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The Effect of a Novel Complex, Composed of Ceramide, Energizing Peptide and Camu Camu Extract, on Epidermal Barrier Function and Dermal Antiaging Properties in *Ex Vivo* Human Skin Small Live Cohort

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Abstract

The skin's primary function is to protect the body against a spectrum of environmental stressors, including mechanical insults, microorganisms, chemicals, and allergens. Located in the outermost layers, the primary structures and components responsible for the skin's barrier function are susceptible to environmental variables, dermatological conditions, and the aging process. The ensuing alterations to structure, composition, and organizational attributes of the epidermal barrier can impact its integrity and functionality. The aim of this study was to assess the effect of a novel complex composed of a ceramide, energizing peptide, and Camu Camu extract (SUPCERAT™ complex) on specific markers of epidermal barrier integrity, as well as epidermal and dermal function. All the experiments were conducted on fresh human abdominal skin explants. Intradermal production of hyaluronic acid, epidermal claudin-1, and ceramide synthase 3 expressions, as well as epidermal lipids content were assessed using specific fluorescent stainings on *ex vivo* skin after the application of the complex or placebo. Additionally, dermal elastase and collagenase activities were assessed using *in tubo* enzymatic assays. Lastly, the effect of a cosmetic cream containing SUPCERAT™ complex was assessed using subjective Global Aesthetic Improvement Scale (GAIS) in a small cohort of patients after 60 days of use. The application of the SUPCERAT™ complex on *ex vivo* skin led to significant increase in dermal hyaluronic acid content and epidermal activity of claudin-1, ceramide syn-

thase 3 and epidermal ceramide content. Furthermore, *in tubo* enzymatic assays demonstrated inhibition of both dermal elastase and collagenase activities. In addition, the patient-reported results indicated significant improvements in skin quality and appearance.

Keywords

SUPCERAT™ Complex, Epidermal Skin Barrier, Moisturization, Antiaging, Ceramide, Camu Camu Extract

1. Introduction

Human skin acts as a primary barrier between the body and its environment. It is composed of three main layers: the epidermis, the dermis, and the underlying subcutaneous fat tissue. Each layer differs significantly in terms of composition and function [1].

In the epidermis, keratinocytes differentiate and progress from the basal layer to the uppermost layers, undergoing an intricate differentiation process that culminates with the transformation into corneocytes and the formation of the *stratum corneum* (SC) [2]. Protein members of the epidermal differentiation complex (EDC) are responsible of the terminal differentiation program of the keratinocytes. These include involucrin, loricrin and small proline-rich proteins, responsible for the cornified envelope of the skin and also a number of calcium binding proteins including intermediate-filament associated profilaggrin and trichohyalin, and several S100A proteins [3]. The SC barrier protects against environmental challenges such as physical and chemical insults, UV radiation, and environmental pollution [4]. Additionally, it regulates and limits the loss of fluids and electrolytes [5]. In its compositional profile, the SC is a complex matrix comprising predominantly proteins (75% - 80%) and lipids (5% - 15%) [6]. It is made up of protein-enriched enucleated corneocytes surrounded by a complex extracellular lipid matrix dominated by lipids, mostly ceramides, acylceramides, cholesterol, cholesterol esters, and non-esterified fatty acids (NEFA, commonly referred to as free fatty acids) [2] [7]. Ceramides, integral to this lipid environment, consist of a hydrophobic fatty acid chain linked to an amino containing sphingoid base by a ceramide synthase (CerS) [8]. Of notable significance, CerS3 is highly concentrated in the skin, particularly during keratinocyte differentiation. Granular keratinocytes secrete ceramides via lamellar bodies, contributing to the intercellular matrix of the SC and providing an effective barrier that impedes the passage of water [9]. The integrity of the skin barrier is also dependent on the tight junctions (TJ) that seal the intercellular spaces between adjacent keratinocytes in the *stratum granulosum*, found below the SC [2]. The "tightness" of this structure determines the trafficking patterns of ions, proteins and even the penetration of the dendrites of Langerhans or dendritic cells [10] [11]. Notably, the transmembrane protein claudin-1 assumes a pivotal role within TJ; its

depletion beyond a critical threshold results in impairment of both TJ and epidermal barrier function, leading to subsequent inflammation in the human epidermis [12].

