Restoring skin with a novel Ca^{2+} delivery system

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ABSTRACT

Ageing and metabolic disorders such as diabetes are key drivers that can lead to skin barrier disruption. Despite the increasing demand for cosmetic treatments for old/diabetic skin, current options are limited and often include occlusive formulations. A major characteristic of a disrupted skin barrier is a defective calcium gradient in the epidermis. Therefore, replenishing the aged/diabetic skin's calcium stores with topical calcium could be a potential therapeutic approach. Both *in vitro* and clinical studies have shown, that a novel calcium ion (Ca^{2+}) vector system enables the successful delivery of bioavailable Ca^{2+} ions into the skin, aiding not only in recovery but also in protection of the skin from SLS stress. This highlights the use of this vector system as a new and superior approach to treat a damaged barrier present in diabetic, aged, or atopic skin.

Our skin's epidermis acts as an essential and strong natural barrier that protects our body. It prevents the entry of pathogens, allergens, and environmental pollutants while at the same time sealing in water, ensuring proper moisture levels in the skin. Damage to the epidermal barrier can have dramatic effects on our skin, leading to dry, irritated, and scaly skin, in extreme cases even xerosis.

Numerous external factors but also internal metabolic changes can affect our skin barrier. Ageing is one of the key drivers that slowly but steadily leads to an impaired skin barrier function.[1] Furthermore, diabetes is also an important factor that can negatively impact the skin's barrier.

Diabetes is an increasingly prevalent disease in our ageing society. In 2019, the global diabetes prevalence was estimated to be 9.3% (463 million people) and is predicted to further rise to 10.2% (578 million) by 2030.[2] Given the high prevalence of diabetic patients and our ageing population, cosmetic treatments to prevent or alleviate problems in old/diabetic skin are a growing market.

Despite the increasing demand for cosmetic treatments, current options remain rather limited, and often include urea and greasy occlusive formulations. More advanced prescriptive medication may even require the assistance of medical professionals.

Moreover, such formulations generally 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 ageing/diabetic skin barrier dysfunction.

The epidermal calcium gradient underlies skin health

The skin barrier is established in the epidermis, with the key molecule driving the epidermal barrier formation being calcium.[3] Calcium ions (Ca^{2+}) serve as essential signalling molecules, regulating numerous cellular functions in the skin, most importantly keratinocyte proliferation, and differentiation.

As keratinocytes need different calcium concentrations for various cellular stages (low calcium for proliferation, high calcium for differentiation) an epidermal calcium gradient is formed. In the lower levels of the epidermis (stratum basale and spinosum), the calcium gradient is at its lowest, but it rises, reaching its peak in the stratum granulosum, and declining again towards the outermost layer of the stratum corneum, where the keratinocytes reach their final differentiation status (Figure 1). Calcium also regulates the expression of differentiation-specific proteins such as loricrin, involucrin and filaggrin,[4] and regulates keratinocyte migration as well as wound healing.[5] Taken together, calcium gradient and signalling are key for a healthy skin barrier.

Replenish the skin's calcium stores with bioavailable Ca^{2+}

Unsurprisingly, a major characteristic of a disrupted skin barrier is a reduced or defective calcium gradient in the epidermis.[6] Indeed, during the ageing process calcium signalling is impaired and the calcium gradient collapses,[7] which may in part underly the ageing skin's reduced barrier function and epidermal thinning. Therefore, a potential therapeutic approach could be to replenish the skin's calcium stores of not only aged, but also diabetic skin, with topically applied calcium.

We developed a new and effective cosmetic active, by encapsulating calcium ions with phospholipids, thereby making calcium optimally bioavailable to the skin. Both *in vitro* and clinical studies demonstrated that our innovative Ca^{2+} vector system not only protects and repairs damaged skin, but also ensures a faster skin recovery.

