pastes of milled macroalgae, or in Irish hot seaweed detox baths.¹

In addition to their cosmetic role, macroalgae have long provided mankind with sources of food and medicine. Their extracts such as agar and carrageenan have been used for nutritional and nutraceutical benefits and have provided the impetus for their inclusion in successful cosmetic formulations.

This article explores the bioactivity and historical applications of macroalgae with a particular focus on fucoidans—sulfated polysaccharides currently emerging as popular nutraceutical ingredients. The article will outline the research underlining the growing nutraceutical use of fucoidans and discuss the potential for these natural ingredients to be used in the development of novel cosmetic formulations.

A Natural Diversity

Macroalgae make an excellent food supplement, being nutrient and micronutrient dense, with protein yields exceeding 30% in some cases. Macroalgae also contain all the essential amino acids, omega-3 lipids and soluble fiber,² as well as a wide variety of bioactives including lipid-modulating, blood pressure lowering and glucose metabolism-modifying compounds.³

Oral ingestion of seaweed was a popular Victorian remedy for weight loss and considered to be an effective topical treatment for scrofula or mycobacterium infection of the skin, in addition to arthritis, sprains and bruises, and as embrocations for the “limbs of rickety children.”⁴ Such recommendations can be traced back through the ages to Gerard’s *On the History of Plants*⁵ published in 1633 and further back to ancient texts such as that by the Greek, Pliny the elder,⁶ who wrote:

But it is the phycos thalassion, or sea-weed, more particularly, that is so excellent a remedy for the gout Used before it becomes dry, it is efficacious as a topical application not only for gout, but for all diseases of the joints

There are several traditional Chinese medicines that include macroalgae such as “concoction of the Jade flask,” which includes *Ecklonia* and *Sargassum* and is used to treat goitre (iodine deficiency).⁷ Today, in the Western market, there are traditional nutraceuticals available such as kelp granules, and there is also an increasing number of novel products containing either whole macroalgae or fucoidan extracts. The preparations can help to maintain skin health in a number of ways, including the supplementation of essential micronutrients.³

The term *macroalgae* refers to a diverse family of marine plants. These can be divided into three main groups classified as *Phaeophyta* (brown), *Rhodophyta* (red) and *Chlorophyta* (green). Readers may be familiar with the very long brown seaweeds such as *Macrocystis* that make up kelp forests, in addition to the smaller, commonly eaten red seaweed *Porphyra* (Nori) or green seaweed *Ulva* (sea lettuce). The bioactivity of macroalgae is equally as diverse and includes anti-viral properties, lipid-lowering activity, heavy metal and organic toxin elimination, enzyme inhibition, growth factor modulation and even anti-cancer activity.⁸

The active components of macroalgae are varied as well, including bioactive carbohydrates such as fucoidan and laminarin, pigments such as polyphloroglucinols and fucoxanthin,⁹⁻¹² and minerals, including iodine. Many of these are generally known to have antioxidant qualities and contribute to the antioxidant nature of aqueous and nonaqueous extracts. Glutathione, an antioxidant sometimes used as an orally delivered skin whitening agent, is a constituent of all macroalgae with some species containing as much as 3,082 mg/100g.¹³

Additionally, omega-3 fatty acids such as stearidonic acid and hexadecatetraenoic acid are found in edible marine algae such as *Undaria pinnatifida* and *Ulva*, contributing up to 40% of the plants' total fatty acid content.¹⁴ Fucoidan fractions, alginates and phloroglucinols isolated from marine algae have enzyme inhibitory properties against hyaluronidase, heparanases, phospholipase A2, tyrosine kinase and collagenase expression.¹⁵⁻¹⁹ Also, profound anti-viral activity against coated viruses such as herpes and HIV is exhibited by the sulfated polysaccharides found in macroalgae.^{20,21}

Supplementing

The most common use of whole macroalgae in the current nutraceutical market is as iodine supplements. Iodine is essential for formation of thyroxine (T_4) and triiodothyronine (T_3), hormones essential for regulating the metabolic rate, body heat, and energy.²² It is important for manufacturers to consider the iodine levels in products since concentrations vary widely in the various species, from relatively low levels in *Porphyra* and *Undaria* (up to 50 mcg/g),

to extremely high levels (1,000s of micrograms/g) in some of the kelps.²³

The upper limit for intake is 300 mcg/day, and the upper limit for toxicity is 1,000 mcg/day. There are also instances where high iodine levels are desirable: the non-radioactive iodine in macroalgae inhibit radioactive iodine uptake, and the polysaccharide components assist in the chelation and elimination of radioisotopes.²⁴ Sodium alginate from *Sargassum* reduced strontium absorption when added to bread at 6% level, while alginate syrup has a more rapid action.²⁵

While iodine is an important and often neglected essential trace element, the benefits of ingesting macroalgae extend to general detoxification. Dioxins are common organic toxins that can accumulate in tissues. Ingestion of *Porphyra* has been shown to increase the rate of excretion of dioxins in animal experiments.²⁶ This detoxification activity via oral ingestion of whole macroalgae is boosted by the additional micronutrient and immunomodulating effects of the bioactive carbohydrates, pigments and omega-3 lipids found in the plants. Whole *Undaria* macroalgal supplements have been shown to lessen the effects of herpes infections,²⁷ and ingestion of macroalgae as a regular part of the diet appears to have many health benefits including inhibition of cancer²⁸ and a lower incidence of allergic rhinitis.²⁹

Fucoxidans

Fucoxidan or *fucan* is a generic term for a class of bioactive carbohydrates found only in brown marine macroalgae and echinoderms. These polysaccharides have a high-fucose polymer backbone; are usually highly branched—in algae, whereas echinoderm fucoidans contain linear structures; and are both sulfated and acetylated.

Extraction methods for fucoidan vary in the literature but it is possible to produce high molecular weight materials that can be fractionated in a controlled fashion to produce lower molecular weight derivatives. Levels of sulfation and acetylation also can be controlled that will enable creation of new substances with increased efficacy. Pure fucoidan extracts are generally off-white or brown soluble powders with no strong odor or taste (see **Figure 1**).



Figure 1. Fucoïdan extracted from Tasmanian *Undaria pinnatifida*

Fucoïdians are sometimes considered to be plant homologues of the mammalian compound heparin. While the sugar backbone structure of heparin is different, it also is highly sulfated. Both fucoïdians and heparins exhibit an effect on the coagulation cascade^{30,31} and share other common bioactivities such as the potentiation of growth factor activity.³² The two families of polysaccharide, however, are different. For example, both fucoïdan and heparin exhibit anti-proliferative effects on vascular smooth muscle but the activity is via subtly different mechanisms.³³

Each brown macroalgae contains its own specific fucoïdan.³⁴ The molecules tend to vary in their natural sugar composition, molecular weight and levels of sulfation. Comparative studies on fucoïdians from different sources recently were carried out, illustrating subtle but important targets for activity.³⁵

Fucoïdians can both inhibit and promote enzymes. A marked example of inhibition is that of phospholipase A2 (PLA2) in snake venoms.³⁶ Snake venom PLA2 is a toxin that causes muscle necrosis in the snake's prey. The mammalian homologue PLA2 is an important part of the eicosanoid cascade and is partly responsible for the regulation of prostaglandin production. The latter are inflammatory

mediators important in many conditions, from atherosclerosis to neural degenerative diseases.³⁷

Bioactivity in Fucoidans

Marinova has designed a solvent-free coldwater process to extract fucoidan and is currently developing extracts from a range of brown macroalgae species sourced from Tasmania, Australia; Nova Scotia, Canada; The Kingdom of Tonga; and Patagonia, South America. Traditionally the extraction of fucoidan has relied on ethanol recipitation and high temperatures, resulting in extracts with unpredictable molecular weights and solvent residue; however, the new process yields extracts with defined molecular weight ranges and high levels of purity. The company's research, described later, has focused on a range of indications and has demonstrated that, much like heparin, fucoidans have relevance to an array of diverse applications.

The company's initial research, in partnership with the University of Chicago, investigated the antiherpetic activity of fucoidans. A small clinical trial involving 17 patients demonstrated the significant efficacy of Tasmanian *Undaria* fucoidan in the inhibition of both normal and acyclovir-resistant strains of HSV-1 and HSV-2.²⁷

In recent unpublished experiments, fucoidan extracts were assessed for α -glucosidase activity. The action of α -glucosidase inhibitors is to delay carbohydrate absorption, reducing the post-prandial increase in blood glucose, making them a suitable treatment for noninsulin dependent diabetes mellitus.³⁸ *Undaria* and *Fucus vesiculosus* fucoidans were shown to be particularly efficacious by inhibiting the activity of α -glucosidase by 50% at a concentration of less than 20 $\mu\text{g}/\text{mL}$, in comparison with luteolin, which inhibited α -glucosidase activity at approximately 18 $\mu\text{g}/\text{mL}$.

Fucoidans were shown to exhibit excellent immunomodulating activity, having a modulatory role on various biomarkers affecting the inflammatory and immune response. Fucoidans have also been demonstrated, in animal trials, to inhibit the development of tumors.^{39,40} In some instances, immunological effects appear to be governed by molecular weight. Mice fed on a high molecular weight fucoidan ($2\text{-}3 \times 10^5$) from *Cladosiphon okamuranus*, at a level of 5%

in the diet, exhibited increased T cells while lower molecular weight fucoïdians from the same source had no effect.⁴¹

This variability of activity led the company to further investigate the uptake of fucoïdan into the blood serum after oral ingestion. Although it was unknown whether absorption or uptake by the gut lymphatic system occurred, evidence that other high molecular weight sulfated polysaccharides, such as chondroitin sulfate, are absorbed whole in small amounts in the small intestine⁴² led researchers to postulate that serum uptake of fucoïdan would occur similarly. In a clinical study, a low but significant serum uptake of a high molecular weight *Undaria*-derived fucoïdan was measured using a novel antibody technique.⁴³

To summarize the methods used, healthy human volunteers of either sex, aged 20–46 years (average 29), were divided into three groups. The first (n = 6) ingested 3g of guar gum as placebo. The second (n = 6) ingested 3g of whole *Undaria* containing 10% fucoïdan w/w and the third group (n = 40) ingested 3g of 75% fucoïdan w/w daily for 12 days.