The dermis, which underlies and is connected to the epidermis at the basement membrane level, imparts elasticity and strength to the skin through the synthesis of two major extracellular matrix (ECM) proteins: elastin and collagen [13]. During the aging process, mitochondrial dysfunction, associated with excessive production of reactive oxygen species (ROS), induces a cascade of transcription factor activation that culminates in the over-expression of matrix metalloproteinases (MMPs). MMPs 1, 3 and 9 play a significant role in the degradation of the dermal ECM. Specifically, collagen undergoes an initial cleavage by MMP 1, followed by complete degradation facilitated by MMPs 3 and 9. Additionally, MMP 9 is capable of degrading elastic fibers [14] [15]. Diminished levels of these functional dermal components precipitate clinically observable features, including the development of wrinkles and reduced skin elasticity [16].

Furthermore, the content of hyaluronic acid (HA) in both the epidermis and the dermis serves as a reliable indicator of hydration. HA, a high molecular weight glycosaminoglycan, stands as the predominant component of the skin extracellular matrix, with water-binding and electrolyte control properties. The hydration status of the skin critically depends on the water tightly bound by HA within both the dermal and epidermal layers [17] [18].

The hypodermis is deep to the dermis and is also called subcutaneous fascia. It is the deepest layer of skin and contains adipose lobules along with some skin appendages like the hair follicles, sensory neurons, and blood vessels [1].

In accordance with these data, we investigated the efficacy of SUPCERAT™ complex comprising three active ingredients: ceramide, Camu Camu superfruit extract and an energizing peptide.

Ceramides, extracted from a rice extract utilizing a process designed to optimize the sphingolipids and phospholipids fraction, was previously described as a booster of skin hydration. Camu camu, acknowledged as a "superfruit," is distinguished for its superior vitamin C content. Vitamin C is naturally present in skin dermis and epidermis, and it acts as a potent antioxidant that can neutralize and remove oxidants, such as those found in environmental pollutants and after exposure to ultraviolet radiation. Besides, it acts as a co-factor for the proline and lysine hydroxylases that stabilize the collagen molecule tertiary structure, and it also promotes collagen gene expression. Vitamin C level is lower in aged or photodamaged skin [19] [20]. Furthermore, the energizing peptide was designed to enhance cell energy rebound. Its application on *in vitro* cells resulted in an increase in ATP cellular levels and triggered intracellular calcium flashes (data not shown), subsequently boosting fundamental physiological processes.

New insights of SUPCERAT™ efficacy were garnered concerning skin barrier by evaluating claudin-1 expression, epidermal lipids by Nile Red staining and CerS3 expression on *ex vivo* skin biopsies. Skin hydration was studied through

HA level on *ex vivo* skin. Complementary *in tubo* enzymatic assays were employed to assess collagenase and elastase inhibition properties. Finally, clinical testing conducted on human volunteers demonstrated a global healthy-looking skin.

2. Materials and Methods

Skin culture

Normal human skin, obtained from abdominal plastic surgery of a 39-year-old female donor, was utilized for this study. Skin biopsies were obtained with a 6 mm diameter punch (pfm medical, Cologne, Germany) and maintained in organotypic culture with 50% of Dulbecco's modified Eagle's medium (DMEM) 1 $g{\cdot}L^{-1}$ glucose (Lonza, Bazel, Switzerland) and 50% of Ham's-F12 (Lonza) medium supplemented with 10% of fetal bovine serum (Lonza), 2 mM L-Glutamine (Lonza) and 100 $\mu g{\cdot}mL^{-1}$ Primocin™ (InvivoGen, San Diego, CA, U.S.A.). The skin biopsies were maintained at 37˚C and 5% CO_2 in a humidified atmosphere.

A 20 µl volume of the SUPCERAT™ complex was applied to the top of the biopsies, twice a day for 24 hours or 48 hours. Phosphate Buffer Saline (PBS) served as the control.

Chemicals and antibodies

Several antibodies were used for these studies. The primary antibodies utilized included rabbit anti-ceramide synthase 3, diluted at 1/200 (Bio-Techne, Minneapolis, U.S.A.), and rabbit anti-claudin 1, diluted at 1/200 (Abcam, Cambridge, UK). The secondary antibodies consisted of donkey anti-rabbit or streptavidin, conjugated to Alexa Fluor® 488, and were diluted at 1/1000 (Molecular Probes, Thermo Fisher Scientific).

