Diabetic skin: New cosmetic treatments with a Ca^{2+} double cone vector system

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Abstract

Background: Diabetic or very aged skin is predominantly characterized by a malfunctioning skin barrier. Underlying skin barrier malfunction is a reduced or defective calcium gradient in the epidermis. Consequently, replenishing the aged/diabetic skin's calcium stores with topical calcium could be a potential therapeutic approach.

Methods: We investigated the effect of our novel Ca^{2+} double cone vector system on improving the differentiation and barrier function of reconstructed human epidermis (RHE), cultured at low basal calcium (0.3 mM) to represent diabetic or aged skin. Further, in a randomized placebo-controlled clinical study the skin barrier of 20 healthy volunteers was challenged with 2% sodium lauryl sulfate (SLS) for 24 hours under occlusion, following and/or prior to treatment with a gel containing 2% of our calcium vector system.

Results: Culture in reduced basal calcium conditions (0.3 mM) strongly impeded the formation of a dense stratified epidermis. The apical treatment with 1.1 mM $CaCl_2$ was not able to restore a functional differentiation. Treatment with 0.1 % of the Ca^{2+} delivery system rescued the differentiation process and resulted in a normal stratified epidermis. Clinically, application of the Ca^{2+} double cone vector system prior to and following SLS stress prevented increases in skin irritation and transepidermal water loss (TEWL) compared to placebo controls. Importantly, the treatment also significantly accelerated the recovery time following SLS stress.

Conclusion: With our novel Ca^{2+} vector system, we highlight the delivery of bioavailable Ca^{2+} ions into the skin as a new and successful approach to treat a damaged barrier present in diabetic, aged, or atopic skin.

Keywords: Skin protection; barrier function; diabetic skin; Ca^{2+}; phospholipids; vector system.

Introduction. An increasingly prevalent disease in our aging society is diabetes. In 2019, the global diabetes prevalence was estimated to be 9.3 % (463 million people) and is predicted to rise to 10.2 % (578 million) by 2030 (1). Both aging and diabetes display a common skin pathology: reduced epidermal barrier function if not barrier dysfunction or loss (2-4). Damage to the epidermal barrier can lead to dramatic effects on the skin including dryness, irritation, inflammation, delayed wound healing and in extreme cases xerosis, atopic dermatitis and eczema (2, 5). Therefore, maintaining and supporting a healthy skin barrier is essential for overall skin health. Despite the prevalence of damaged diabetic/aged skin, current treatments are limited, and often include urea and greasy occlusive formulations, and more advanced prescriptive medication may even require the assistance of medical professionals. Moreover, such formulations often only treat the symptoms without addressing or preventing the underlying cause of damaged skin. It is therefore evident that novel effective treatment options are required that target the underlying mechanism of aging skin barrier dysfunction.

The skin barrier is established in the epidermis, with the key molecule driving the epidermal barrier formation being calcium. Calcium ions (Ca^{2+}) serve as essential signalling molecules, regulating numerous cellular functions in the skin, most importantly keratinocyte proliferation, and differentiation (6, 7). Different calcium concentrations are required for various keratinocyte functions: low levels of calcium for proliferation and division of stem cells, and high levels of calcium to initiate the differentiation program and the formation of the cornified envelope (8, 9). Consequently, an epidermal calcium gradient is formed to meet these differential demands, with low levels of calcium in the *stratum basale* and *stratum spinosum*, rising and reaching its peak in the *stratum granulosum*, and declining again toward the outermost layers of the *stratum corneum,* where the keratinocytes reach their final differentiation status (10, 11) (Fig. 1). Calcium also regulates the expression of differentiation-specific proteins such as loricrin, involucrin and filaggrin (12, 13), and regulates keratinocyte migration and wound healing (14).