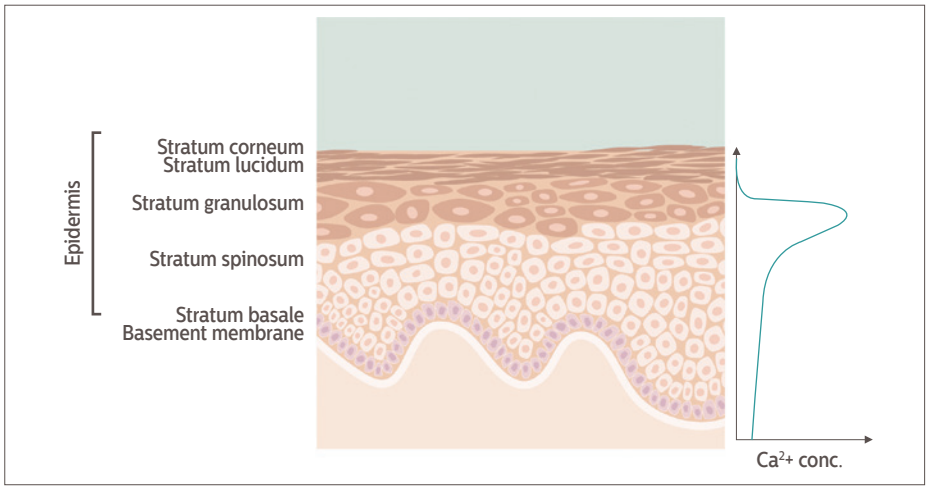

Figure 1: The epidermal calcium gradient

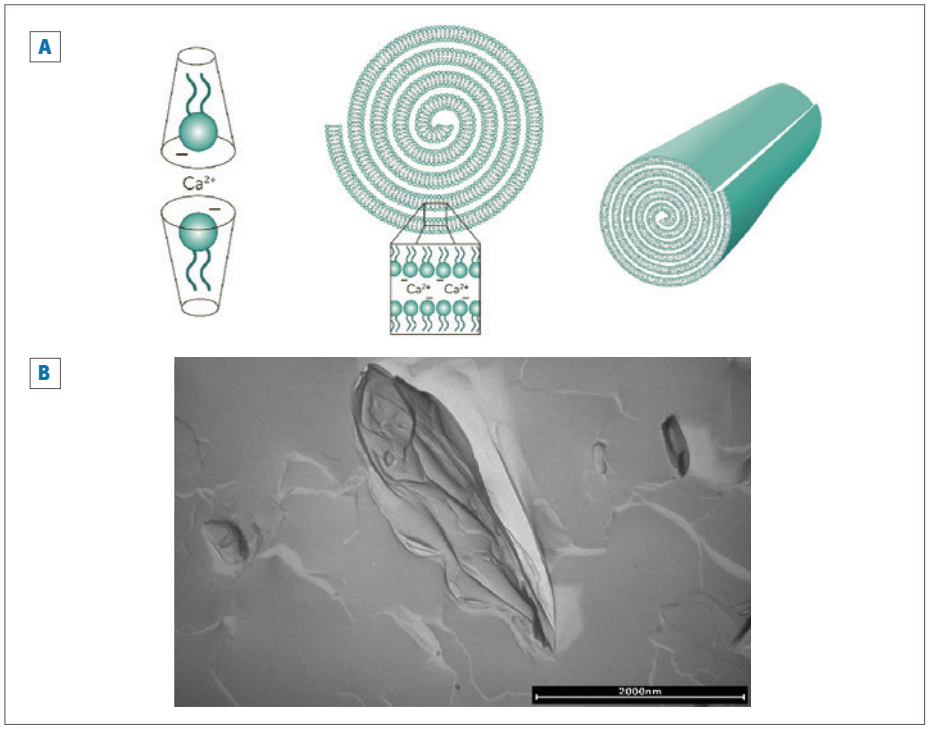

Figure 2: Structure and EM image of the Ca^{2+} vector system

Methods

Preparation of the Ca^{2+} double cone carrier system

To prepare the novel Ca^{2+} delivery system, glycerol, pentylene glycol and ddH_2O were mixed at 60°C, soy lecithin was added, then the dispersion pumped repeatedly through a high-pressure homogeniser (M700, MicrofluidicsTM, USA) at 1200 bar.

Then, 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. The final structure of the Ca^{2+} vector system was confirmed by freeze-fracture transmission electron microscopy (FFTEM).