HA was stained using biotinylated anti-hyaluronic acid binding protein, diluted at 1/400 (Sigma-Aldrich, MO, U.S.A.).

Epidermal lipids were stained using Nile red (Sigma-Aldrich). A stock solution containing 0.05% (wt/vol) Nile red in acetone was stored at 4˚C, protected from light. Prior to each staining, the stock solution was diluted to 100 µM with acetone and further diluted to 100 nM with PBS.

Biopsy preparation

To facilitate preservation and further skin sectioning, tissues were fixed for 4 hours in 10% buffered formalin. Subsequently, samples underwent a series of treatments: immersion in baths with increasing concentrations of ethanol to remove water, followed by transfer to two baths of xylene to eliminate the alcohol. Finally, the samples were embedded in molten paraffin wax. The embedded skin biopsies were then sliced into 4 µm thick sections using a microtome (Shandon) and placed on glass slides.

Immunofluorescent stainings on paraffin-embedded *ex vivo* skin

Paraffin-embedded sections of 4 µm thickness were dewaxed in xylene and rehydrated through a graded series of ethanol and water applications, as per routine histology protocol. The sections were then washed in PBS, and antigen

retrieval was achieved through enzyme digestion and/or microwave exposure in citrate buffer (0.01 M, pH 6). After blocking non-specific sites with 5% Bovine Serum Albumin (BSA) (Sigma-Aldrich) for 30 minutes, the sections were incubated with a primary antibody. Following washing with PBS, a secondary antibody was applied. Finally, after further washing, the slides were mounted with Fluoromount-G® containing DAPI for blue nuclei staining (Electron Microscopy Sciences, Hatfield, PA, U.S.A.).

Nile red staining

Paraffin-embedded sections, measuring 4 µm in thickness, underwent dewaxing in xylene and subsequent rehydration through a graded series of ethanol and water applications, as per routine histology protocol. The sections were washed in PBS, and 100 nM Nile red solution was applied for 10 minutes. Finally, after washing, the slides were mounted with Fluoromount-G® avec DAPI (Electron Microscopy Sciences).

Fluorescence microscopy

Tissue sections were examined by fluorescence microscopy using a Nikon Eclipse 80i microscope (Nikon, Champigny sur Marne, France) with a 20x objective. Photomicrographs were taken with a Nikon Digital Camera DMX1200F (Nikon) and captured using Q-Capture Pro 7™ acquisition software (QImaging®, Canada).

Image quantification methodology

Five images per condition were analyzed with Volocity®. This software quantifies the luminosity of each pixel, and all the values obtained were added. The resultant luminosity values were normalized to the area of the examined zone of each photograph. Statistical significance was assessed using a one-tailed unpaired Student's t-test with $p < 0.05$ considered significant, $p < 0.01$ very significant, and $p < 0.005$ highly significant.

Collagenase inhibitory assay

The employed assay was based on fluorescent spectrophotometric methods. The complex was incubated with the assay system following manufacturer's instructions (E12055, EnzChek™ Gelatinase/Collagenase Assay Kit; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with some modifications tailored for use in microplate reader use. The assay was initiated by adding collagenase (type IV from Clostridium histolyticum), reaction buffer, and the complex to each well, with a final enzyme concentration of 0.2 units/ml. The complex was incubated with the enzyme and reaction buffer for 25 minutes at room temperature. Following incubation, the substrate of the enzyme, DQ™ gelatin, was added to start the reaction. The fluorescence of the digested product of the DQ™ gelatin was measured immediately after adding substrate and then continuously monitored for 30 minutes at 495 nm absorbance using a microplate spectrophotometer (Bio Tek Synergy 2). Gallic acid at 0.1% served as a positive control for the inhibitory effect. Negative controls were conducted by replacing the complex with reaction buffer.