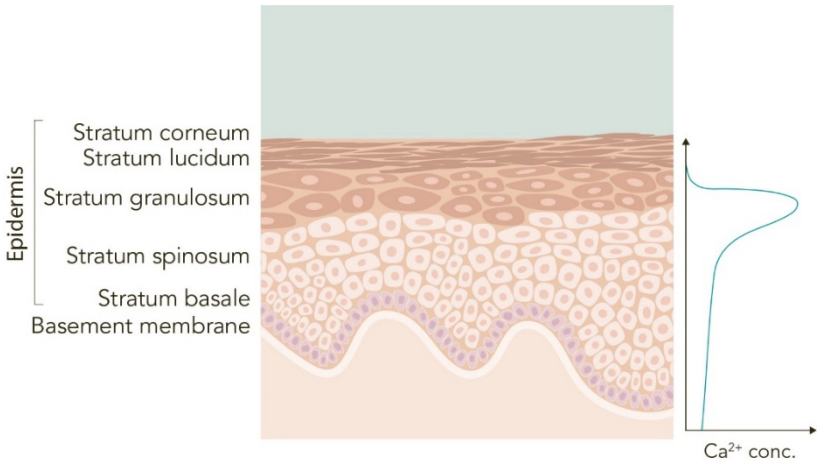

Figure 1: The Epidermal Calcium Gradient

Taken together, calcium gradient and signalling are key for a healthy skin barrier and barrier homeostasis. Unsurprisingly, a major characteristic of a disrupted skin barrier is a reduced or defective calcium gradient in the epidermis (10). Indeed, during the aging process calcium signalling is impaired (15) and the calcium gradient collapses (11, 16), which may in part underly the aging skin's reduced barrier function and epidermis thinning. Consequently, a potential therapeutic approach could be to replenish the calcium stores of not only aged, but also diabetic skin, with topically applied calcium. To achieve this, optimal Ca^{2+} bioavailability must be ensured, which may be achieved by appropriate phospholipid encapsulation of the ions (17). This study investigated the potential of a novel Ca^{2+} double cone carrier system, based on a nanocochlear molecular structure, in improving epidermal barrier function and protection *in vitro* and in a clinical trial *in vivo*.

Materials and Methods.

Materials

Loricrin antibodies were purchased from BioLegend (USA, cat. no. 905104) and Involucrin antibodies from Sigma Aldrich (USA, cat. no. SAB4200794). Chemicals and solvents were purchased from Sigma Aldrich, soy lecithin (containing 50% phosphatidylcholine) was obtained from NIKKOL (Japan, Lecinol S-10M). Human keratinocyte progenitor cells were isolated from donor tissues, human full skin biopsies were obtained from donor tissues from elective cosmetic surgery and were anonymized and coded prior to use. All human tissues

and cells were obtained and processed in accordance with the Swiss research ethics committee.

Preparation of the Ca2+ double cone carrier system.

To prepare the novel Ca^{2+} delivery system, glycerol, pentylene glycol and ddH_2O was mixed at 60°C and soy lecithin was added, then the dispersion pumped repeatedly through a high-pressure homogenizer (M700, Microfluidics™, USA) at 1200 bar. After, a $CaCl_2$ solution is slowly added to the liposomal dispersion, forming a Ca^{2+}-phospholipid double cone complex, which is again homogenized at high-pressure (1200 bar). The final nanocochleate structure of the Ca^{2+} vector system was confirmed by freeze-fracture transmission electron microscopy (FFTEM).

RHE culture and treatment

Human keratinocyte progenitor cells were expanded in growth medium (TAK-GM) at low calcium (0.05 mM). To reconstruct the 3D epidermis, the keratinocyte progenitor cells were grown in porous membrane inserts for 2 days in TAK-GM. The medium was then changed to 3D differentiation medium (TAK-3D) containing 1.1 mM calcium and the cells were grown for 24 h in submerged condition. 3D cultures were then airlifted, and the different treatments started, for 9 days, with medium changes every alternate day. Cells were either treated with 1.1 mM calcium basal and apical (control), 1.1 mM calcium basal and 0.3 mM calcium apical (reduced calcium), 1.1 mM calcium basal and 1.1 mM $CaCl_2$ apical, or 1.1 mM calcium basal and 0.1 % Ca^{2+} vector system apical. 3D models were harvested at day 7 and day 9, fixed and processed for histological and immunohistochemistry analysis.

Skin explant treatment

Skin explants were treated topically with 20 mg of a gel containing either 2 % Ca^{2+} vector system, 0.884 mM $CaCl_2$, or a placebo gel, for 24 hours using Franz diffusion cells. After exposure, the explants were washed with 3 mL of ddH_2O and frozen at -80 °C.

