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The First Self-delivered Ingredient: A Novel Bioinspired Electroporation-like Mechanism via Transient Membrane Pores for a Radiant Skin.

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1. Introduction

The skin, the body's largest organ, is a sophisticated barrier that shields against environmental aggressors while preserving internal balance. Beyond its classical protective role, growing evidence highlights that the skin is also an electrically active tissue, capable of generating and responding to endogenous and exogenous bioelectric signals, positioning the skin as a newly recognized bioelectrical tissue. These electrical phenomena are emerging not only as critical regulators of processes such as wound healing, inflammation, and tissue regeneration, but also as novel mechanisms for delivery [1-3]. The same structural complexity that protects the skin also restricts the penetration of external substances, making transdermal delivery challenging. To address this, modern delivery strategies aim to enhance skin permeability through the application of electric pulses. Among these, electroporation has gained increasing attention: it uses short, high-voltage electrical pulses to transiently disrupt the lipid bilayer of cell membranes, opening pores of 1 to 10 nm [4], facilitating the uptake of bioactive molecules through the skin and their intracellular transport and assimilation [5-7].

Beyond its established role as a delivery system, electroporation or needle-free mesotherapy, and other bioelectrical techniques like microcurrents, have also demonstrated regenerative and rejuvenating benefits. Electrostimulation-based therapies have been shown to promote epidermal proliferation, neovascularization and collagen synthesis. Specifically, *in vitro* studies have demonstrated enhanced proliferation, metabolic activity, secretion of growth factors and migration of keratinocytes and fibroblasts, key processes in skin repair [8-10]. Other studies also describe that electrostimulation enhances cellular processes such as ATP production, protein synthesis, and collagen formation, while also modulating inflammation and cellular migration [3,9-11]. Despite its benefits, electroporation remains a developing technology, limited by device complexity, safety concerns, and lack of standardized guidelines [6,9,12-15].

Inspired by the principles of bioelectrical stimulation we set out to develop a biologically based alternative capable of emulating the beneficial effects of electrical stimulation, with particular emphasis on reproducing the mechanism of electroporation. At the core of this strategy lies the P2X receptors (P2XR) – an ATP-gated, ionotropic receptors expressed on epithelial cell membranes and various other tissues [16]. Upon activation by extracellular ATP (eATP), P2XR facilitate cation influx, resulting in membrane depolarization and the activation of intracellular signalling cascades. Moreover, this activation may lead to a pore dilation of up to 1 nm, enabling the passage of large molecules into the cell [17-19]. Among its downstream effects, P2XR have been implicated in wound healing through the regulation of cell migration and the

induction of VEGF secretion [20,21]. These features make P2XR an attractive molecular target for mimicking the delivery benefits traditionally achieved mechanically through electrical devices.

In this study, we present a bioinspired, electroporation-like self-delivery system derived from *Hylocereus undatus* stem cells (Hu). This biotechnological ingredient is naturally enriched in glycoconjugates – identified as positive allosteric modulators of the P2XR – as well as polyphenols and pigments known for their potent antioxidant and regenerative activities [22,23]. By harnessing this unique composition, we propose a system capable of activating P2X7R promoting efficient intracellular delivery of bioactive compounds, thereby enhancing its own biological efficacy without the need of external devices. To evaluate the electroporation-mimetic effect of Hu, an optimized *in vitro* model utilizing HaCaT cells was developed. This model included the evaluation of Hu's effects, using electroporation and eATP as positive controls to validate the proposed mechanism of action. Finally, *in vivo* studies were conducted to validate the ingredient's efficacy in skin rejuvenation applications.

2. Materials and Methods

2.1 *In vitro* studies

2.1.1 Experimental system and culture conditions

HaCaT (cat no. 300493; CLS, Eppelheim, Germany) were maintained in complete culture medium (DMEM + 10% FBS). HDF (cat no. P10856; Innoprot, Biscay, Spain) were cultured in fibroblast medium with 2% FBS. Cell cultures were cultured in standard conditions (37ºC, 5% CO_2 and 95% humidity). 10^5 cells/well were seeded into 96-well for the different assays.

2.1.1.1 HaCaT cell electroporation: protocol development

Electroporation was set up on HaCaT cells using a Gene Pulser Xcell Electroporation System (cat no.1652660; Bio-Rad, California, USA). Cells were trypsinized, washed with PBS and resuspended in electroporation buffer. 10^5 cells were introduced in electroporation cuvettes (0.2 cm gap) and BLOCK-iT™ dye (1 µM) was added. Different voltages (80, 90, 100, 110, 130, 160V) and pulse lengths (15, 20, 25 ms) were studied. The negative control (C−) consisted of adding 1 µM BLOCK-iT™ dye to cells without applying electroporation. Cell viability and dye cell penetration efficacy were analysed by flow cytometry. The efficacy was evaluated based on the dye's ability to penetrate the cells and quantified as a % of fluorescence relative to C-.