Elastase inhibitory assay

The assay employed utilized fluorescent spectrophotometric methods. The complex was incubated with the assay system following manufacturer's instructions (E12056, EnzChek™ Elastase Assay Kit; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with some modifications for microplate reader applications. The assay was initiated by adding elastase (derived from pig pancreas), reaction buffer, and the complex to each assay well, with a final enzyme concentration of 0.1 units/ml. The complex was incubated with the enzyme and reaction buffer for 25 minutes at room temperature. Following incubation, the substrate of the enzyme, the DQ™ elastin, was added to start the reaction. The fluorescence of the digested product of the DQ™ elastin was measured immediately after adding substrate and continuously monitored for 30 minutes at 485 nm absorbance using a microplate spectrophotometer (Bio Tek Synergy 2). Gallic acid at 0.1% served as a positive control for the inhibitory effect. Negative controls were performed by replacing the complex by reaction buffer.

Clinical study

Ten female participants, with ages ranging from 28 to 70, were enrolled in the study. Instructions were provided directing them to abstain from the use of any cosmetic product, except for moisturizing cream containing 5% SUPCERAT™ complex, applied twice daily over the span of 60 days. Following the intervention period, participants were questioned regarding the perceived changes in their skin quality, with the following response options: their skin quality became worse, no change, improved, much improved, or very much improved.

3. Results

Elastase and collagenase inhibition properties of the complex

The intrinsic properties of the complex to inhibit elastase and collagenase activities were assessed through *in tubo* enzymatic studies. Results showed a strong inhibition of the collagenase activity by 93% and elastase activity by 36%. Comparable effects were observed with the positive control, gallic acid, administered at 1 mg/mL (**Figure 1**).

Effect of the complex on skin barrier integrity and moisturization

The complex was applied twice a day over a 24-hour period on human *ex vivo* skin, and its direct impact on skin barrier integrity was assessed through the immuno-histochemical analysis of claudin-1 and CerS3. An increase of 37%*** and 55%***, respectively, in staining intensity was observed in the treated condition compared to the control. Furthermore, Nile red staining revealed a higher content in epidermal lipids by 185%*** after a 48-hour application of the complex, in comparison to the control condition (**Figure 2**).

Similarly, the effect of the complex on skin moisturization was highlighted by an enhancement of hyaluronic acid production (64%***) observed in the treated samples (**Figure 3**) after 24 hours of application, as compared to the control.

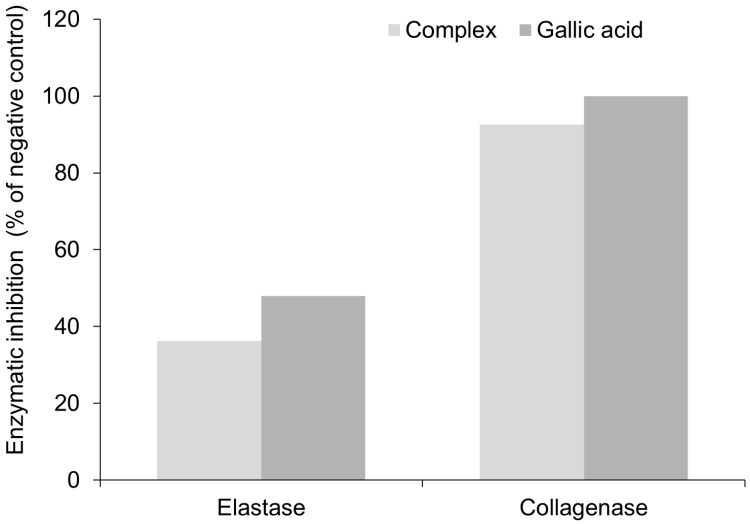

Figure 1. Inhibition of the collagenase activity by SUPCERAT™ complex. Positive control by gallic acid 1 mg/ml shows comparable effect.