Histology and immunohistochemistry

The reconstructed human epidermis (RHE) were fixed, and morphology and stratification analysed by hematoxylin and eosin (H&E) staining. For the expression of loricrin, immunohistochemistry expression was performed on all day 9 cultured RHE. Deparaffinized tissue sections were processed for antigen retrieval, peroxide blocking and immune-histochemical staining using Bond Polymer Refine Detection kit (Leica Biosystems, Germany, cat. no. DS9800). Sections were counterstained and mounted on coverslips before being imaged with high-resolution microscopy.

The frozen skin explants were thawed, fixed in formaldehyde, and embedded in paraffin. 5 µm tissue sections were prepared and mounted on glass slides prior to deparaffinization. Slides were then stained with H&E for tissue structure analysis. Protein expression of involucrin was assessed by immunohistochemistry staining following antigen retrieval and peroxide blocking, using Bond Polymer Refine Detection kit (Leica Biosystems, Germany, cat. no. DS9800). Quantification of involucrin was performed with Image J (NIH, USA) using customized plugins and filters.

Clinical study

To evaluate the clinical efficacy of the Ca^{2+} vector system, a monocentric, double blinded randomized and placebo-controlled study was performed. 20 female volunteers aged 23 – 65 years (mean 42.2 y) with normal skin (Fitzpatrick II to III) were included. The study involved two parts, evaluating both the protective and regenerative effects of the Ca^{2+} vector system prior to/after 2 % sodium lauryl sulfate (SLS) stress using occlusive patches (Finn Chamber Large®, SmartPractice, Germany). Protection phase: application of the test product twice daily in one area, randomly selected (test area), for 7 days followed by a challenge with SLS in two areas (test and control) and evaluation of the skin effects until complete recovery. Regeneration phase: challenge with SLS in two areas (test and control) followed by the twice daily application of the test product in one area randomly selected (test area) and evaluation of the skin effects until recovery of the two areas. The skin parameters were measured at baseline, 24 h after stress and every 2-3 days until recovery. Parameters measured were skin microcirculation (Periflux PF5000, Perimed, Sweden), skin redness according to a* parameter (Chromameter® CR-400, Minolta, Japan), and TEWL (Tewameter® TM300,

Courage +Khazaka, Germany). The study was performed at PhD Trials, Lisbon, Portugal in accordance with the ethical principles and guidelines established for the performance of Human test projects. All participants gave informed consent.

Statistical analysis

Statistical comparisons were performed using GraphPad Prism 8 software (GraphPad, USA) and all data are expressed as mean ± SD of at least 3 independent experiments. Comparisons among different groups were made using Student's t-test with homoscedasticity. P values < 0.05 were considered significant.

Results.

The mixing of negatively charged phospholipids with Ca^{2+} ions by high pressure homogenization resulted in a supramolecular nanocochleate with a double cone structure (Fig. 2A), a structure that was firstly described by Papahadjopoulas *et al.* in 1975 (18). The structure was further confirmed by our own observations using FFTEM (Fig. 2B). Such an

