STUDY OF THE SOOTHING AND REPAIR EFFECTS OF AN INNOVATIVE ACTIVE INGREDIENT COMPLEX

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Abstract

The skin barrier function has emerged as a cosmetics research hotspot in recent years. Impairment of the skin barrier increases the likelihood of inflammation and may potentially lead to skin allergies and chronic inflammatory disorders. Therefore, this research attempts to verify the role of an innovative complex in protecting skin barrier and repairing impaired skin. In this study, the moisturizing, soothing and repairing effects of the complex was evaluated from different perspectives (in vitro reconstructed epidermis, cell culture, clinical trials, etc.). Specifically, the complex's moisturizing effect was assessed by measuring retained water and transepidermal water loss on the reconstructed epidermis. The soothing effect research was carried out by detecting IL-6, IL-1alpha, and TNF-alpha through ELISA test. Likewise, the repairing effect was obtained by investigating the expression of Ki-67 on the epidermis through immunohistochemical staining as well as the formation of glycosaminoglycan and collagen in human fibroblasts. Moreover, 30 volunteers were recruited to test the complex's clinical effects.

Our results showed an increase in water retention, Ki-67, global glycosaminoglycans and collagen in vitro experiments, and a decrease in the transepidermal water loss, IL-1alpha, IL-6 and TNF-alpha. The complex presents a good soothing and repair effect on volunteers after 28 days' application. In general, the innovative complex helps the water retention and minimizes TWEL, which can also reduce skin inflammation and stimulate dermis regeneration, implying that the complex benefits in skin barrier protection and regeneration, with good efficiency of moisturizing, soothing and

repairing.

Key words: water retention, soothing, repair, innovative active ingredient complex

Introduction

The skin serves as a barrier between the human body and the surrounding environment, preventing microbial infection, limiting passive water loss, and minimizing environmental aggression (such as chemicals, ultraviolet rays, allergens...)[1,2]. Functionally, the skin barrier can be separated into four different layers, these highly interdependent layers are the microbiome, chemical, physical, and immune layers[3]. Although each layer has a particular function they all contribute to the overall integrity of the skin barrier[4]. However, as a result of internal (such as keratinocytes and the structure change of lipids, influence of metal ions, the regulation of various enzymes and hormones) and external (such as dry environment, UV radiation, poisonous compounds, particles) factors, an increasing number of people are experiencing skin barrier impairment[5-8]. According to several research[9-11], sensitive skin is a result of impaired barrier function. Moreover, skin barrier dysfunction is frequently linked to a variety of skin and sebaceous gland disorders[12-14], including atopic dermatitis, psoriasis, ichthyosis, acne, and others, these disorders are typically accompanied with skin lesions. Take acne, a pretty prevalent condition, as an example, which is characterized by several lesion types: noninflammatory open or closed comedones (blackheads or whiteheads) and inflammatory lesions (red papules, pustules, or nodules)[15,16]. In general, the protection of the skin barrier and the restoration of impaired skin are the topics of this essay.

Proper skin hydration helps to recover and maintain the skin protection barrier[17]. A decrease in skin moisturizing capacity will lead to a decrease in skin barrier function, which in turn leads to a further drop in skin moisturizing ability, establishing a vicious loop. Although all layers of the skin are connected to the moisturizing function, the stratum corneum is more closely associated with skin moisturizing (nearly 90% of the skin barrier function exists in the stratum corneum[18]). As the outermost layer of the

skin, the stratum corneum (SC) is also an important component of the physical barrier[19] and consists of two different structural components: corneocytes and intercorneocyte lipids (ceramide, cholesterol, and free fatty acids). These two structural components are derived from keratinocytes through the terminal differentiation process[4,20], and both of them, the SC's lipid-filled extracellular compartment and humectant/water-filled intracellular compartment, are critical for the skin's barrier function and water-holding properties[21,22]. One of the most significant indexes of skin barrier function is transepidermal water loss (TEWL), which is defined as the flux density of water that diffuses from the dermis and epidermis through the stratum corneum to the skin surface[23]. High and low TEWL levels indicate skin barrier dysfunction and intact or recovered skin barrier, respectively[24]. Furthermore, skin pH, as a symbol of the chemical barrier, also plays a central role in the function of SC and skin barrier. The pH of normal human stratum corneum varies from 4 to 6 and is associated with physical barrier homeostasis, SC cohesion, microbiome modulation, and proinflammatory cytokine signal down-regulation[25,26]. For example, both lipid organization and lipid metabolism in the stratum corneum require an acidic pH; the diversity of skin microbiota was maintained by acidic pH; the changes in natural moisturizing factors (NMFs) alter the epidermis pH and lipids; and elevated pH is also present in skin diseases associated with skin barrier dysfunction[27-29]. It can be seen that although different barriers have their unique features and composition, these layers are highly interconnected. However, if one barrier compartment is disordered, it may lead to a vicious circle of inflammation and skin disease development[4,30].