RHE culture and treatment

Using human keratinocyte progenitor cells, 3D reconstructed human epidermis (RHE) models were established for the experiment. During the differentiation process, the RHE were either treated with 1.1 mM calcium basal (control), 0.3 mM calcium basal (reduced calcium), 0.3 mM calcium basal and 1.1 mM $CaCl_2$ apical, or 0.3 mM calcium basal and 0.1 % Ca^{2+} vector system apical.

The 3D models were harvested at day 9, fixed and the morphology analysed by hematoxylin and eosin (H&E) staining, whilst the expression of loricrin (antibody No.905104, BioLegend, USA) was determined by immunohistochemistry analysis.

Skin explant treatment

Human full skin explants, obtained from donor tissues of elective cosmetic surgery, 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 ddH_2O, fixed in formaldehyde, and embedded in paraffin.

Then 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 (antibody

No. SAB4200794, Sigma Aldrich, USA) was assessed by immunohistochemistry staining. 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 double blinded randomized and placebo-controlled study was performed. 20 female volunteers aged 23 to 65 years (mean 42.2 years) 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 (2%, formulated into a gel emulsion) prior to/after 2% sodium lauryl sulfate (SLS) stress using occlusive patches (Finn Chamber Large®, SmartPractice, Germany).

For the protection phase, the application of the test products (2% Ca^{2+} vector system and placebo gel control) twice daily in one area, randomly selected (test area), for seven days followed by a challenge with SLS in three areas (untreated, 2% Ca^{2+} vector system and placebo creme control) and evaluation of the skin effects until complete recovery.

For the regeneration phase, challenge with SLS in three areas (untreated, 2% Ca^{2+} vector system and placebo creme control) was followed by the twice daily application of the test product in one area randomly selected (test area) and placebo in another area (control area), and evaluation of the skin effects until recovery of the three areas.

The skin parameters were measured at baseline, 24 hours after stress and every two to three days until recovery. Parameters measured were skin microcirculation (Periflux PF5000, Perimed, Sweden), skin redness (Chromameter® CR-400, Minolta, Japan), and trans-epidermal water loss (TEWL, Tewameter® TM300, Courage +Khazaka, Germany).

Results and discussion

The mixing of negatively charged phospholipids with Ca_2+ ions by high pressure homogenization resulted in a supramolecular nanocochleate