2.1.1.2 Electroporation-mimetic mechanism assessment

HaCaT cells were treated with three concentrations of Hu (1, 5, 20% (v/v)), two concentrations of ATP (5, 10mM) or were electroporated with the optimal conditions (110 V for 20 ms). Cell viability, dye penetration and VEGF production were assessed at different incubation times: 1, 4 or 24 hours. A C-, as previously described, was also included in the study. Six biological replicates were analysed for each tested concentration and time point for cell viability (MTT) and dye cell penetration (flow cytometry) and four biological replicates for VEGF production.

2.1.2 Assessment of Cell viability

MTT assay (cat no. M2128; Sigma-Aldrich, Massachusetts, USA) was used to determine cell viability. The analysis included washing the cells with PBS, staining with MTT solution, and incubating at 37ºC for 2 hours. After the incubation period, the staining medium was removed, 100 µL of DMSO/well were added, and the absorbance was read at 570 nm using a Varioskan

LUX spectrophotometer plate reader (Thermo Fisher Scientific, Massachusetts, USA). Cell viability was calculated relative to negative control.

2.1.3 Evaluation of Cell Membrane Permeability via Dye Uptake and Imaging

A 1 µM BLOCK-iT™ dye was used to assess cell membrane permeability and was added prior to either electroporation or electroporation-like treatments. At each designated time point, cells were washed twice with PBS and analysed using a CytoFLEX S Flow Cytometer (Beckman Coulter, California, USA). The efficacy of electroporation-like treatments was evaluated based on the dye's ability to penetrate the cells and quantified as median fluorescence intensity (MFI). A high content imaging system with an Operetta CLS microscope (PerkinElmer, Massachusetts, USA) was used to visualize the dye incorporation in the different conditions analyzed. Beyond BLOCK-iT™ dye, cells were stained with 2 µM Hoechst dye to label the nuclei.

2.1.4 VEGF stimulation by electroporation-like treatments

VEGF production was analysed at 4- and 24-hours following treatment with Hu (5 and 20% v/v), ATP (5 and 10 mM), or electroporation (110 V for 20 ms). Supernatants from HaCaT cells were collected, and VEGF quantification was performed using an ELISA assay (Human VEGF DuoSet ELISA Kit, cat no. DY293B-05; R&D Systems, Minneapolis, USA). VEGF values were normalized by cell viability of each condition.

2.1.5 Measurement of eATP in HaCaT and HDF Cells

HDF and HaCaT cells were treated with three concentrations of Hu (0.3, 1.25, 5%(v/v)) and incubated in complete media for 24 hours at 37ºC and 5% CO_2. Then eATP levels were measured using the RealTime-Glo™ Extracellular ATP Assay Kit (cat no. GA5010; Promega, Wisconsin, USA). No treated cells were included as a negative control. Three biological replicates were analysed for each tested concentration.

2.1.6 Statistical analysis

A two-sample, two-tailed Student's T-test (equal variance assumed) was used to determine statistical significance of the data. A Fisher's F-test was performed to compare the variances of the samples.

2.2 *In vivo* studies

Two independent *in vivo* studies were conducted to evaluate the short- and long-term effects of Hu formulations on skin aging parameters. The first study involved 40 healthy female subjects (aged 40–65, mean age = 56.7 ± 2 years) with dry skin and visible signs of aging (mild to moderate wrinkles). Participants were randomized into two groups (n = 20): one group received a cream containing 1.5% Hu, and the control group received a placebo. Formulations were applied twice daily, and skin was assessed at baseline (T0), day 14 (T14), and day 28 (T28). Evaluations included wrinkle depth (crow's feet area) via PRIMOS 3D (Canfield Scientific Europe, Utrecht, Netherlands) and moisturization via Corneometer® (Courage+Khazaka, Cologne, Germany). The second study included 60 healthy subjects (same age range) with clinical signs of sagging, loss of elasticity and eyebags. Subjects were randomized (n = 30 per group) to receive either Hu formulations (1.5% for the face, 3% for the eye contour area) or placebos. Assessments were performed at T0, T28, and T56. Eyebags volume was measured via PrimosCR SF (Canfield Scientific Europe, Utrecht, Netherlands), elasticity (R2 parameter) and firmness (R0 parameter) using the Cutometer® MPA 580 (Courage+Khazaka, Cologne, Germany). Instrumental data were analysed using two-way Student's t-test for paired data. A p-value < 0.05 was considered indicative of statistical significance.