The skin barrier (stratum corneum) protects the underlying epidermis and Langerhans cells from exposure to environmental chemicals and symbiotic bacteria when the barrier function is complete, however, when barrier function is disordered, bacteria, fungi, viruses, and other symbiotic bacteria gain access to the epidermis and then taken up by Langerhans cells which present symbiotic bacteria antigens to the immune system, activating it and inducing an inflammatory response[31]. Studies have shown how stratum corneum damage causes Interleukin-1α (IL-1α)、Interleukin-1β (IL-1β) and

tumor necrosis factor-α (TNF-α) expression, ultimately triggering the cytokine cascade that could regulate cytokine and cytokine receptor production and /or inflammatory responses[32]. We already know that IL-1, TNF, and IL-6 are effective mitogens and lipid synthesis stimulators, and the expression of TNF, IL-1 and IL-6 in the epidermis increased following acute physical barrier disruption[2]. To be more specific, IL-1α is a critical inflammatory mediator in the skin and is considered to be the primary switch in the initiation of inflammation. By binding to IL-1 receptor I, IL-1α stimulates the production of itself and other pro-inflammatory cytokines such as IL-6 and IL-8[33]. In addition, IL-1α is a potent regulator of the AP-1 and NF-κB pathways, and the latter regulates the expression of multiple genes involved in epidermal homeostasis and inflammatory response[34,35]. In this article, we hope to restore the immune barrier by lowering the response of these characteristic inflammatory factors, to achieve a soothing effect. It's worth noting that minimizing inflammatory reactions is not only vital for skin barrier restoration, but also for imperfection healing.

The imperfections healing process occurs in three stages: reduced overall inflammation, increased cellular activity, and cellular tissue regeneration[36,37]. Therefore, the migration and proliferation of epithelial cells and the deposition of extracellular matrix (ECM) are essential for effective wound closure[38]. Glycosaminoglycans such as hyaluronan and chondroitin sulfate are the major ECM components, and studies suggest that exogenous hyaluronic acid may accelerate the wound healing process[39,40].

In this study, we evaluated the ability of an innovative active ingredient complex to protect the skin barrier and repair impaired skin by testing its moisturizing, soothing and repairing effects. The complex contains hydrolyzed hyaluronic acid, hydrolyzed sodium hyaluronate, sodium acetylated hyaluronate, adenium obesum leaf cell extract, and vitis vinifera (Grape) flower cell extract. The cell extracts are acquired from dedifferentiated plant cells. And due to the phospholipid bilayer of these cells, they may be able to assist hydrolyze hyaluronic acid and sodium hyaluronate to be better absorbed by the skin. Since sodium acetylated hyaluronate is both hydrophilic and lipophilic, it may absorb

water while being absorbed by the lipid membrane of the skin. The efficacy of this innovative active complex is verified as follows:

Materials and Methods

1. Preparation of active substances

The complex is comprised of hydrolyzed hyaluronic acid, hydrolyzed sodium hyaluronate, sodium acetylated hyaluronate, adenium obesum leaf cell extract, vitis vinifera (Grape) flower cell extract, and vegetable glycerin.

2. Principle of cell culture

2.1 Monocytes

The macrophage cell line called MM6 (Mono-Mac-6) is established from the blood cells of a patient with monoblastic leukemia.

2.2 Fibroblasts

Cultures of fibroblasts are established from the skin of human foreskins harvested during circumcision and are amplified in culture medium RPMI 1640 supplemented with fetal calf serum, L-glutamine and gentamicin. The tests are carried out on fibroblasts, between the 2^{nd} and 4^{th} passage, in order to ensure reproducibility between the different experiments.

2.3 The reconstituted epidermis SKINETHIC®

Human keratinocytes were seeded onto 0.5 cm^2 polycarbonate filters in a defined medium (modified MCDB 153) and supplemented. The cells were cultured for 14 days at the air/liquid interface, the culture medium being changed every two days. The epidermis thus formed was used to conduct the study from the 17th day of culture.