Figure 2 illustrates the relative amounts of free fucoïdan in the serum for each group. The effect was only significant when 75% fucoïdan was ingested, although the increase could also be seen in the “whole *Undaria*” group (see **Figure 2**). In addition, the researchers were able to demonstrate a small but measurable increase in fucoïdan or its breakdown products in urine.

Although it was unknown whether absorption or uptake by the gut lymphatic system occurred, evidence that other high molecular weight sulfated polysaccharides, such as chondroitin sulfate, are absorbed whole in small amounts in the small intestine⁴² led the company to postulate that serum uptake of fucoïdan would occur similarly. In a clinical study, a low but significant serum uptake of a high molecular weight *Undaria* derived fucoïdan was measured using a novel antibody technique.⁴³ The percentages shown in **Figure 3** represent the averages of 23 human subjects. Subjects ingested 3g of the test *Undaria pinnatifida* fucoïdan per day.

This study confirmed that systemic effects can be attributed to serum uptake, even from a high molecular weight fucoïdan. The company has recently developed a low molecular weight standardized

fucoïdan from Nova Scotian *Fucus vesiculosus* to hopefully maximize this uptake. In a follow-up clinical study by the company, the effects of orally ingested *Undaria* fucoïdan on stem cells was investigated.

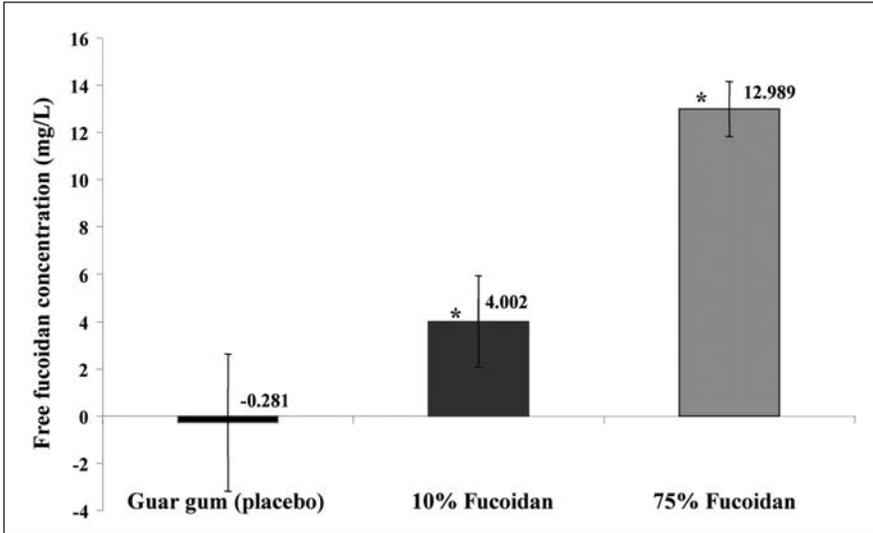


Figure 2. Concentration of free fucoïdan in blood plasma (median) at 4, 8, and 12 days for each group (placebo, 10% fucoïdan w/w, and 75% fucoïdan w/w); ± 95% confidence intervals; * p-value < 0.05 (ANOVA).

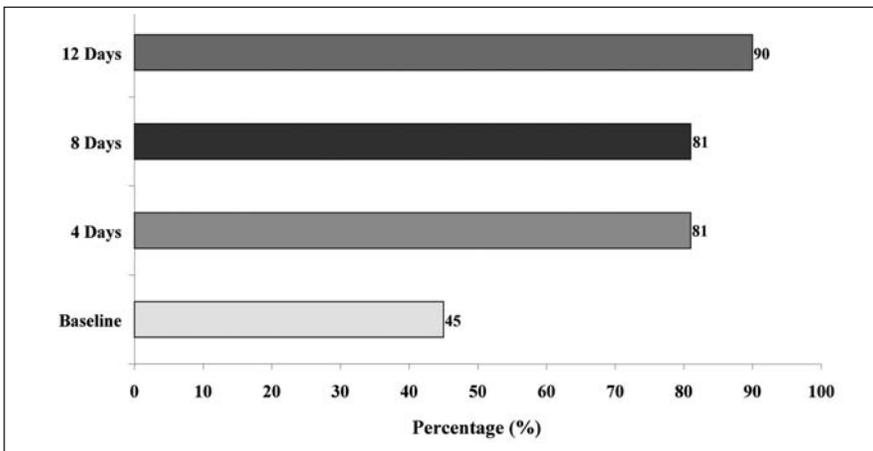


Figure 3. Serum uptake of fucoïdan

Previous research has shown that intravenous fucoïdan has a pronounced and extended mobilizing effect on a class of stem cells that circulates in the blood. These cells are called haemapoietic stem cells (HPCs) and they give rise to immune cells and potentially other cell

types in the body. A sticky receptor on the stem cells called CXCR4 helps the cells to settle wherever they are required by adhering to the chemokine stromal derived factor-1 (SDF-1).

After ingestion of *Undaria* fucoïdan, a profound increase in the sticky receptor CXCR4 occurred on the cells, in addition to a significant increase in the total numbers of HPCs. This was accompanied by healthy changes in cholesterol levels, and an increase in the immune regulating cytokine interferon gamma.⁴⁴

Stem cells are a popular topic in health today. Stem cell technology is creating advances in regenerative therapies in which damaged or diseased organs are given a new lease on life with infused stem cells. The significant effects of orally ingested fucoïdan suggest potential for rejuvenation nutraceuticals designed to give the body a boost of immune function.

Fucoïdians in Nutraceuticals

With a growing body of related research undertaken by private companies and research institutes in Japan, Korea and the United States, fucoïdians are attracting increasing interest from nutraceutical formulators. Increasingly touted as the active component of edible seaweeds such as *Laminaria japonica* (*kombu*) and *Undaria pinnatifida* (*wakame*), fucoïdians can be used in formulations ranging from beverages to tablets. Like their colloidal cousins, alginates and carrageenans, fucoïdians are relatively stable molecules.

In Korea, products such as FucoWell, a fucoïdan-containing beverage, are increasing in popularity and in the United States, fucoïdians are being integrated into immune-boosting and anti-inflammatory formulations as well as in nutritional beverages and functional foods such as those produced by Takara in Japan, or Mannatech in the United States.

With the development of low molecular weight fucoïdians, Marinova will be initiating a set of clinical studies in 2007 specifically focused on investigating the immunomodulatory and anti-inflammatory properties of fucoïdians, currently of interest to nutraceutical developers. The trials will aim to underline the relevance of fucoïdians as complements to other ingredients such as glucosamine.

Fucoidans as Novel Cosmetic Ingredients

Throughout the past 20 years there has been a growing body of research focused on the bioactive properties of fucoidans. Many of these studies have focused on the potential for fucoidan as a cosmetic or cosmeceutical ingredient. The key focus of this research has been on the inhibitory effects of topically applied fucoidan on aging and photo-damaged skin. Relevant research papers have focused on the following activities:

1. Increasing dermal fibroblast numbers and matrix production;⁴⁵
2. Inhibition of matrix metalloproteases (collagenase) and the serine protease elastase;⁴⁵
3. Contraction of fibroblast-populated collagen gels, increasing integrin expression on fibroblasts;⁴⁶
4. Increased skin thickness and elasticity in clinical trials;⁴⁷
5. Modulation of growth factor activity;³¹
6. Inhibition of inflammation;⁴⁸ and
7. Inhibition of tyrosinase.⁴⁹

Fucoidan acts to enhance dermal fibroblast proliferation and deposition of collagen and other matrix factors.⁴⁵ Matrix metalloproteases, which modulate connective tissue breakdown, are inhibited by low molecular weight fucoidan due to increased association with their inhibitors. In addition, the serine protease leukocyte elastase was inhibited, which protected elastic fiber network in human skin cultures.⁴⁶ *Undaria*-derived fucoidan inhibited the enzyme hyaluronidase,¹⁵ as did a smaller *Sargassum*-derived fucoidan.²⁷ Tyrosinase also is inhibited by fucoidan fractions, indicating potential for inclusion as a whitening agent.⁴⁹

Fujimura initially demonstrated that a fucoidan extract of *Fucus vesiculosus* (up to 30,000 MW) promoted fibroblast-populated collagen gel contraction and that the promotion of the gel contraction was due to the increased expression of integrin alpha2beta1 on the surface of the fibroblasts.⁴⁶

A clinical study followed in which a gel formulation with 1% of the fucoidan was applied topically to human cheek skin twice daily for five weeks. A significant decrease in skin thickness measured by B-mode ultrasound was elicited, as was a significant improvement in

elasticity measured with a Cutometer as compared with controls.⁴⁷ It appears that the activity in skin is in part modulated by growth factor potentiation. Heparin, the mammalian analogue for fucoïdan, is an essential cofactor for particular growth factor activities, including that of fibroblast growth factor.

Marinova observed, in an unpublished study, that crude fucoïdan isolated from *Undaria* also was able to potentiate fibroblast growth factor activity, at concentration levels similar to heparin. Transforming growth factor beta (TGF beta) is an important regulator of wound healing. O'Leary et al demonstrated that concentrations >1mg/mL of fucoïdan were able to modulate the activity of inhibitory effects of TGFb1 in a tissue culture model, increasing the migrating of cells into wounded defects, reaching normal levels at 10mg/mL.³² These results using a relatively unrefined fraction of fucoïdan indicate a promising future for research into both cosmetic applications and pharmaceutical applications such as wound healing.

Fucoïdan is also an effective inhibitor of CXCR4 and inhibits the accumulation of eosinophils in models of allergic skin inflammation.⁴⁸ Topical heparin is useful as a treatment in burns,⁵⁰ where modulation of the CXCR4 interaction may result in superior healing.⁵¹ Fucoïdan has potential as a topical and oral anti-inflammatory choice for cosmetic aftersun, allergic skin condition-soothing products, or speciality postsurgical products to inhibit eosinophilia. Fucoïdan has greater stability than heparin, and is a plant product, conferring certain advantages.⁵²

No data is available to date on the penetration of fucan fractions into the skin. Heparin, however, is formulated into topical lotions for the treatment of bruises where it acts as a fibrinolytic agent. It has demonstrable penetration,⁵³ which could potentially be mimicked by similar formulations using fucoïdan fractions of comparable molecular weight.