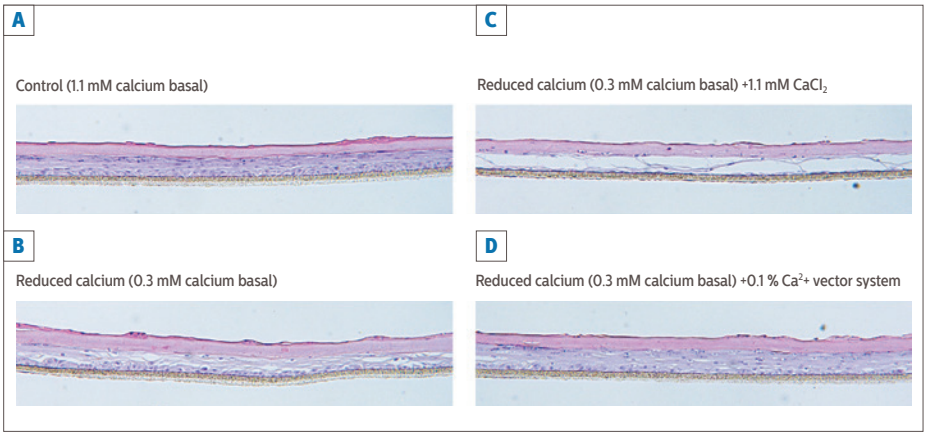

Figure 3: Rescuing of RHE stratification with the Ca^{2+} vector system

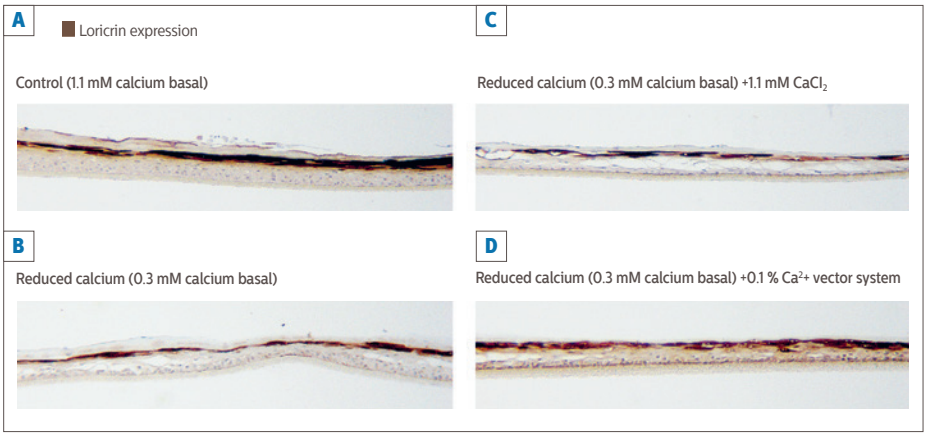

Figure 4: Ca^{2+} vector system increases the expression of loricrin

with a double cone structure (Figure 2A). This structure, resembling a snail shell (cochlea is Greek for snail), was firstly described by Papahadjopoulas *et al.* in 1975,[8] and we further confirmed it by our own images taken with FFTEM (Figure 2B).

Such an encapsulation technology can make impermeable molecules, such as Ca^{2+} ions, bioavailable to tissues, including the skin.[9]

The effect of this Ca^{2+} delivery system on epidermal differentiation was then investigated *in vitro* in RHE. The RHE grown in standard conditions (1.1 mM calcium basal) formed all stratified layers of the epidermis (basal, squamous, granular and cornified) (Figure 3A) with well-established loricrin expression (Figure 4A).

Reducing calcium levels during the differentiation process to 0.3 mM basal calcium, mimicking a very aged or diabetic skin, strongly impaired the formation of a dense, stratified epidermis and led to the formation of vacuoles (Figure 3B). Treating the differentiating keratinocytes with 1.1 mM $CaCl_2$ from the apical side could not restore a functional differentiation

but resulted in the further deterioration of the epidermis formation (Figure 3C).

Correspondingly, loricrin expression and thus the barrier of the epidermis was also strongly impaired (Figures 4B and 4C). On the other hand, treatment with 0.1 % of the novel Ca^{2+} vector system rescued the differentiation process, prevented the formation of vacuoles (Figure 3D), and increased the expression of loricrin (Figure 4D), resulting in a normal stratified epidermis.

These results highlight the potent action of the Ca^{2+} delivery system, not only in making calcium bioavailable to calcium-deprived and stressed skin but also rescuing the negative effects of low skin calcium levels.

The beneficial effects of topical application of our novel Ca^{2+} vector system was also investigated in a clinical study. Applying 2% of the the Ca^{2+} vector system in a gel formulation 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 (Figure 5). Furthermore, the increase in TEWL was significantly prevented by 39.5% compared to untreated controls.

In contrast, pre-treatment with a placebo gel did not have protective effects and, moreover, treatment with the Ca^{2+} vector significantly improved all measured skin parameters compared to the placebo (Figure 5).

However, often a skin irritation cannot be foreseen, and one needs to apply regenerative treatment after barrier disruption has already occurred. Importantly, treatment with 2% of the Ca^{2+} vector system also had a significant regenerating effect on the skin. TEWL recovered 15 % faster than untreated controls, with a mean recovery of 15 days (Figure 6A).

The recovery time of skin microcirculation was 19% shorter than untreated controls and the skin redness recovered 24.1% faster (Figure. 6B). All effects were significant compared to the placebo treatment, further highlighting the strong regenerative action of our novel Ca^{2+} vector system.