3. Moisturizing assessment on recombinant epidermis SKINETHIC®

3.1 Drying of epidermis

Epidermis was placed under a laminar flow hood for 15 minutes to induce superficial dryness, mainly of the stratum corneum, without altering the epidermal cells.

3.2 Evaluation of static and dynamic hydration by tritiated water

This study consisted in using the products under consideration and tritiated water (1µCi) to treat the reconstituted epidermis SKINETHIC® previously dried at the level of the stratum corneum. Quantity of product applied is 10 µl/cm². Final concentration of the innovative active ingredient complex is 0.5%. Study performed in triplicate.In the study of "Reservoir effect – bound water", product contact time with epidermis is 15 and 30 minutes. The negative control is untreated and the test group is applied with products. At the end of each incubation time, the epidermis was washed quickly on the surface, peeled from the filter and sonicated. 1 ml of cell homogenate was mixed with 5 ml of scintillation fluid and the radioactivity was counted in a Packard counter. In the study of "Dynamic effect – Free Water", product contact time with epidermis is 15, 30 and 60 minutes. The negative control is untreated and dried and the test group is applied with products after drying. At the end of each incubation time, 20 µl of medium were collected, mixed with 5 ml of scintillation fluid and the radioactivity was counted in a counter.

4. Ki-67 assessment

In order to determine the times of contact product/epidermis not inducing cytotoxicity, the product was put in contact with the recombinant epidermis for 24 hours. After that, the epidermises, fixed in formaldehyde solution (10%), were included in blocks out of paraffin. The vertical cuts of 4 microns were stained with hematoxylin/eosin (HES) and were photographed under optical microscope. The mitotic index was evaluated by counting the stained nuclear sites, at a rate of 10 fields per slide by the optical microscope, enlargement x250, comparatively with the epidermis control. To achieve this purpose, the epidermises control not receiving any product and the epidermises receiving the products being studied during 24 hours of contact were fixed in the formaldehyde 10%. After inclusion in blocks of paraffin, these epidermises were cut then treated by immunohistochemistry.This reaction was carried out with antibody MIB1 (Immunotech), recombining peptide of the nuclear antigen by Ki-67 pretreatment. The revelation was made by the method peroxydase-antiperoxydase after antigenic

unmasking by pretreatment with heat. The staining by DAB chromogene reveals in brown the Ki-67 nuclear sites of the cells fraction in growth expressed in phases. The epidermises control not receiving any product and the epidermises receiving the product being studied during 24 hours of contact, were frozen at -180°C. After inclusion in blocks of paraffin, these epidermises were cut then treated by immunohistochemistry.

5. Anti-inflammatory assessment

Final concentration of the innovative active ingredient complex is 0.5%, 1.0%, 2.5%. The negative control is untreated, the positive control treated with Lipopolysaccharids (LPS). Product contact time with epidermis is 24 hours. 24 hours after the treatment of reconstituted epidermises;the culture mediums were taken, and the assessment of inflammatory mediators was performed according to the protocols described in the Interleukin 1-α (IL1-α) kit、Interleukin 6 (IL-6) kit and Tumor Necrosis Factor α (TNFα) kit.

6. Assessment of the glycosaminoglycans content

6.1 treatment and cell extraction

The fibroblasts were distributed in multiwell plates (6 wells) at a rate of $2x10^5$/ml, and then were maintained 24 hours in the incubator with CO_2. The radioactive precursor ([^{3}H]-Glucosamine) was added to the cultures 18 hours. The time of contact of the cells with the treatments was 24 hours at 37°C. After having eliminated the medium by aspiration, the cells were washed 2 times with medium without serum, then the cells were detached from the culture surface. The cells were washed once again with medium then centrifuged at 600g during 5 minutes.

6.2 FPLC method

This base was realized the proteoglycans assay by FPLC (Fast protein liquid Chromatography). The base was suspended in 250ml of Tris-HCl buffer (50 mM pH 7.4). The sepharose gel CL-6B (DEAE) was run in a column K 10/40. 100 µl of each sample were injected, elution is followed by the spectrofluorometer detection at 280 nm wavelength. The peak containing the proteoglycans is eluated with NaCl (1M).

6.3 glycosaminoglycan measurement

A second fraction of the cellular bottom was treated by the pronase 1mg/ml during 24h at 60°C. The reaction was stopped by cooling the tubes. The proteins were precipitated by TCA 12% at 4°C for one night. The precipitates were then centrifuged to 12,000 G during 30 min. the supernatants containing the glycosaminoglycan's were recovered, dialysed against a pure ultrawater (MilliQ more), then freeze-dried. The lyophilisats were then suspended again in buffer TrisHCl pH 7.4 containing protease inhibitors. An aliquot of 50µl was taken for the counting of the radioactivity incorporated in the total glycosaminoglycans.