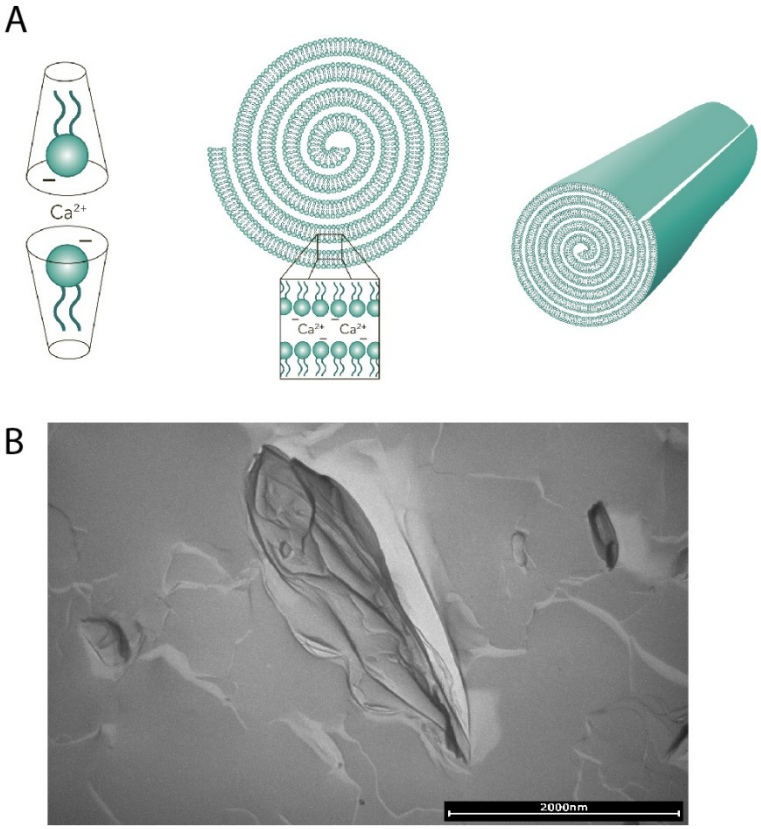

Figure 2: Structure and EM Image of the Ca^{2+} Vector System

encapsulation technology can make impermeable molecules, such as Ca^{2+} ions, bioavailable to tissues, including the skin (19).

The effect of this Ca^{2+} delivery system on the differentiation of RHE was investigated at a low basal calcium condition (0.3 mM) to represent a diabetic or aged skin. After 9 days of growth, the 3D RHE grown in standard conditions (1.1 mM calcium basal) formed all stratified layers (basal, squamous, granular and cornified) (Fig. 3A) with established loricrin expression (Fig. 4A). Reduction of the calcium concentration during the differentiation process to 0.3 mM basal calcium strongly impaired the formation of a dense, stratified epidermis and led to the formation of vacuoles (Fig. 3B). Treatment of differentiating keratinocytes with 1.1 mM $CaCl_2$ from the apical side could not restore a functional differentiation but further deteriorated the process (Fig. 3C). Correspondingly, loricrin expression was also strongly impaired in both conditions (Fig. 4B and 4C). In contrast, treatment with 0.1 % of the novel Ca^{2+} vector system rescued the differentiation process, prevented the formation of vacuoles (Fig. 3D), and increased the expression of loricrin (Fig. 4D), resulting in a normal stratified epidermis.