Toxicity is always a consideration for any nutraceutical or cosmetic ingredient. Fucoïdians can be considered to be a dietary fiber and are nontoxic in cell culture. There were no toxicological changes observed in rats given up to 300mg/kg orally of fucoïdan from *Laminaria japonica*; anticoagulant effects were observed at doses of 900 to 2,500mg/kg, but no other signs of toxicity were noted.⁵⁴ Marinova

has undertaken a safety trial in cancer patients in Australia and demonstrated that ingestion of up to 6 g per day of *Undaria* containing 10% w/v fucoidan has no observable side effects. This study is currently unpublished.

Formulating with Fucoidan

Fucoidans are, in essence, natural aqueous extracts of marine macroalgae. A number of these extracts are already INCI listed and available for use in cosmetic products, as outlined in the **Table 1**.

Macroalgal Species	CAS Number	INCI Listing Functions
<i>Laminaria japonica</i>	223751-72-2	Skin protecting
<i>Ascophyllum nodosum</i>	84775-78-0	Skin conditioning
<i>Fucus vesiculosus</i>	84696-13-9	Soothing, smoothing, emollient, skin conditioning
<i>Undaria pinnatifida</i>	223751-81-3	Skin protecting
<i>Durvillea antarctica</i>	223749-87-9	Skin protecting
<i>Macrocystis pyrifera</i>	---	Viscosity controlling

As natural marine products, these extracts are ideal for inclusion in cosmetics. But how easy are they to formulate? Fucoidans are effectively water-soluble and create relatively nonviscous solutions in water or dilute salt solutions. Thus, in contrast to alginates or agar, they will not add significant body to formulations. Generally, fucoidans are heavily sulfated and thus anionic, which should be taken into account during formulation. Since they can be formulated in many different molecular weights and from several different source macroalgae, it is difficult to give an indicator for viscosity. In one example, viscosity of a high molecular weight *Undaria* derived fucoidan ranged from ~4.5cP in a 1% solution, to ~40cP in a 5% solution. Bioactivity for fucoidan may occur in 1mg/mL range, indicating formulation concentrations at around 0.1–1% level, depending on other factors such as molecular weight.

The use of fucoïdan also sits well with the recent trend for “beauty from within” nutraceutical formulations and provides the opportunity to offer a cosmetic solution from a systemic, as a well as a topical approach.

Conclusion

Fucoïdians are interesting nutraceutical ingredients with significant potential for topical cosmetic formulations. Purified fractions can be easily incorporated into creams and lotions, providing cosmetic antiaging benefits such as inhibition of matrix enzymes and anti-inflammatory activity, in addition to increasing numbers of dermal fibroblasts and collagen tightness. As nutraceutical ingredients they offer unique opportunities in immunomodulation and rejuvenation.

Sourced from some of the most pristine waters in the world, fucoïdians combine excellent bioactivity, physical protective properties and a natural organic source, thus bringing together the best elements of both science and marketability.

While macroalgae remain a staple of the spa industry, fucoïdan-rich macroalgal extracts have a growing role to play in the nutraceutical market and strong potential for commercial success in cosmetics.

Published August 2007 *Cosmetics & Toiletries* magazine.

References

1. Celtic Seaweed Baths Web site; Available at: www.celticseaweedbaths.com (Accessed Apr 25, 2007)
2. S Aaronson, Algae in *The Cambridge World History of Food*, K Kiple and KC Ornelas, eds, Cambridge University Press, vol I, chapter II.C.I (2000) pp 231–249
3. M Richelle, M Sabatier, H Steiling and G Williamson, Skin bioavailability of dietary vitamin E, carotenoids, polyphenols, vitamin C, zinc and selenium, *Br J Nutr* 96(2) 227–38 (Aug 2006)
4. M Grieve, *A Modern Herbal*, Dover Publications Inc, NY, NY (1971)
5. J Gerard, *The Herbal or General History of Plants*, NY, NY, Dover Publications Inc. (orig. published 1633; 1975) Lib 3:1566–1615
6. J Bostock, MD, FRS and HT Riley, *The Natural History. Pliny the Elder*, London, Taylor and Francis (1855)
7. C Tseng, Algal biotechnology industries and research activities in China, *J Appl Phycol* (13)375 (2001)
8. J Ara, V Sultana, R Qasim and VU Ahmad, Hypolipidaemic activity of seaweed from Karachi coast, *Phytother Res* 16 479–483 (2002) and IBID References 11–14, 16, 17, 20, 22, 24 and 28.

9. H Maeda et al, Fucoxanthin from edible seaweed, *Undaria pinnatifida*, shows antiobesity effect through UCP1 expression in white adipose tissues, in *Biochemical and Biophysical Research Communications* 332 (2005) pp 392–397
10. H Maeda, M Hosokawa, T Sashima, N Takahashi, T Kawada and K Miyashita, Fucoxanthin and its metabolite, fucoxanthinol, suppress adipocyte differentiation in 3T3-L1 cells, *Int J Mol Med* 18(1)147–52 (Jul 2006)
11. X Yan et al, Fucoxanthin as the major antioxidant in *Hijikia fusiformis*, a common edible seaweed, *Biosci Biotechnol Biochem* 63(3)605–607 (1999)
12. K Shiratori et al, Effects of fucoxanthin on lipopolysaccharide-induced inflammation *in vitro* and *in vivo*, *Exp Eye Res* 81(4)422–8 (Oct 2005)
13. M Kakinuma, CS Park and H Amano, Distribution of free L cysteine and glutathione in seaweeds, *Fisheries Science* (67)194 (2001)
14. K Ishihara et al, Purification of stearidonic acid (18:4(n-3)) and hexadecatetraenoic acid (16:4(n-3)) from algal fatty acid with lipase and medium pressure liquid chromatography, *Biosci Biotechnol Biochem* (64)2454 (2000)
15. T Katsube, Y Yamasaki, M Iwamoto and S Oka, Hyaluronidase-inhibiting polysaccharide isolated and purified from hot water extract of *Sporophyll of Undaria pinnatifida*, *Food Sci Technol Res* (9)25 (2003)
16. CR Parish, DR Coombe, KB Jakobsen, FA Bennett and PA Underwood, Evidence that sulphated polysaccharides inhibit tumour metastasis by blocking tumour cell derived heparanases, *Int J Cancer* (40)511 (1987)
17. T Shibata, K Nagayama, R Tanaka, K Yamaguchi and T Nakamura, Inhibitory effects of brown algal phlorotannins on secretory phospholipase A₂s, lipooxygenases and cyclooxygenases, *J Appl Phycol* (15)61 (2003)
18. M Wessels, G Konig G and A Wright, A new tyrosine kinase inhibitor from the marine brown alga *Styopodim zonale*, *J Nat Prod* (62)927 (1999)
19. MJ Joe et al, The inhibitory effects of eckol and dieckol from *Ecklonia stolonifera* on the expression of matrix metalloproteinase-1 in human dermal fibroblasts, *Biol Pharm Bull* 29(8)1735–9 (Aug 2006)
20. DJ Schaeffer and VS Krylov, Anti HIV activity of extracts and compounds from algae and cyanobacteria, *Ecotoxicology and environmental safety* (45)208 (2000)
21. KD Thompson and C Dragar, Antiviral activity of *Undaria pinnatifida* against herpes simplex virus, *Phytother Res* (18)551 (2004)
22. SL Lee MD, PhD, Iodine deficiency, *Emedicine*, available at: www.emedicine.com/med/topic1187.htm (2006)
23. J Teas, S Pino, A Critchley and LE Braverman, Variability of iodine content in common commercially available edible seaweeds, *Thyroid* (14)836 (2004)
24. R Hesp and B Ramsbottom, Radiobiology—Effects of sodium alginate in inhibiting uptake of radiostrotrium by the human body, *Nature* 1341–1342 (1965)
25. YF Gong et al, Suppression of radioactive strontium absorption by sodium alginate in animals and human subjects, *Biomed Environ Sci* (4)273 (1991)
26. K Morita and K Tobiishi, Increasing effect of nori on the fecal excretion of dioxin by rats, *Biosci Biotechnol Biochem* 66(11) 2306–13 (Nov 2002)
27. R Cooper et al, A preparation of Tasmanian *Undaria pinnatifida* is associated with healing and inhibition of reactivation of Herpes, *BMC Complement Altern Med* (2)11 (2002)
28. H Funahashi et al, Seaweed prevents breast cancer? *Jpn J Cancer Res* (92)483 (2001)
29. Y Miyake et al, Dietary intake of seaweed and minerals and prevalence of allergic rhinitis in Japanese pregnant females: Baseline data from the Osaka Maternal and Child Health Study, *Ann Epidemiol* 16(8) 614–21 (Aug 2006)
30. O Berteau and B Mulloy, Sulfated fucans, fresh perspectives: Structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide, *Glycobiology* (13)29 (2003)
31. S Soeda, S Sakaguchi, H Shimeno and A Nagamatsu, Fibrinolytic and anticoagulant activities of highly sulfated fucoidan, *Biochem Pharmacol* (43)1853 (1992)