3. Results and Discussion

3.1 In vitro studies

The electroporation-like effect of Hu on a HaCaT cell line was assessed based on its ability to facilitate the intracellular delivery of BLOCK-iT™ dye. As a first step, an electroporation protocol was specifically optimized for HaCaT cells to ensure effective membrane permeabilization while maintaining cell viability. Once the optimal electroporation conditions were established, a comparative analysis was carried out to evaluate the effects of Hu or ATP relative to dye uptake and cell viability. VEGF production and eATP were also studied.

3.1.1 HaCaT cell electroporation: protocol development

The electroporation set-up aimed to identify conditions that maximize electroporation efficiency, understood as a maximum dye uptake. Six voltages ranged from 80 to160 V were tested at three pulse durations (15, 20, and 25 ms). As shown in Figure 1, electroporation efficiency increased in a dose-dependent manner with both voltage and pulse duration, reaching a maximum at 130 V. However, this condition also led to a marked decrease in cell viability, highlighting an inverse relationship between efficiency and cell survival. Skorupska and Grabowska-Jadach (2019) likewise highlighted the need to optimize electroporation at the minimal effective voltage to ensure cell viability. [4].

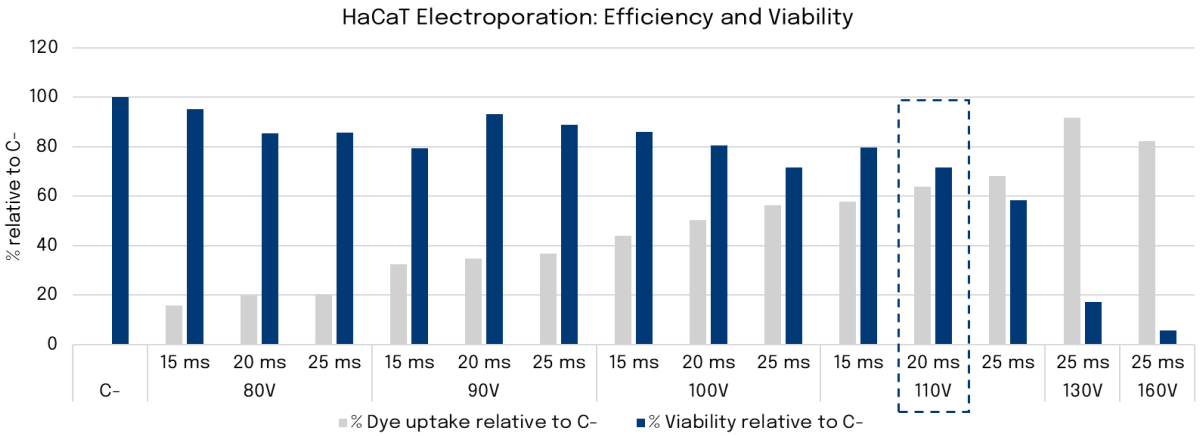

Figure 1. HaCaT electroporation: Efficiency and Viability. Dye uptake (%) and cell viabily (%) vs C- after different voltages (80, 90, 100, 110, 130, 160V) and pulse lengths (15, 20, 25 ms). The negative control (C-) consisted of cells treated with 1 µM BLOCK-iT™ dye but not subjected to electroporation.

Based on these findings, the optimal electroporation conditions were identified as 110 V with a pulse duration of 20 ms, balancing an immediate effective electroporation (64%) with cell viability (72%). Therefore, electroporation must be carefully optimized to balance delivery efficiency and cell viability, as high voltage levels can cause irreversible cell damage [4,24]. Skorupska and Grabowska-Jadach (2019) reported a protocol using 175 V pulses for 10 ms in HaCaT cells, achieving ~80% viability [4]. Although this approach is more aggressive than ours, the comparable viability may reflect differences in culture conditions and assessment methods. Moreover, in DNA transfection protocols, voltages between 250 and 500 V are commonly applied to maximize transfection efficiency; however, cell survival typically ranges from only 20% to 50% [24]. While many electroporation studies prioritize penetration over viability, our goal is to ensure efficient delivery without compromising cell survival.

3.1.2 Electroporation-mimetic mechanism assessment

To further explore the electroporation-like behaviour of Hu, we conducted a comparative study in HaCaT cells to evaluate its membrane-permeabilizing effects and the potential involvement of eATP, using optimized electroporation as a reference.