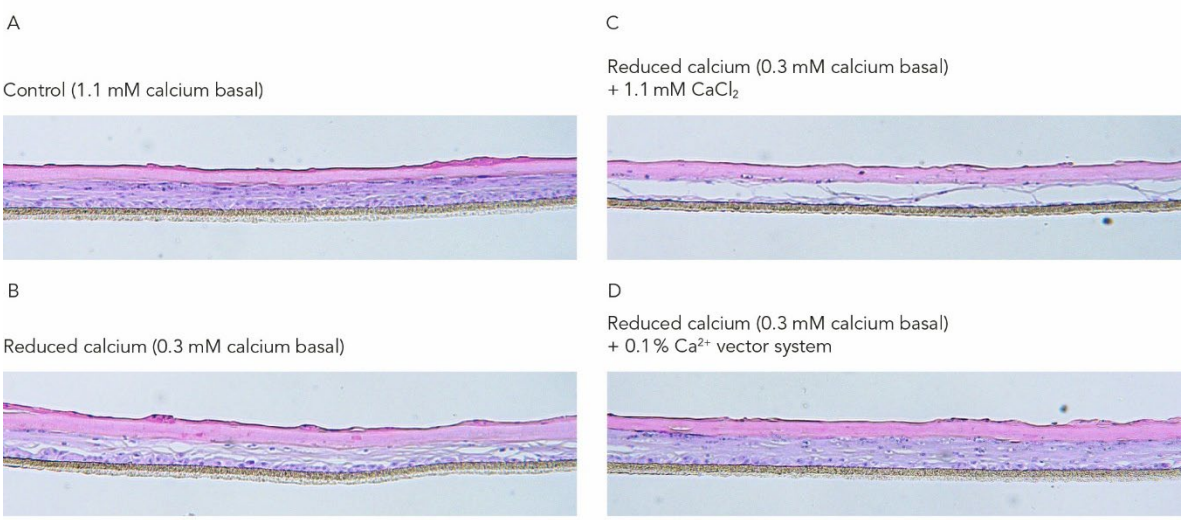

Figure 3: Effects of the Ca^{2+} vector system on RHE stratification

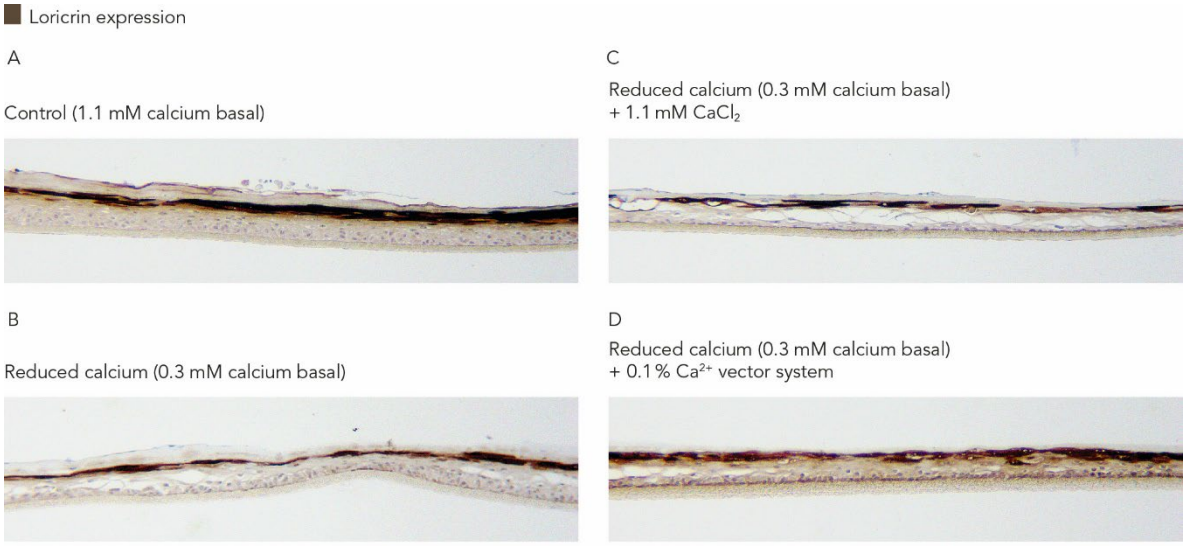

Figure 4: Increased Loricrin Expression after Treatment with Ca^{2+} Vector System

In a further experimental setup using human skin explants, a model closer to the clinical setting, the topical treatment with 2 % of the Ca^{2+} vector system led to increased expression of the epidermis differentiation marker involucrin by 22.0 % (Fig. 5). In contrast, the treatment with the corresponding concentration of free $CaCl_2$ induced a significant decrease of involucrin expression by 91.1 %. These observations correspond with the results of the RHE model and highlight the superior action of our novel Ca^{2+} vector system, compared to use of topical $CaCl_2$ alone. Overall, the *in vitro* and *ex vivo* results demonstrate the strong potential of our novel Ca^{2+} vector system, not only in making calcium bioavailable to calcium-deprived and stressed skin but also in rescuing the negative effects of low skin calcium levels.