32. R O'Leary et al, Fucoidan modulates the effect of transforming growth factor (TGF)— β 1 on fibroblast proliferation and wound repopulation in *in vitro* models of dermal wound repair, *Biol Pharm Bull* 27(2)266–270 (2004)
33. MK Patel, B Mulloy, KL Gallagher, L O'Brien and AD Hughes, The antimitogenic action of the sulphated polysaccharide fucoidan differs from heparin in human vascular smooth muscle cells, *Thromb Haemost* (87)149 (2002)
34. B Kloreg and RS Quatrano, Structure of the cell walls of marine algae and ecophysiological functions of the matrix polysaccharides, *Oceanogr Mar Biol Ann Rev* (26)259 (1988) (Is this the correct matchup with the reference?)
35. A Cumashi et al, A comparative study of the anti-inflammatory, anticoagulant, antiangiogenic and antiadhesive activities of nine different fucoidans from brown seaweeds, *Glycobiology* 12 (Feb 2007)
36. Y Angulo and B Lomonte, Inhibitory effect of fucoidan on the activities of crotaline snake venom myotoxic phospholipases A_2 , *Biochem Pharmacol* (66)1993 (2003)
37. SP Khanapure, DS Garvey, DR Janero and LG Letts, Eicosanoids in inflammation: Biosynthesis, pharmacology and therapeutic frontiers, *Curr Top Med Chem* 7(3)311–40 Review (2007)
38. HP Rang, MM Dale and JM Ritter, *Pharmacology*, 3rd edn, Churchill Livingstone, USA (1995)
39. H Maruyama, H Tamauchi, M Iizuka and T Nakano, The role of NK cells in antitumor activity of dietary fucoidan from *Undaria pinnatifida* sporophylls (Mekabu), *Planta Med* 72(15)1415–7 (Dec 2006)
40. I Yamamoto, H Maruyama, M Takahashi and K Komiyama, The effect of dietary or intraperitoneally injected seaweed preparations on the growth of sarcoma-180 cells subcutaneously implanted into mice, *Cancer Lett* (30)125 (1986)
41. J Shimizu et al, Proportion of murine cytotoxic T-cell is increased by high-molecular weight fucoidan extracted from *Okinawa mozuku* (*Cladosiphon okamuranus*), *J of Health Sci* (51)3 (2005)
42. L Barthe et al, *In vitro* intestinal degradation and absorption of chondroitin sulfate, a glycosaminoglycan drug, *Arzneimittelforschung* (54)286 (2004)
43. MR Irhimeh, JH Fitton, RM Lowenthal and P Kongtawelert, A quantitative method to detect fucoidan in human plasma using a novel antibody, *Methods Find Exp Clin Pharmacol* 27(10)705–710 (Dec 2005)
44. MR Irhimeh, JH Fitton and RM Lowenthal, Fucoidan ingestion increases the expression of CXCR4 on human CD34(+) cells, *Exp Hematol* 35(6)989–94 (Jun 2007)
45. K Senni et al, Fucoidan a sulfated polysaccharide from brown algae is a potent modulator of connective tissue proteolysis, *Arch Biochem Biophys* 1,445(1):56–64 (Jan 2006)
46. T Fujimura, K Tsukahara, S Moriwaki, T Kitahara and Y Takema, Effects of natural product extracts on contraction and mechanical properties of fibroblast populated collagen gel, *Biol Pharm Bull* 23(3)291–7 (Mar 2000)
47. T Fujimura, Treatment of human skin with an extract of *Fucus vesiculosus* changes its thickness and mechanical properties, *J Cosmet Sci* 53(1)1–9 (Jan-Feb 2002)
48. MM Teixeira and PG, The effect of the selectin-binding polysaccharide fucoidin on eosinophil recruitment *in vivo*, *Br J Pharmacol* 120(6)1059–66 (Mar 1997)
49. XJ Kang, FX Wang, CM Sheng and Y Zhu, *Undaria pinnatifida* stem fucoidan biological activity of the composition and bioactivity, *Chinese Pharma Journal* 41(22) 1748–1750 (2006)
50. MJJ Saliba, Heparin in the treatment of burns: A review, *Burns* 27(4)349–358 (2001)
51. S Avniel and Z Arik, Involvement of the CXCL12/CXCR4 pathway in the recovery of skin following burns, *J of Invest Dermatol* 126 468–476 (2006)
52. C Michel, M Lahaye, C Bonnet, S Mabeau and JL Barry, *In vitro* fermentation by human faecal bacteria of total and purified dietary fibres from brown seaweeds, *Br J Nutr* 75(2) 263–80 (Feb 1996)

-
53. G Betz, P Nowbakht, R Imboden and G Imanidis, Heparin penetration into and permeation through human skin from aqueous and liposomal formulations *in vitro*, *Int J Pharm* (9)228(1-2)147-59 (Oct 2001)
 54. N Li, Q Zhang and J Song, Toxicological evaluation of fucoidan extracted from *Laminaria japonica* in Wistar rats, *Food Chem Toxicol*, 43 421 (2005)

Balancing Skin's Microflora with Probiotics

Katie Schaefer

Cosmetics & Toiletries *magazine*

KEY WORDS: *microflora, probiotics, skin, nutrition, antiaging*

ABSTRACT: *This chapter reviews probiotics, their uses and their benefits as nutritional supplements.*

In the food and nutrition world, a probiotic is an ingredient that, when ingested, helps aid various ailments. The World Health Organization and the Food and Agriculture Organization of the United Nations describe probiotics as, “Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host.”¹ Probiotics benefit the body as nutritional supplements because they help it produce its own natural or gut flora.

These healthy bacteria reportedly allow the body to boost its own immune system, reduce inflammation, lower cholesterol and manage lactose intolerance, among other benefits that are not entirely proven since research is still in its infancy. Although the specific benefits of probiotics are not concrete, and testing has shown the ingredient can produce overall positive results. Soon after the nutrition industry recognized the benefits of probiotics, the personal care industry began researching its application in skin care. What has been discovered so far may reinvent antiaging formulations.

Aea Marc, the vice president of product and business development for Bioelements Inc., had a hand in her company's effort to incorporate probiotics into one of its products.

Creating a Probiotic Serum

Bioelements Inc. stumbled across probiotics when Barbara Salomone, its president and founder, noted the ingredient's popularity in food.

"Our president was seeing a lot of information that showed how probiotics strengthened the immune system. She then began to investigate whether probiotics could be applied topically to the skin," said Marc.

During investigation, the company found a supplier that developed a complex incorporating bifidus ferment lysate in milk proteins. "The [engineered] bifidus ferment lysate is immersed in milk proteins to strengthen the skin," said Marc.

Although the actives are no longer alive, they are still active. The company formulates the complex at 5%.

Also formulated into the product is soybean seed extract and hydrolyzed rice protein. These ingredients were added for their claimed ability to help protect the skin barrier and build collagen.

In vivo and *In vitro* Testing

As most cosmetic manufacturers know, science sells. Thus during its ingredient search, the company placed heavy emphasis on efficacy testing and found that, with the particular ingredient chosen, four different tests were performed, two of which relating to the ingredient's interaction with ultraviolet (UV) light.

"The tests showed that the bifidus complex strengthened the skin against UV light, meaning that the chemical changes that occur when the skin interacts with the sun were reduced," added Marc.

Additionally, an test showed an increase in cellular function.

The fourth, test examined the ingredient's potential to cause irritation and redness. "Because [the material] strengthens the skin, irritation and redness are reduced. This [benefit] is attributed to the ingredient's ability to balance the good microflora in the skin," said Marc, who added that balancing microflora on the skin is similar to balancing it in the gut.

Misconceptions

Probiotics were initially linked to skin care because of their help with acne. According to Marc, this effect is misunderstood.

“[Probiotics] are not really an acne treatment. They can help keep the good bacteria on the skin, thereby helping skin to heal and the acne to clear. Therefore, they are not as much a help in clearing the acne as they are in balancing the skin,” explained Marc.

Who Uses Probiotics

Although the company labels its probiotic serum as antiaging, it recommends that young women in their 20s and 30s use the product.

“Because probiotics help strengthen skin and prevent UV and environmental damage, we recommend the product as a preventive product—as the first step in antiaging skin care,” said Marc. The product is also recommended for overprocessed and irritated skin.

Marc has seen a growth in the use of probiotics in skin products; however, she notes their use is mainly confined to the professional/spa industry.

“Probiotics have not made their way into mass-market products because marketers for cosmetic companies have a difficult time explaining what these ingredients do. Estheticians can better explain to their clients how they can help their skin,” added Marc.

She has noticed that probiotics have branched into different arenas of personal care. “I am hearing they are being formulated into toothpaste, body products and deodorant. I really think that consumers are going see their uses grow in personal care.”

Published October 2007 *Cosmetics & Toiletries* magazine.

References

1. Food and Agriculture Organization (FAO)/World Health Organization (WHO), *Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria*. Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. (2001)

INDEX

#

8-oxo-dG 399, 402-7
8-oxo-guanine 125, 399-400

A

AAR (Aspartic acid racemization) 156, 161-2
absorbances 356, 362
ABTS oxidation 355-6
Acad Dermatol 18, 154, 201, 334, 397
açai 353-4, 356
ACE (angiotensin-converting enzyme) 432
acetate 103, 107, 110, 116, 230, 241, 402
Acetyl-L-carnitine (ALC) 116
acids
 behenic 369, 374
 glutamic 273, 420
acne 74, 140, 426, 469
active ingredients 20, 28, 41, 68, 99, 102, 113-14, 171-2, 179, 191,
 197, 215-16, 219, 227-8, 285, 426
active MMPs 134, 137
actives 109, 187, 228-9, 235, 240-1, 252-3, 283, 392, 423, 447, 468
AcTP11 191-7, 199-200
adenine 114, 116, 121

- adhesion, initial 259-61
- aged cells 67-9, 174, 184, 340, 342, 345-6
- aged fibroblasts 266-7, 340-3
- aged human fibroblasts 262-4, 266-9
- aged skin 11, 78, 153, 156-7, 159-62, 166, 169, 182, 248, 283, 287, 367-8, 374
- aging 5-6, 18, 50-3, 55-8, 62-5, 70-1, 79-80, 111-14, 116-18, 151-2, 154-5, 162-3, 167-8, 187-9, 210, 342-4
 - biological 64, 73
 - cellular 286, 340, 344
 - cutaneous 121, 154, 187, 203, 229, 388
 - free radical theory of 62, 111
 - mitochondrial theory of 113, 120
 - premature 40, 49, 58, 62, 106, 115, 120, 305
 - programmed 57, 156
 - sign of 187, 246
- aging cells 66, 69, 120, 173-4, 179, 184, 263
- ALC (Acetyl-L-carnitine) 116
- algae 39, 48, 62, 335-6, 439-40, 454, 463-4
- alpha-SMA 258-60, 262-4, 267, 269
- amino acid sequences 136, 238, 272, 274, 277
- amino acids 119, 136, 148, 156, 191, 216-17, 221, 271-2, 274-5, 277-8, 378, 382-3, 390, 415, 448
 - basic 216, 221
 - free 159, 378, 382
- angiotensin-converting enzyme (ACE) 432
- ANOVA (Analysis of Variance) 25, 29, 32-3, 458
- ANOVA test 27-8, 31
- anti-wrinkle effects 20, 219, 223-5
- antiaging 5, 7, 9, 11, 13, 15, 17, 39, 51, 57, 60-1, 63-4, 77, 111, 229, 271
- antiaging actives 227-9, 231, 233-5, 237, 239-41, 243
- antiaging benefits 60, 111, 215, 335, 389, 413, 422, 426, 431
- antiaging effects 111, 113, 115, 117, 119, 121, 184, 197, 200, 245, 247, 249, 251, 253, 255, 278-9
- antiaging efficacy 271, 283, 390
- antiaging ingredients 273, 438
- antioxidant activity 27, 36, 116, 232, 297, 313, 318, 336, 347, 353-8

antioxidant enzymes 117, 120, 232, 234, 242, 366
antioxidants 39-40, 43, 62, 105, 114-17, 230-1, 240, 303-4, 307,
312-13, 317-20, 353-5, 358-9, 424-5, 434-5, 446-7
antiradical activity 319
ApoB protein 144
apoptosis 65, 69, 93, 111, 113, 303, 401
aqua 8, 11, 21, 47, 58, 60, 81, 85, 197, 250, 252-3, 265, 273, 284,
345, 348-50
aqueous phase 19, 21, 24, 239, 383
arabinoxylans 257, 262, 269
arbutin 12, 46-7, 50
arrays 41, 281, 327, 336-7, 456
ATP 103-4, 111-12, 115, 275, 322, 389