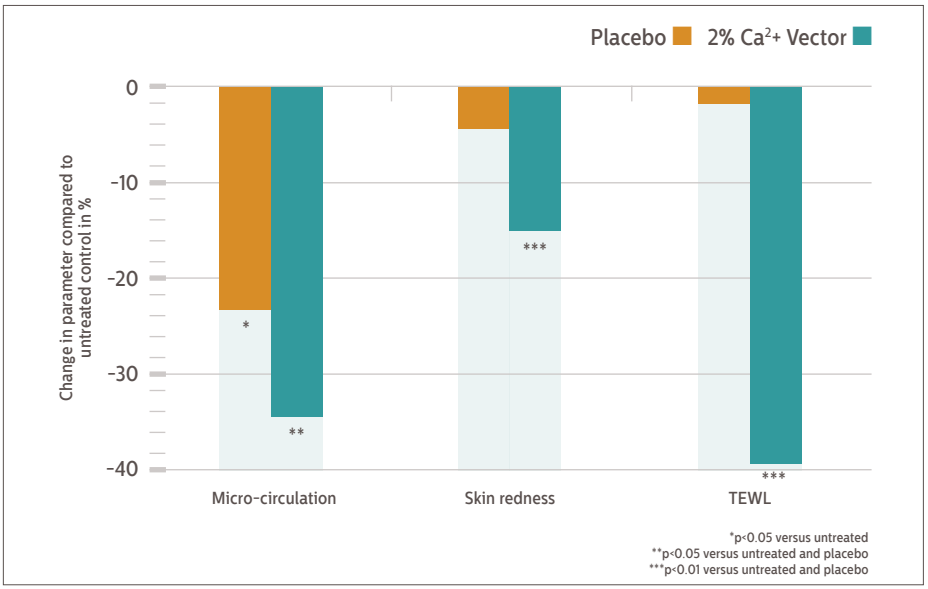

Figure 5: Clinical: protective effect with Ca^{2+} vector pre-treatment

Conclusion

The delivery of bioavailable Ca^{2+} ions to the skin is a new approach to treat damaged barrier functions of the epidermis present in diabetic, aged, or atopic skin. We believe that the treatment of diabetic skin will be a new important market for the cosmetic industry and that the provision of novel active ingredients such as this Ca^{2+} vector system will contribute to a better healthy ageing.

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 ageing, which is affordable and accessible to all. **PC**

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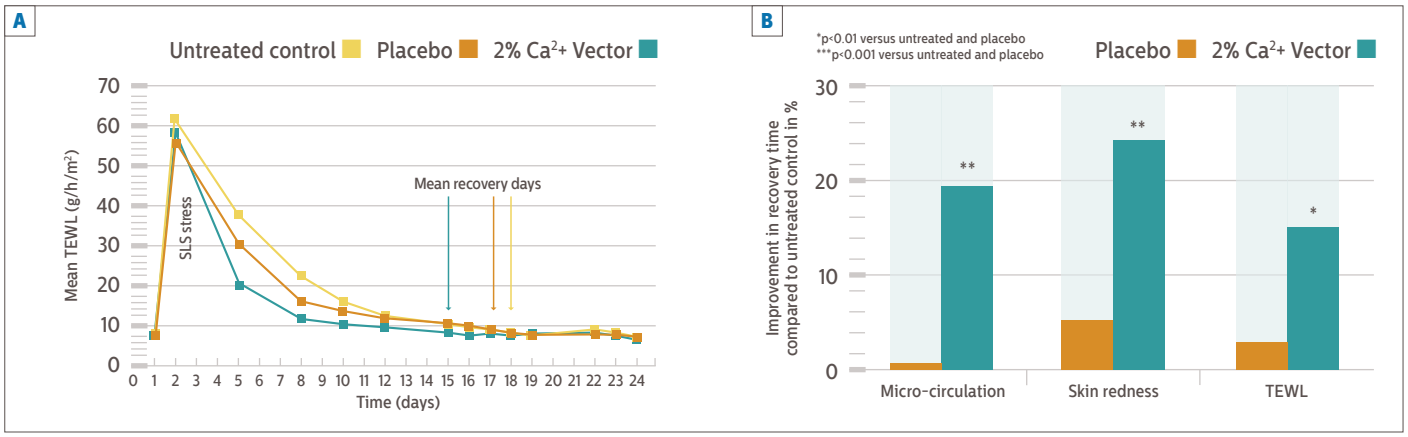

Figure 6: Clinical: faster regeneration with Ca^{2+} vector system after SLS stress