3.1.2.1 Dye uptake though electroporation-mimetic mechanisms

The results shown in Figure 2 and 3 indicate that both Hu and eATP facilitate intracellular dye uptake in HaCaT cells. Specifically, Hu exhibited a dose-dependent electroporation-like effect, with the highest dye uptake observed at 24 h and at a concentration of 20%. At this concentration, Hu induced an MFI of 5797 at 1 h, 11693 at 4 h (+102% vs 1 h), and 26419 at 24 h (+356% vs 1 h). Lower concentrations of Hu (1% and 5%) promoted dye uptake, although the effect was less pronounced. Thus, dye uptake appears to increase, not only with Hu concentration, but also over time. Unlike electroporation, where membrane pores begin to close within seconds and fully reseal after 10 minutes [25], Hu treatment may involve prolonged pore opening or repeated cycles of opening and closing, allowing a cumulative intracellular entry throughout the incubation period. Regarding ATP treatment, maximum dye uptake was observed at 4h with 10 mM, showing levels comparable to those obtained with 20% Hu at 24 h. However, this effect declined at 24 h, likely due to reduced cell viability. In contrast, at a concentration of 5 mM, eATP exhibited a dose-dependent effect like the observed with 20% Hu, with MFI of 5805 at 1h, 16886 at 4 h (+190% vs 1 h), and 19061 at 24 h (228% vs 1 h).

While both Hu and ATP showed notable effects, the highest BLOCK-iT™ cellular uptake was achieved through electroporation (110 V, 20 ms), which served as the positive control. This condition resulted in an MFI of 83262 units—representing a 3.15-fold and 2.92-fold increase compared to the peak effects of Hu and eATP, respectively. Unlike eATP and Hu, which require incubation to exert their effects, electroporation induces immediate dye uptake due to its physical nature; thus, subsequent monitoring focuses on cell viability recovery. Figure 3 corroborates dye incorporation and supports the fluorescence differences observed.

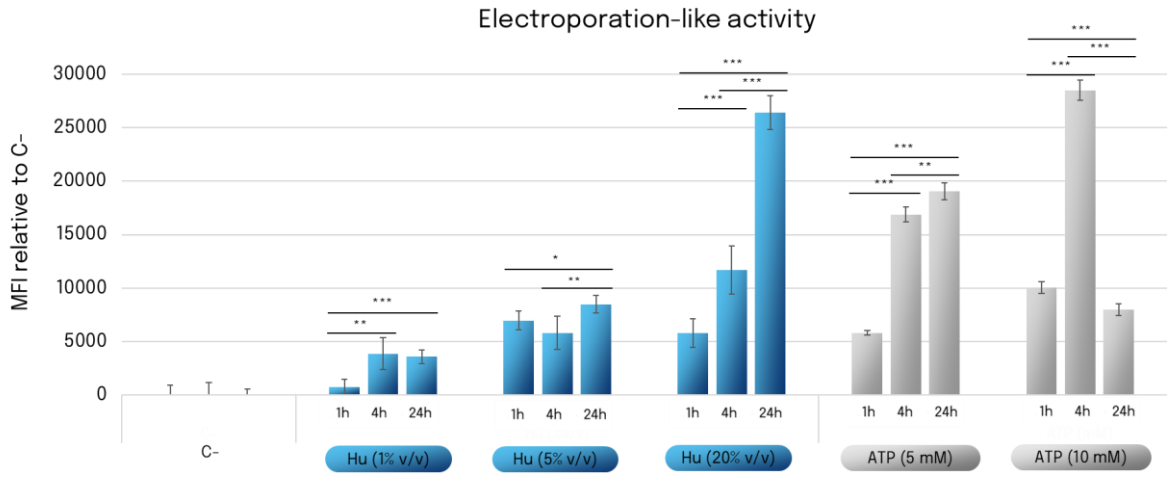

Figure 2. Electroporation-like activity in HaCaT. Median fluorescence intensity (MFI) of internalized BLOCK-iT™ at 1, 4, and 24 h of Hu (1, 5, 20% (v/v) or ATP (5,10 mM) incubation. Data represent the mean ± SD of n=6. Asterisks indicate statistically significant differences (*p<0.05, **p<0.001, ***p<0.0001).