7. Collagen content assessment

After incubation time, fibroblasts were recovered by centrifugation. The pellets were digested by collagenases (1 mg/cellular pellet) in acetic acid (0.5ml/l) during 24 hours at 4°C. After centrifugation at 10000g, collagens were precipitated in NaCl at 1 M, the precipitate being suspended and dialyzed. Primary amino acids were derivated by ophtaldehyde acid (OPA), eliminating their interference. Hydroxyproline and proline were the derivated by NBD-Cl by coupling with NBD-Cl amino groups. The NDB-Hyp was separated and identified by HPLC in reverse phase. For the tuning of amino acids derivatives, a coupling with NBD-Cl of a standard containing hydroxyproline was firstly realized. Hydroxyproline was dosed by fluorescence measure after separation of HPLC in reverse phase.

8. Clinical test

8.1 Subjects

The recruitment requirement is at least 20 subjects with oily skin or oily combination skin with acneic tendencies to the face, with at least 10 retentional lesions, 5 inflammatory lesions and brown areas (scars). These subjects must be within the range of 18 to 35 years old. There are 20 eligible Caucasian women were included in this clinical test.

8.2 Assessment of the anti-redness effect and pigmentary spot

The clinical scoring consists of a visual assessment of the cutaneous state before and after the application of the investigational product to the experimental area, in order to determine its cosmetic efficacy. For this purpose, the Head Technician sits the subject in front of the table Evalux bench® (Orion concept) with a long-life and calibrated lighting (leds - 6000°K).

8.3 pH measurements

The measurements are performed with Skin-pH-Meter pH 905 ® (C&K). The surface of measurement for the probe is 78.5 mm². The effect on the skin is evaluated by comparing measurements taken on the control area (neck) and the treated area (one cheek).

8.4 Self-assessment

The subjects answered a self-assessment questionnaire about the investigational product after 28 days (D28) of application in conditions of normal use. The answering modalities were the following: “agree”, “somewhat agree”, “somewhat disagree”, “disagree”. For each item, the satisfactory percentage (= percentage of “agree” + percentage of “somewhat agree”) was calculated.

Results

1. The effect of the innovative active ingredient on skin barrier function through reconstituted epidermis SKINETHIC®

The results (Fig 1) shows that treatment with the 0.5% innovative active ingredient complex led to an increase in water retention in the epidermis at 15 minutes and 30 minutes of 24% and 35% respectively, compared to each respective untreated control.

The trans-epidermal passage of tritiated water was collected and the results show that treatment with the innovative active ingredient complex resulted in a decrease in the trans-epidermal passage of tritiated water of 27%, 24% and 21% respectively compared to untreated control at times t0 + 15 minutes, t0 + 30 minutes and t0 + 60 minutes (Fig 2).

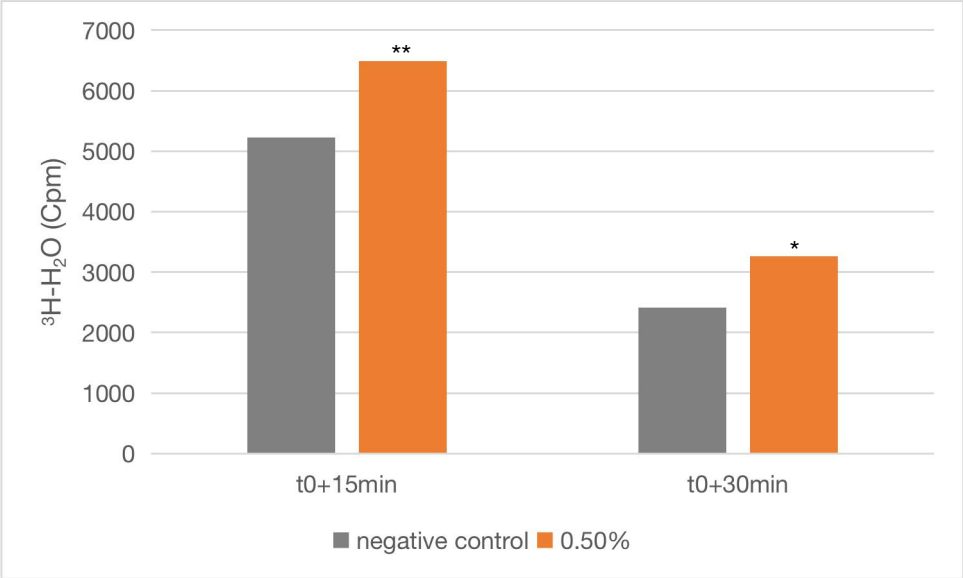


Figure 1： Evaluation of the levels of radioactivity in epidermis. Statistical analysis: t-test. * p <0.05; ** p <0.01; *** p <0.001.

