An Aquaporin-inspired Lipid Concentrate for Mature Skin

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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 profilaggrin 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 in aged skin are accompanied by reduced levels of ceramides in the SC, especially ceramide EOS-linoleate.3

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, hydroxymethyl-glutarylcoenzyme-A reductase, and acetylcoenzyme-A carboxylase.4 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 photodamaged 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 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

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.
and skin protective characteristics\textsuperscript{10}, whereas the latter were chosen for their effects as epidermal cell signalling molecules\textsuperscript{11,12} that aid epidermal repair and regeneration.

**Materials and Methods**

Human SC lipids were isolated, as previously described.\textsuperscript{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\textsuperscript{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.\textsuperscript{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 structure of 5–6 nm each called the short periodicity phase (SPP), separated by a narrow central lipid layer with fluid domains.\textsuperscript{15}

**Aquaporin-3 is an aquaglyceroporin; it can co-transport glycerol.**

**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\textsuperscript{a} under evaluation was topically applied daily, 2 mg/cm\textsuperscript{2} 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.\textsuperscript{13} Briefly, after reverse transcription with random hexamers, the PCR reactions were carried out on a monitor\textsuperscript{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\textsuperscript{(-ΔΔC(T))} method was used.\textsuperscript{14}

**Skin Bioengineering Tests**

TEWL was measured, SC moisturization was determined quantitated as changes in electrical capacitance in arbitrary units (AU), and skin viscoelasticity was evaluated\textsuperscript{c} over the four-week study.

**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.

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 loricin; rate-limiting steps of ceramide biosynthesis including serine palmitoyl

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\textsuperscript{a}Skinmittics (INCI): ceteareth-25 (and) glycrrin (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.

\textsuperscript{b}The Opticon 1 monitor is a device from MJ Research, Waltham, MA, USA.

\textsuperscript{c}The Cutometer MPA 580 is a device of Courage and Khazaka.

\textsuperscript{d}The Corneometer CM825 is a device of Courage and Khazaka.
transf erase 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).

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.

The present work evaluated a lipid mixture and behenic acid, together with caprooyl-phytosphingosine and caprooyl-sphingosine.

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; 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.

The described combination of lipids could allow formulators to target different water management aspects in aged skin.

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.

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.

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