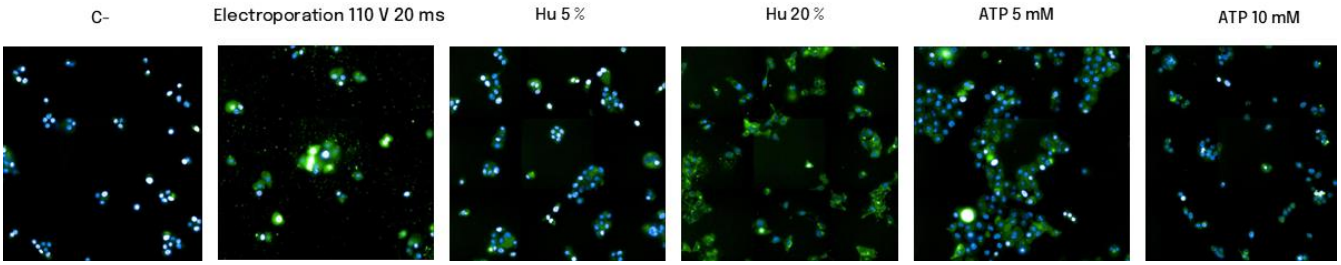

Figure 3. Electroporation-like activity in HaCaT after 24 h of Hu (5, 20% (v/v)) or ATP (5,10 mM) incubation (High-content imaging). Green: BLOCK-iT™ uptake; blue: Hoechst nuclear staining.

As previously mentioned, evaluating electroporation efficacy requires the simultaneous assessment of dye uptake and cell viability, thus viability was monitored at 1, 4, and 24 h for each treatment. (Figure 4).

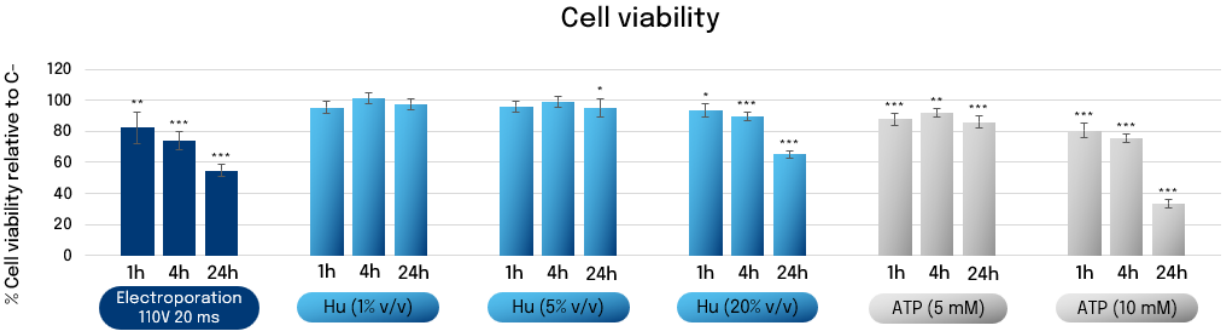

Figure 4. Cell viability (%) relative to C- was measured at 1, 4, and 24h after electroporation (110 V, 20 ms), Hu (1%, 5%, 20%), or ATP (5 mM, 10 mM) treatment. Asterisks indicate statistically significant differences vs C− (*p < 0.05, **p < 0.001, ***p < 0.0001). Data represent the mean ± SD of n= 6.

The results indicate that electroporation caused a moderate reduction in cell viability after 1 to 4 h post-electroporation, followed by a pronounced decline after 24 h, with viability dropping to 55%. This decline indicates that electroporation may trigger irreversible damage, with insufficient repair responses leading to delayed cell death [26,27]. Hu treatment at 1% and 5% did not cause significant reductions in HaCaT cell viability at any of the assessed incubation time points. In contrast, treatment with 20% Hu resulted in a reduction of cell viability after 24 h, reaching 65%. Similarly, eATP at 5 mM had no effects, whereas eATP at 10 mM induced a significant decrease at 24 h, with viability declining to 34%.

Electroporation relies on a biophysical process involving transient aqueous pore formation [28], whereas eATP induces a biological response mediated by receptor interaction, notably through P2X7R activation [29,30]. In our model, electroporation induces strong membrane permeabilization without immediate loss of viability (Figure 1), although a marked reduction is observed 24 h post-treatment (Figure 4). It is already described that electroporation induces membrane damage that may initially be reversible but can lead to delayed cell death due to irreversible membrane disruption or the activation of cellular stress and injury pathways [26]. On the other hand, several authors have reported that eATP can activate P2X7 receptors at high doses and provokes a conformational change in the receptor that led to the formation of large membrane pores that can allow the entrance of hydrophilic molecules [19,31,32]. Interestingly, this mechanism has been studied for compound delivery into ganglion cells [33]. In this context, eATP levels must be tightly regulated under physiological conditions, with intracellular ATP concentrations typically around 10 mM and extracellular levels maintained near 10 nM, and only a 1%

release of the intracellular ATP pool is sufficient to trigger extracellular signalling [34]. Therefore, the cytotoxicity observed at 10mM is likely attributable to the excessive ATP applied.