B

bacillus subtilis 419-20
BaP 402-6
base excision repair (BER) 324
BCA Assay 360-1
BCA method 360, 362
BCAAs (branched-chain amino acids) 381
BER (base excision repair) 324
beta-carotene 49, 319, 425, 434, 446
BHT 43-4, 250, 253
bioactivity 441, 451-3, 456, 462, 465
bioavailability 238, 423, 425
biomolecules 97-8
blood flow 90, 151-3, 446
botanicals 229, 232, 235, 239-40
Botox 221, 277, 449
branched-chain amino acids (BCAAs) 381
buttocks 157-8, 167-8, 370, 443

C

calcium 158, 236, 382, 384
calibration 117-18

- calmodulins 384
- caprooyl-phytosphingosine 369
- caprooyl tetrapeptide-3 81-90
- carotenoids 39-42, 46, 49-50, 422-4, 431, 434, 439, 446, 463
- catalase 105, 118-19, 227, 232-4, 242, 300-1, 340-1, 359-60, 366
- catalase activity 118, 242, 360-1, 363-4
- catalytic domain 134-6, 142
- CDC (Center for Disease Control) 450
- Cdel 107-9
- CDKs (cyclin-dependent kinase) 183-4
- cell cycle 58, 64, 69, 71, 183, 322, 400
- cell divisions 173, 181-4
- cell proliferation 36, 57-9, 61, 210, 216, 254, 351, 397
- cell surfaces 135, 137-8, 140, 389
- cell survival 65-6, 69-70, 270
- cells
 - blocking tumor 440, 464
 - damaged 124, 302, 322, 327, 362
 - immune 302, 458
 - inflammatory 8, 138
 - mononuclear 139
 - nonirradiated 94-5, 98
 - peptide-treated 364-5
 - senescent 181-2, 185
- cellulite 443-6, 448-50
- cellulite formation 444
- cellulose 203-4
- Center for Disease Control (CDC) 450
- centrifugation 23, 318
- ceramides 54, 163, 208, 212, 227, 235-6, 243, 367-8, 372, 374, 378, 380-1, 385
- cetearyl alcohol 11, 60, 250, 252-3, 265, 276, 317, 348-50
- cetearyl glucoside 265, 276, 348-50
- Cetyl PEG 20-1
- Chinese herbs 279-80
- chitin 203-7, 209, 212, 214
- chitin nanofibrils 203-7, 209-13
- cholesterol 7, 208, 368-9, 378

- chromophores 5-7, 9, 14, 18
chronological aging 77-8, 169
CO₂ 94-5, 98, 126, 191, 218, 263-4, 309, 326-7, 360
coenzyme 105, 121, 352
coenzyme Q₁₀ 103, 105-6, 110, 115, 206, 423-4, 446
collagen 8-11, 78-9, 106-7, 156-7, 161, 176, 187-8, 190-2, 194,
200, 210, 214-15, 218, 237-8, 289-92, 413-14
collagen degradation 41, 44, 62, 123, 130
collagen distribution 15-16
collagen encoding gene 190, 193-4
collagen fibers 29, 156, 161, 176, 209, 229, 257-9, 342, 387
collagen fibrils 124, 206
collagen molecules 7, 156
collagen production 218, 272, 291, 296, 389, 433
collagen protein 194, 293
collagen synthesis 36-7, 156, 176, 179, 209, 219, 226, 273, 275,
295, 397
collagen VII 77, 79, 83-4, 90
collagen VII expression 84, 89
colorless carotenoids 39, 43-4, 49-50, 57, 62, 64
comet 326, 328, 362, 402-4
comet assay 326, 328-30, 365, 399, 402-5, 407
components, cellular 305-6, 308, 312
compounds, mitochondria-nourishing 111, 113
connective tissue (CT) 87, 124, 139, 162, 237, 246, 257, 275,
443-6, 448
contraction 223, 260, 391, 465
contraction frequency 222-3
copper 43, 215, 271-2, 388-9
CoQ₁₀ 105, 107, 110, 115
corneocytes 61, 207, 209, 211, 213, 377-8, 381-3, 385
corticoids 82-3
cosmetic antiaging benefits 439, 463
cosmetic chemists 52, 54, 140, 379-80, 383, 385
cosmetic formulations 46, 120, 206, 305, 309, 313, 347, 389, 413,
416, 420, 448, 451
cosmetic ingredients 18, 91, 110, 215, 218, 228, 235, 387-8,
397, 461

- cosmetic products 109, 114, 149, 234, 307, 312, 390-1, 395, 419, 462
- cosmetics 10, 19, 36, 50, 55, 64, 70, 73, 212-13, 229-30, 278, 317, 383-4, 421-3, 427-8, 462-3
- cotton extract 91, 99, 101-2
- CPD (cyclobutane-pyrimidine dimers) 91-9, 101, 124, 129, 323-4
- CPD, quantity of 93, 95, 99-101
- CPD lesions 95, 99, 101-2, 130
- crow's-feet 83, 87, 221, 224-5, 265-6, 268-70, 391-2
- crow's-feet area 127, 129, 268, 396-7
- crustaceans 203-5
- CT *see* connective tissue
- cyclobutane-pyrimidine dimers *see* CPD
- Cytokeratin 178, 338
- cytokines 123-4, 178, 210
- cytosol 103-4, 133, 135

D

- darutoside 5, 8, 12, 18
- deanol 398
- DEJ (dermal-epidermal junction) 77-81, 83-4, 89-90, 134, 345
- delivery 3, 75, 109, 113, 125, 143-5, 215, 423
- dermal 18, 124, 166, 171-3, 175, 177, 179, 225, 246, 260, 289, 449
- dermal cells 71, 323
- dermal-epidermal junction *see* DEJ
- dermal fibroblasts, human 180, 242, 270, 290, 293, 327, 329-30, 440, 464
- dermal thickness 164, 167
- dermis 14-15, 29, 53, 74, 79, 81, 87, 123-5, 156-8, 161, 166, 187, 257-9, 323-4, 387-8, 444
 - papillary 158-9, 295, 397
- desmosomes 377, 384-5
- DHEA 164
- DHR 308
- DHT 164
- Dicaprylyl carbonate 348-50
- Dicaprylyl ether 197, 348-50

dimer 91, 359, 364-5
 cyclobutane-pyrimidine 91-2
dimer peptide 359-60, 363-5
dimethylaminoethanol 387-9, 391, 393, 395, 397-8
Discoidin Domain Receptor 193, 201
diseases 7, 54, 121, 140, 145-6, 247, 305-6, 379, 408, 452
DMAE 388-91, 395, 397-8
DNA 45, 62, 91-3, 98, 101-2, 111-12, 115-16, 123-7, 183-4, 247,
 254-5, 301-2, 321-2, 324-6, 358-9, 399-403
DNA array 200, 281
DNA damage 41, 44-6, 49, 91, 102, 123-5, 129, 183, 321, 329, 358,
 362, 365, 399-400, 402-7
DNA-Na 248, 251, 254
DNA protection 70, 235, 334, 401-2, 404, 407
DNA repair enzymes 123, 125-6, 130-1, 355
DNA Repair System 322-5
dormins 57-8, 60-1
DRMC 390-1, 393, 395-6
dsRNAs 141-2

E

ECM 78, 80, 82, 193, 258, 260-1, 266, 289-90
ECM proteins 289-90, 295-6
EGF (epidermal growth factor) 94, 342-3, 345-6
elastases, neutrophil 137-8
elasticity 79, 198-200, 245, 249-51, 254, 283, 290, 367-8, 375, 413,
 434-5, 460-1
elastin 78, 106, 155-8, 161, 210, 215, 237-8, 257, 283, 387, 422,
 434, 446
electrolytes 302, 383, 416
electron transfer 298, 300
electron transfer chain 104-5
electrons 62, 104-5, 282, 301, 306, 355
 unpaired 298, 301, 303, 353
electrophoresis 326, 328, 361-2, 401-3
emulsions 19-25, 31, 35-6, 149, 212, 225, 239, 249, 251, 253, 265,
 268-9, 276, 315, 383

- endogenous antiproteinases 139-40
- endogenous stem cells 147-8
- enzymes 2, 75, 116, 124, 135-9, 206, 208, 229-30, 232-4, 239-40, 282-3, 300-1, 325, 359-60, 388-9, 413
- epidermal 127-8, 167, 179, 368, 370, 374, 384, 434
- epidermal differentiation 372, 374, 398
- epidermal growth factor *see* EGF
- epidermal keratinocytes, human 194, 197, 389
- epidermal metabolism 171-3, 175, 177, 179
- epidermal thickness 164-7, 169, 193, 230
- epidermis 18, 26, 66, 79, 81, 123-5, 166-7, 172, 174, 187-9, 198-200, 246, 312-13, 368, 377, 388
 - reconstructed 173-5, 340-1
- erythema 7, 19-20, 25-8, 50, 124, 230, 315, 325
- erythema formation 44, 62
- extracellular matrix 7, 44, 78, 82, 124, 133-4, 177, 179, 187-8, 190, 193-4, 229, 243-4, 258, 273, 283
- extracellular proteolytic enzymes 133, 136-7