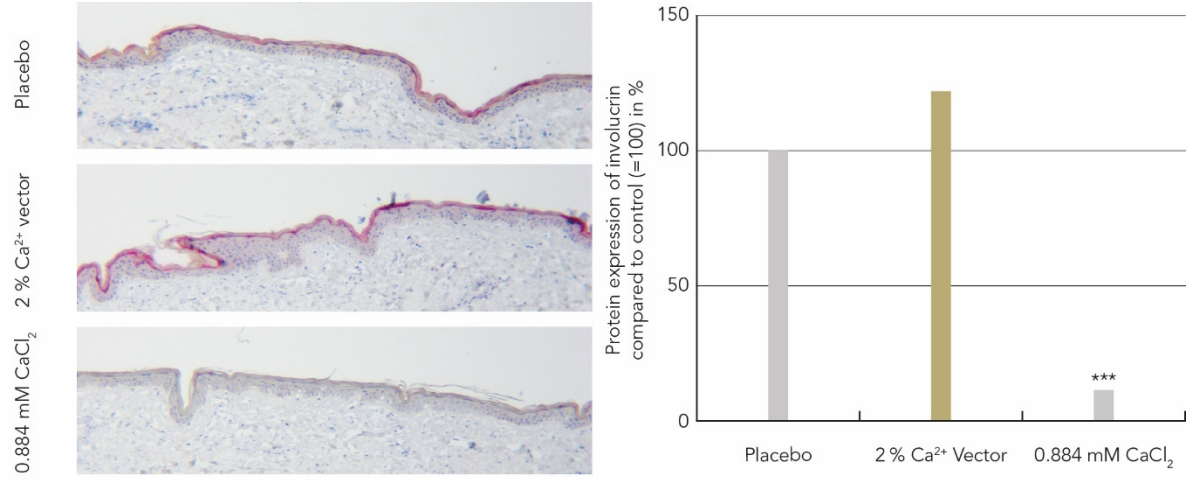

Figure 5: Ca^{2+} Vector System Improves Involucrin Protein Expression

To substantiate our preclinical observations, the effects of topical application of our novel Ca^{2+} vector system was also investigated in a clinical study. Applying our novel Ca^{2+} vector system before SLS stress had a significant protective effect on the skin. Increases in skin microcirculation and skin redness, which are indicative of irritation, were significantly reduced by 34.4 % and 15 %, respectively, directly after the removal of the SLS patch, compared to untreated controls (Fig. 6). Furthermore, the increase in TEWL was significantly prevented by 39.5 % compared to untreated controls. In contrast, pre-treatment with placebo gels could not exert such protective effects and, moreover, treatment with the Ca^{2+} vector significantly improved all measured skin parameters compared to the placebo (Fig. 6).

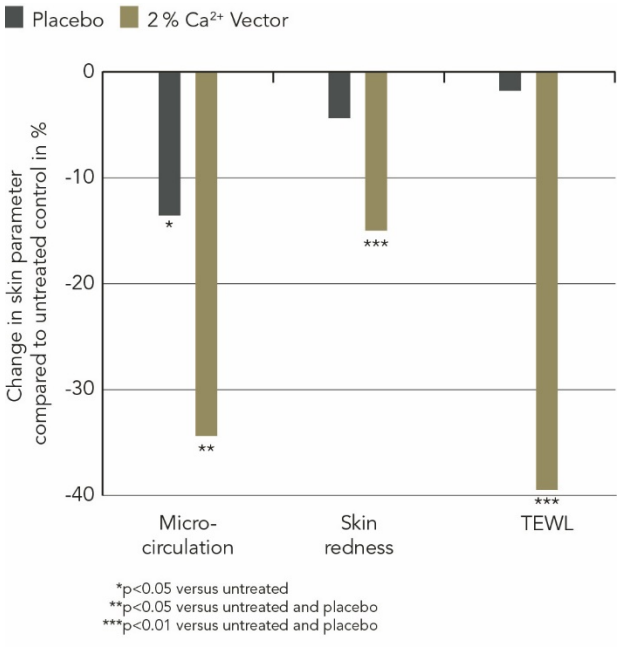

Figure 6: Protective Effect with Ca^{2+} Vector Pre-treatment

Topical application of the Ca^{2+} vector system further had a significant regenerating effect on the skin. TEWL recovered 15 % faster than untreated controls, with a mean recovery of 15 days (Fig. 7A). The recovery time of skin microcirculation was 19 % shorter than untreated controls and the skin redness recovered 24.1 % faster. All effects were significant compared to the placebo treatment, highlighting the strong regenerative action of our novel Ca^{2+} vector system.