This work provides the first evidence that a plant-derived bioactive can mimic electroporation, achieving efficient intracellular delivery without physical intervention. Strikingly, 5% Hu and 5 mM eATP emerged as optimal conditions, combining high uptake with preserved cell viability.

3.1.2.2 VEGF production

VEGF is a key marker of cellular stress and tissue remodelling, often used to evaluate regenerative responses [35]. VEGF secretion was evaluated at 4 and 24 h post-electroporation or following treatment with Hu or ATP (Figure 5). Under control conditions, a time-dependent increase in VEGF production was observed, reaching up to 179 pg/ml at 24 h (a 15-fold increase vs 4 h). Electroporation reduced VEGF productivity by 55% at 4 h compared to the C-, while at 24 h, levels increased by 29%. The increase in VEGF levels may reflect a cellular attempt to adapt to damage and activate protective or regenerative processes. This is consistent with previous reports showing that electroporation-induced membrane injury triggers complex stress responses [26]. Similar results were reported by Golberg et al. (2015), demonstrating that electric field treatment significantly increased levels of various growth factors, including IL-6, EGF, and VEGF [10]. In contrast, ATP treatment led to increased VEGF productivity at 5 mM, but not at 10 mM. This result also confirmed that 10 mM eATP acted as a stronger stimulus, reducing cell viability and impairing VEGF secretion. At 5 mM, a 69% increase was observed after 4 h compared with C-; however, no effect was observed at 24 h. Previous reports indicate that eATP, through activation of P2X7 receptors, can enhance VEGF secretion in various cell types [21]. Finally, Hu treatment resulted in a dose- and time-dependent increase in VEGF production, with levels rising by 58% and 187% after 4 and 24 h of incubation, respectively. These results suggest that Hu induces early activation of VEGF production, and that its effect is sustained over time, possibly due to reduced cellular damage compared to other treatments. Interestingly, the relationship between membrane permeabilization and VEGF production was not strictly proportional, suggesting more complex mechanisms, possibly involving pore formation upon P2X7R activation and the entry of Hu-derived compounds, which may further enhance VEGF secretion.

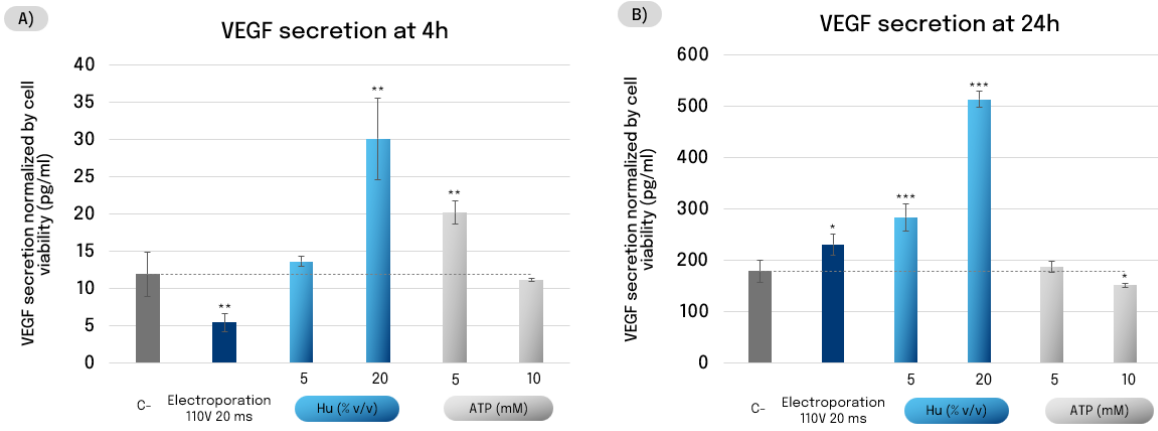

Figure 5 VEGF secretion in HaCaT at **(A)** 4h and **(B)** 24h after electroporation (110V, 20ms), Hu (5, 20%) or ATP (5, 10 mM) treatments. Data is normalized by viability and expressed as pg/mL, bars represent the mean ± SD of n=4. Asterisks indicate statistically significant differences vs C- (*p<0.05, **p<0.001, ***p<0.0001).