F

- FADH₂ 104-5
- FCS (fetal calf serum) 176, 191-2, 290, 360
- fetal calf serum *see* FCS
- fibroblasts 7, 68, 78, 81-2, 89, 106-7, 109, 123-4, 128-30, 171, 209-10, 246, 248-9, 257-61, 270, 460
 - attachment of 258-9
- fibronectin 77, 79-80, 82, 89, 134-5, 162, 215, 237, 273, 289-90, 292, 296, 342, 446
- filaggrin 173-5, 178-9, 372, 378, 382
- fluorescence 45, 95, 117, 308-9, 342
- fluorescence microscopy 305, 308, 312
- Fluorescence Microscopy Chapter 307, 309, 311, 313
- Food and Agriculture Organization (FAO) 467, 469
- formulas 20, 24-8, 30-3, 234, 238-9, 252, 262, 279, 390
- formulations 19, 23-4, 28-9, 31, 33-6, 48, 74, 83, 87, 109, 179, 206, 239-40, 309-10, 319, 462
 - slimming 446-8

free fucoidan 457-8
free radical damage 62, 114, 312
free radicals 26, 43-5, 48-9, 61-3, 115, 117, 210-11, 279-80, 282,
301, 303-6, 308-9, 335, 353, 355, 422
fruits 35-6, 50, 85, 172, 353-4, 421, 425-6, 445
fucoidan extracts 436, 438, 452, 456, 460
fucoidan fractions 435, 453, 460
fucoidans 336, 431-2, 435-41, 451-63, 465-6
fucoxanthin 434-5, 440, 453, 464

G

gadd45 α 321, 325, 327, 334
gadd45 α gene expression 327, 329, 334
GAGs 78, 160-1, 187-8, 209, 446
GEKG 290, 292-6
gene expression 9, 80, 178, 327, 329, 334, 336-40, 342, 344, 366
collagen encoding 193-4
gene expression profile 172, 177, 179
genes 66, 69, 71, 102, 104, 141, 177-9, 193, 280-3, 290-1, 322, 327,
330, 332, 336-8, 341-2
genome 93, 321-2
GHK 215, 271-2
glutathione 7, 36, 282-3, 301, 435, 440, 453, 464
glycation 431, 436-7
glycerin 21, 25, 29, 33, 47, 53, 60, 81, 85, 191, 197, 250, 252-3, 273,
276, 348-50
glyceryl oleate 250, 252-3
glyceryl stearate 47, 85, 250, 252-3, 292, 348-50
glycosaminoglycans 160, 187, 200, 209, 273, 397
grapefruit 20, 35-6, 445
grapefruit extract 19-21, 25-7, 29, 32, 35, 315

H

H-beta-Ala-Pro-Dab-NH-benzyl diacetate 220, 222-5
H-beta-Ala-Pro-Dab-NH-benzyl diacetate solution 223
hair 61, 161, 416, 418-19, 421-2, 427

hair strength 417-18
heme oxygenase 282-3, 341
hemoglobin 6, 12, 17, 153, 282
heparin 455-6, 461, 465
heterogeneity 5, 10, 14-15
histidine 73-4, 136, 300
humectants 29, 381, 385, 409
hyaluronic acid 53, 160, 203-4, 278, 289-90, 292, 412-13
hydrogel 415-16, 420
hydrogen peroxide 7, 43, 112, 232-4, 241, 270, 297-8, 303, 306,
313, 355, 359, 366
hydroxyl 43, 50, 117, 233, 297, 303
Hydroxyl Radical Production 298-9
hydroxyl radicals 42-3, 45, 62, 112, 299
hypodermis 159, 164, 444, 446

I

immunofluorescence intensity 95, 97, 99
inflamm-aging 434, 440
inflammation 12, 41, 44, 46-7, 51, 54-5, 139-40, 237, 248, 280-1,
298, 304, 335, 345, 389, 445-6
inflammation index 9, 13
inflammatory cascade 54-5
ingredients 13, 47, 74, 81, 114, 148-9, 229, 345, 347, 351, 389-90,
412-13, 422-4, 432, 445-8, 467-9
nutricosmetic 424, 426, 435
insulin levels 431, 437-8
integrins 188, 190, 215, 257-9, 261-3, 266, 269
intercellular lipids 53-4, 174
intrinsic aging 57-8, 61, 64, 153-4, 158, 160, 182-3, 258
intrinsic skin aging 180
iodine 432, 453-4
irradiation 48, 93-5, 98, 100-2, 107-9, 118, 125-6, 128-9, 226, 230,
243, 309, 326, 328, 332, 420
isomers 115, 307-8

J

junctions, dermal-epidermal 77-8, 90, 134, 345

K

keratin 215, 377-8, 384-5

Keratinocyte culture 191-2, 194, 352

keratinocyte differentiation 173-5, 179, 236, 338, 374

keratinocyte serum-free medium (KFSM) 94, 173

keratinocytes 1, 5, 7, 12, 53, 94-5, 106, 128, 130, 171, 174, 193-4,
248-9, 283, 345, 405-7

growth of 200, 248-9, 275

metabolic activity of 172-3

nuclei of 93, 99, 101

KFSM (keratinocyte serum-free medium) 94, 173

L

Laminaria japonica 438, 459, 461-2

laminae 205

laminin 79, 82, 89-90, 215

laminin-5 77, 79, 83-4, 89, 190

lanatus 401, 404, 406-7

Langerhans cells 55, 210

Laser Doppler flowmetry (LDF) 152-3

laser Doppler velocimetry (LDV) 152

LDF (Laser Doppler flowmetry) 152-3

LDL (low-density lipoprotein) 144

LDV (laser Doppler velocimetry) 152

lentiginos 5-6, 13

lipid bilayers 377, 380, 385

lipid mixture 368-9, 371, 374

lipid peroxidation 41, 242, 299, 313, 340, 342, 359-60, 362, 365

lipids 123, 158, 161, 214, 230, 242, 297, 299-301, 308-9, 345, 359,
366, 369, 374, 377-8, 380

omega-3 431-2, 452, 454

lipoic acid 115, 121
liposomes 125, 128, 130-1, 313
lotions, liposome 125, 127-8
low-density lipoprotein (LDL) 144
LPP (long periodicity phase) 369, 371
LSD test 29, 31, 33
lycopene 20, 39, 46, 49-50, 424-5, 434, 446
lysine 272-3

M

macroalgae 431-3, 435, 439, 451-4, 463
 historical applications of 451-2
magnesium ascorbyl phosphate (MAP) 126, 128
maintenance, epidermal water 368, 372, 374
mammalian cells 125, 303, 322
mammalian skin epidermis 366
MAP (magnesium ascorbyl phosphate) 126, 128
matrikines 227, 237-8, 243, 296
matrix components 106, 138, 283, 287
matrix metalloproteinases 107, 124, 133, 140, 257, 279, 282, 352,
 445-6
 elevated collagen-degrading 287, 398
mechanical properties 79, 160, 162, 261, 397, 465
mechanical stresses 258-9, 261, 268-9
mechano-receptors 257-61, 266
melanin 6, 12, 19-21, 23, 25-7, 29, 31, 33, 35, 37, 233, 301, 315
melanin content 9, 12, 27, 46-7
melanin formation 26, 426
melanocyte stimulating hormone (MSH) 1-2, 226
melanocytes 1-3, 5-6, 12, 26, 46, 389, 408
melanosomes 5, 9, 12
mesotherapy 443, 448-50
metabolic activity 172-3, 179, 207, 214, 258
microcirculation, improving 443, 445
micrograms 433, 454
micronutrient 431-2, 452, 454
mineral salts 387-8, 390, 395, 397-8

- minerals 265, 276, 348-50, 378, 389, 421, 423-4, 439-40, 448, 453, 464
- Minimum Protection Factor (MPF) 48, 62
- miRNAs 141-3
- mitochondria 64, 103-5, 111-12, 114-17, 120, 125, 321-3, 330
- mitochondrial 107, 112-13, 116, 341
- photoaging-associated 110, 313
- moisture 20, 29, 113, 381, 383, 415
- moisturization 246, 254, 352, 367, 377, 379, 384-5, 396, 409
- moisturizing effect 285, 395-6, 414
- molecules 6-9, 39-41, 61-2, 97, 113-14, 138, 143, 172, 206, 209, 213, 229-34, 271, 301, 353, 388-9
- active 48, 141-2, 325, 327, 423
- fluorescent 117
- toxic 279, 282
- mRNA 141-2, 210, 322, 327, 337, 372, 374
- mRNA coding, expression of 173, 175-6, 179
- mRNA of alpha 263, 266
- MSE (Malva sylvestris extract) 172-9, 437
- MSH (melanocyte stimulating hormone) 1-2, 226
- mtDNA 104-7, 109, 111-12, 321-6, 329-30, 334
- MtDNA damage 113, 322
- mtDNA mutations 106-7
- muscles 52, 277, 421

N

- Na 410-11
- NADH 104-5
- nanoemulsion 103, 107-10
- nanofibrils 204-7, 209, 213
- NER (nucleotide excision repair) 93, 101-2, 324, 333
- neutrophils 136, 139, 210
- NHDF (normal human dermal fibroblasts) 81, 126, 360
- NHEK (normal human epidermal keratinocytes) 126, 128, 339
- NMF (natural moisturizing factor) 213, 368, 378-9, 381-3, 385, 412-13, 416, 420
- nodosum 336, 342, 345, 347, 351

nondepolymerized fucans 345, 347
nuDNA 321, 324, 326, 333-4
nutraceuticals 214, 433, 451, 459, 461
nutricosmetics 421-5, 427-9, 431-2, 436, 438

O

oil 23-4, 265, 276, 317, 348-50
 paraffin 20-1, 31, 33-4
oridonin 5, 8, 12, 18
oxygen 6-7, 104-5, 111, 234, 297, 300, 303, 306, 381
 molecular 297-8, 303