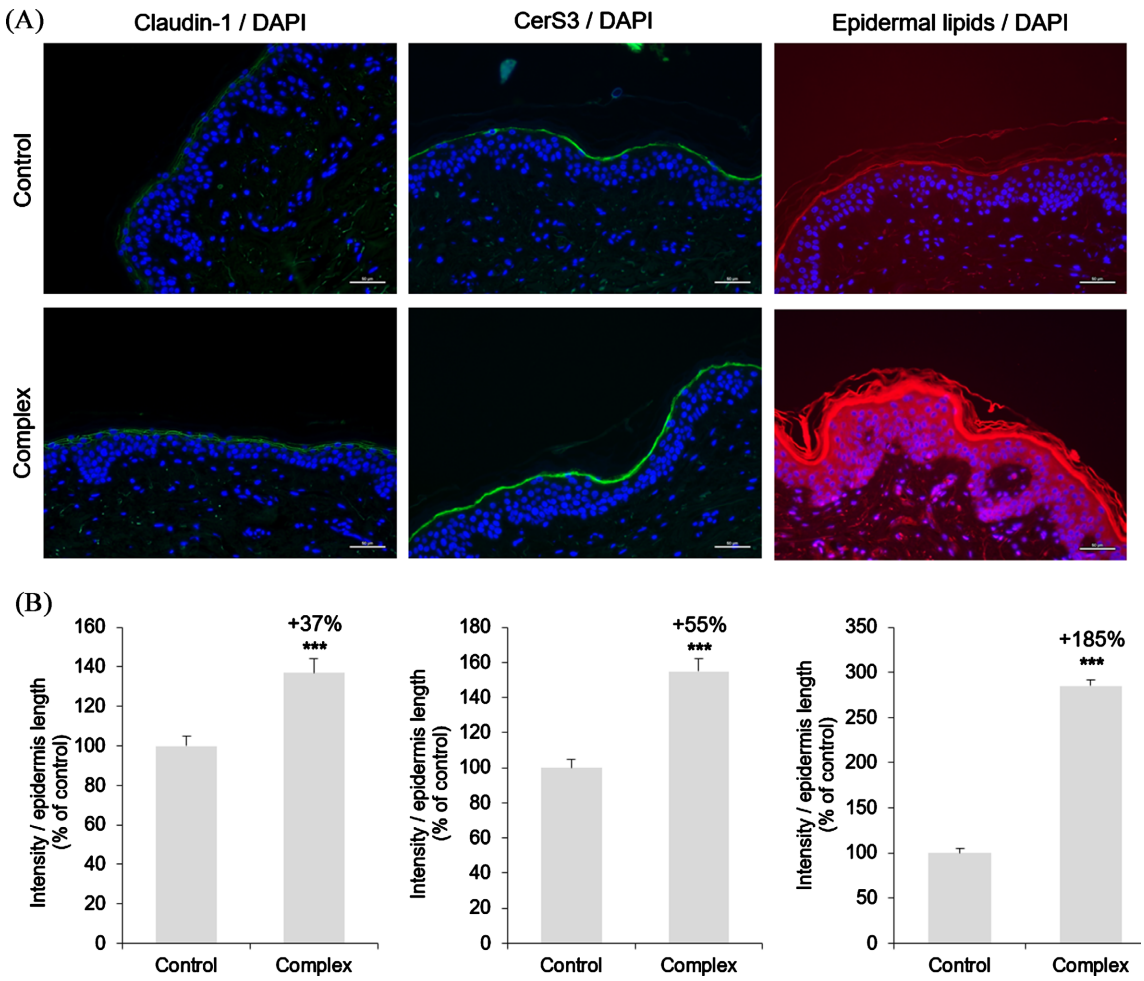

Figure 2. Efficacy of the complex on skin barrier integrity. (A) Representative images of claudin-1 (green staining), CerS3 (green staining), and epidermal lipids (red staining) associated with DAPI blue nuclear counterstain. (B) Corresponding image quantification with Volocity® software. ***: highly significant with Student's *t*-test (mean ± sem; n = 5 images). Scale bars corresponded to 50 µm.

Clinical evaluation

Lastly, the effects of the SUPCERAT™ complex, integrated into a formulated product, were evaluated on human volunteers. Out of the 10 participants surveyed regarding their perception of skin quality, 5 reported a very significant improvement, 3 reported significant improvement, and one participant noted an improvement in skin quality (Table 1 and Figure 4).

4. Discussion & Conclusions

In the present study, we assessed the efficacy of a complex comprising ceramides, an energizing peptide, and a camu camu extract on parameters reflecting dermal aging, epidermal barrier integrity, and hydration, ultimately aiming to enhance the overall appearance of the skin. *In tubo* evaluations revealed that the SUPCERAT™ complex inhibited collagenase and elastase activities, comparable to gallic acid, which served as a positive control in this study. Elastin and collagen, key components of the dermal ECM, have a pivotal role in skin strength