3.1.3 eATP-release in HDF and HaCaT

In this section, we assessed whether Hu induces eATP release in HDF and HaCaT cells, as a potential indicator of P2X pathway activation. Figure 6 showed that Hu treatment induced eATP release in a dose-dependent manner in both HDF and HaCaT cell lines. Notably, Hu treatment at 5% was significantly stronger in HaCaT cells, where eATP levels increased by up to 116% compared to a maximum increase of 42% in HDF cells. This differential activation could be due to the higher expression of P2X7 receptors in keratinocytes compared to fibroblasts [36]. Therefore, Hu can increase eATP levels in the culture medium, which is expected to enhance receptor activation and ultimately facilitate the entry of compounds into the cell.

To better understand the biological basis behind the electroporation-mimetic effect and the observed ATP release, it is essential to consider the unique composition of Hu. Produced through a biotechnological process, Hu is naturally enriched in glycoconjugates (representing approximately 80% of its total protein content), complex sugars, polyphenols, and pigments, components that may synergistically contribute to its biological activity.

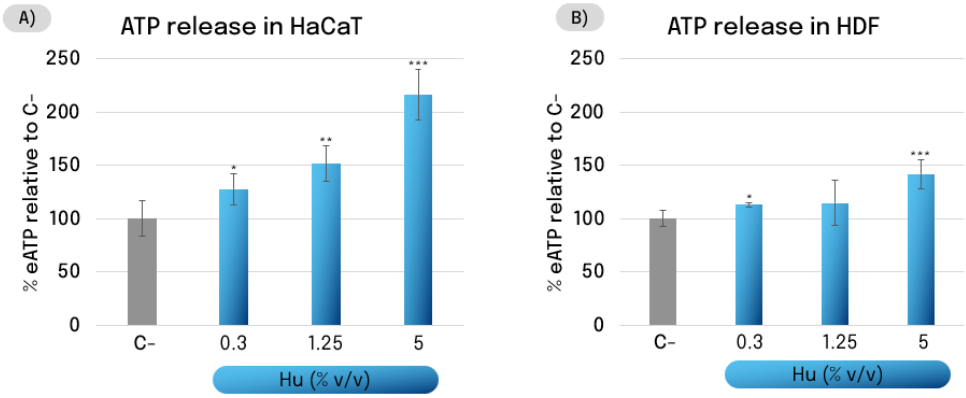

Figure 6. eATP release in **(A)** HDF and **(B)** HaCaT cells after 24 h Hu treatment (0.3–5%). Results expressed as % vs. untreated control (C-). Asterisks indicate statistically significant differences compared to C- (*p < 0.05, **p < 0.001, ***p < 0.0001). Data represent the mean ± SD of n=3.

Glycoconjugates, such as ginsenosides (saponins) and gintonin (a glycoprotein) derived from *Panax ginseng*, have been studied for their potential to enhance P2X receptor signalling and are considered positive allosteric modulators of these receptors. Interestingly, the aglycone forms of ginsenosides, which lack the sugar moiety, failed to potentiate P2X activity, underscoring the critical role of the sugar chains in receptor modulation [23,37–40].

All these results support the hypothesis that glycoconjugates from Hu activate P2XR through an allosteric mechanism by sensitizing the receptors and making them responsive to minimal concentrations of eATP, which in amplifies eATP release. The released eATP may, subsequently, reinforce P2X7R activation through autocrine and paracrine signalling [19], opening the pores and potentially explaining the sustained electroporation-mimetic effect observed. Interestingly, other authors have also exploited P2X7R as a gateway for intracellular delivery of charged molecules, such as photoswitches in retinal ganglion cells [33]. Although in our *in vitro* model the internalization of the fluorescent dye served as proof of concept, it also demonstrates that Hu's own bioactive compounds are also able to penetrate the cell membrane. Since Hu is also rich in polyphenols and pigments, these bioactive compounds could enter cells through the transient pores and exert intracellular effects, contributing to antioxidant protection, stimulation of regenerative processes, and ultimately promoting skin rejuvenation from within.

3.2 In vivo studies

The well-aging efficacy of Hu was evaluated both short- and long-term through clinical assessment of key skin parameters. In the short term, treatment with Hu at 1.5% significantly reduced wrinkle depth (Figure 7A), with a 7% decrease at T14 and 15% at T28, indicating a rapid and cumulative anti-wrinkle effect. Skin hydration also improved significantly (Figure 7B), with a 14% increase at T14 and 21% at T28, supporting immediate and sustained moisturization benefits. These early improvements may be attributed to the effective penetration of bioactive compounds present in Hu, such as polyphenols and pigments, into skin cells, where they can exert antioxidant, hydrating, and rejuvenating effects.