P

palmata 336, 340, 351
palmata extract 340, 343, 347-8, 350-1
palmitoyl pentapeptide-3 273
palmitoyl tripeptide-5 216-18, 220
 solution of 219
PBN (phenyl-butyl-nitrone) 114
PBS (phosphate buffer saline) 94, 98, 108, 126, 264, 402
PCA (pyrrolidone carboxylic acid) 382, 412
PCL 316-17
peptides 73-5, 81, 87, 148-9, 215, 217-19, 222-3, 225-6, 237-40,
 244, 271-8, 289-90, 292, 295, 359, 415
 fusion 277-8
peroxides 7, 26, 182-4
PGA 409-19
PGA hydrogel 410, 415-17
PGE2 8, 345, 347
phenyl-butyl-nitrone (PBN) 114
phosphate buffer saline *see* PBS
phospho-p53 405-6
photo-aging 50, 64, 121, 183, 296, 306
photoaged skin 78, 106, 156-61, 167, 360, 375, 397
photoaging 37, 57, 70, 77-8, 102, 123-31, 149, 151, 154, 157-8,
 161, 169, 227, 321, 324-5, 397-8

- cutaneous 231, 241
photolyase 125, 127-8, 130, 325
physiologic aging 155, 163
phytoene 39-41, 45-6, 49-50, 64, 424-5
phytofluene 39-41, 45-6, 49-50, 62, 64, 424-5, 434
pigmentation 1-3, 5-6, 46, 169, 309, 313
pigments 2, 434, 453-4
placebo 11, 61, 87, 219, 223-4, 236, 265, 268, 285, 309-11, 390,
394, 396-7, 457-8
placebo cream 197, 199, 284, 291, 328, 330, 390, 394, 427
plasma 41, 46, 134, 422
plasmid DNA 45, 401-3
polydeoxyribonucleotides 255
polypeptide 148-9, 274-5, 384, 388, 409
potassium hydroxide 250, 252-3
probiotics 422, 467-9
protein carbonylation 359-61, 363-4
protein complexes 104
protein oxidation 279, 286
proteinases 133, 136-9
proteins 155-6, 159-61, 176-7, 187-8, 204-6, 215, 274-8, 299-301,
303-4, 324-5, 361-2, 368, 374, 384, 400, 432
 hydrolyzed wheat 250, 252-3
 oxidized 286
 receptor 1-2
proteolytic activity 134, 136-7
proteolytic enzymes 133, 135, 182, 378, 382
pyrrolidone carboxylic acid *see* PCA

Q

- quercetin 403-4, 426

R

- R-lipoic acid 115-16
Racemization 156
radiation, solar 228, 298-300, 366

- radicals 103, 301, 316, 354, 407
- Raman spectroscopy 155, 159-60
- reactive oxygen species *see* ROS
- relative humidity *see* RH
- respiratory syncytial virus (RSV) 144-5
- retinoic acid 74, 172, 174-9, 231, 235, 241, 390, 398, 446
- retinoids 171-2, 179, 229, 231, 388, 397
- retinol 231, 238, 278, 387-91, 393, 395, 397-8, 428, 446, 450
- RH (relative humidity) 22, 24-5, 151, 155, 163, 382
- rice bran extract 307-12
- RISC (RNA Interference Silencing Complex) 142-3
- RNA 126-7, 142, 255, 263, 281, 291, 389
- RNA Interference 141, 146
- RNA interference *see* RNAi
- RNA Interference Silencing Complex *see* RISC
- RNAi (RNA interference) 141-3, 145-6
- RNAi therapeutics 145-6
- ROS (reactive oxygen species) 41, 48, 92, 105, 107, 112-13, 233, 297, 301-2, 304-8, 310-13, 315, 322-5, 333, 340, 399
- RSV (respiratory syncytial virus) 144-5
- rye 262, 265
- rye extract 257, 262-70

S

- SAE 325-30, 334
- SAXD 163, 367, 369, 371
- SC (stratum corneum) 29, 32-3, 53, 113, 119, 159, 163, 165-6, 168-9, 174, 203, 207-8, 312, 367-9, 377-82, 384-5
- SC Antioxidant Enzymes 118
- SDS (sodium dodecyl sulfate) 284
- Sea 335, 337, 339, 341, 343, 345, 347, 349, 351
- sebum 20, 30-1
- sebum content 30-1
- selenium 432-3, 463
- senescence 65, 69, 71, 182-6
- senescent 181-4
- septa 444, 446

-
- serine 135-6, 272-3
serine proteinases 133, 135-9
serratus 336, 338
serratus extract 338-9, 347-51
SIA (spectrophotometric intracutaneous analysis) 5, 9-10, 392, 396
SIA analysis of wrinkles 395-6
signaling
 cytokine 123-4
 molecular 124
signals 98-9, 193, 259
siRNA duplex 142
siRNAs 141-6
SIRT1 65-71
SIRT1 expression 65-8, 70-1
SIRT1 induction 67, 70-1
sirtuin expression 65-7, 69
sirtuins 65-7, 69-71
skin
 dry 54, 203, 209, 252, 302, 370, 381, 383-4, 433
 ethnic 47
 murine 131, 313
 photo-aged 126, 160
 photo-damaged 368, 397, 460
 photodamaged 157, 160, 162, 270
 photoexposed 261, 323
skin aging 7, 51, 65, 77-8, 82, 87, 89, 151, 153-5, 171-2, 275,
 295-6, 304, 368, 387-9, 397-8
 reversal of 275, 278
 signs of 305-6
skin barrier 29, 53-4, 207, 213, 236, 243, 281, 284, 374-5, 468
skin barrier function 216, 339, 372-3, 385
skin cancer 2-3, 46, 50, 54-5, 92, 124, 131, 281, 313, 315
skin chromophores 5, 8
skin cohesion 77, 79-80, 90
skin collagen 157, 169
skin color 26, 46, 130, 231, 387
skin damage 54, 62, 123, 229, 384, 431, 439
skin diseases 36, 159, 325, 352

- skin disorders 64, 279, 281, 286, 315
- skin echogenicity 165, 169
- skin elasticity 44, 169, 201, 245, 275, 293, 372-4, 388, 414
- skin erythema 25, 28
- skin explants 83-4, 89, 255
- skin fibroblasts 36, 180, 200, 234, 254, 273, 336
- skin homeostasis 335-6, 351
- skin lipids 214, 302
- skin melanin 20, 26
- skin moisture 20, 245, 383
- skin moisturization 214, 245, 251
- skin morphology 68, 164
- skin penetration 35, 81
- skin physiology 151, 154, 178, 337, 385, 443
- skin pigmentation 46, 49, 426
- skin protection 243, 319, 338, 374, 401
- skin proteins 81, 156, 210
- skin redness 233, 285
- skin regeneration 289, 295
- skin thinning 53, 283
- skin tissues 245, 309
- skin tone 18, 124, 265, 268-9, 347
- skin type 46, 78, 156, 169
- skin-whitening 19, 315
- slimming treatments 443, 445, 447-8
- SOD 118-19, 233-5, 340-1, 355, 359-60
- Sodium cetearyl sulfate 348-50
- Sodium DNA 245, 247-8, 250, 252-4
- sodium dodecyl sulfate (SDS) 284
- sodium polyacrylate 197, 265, 348-50
- sodium salt of -PGA 409-11, 413
- sorbitol 273, 340, 345, 348-50
- spectrophotometric intracutaneous analysis *see* SIA
- SPF (sun protection factor) 48, 62-3, 228, 237, 325
- stem cells 147-9, 458-9
- Stoke's law 22-3
- stratum corneum *see* SC
- sun protection factor *see* SPF

Sunscreen cream 48-9, 63
sunscreen creams 48-9, 62-3
sunscreen filters 228, 306
sunscreens 48, 62-3, 227-37, 239-40, 306, 312-13, 320, 325
superoxide anion 112, 233, 297, 299
superoxide dismutase 105, 118, 227, 233, 242, 253, 300-1, 317, 355, 359, 366
syndecan-1 187-8, 191-2, 194, 200
syndecan-1 expression 189, 191, 201
syndecan-1 synthesis 195-6, 200

T

TAS (Total Antioxidant Status) 353-8
TCM *see* traditional Chinese medicine
TCM blend 280-1, 283-6
 tested 286-7
telomeres 58, 64, 182
tetrapeptide-21 290
tetrapeptides 290
TEWL (trans-epidermal water loss) 20, 32-3, 339, 367, 370, 374, 417
TGF *see* transforming growth factor
TGF-beta 140, 215-16, 218, 226
thioredoxin 282-3, 286, 340-1, 352
threonine 272-3
thymine dimers 323, 326, 329-30
thymine dimers formation 331
tocopherols 60, 230, 241, 301, 305, 307-8, 312, 434
tocotrienols 305, 307-8, 312-13
traditional Chinese medicine (TCM) 8, 131, 279, 433, 452
trans-epidermal water loss *see* TEWL
transforming growth factor (TGF) 90, 216, 226, 440, 461, 465
transglutaminase 338-9, 352
tripeptide 7, 215, 226, 282
tryptophan 298, 300

U

ultrasound 165-8

urea 378, 382-3

UV 1, 8, 39-40, 48-9, 58, 70, 92, 103, 123, 125, 183, 234, 239, 327-9, 359-60, 468-9

UV filters 228, 240

UV-induced DNA damage 125-6, 130, 321

UV-induced skin damage 64, 434

UV irradiation 41, 49, 61-2, 91, 93, 109, 123-5, 177, 230, 232-3, 236, 242-3, 289, 305, 309, 323-4

UV light 2, 44, 62, 183-4, 231, 234, 236, 280, 298, 354, 468

UV radiation 7, 10, 12, 41, 50, 57, 92, 114, 123-4, 130, 231, 258, 283, 298, 305-6, 399-400

UV Toxicity 322-3, 327

UVA 41, 44, 50, 54, 64, 92, 106, 240-1, 258, 303, 309, 311, 321-4, 326-7, 330, 363

UVA irradiation 92, 177, 179, 235, 306, 313, 363-5

UVA stress 176, 363

UVB 41-2, 44, 50, 62, 67, 91, 94-5, 98, 100, 102, 107, 110, 126-8, 321-4, 326, 328-9

UVB doses 69, 92-3, 95, 97, 99, 102

UVB irradiation 91, 93, 96, 99-101, 108-9, 127, 230, 235, 352

V

vascularization 6, 17, 154, 275

VEGF (Vascular Endothelial Growth Factor) 7-9, 12, 18, 145

vinculin 257, 260-3, 266, 269

viscosity 22-3, 36, 60, 252-3, 348-50, 411, 462

vitamin E-containing formulation 318

vitamins 29, 37, 107, 110, 227-34, 239-41, 307, 313, 317-20, 325-6, 329, 412-13, 421-6, 433-5, 439-40, 446