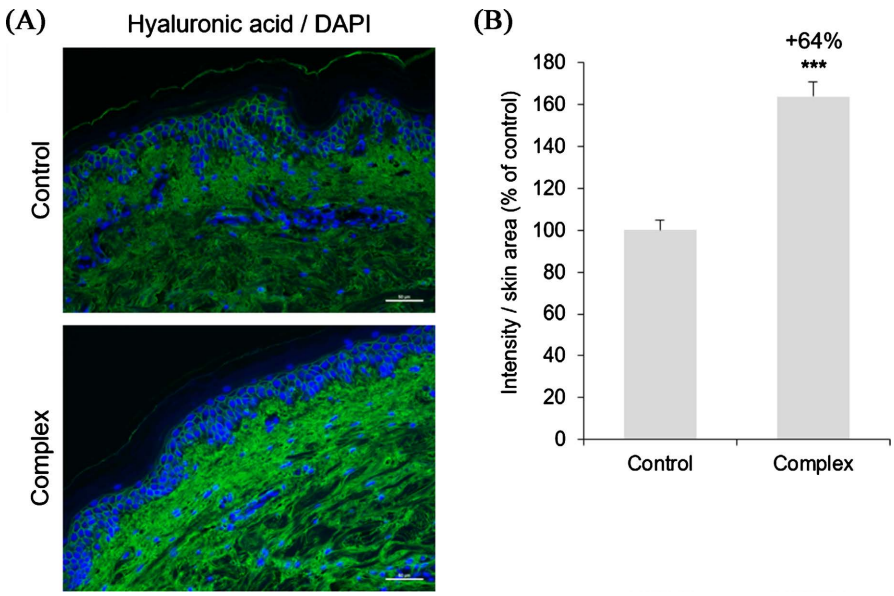

Figure 3. Efficacy of the complex on skin moisturization. (A) Representative images of hyaluronic acid (green staining) associated with DAPI blue nuclear counterstain. (B) Corresponding image quantification with Volocity® software. ***: highly significant with Student's *t*-test (mean ± sem; n = 5 images). Scale bars corresponded to 50 µm.

Table 1. Global aesthetic improvement scale assessment (GAIS).

Subject	1	2	3	4	5	6	7	8	9	10	
Very much improved	X				X				X	X	40%
Much improved		X	X				X				30%
Improved						X		X			20%
No change				X							10%
Worse											0%

Figure 4. Four patients before and 60 days after daily use (two daily applications) of 5% SUPCERAT™ complex, integrated into a formulated product. Subject 1, female 42 years old; Subject 2, female 70 years old; Subject 3, female 35 years old; Subject 4, female 38 years old.

and the elasticity. It is noteworthy that camu camu extract, previously shown to exhibit antioxidant and mitochondrial protection properties, demonstrated potential in preventing ROS-induced expression of MMPs, thereby protecting elastin and collagen fibers from degradation [21]. Moreover, experiments on *ex vivo* skin highlighted the complex's ability to preserve the epidermal barrier function by acting on the 2 major barrier structures: the SC, the outmost layer, and the TJs, intercellular junctions that seal adjacent keratinocytes in the stratum granulosum, found below the SC. Indeed, an increased expression of the major TJs protein claudin-1 was observed after the application of the complex. Tight junctions act as a semipermeable barrier to the paracellular transport of ions, solutes and water, as well as cells, and are considered to function as a fence that divides apical and basolateral domains of plasma membranes [22]. In addition, an elevated level of epidermal lipids associated with an increase of CerS3 expression showed after the application of the complex emphasized the potential of SUPCERAT™ to reinforce the extracellular lipid matrix of the SC. CerS3 has been determine as essential for the synthesis of cornified lipid envelope and extracellular lipid and importantly for Ceramide-derived sphingosines. Hence, CerS3 determines the quality of the water permeability barrier and antimicrobial function as well as final cornification [23]. This result is also aligning with the composition of the complex that includes a ceramides fraction. Finally, the increased expression of HA in *ex vivo* skin suggested a potential enhancement in

moisturization capacity induced by the complex [17] [18]. When SUPCERAT™ complex was integrated into a cosmetic cream and used by patients, all participants reported an improvement/significant improvement/very significant improvement in their skin quality and appearance after 60 days of twice-daily use.

In conclusion, this study demonstrates the positive effects of a newly developed complex on both epidermal barrier integrity and function, showcasing anti-aging potential in human skin explants and an enhancement in skin quality for real patients.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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