Regarding long-term effects, treatment with Hu at 3% significantly reduced under-eye bag volume (Figure 8A), with a 4.7% decrease at T28 and 5.3% at T56, suggesting a firming effect in the periorbital area. In parallel, Hu at 1.5% improved skin firmness, as shown by reductions in the R0 parameter (−6% at T28, −9% at T56; Figure 8B), and enhanced elasticity, with R2 increases of 6% at T28 and 8% at T56 (Figure 8C). All effects were significantly greater than placebo, highlighting the progressive and multi-targeted benefits of Hu on skin care.

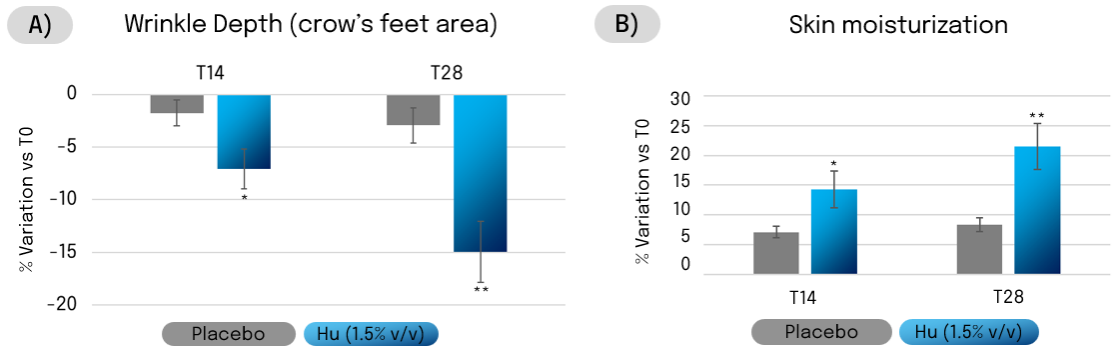

Figure 7. Short-term effects of Hu on **(A)** wrinkle depth and **(B)** skin moisturization. Graphs show the mean percentage ± SE change from baseline (T0) after 14 and 28 days of treatment. Asterisks indicate statistically significant differences compared to the placebo group (*p < 0.05, **p < 0.001).

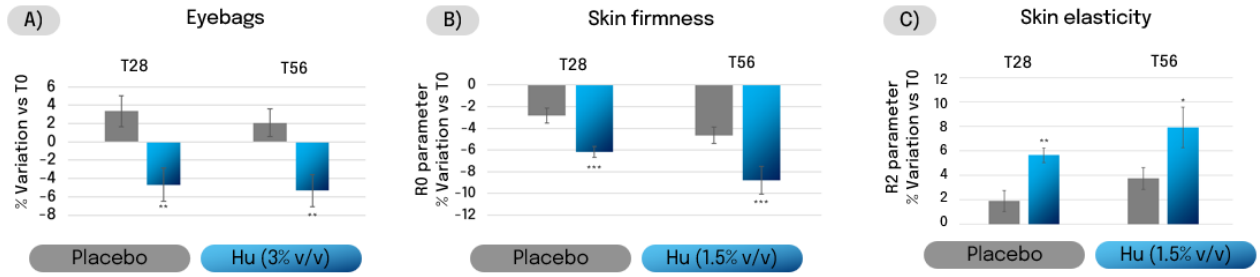

Figure 8. Long-term effects of Hu on **(A)** eyebags volume, **(B)** skin elasticity and **(C)** skin firmness. Graphs show the mean percentage ± SE change from baseline (T0) after 28 and 56 days of treatment. Asterisks indicate statistically significant differences compared to the placebo (*p < 0.05, **p < 0.001).

Collectively, these findings highlight both the rapid and long-lasting efficacy of Hu in improving key indicators of skin health and well-aging, reinforcing its strong bioactive potential. The clinical outcomes align with *in vitro* results, which demonstrated Hu's ability to activate an electroporation-mimetic mechanism, enhancing cellular penetration of Hu compounds and stimulating VEGF secretion.

5. Conclusion

Altogether, these results position *Hylocereus undatus* stem cell extract as a pioneering self-delivery system that biologically mimics electroporation, enhancing membrane permeability without external devices. Through P2X receptor activation by eATP release—likely mediated

by its unique glycoconjugate profile —Hu promotes transient pore formation, enabling effective intracellular delivery while preserving cell viability. Beyond its mechanistic innovation, Hu stimulates key biomarkers and demonstrates remarkable *in vivo* efficacy, significantly improving multiple signs of skin aging. These findings highlight Hu's auto-delivery role offering a novel and highly effective approach to skin regeneration and well-aging.

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