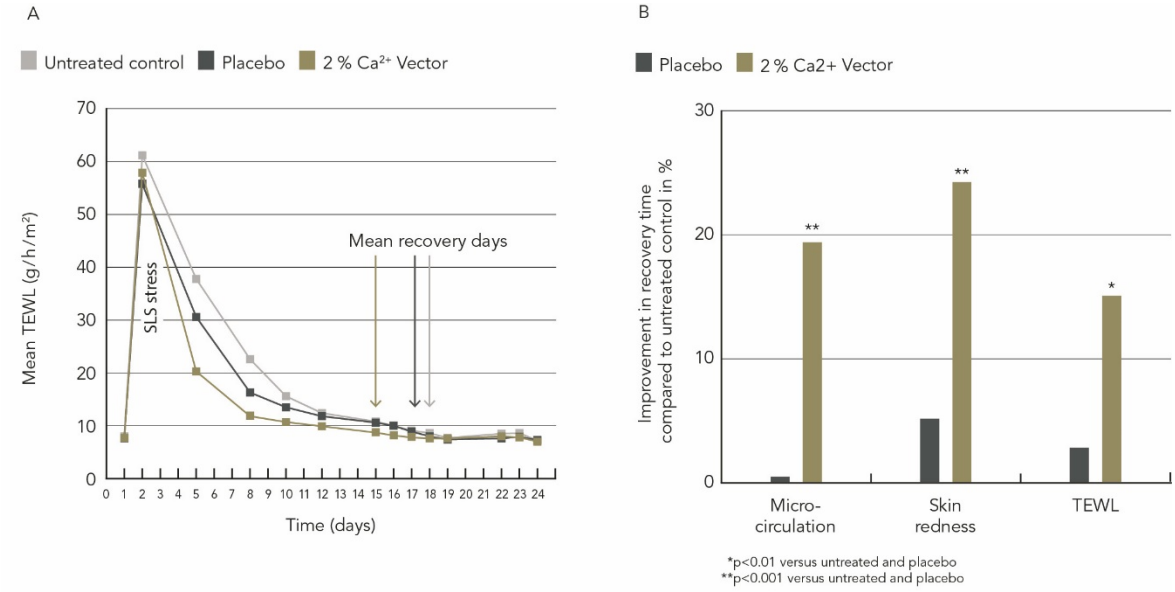

Figure 7: Faster Regeneration with Ca2+ Vector System after SLS Stress

Discussion.

In the present study, we were able to demonstrate that our novel Ca^{2+} vector system is effective in restoring a functioning barrier, both *in vitro* and *in vivo*. The molecular design of the vector system appears to underlie the successful delivery of bioavailable calcium to the skin. In the *in vitro* experimental setting of diabetic skin, supplementation with $CaCl_2$ alone had apparent detrimental effects, which implements that the Ca^{2+} may not have been able to penetrate the RHE to restore the calcium gradient. In contrast, the phospholipid-based nanocochleate Ca^{2+} vector system was successful in the restoration of a functioning barrier, demonstrating that the functional design of the vector system enables appropriate calcium delivery. Indeed, the use of phospholipid-based nanocochleate carrier systems is of emerging interest in the field of drug delivery systems (19). The first calcium-based nanocochleeate structures were discovered in 1975 by the research group of Papahadjopolous *et al*., who also coined the name "nanocochleates" (18). The terminology refers to a spirally rolled-up structure, which are formed by the interaction of negatively charged phospholipid bilayers and the bivalent positively charged calcium ions (20). The phospholipid-Ca^{2+} interactions generate stable bonds, resulting in a complex spiral structure that resemble a snail (Latin: *cochlea*). As such, the nanocochleates have a higher stability compared to other nanoparticle delivery systems (21). Further, being composed of natural lipids, they represent a biocompatible, and cell-permeating delivery system (21, 22).

Their overall beneficial characteristics make the use of such phospholipid-nanocochleates an interesting approach for the treatment of various disorders. As mentioned, the use of nanocochleate carrier systems for drug delivery is gaining significant interest, particularly in the context of delivering unstable and low-soluble agents (19). To the best of our knowledge, the present study and the Ca^{2+} vector system used herein demonstrates the first application of such a carrier system for skin cosmetic treatments. In our aging society, the prevalence of diabetes is continuously increasing (1), which yields the need for novel and effective treatments specific for diabetic skin. A major cause for a disrupted skin barrier in diabetes is a diminished calcium gradient and defective calcium signalling (16, 23, 24). With our novel Ca^{2+} vector system, the skin's calcium gradient can be restored, both as a preventive and as a restorative treatment. This opens the possibility not only for diabetic skin treatments, but also restorative cosmetic treatments for very dry, atopic, and psoriatic skin. Indeed, a diminished calcium gradient is apparent in psoriatic skin (25, 26), the application of a topical calcium source such as the Ca^{2+} vector system could therefore be a promising approach. Further clinical investigations in specific panels will certainly provide more insight.

Finally, the simple application and use of our novel Ca^{2+} vector system (patent-pending application) opens up new and beneficial perspectives for daily consumer-friendly products, without the need for medical or dermatological expertise (at-home treatment). As such, the Ca^{2+} vector system aids not only in restoring a strong skin barrier, but also supports healthy skin aging, which is affordable and accessible to all.

Conclusion.

The study demonstrated the successful application of a novel Ca^{2+} vector system on diabetic and stressed skin (patent-pending application). The delivery of bioavailable Ca^{2+} ions to the skin is a new and promising approach to treat damaged barrier functions of the epidermis in diabetic, aged, psoriatic, or atopic skin. Such potential treatment avenues provide exciting new foundations for cosmetic treatments of skin impairments. Diabetic skin-specific treatment options will be a new important market for the cosmetic industry and novel active ingredients such as this Ca^{2+} double cone vector system will contribute to healthy aging.

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Conflict of Interest Statement. NONE

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