W

water 11, 23-4, 32, 47, 53, 60, 85, 159-62, 209, 212-13, 252-3, 273, 284, 348-50, 377-82, 384-5

water retention 416, 445
water-soluble 20, 117, 381, 447, 462
watermelon 399-402, 404, 407
whitening agents, delivered skin 435, 453
wrinkles 5, 51-3, 77, 79, 85-6, 89-90, 127, 129-30, 172, 219, 235-6,
238-9, 246-7, 268, 387-8, 390-6
 gravity 257-8
wrinkling 77-8, 90, 123-4, 126, 306, 387, 439

X

xanthines 443, 447

Z

zinc 388, 398, 432, 463

Alluredbooks

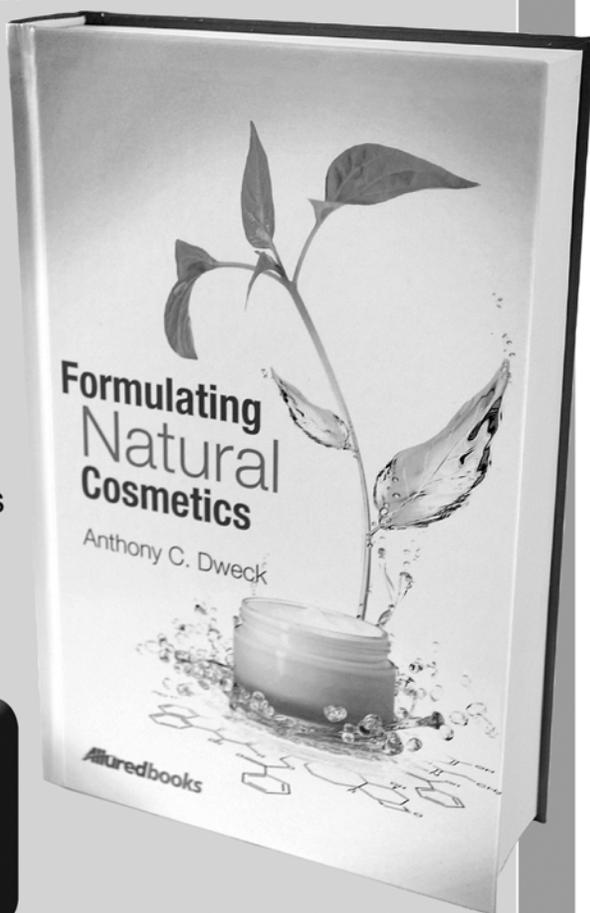
Specialty Science Books

Top seller!

A concise overview for both formulator and marketer of much of the chemistry of the natural world.

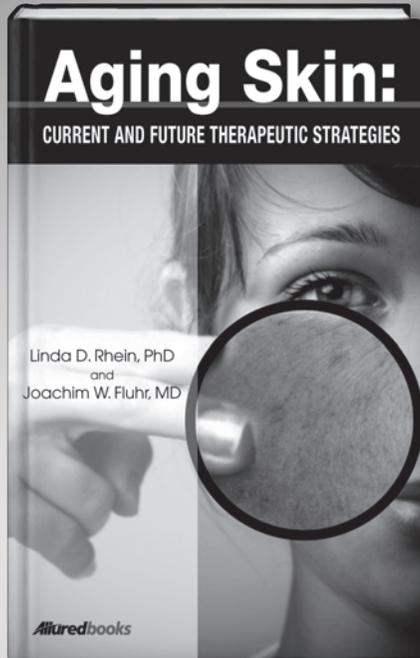
This volume looks at the entire portfolio of natural raw materials and explains, in simple terms, the chemistry, folklore and traditional uses that nature has provided.

**Order
Today!**



www.AlluredBooks.com

Alluredbooks
presents:



Aging Skin:

CURRENT AND FUTURE THERAPEUTIC STRATEGIES

Linda D. Rhein, PhD
and Joachim W. Fluhr, MD

- Innovative treatments for aging skin
- Novel science-based antiaging strategies
- Multiple diverse actives for prevention and repair of photodamage

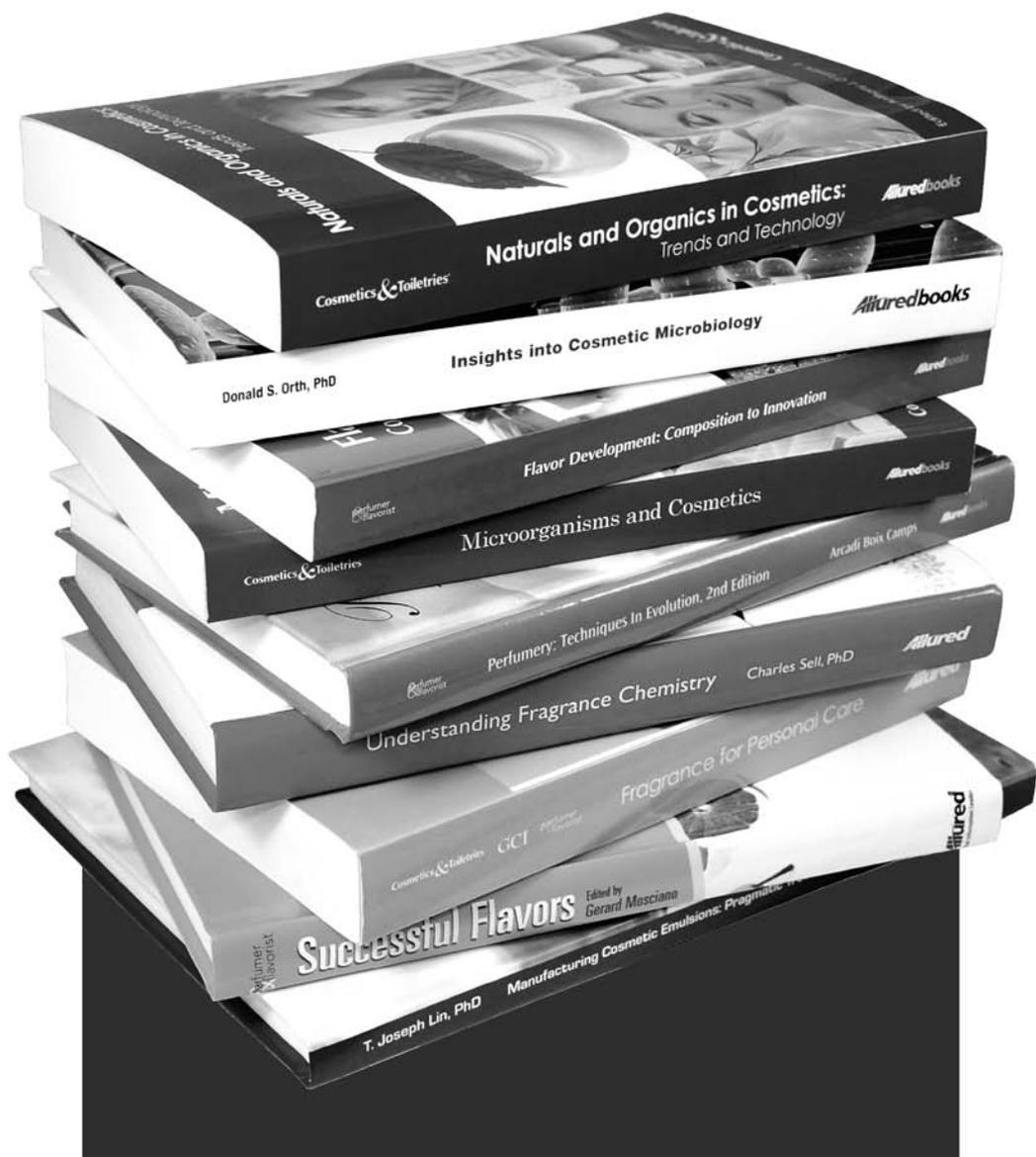
Order Today!

www.AlluredBooks.com

Alluredbooks

Specialty Science Books

www.AlluredBooks.com



Skin Care and Aging

This collection of peer-reviewed articles scientifically examines antiaging from a functional, genomic, and physiologic standpoint while presenting cosmeceutical, antioxidant and moisturization topical agents and nutricosmetic oral agents to achieve the one goal desired by all ... to be perceived as beautiful!

Skin Care and Aging looks at the behavior of aging skin and how this can be modified with cosmeceuticals, antioxidants, moisturizers and nutritional supplements. The latest in the Formulators' Resource Series, this 47-chapter volume presents the best techniques and ingredients to achieve desired antiaging effects. The industry's top researchers present studies of specific active ingredients that impact the skin's cell defenses and other mechanisms.

Topics include

- **Skin pigmentation**

The authors examine the melanocyte and skin color perception while presenting some new ingredients and their role in skin color normalization.

- **Novel cosmeceutical ingredients**

Many novel ingredients have been brought forth to accomplish these ends including peptides, tripeptides and tetrapeptides, all of which are covered along with several others.

- **Skin moisturization**

Five chapters are devoted to newly discovered aquaporins and other new ingredients with moisturization properties.

- **Antioxidants and skin care**

This section examines the nature of reactive oxygen species and elucidates measurement techniques while presenting some new ingredients for oxidative protection.

- **Nutricosmetics and skin health**

This section examines the concept of nutricosmetics and uncovers the role that several ingredients may play in skin health.

Edited by Zoe Diana Draelos, MD

Authors include Howard Maibach, Luigi Rigano, Philippe Mondon, Zoe Draelos, Diane Bilodeau, Daniel Schmid, Mindy Goldstein, Jeanette Waller, and more.

Alluredbooks

Allured Business Media
336 Gundersen Drive, Suite A
Carol Stream, IL 60188
www.AlluredBooks.com
books@allured.com
Phone: 630-653-2155
Fax: 630-653-2192