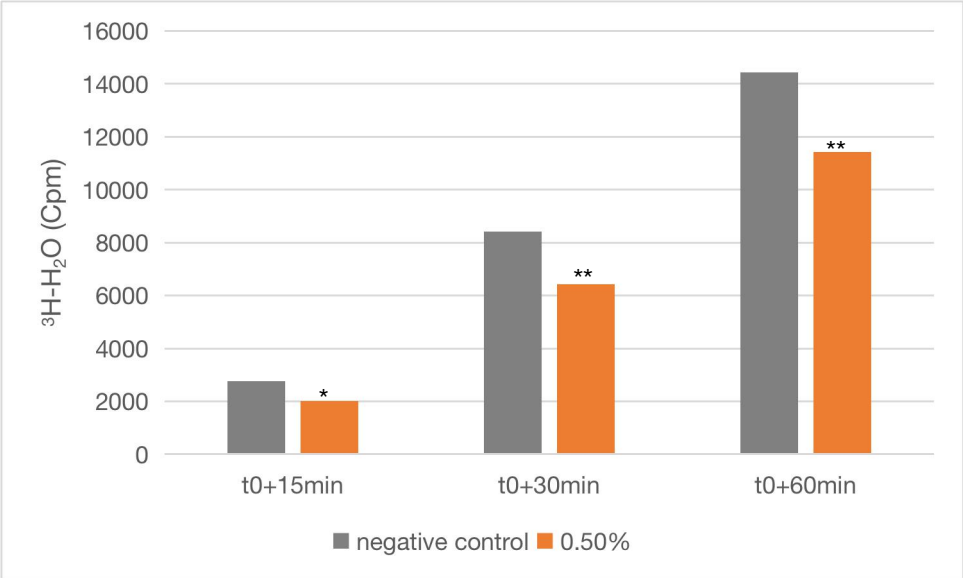
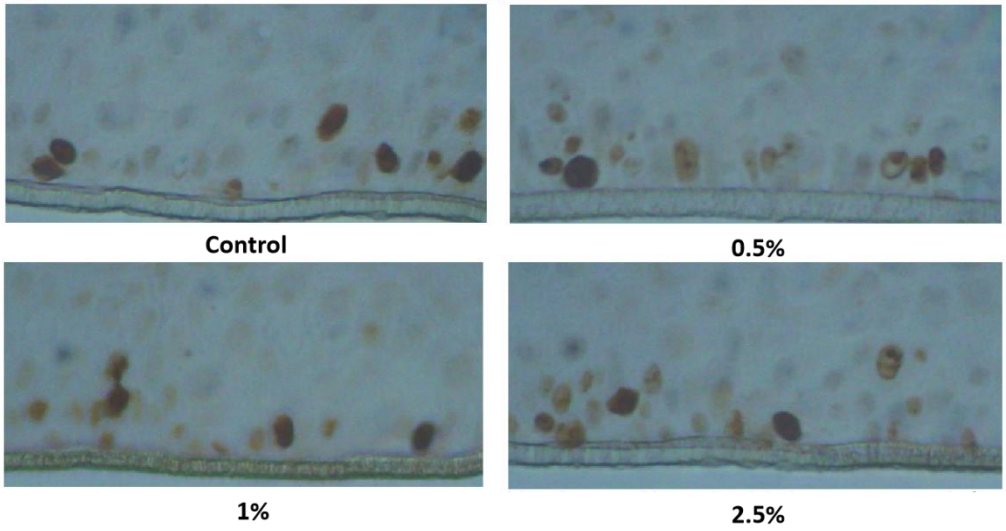


Figure 2： Evaluation of the transepidermal passage of tritiated water. Statistical analysis: t-test. * p <0.05; ** p <0.01; *** p <0.001.

2. Evaluation of the innovative active ingredient complex's effect on the epidermis stimulation and regeneration

The innovative active ingredient complex (0.5%; 1.0% and 2.5%) induced an increase of the KI-67 expression respectively by 11%, 17% and 23%, which translated a

stimulation of the epidermis regeneration (Fig 3).



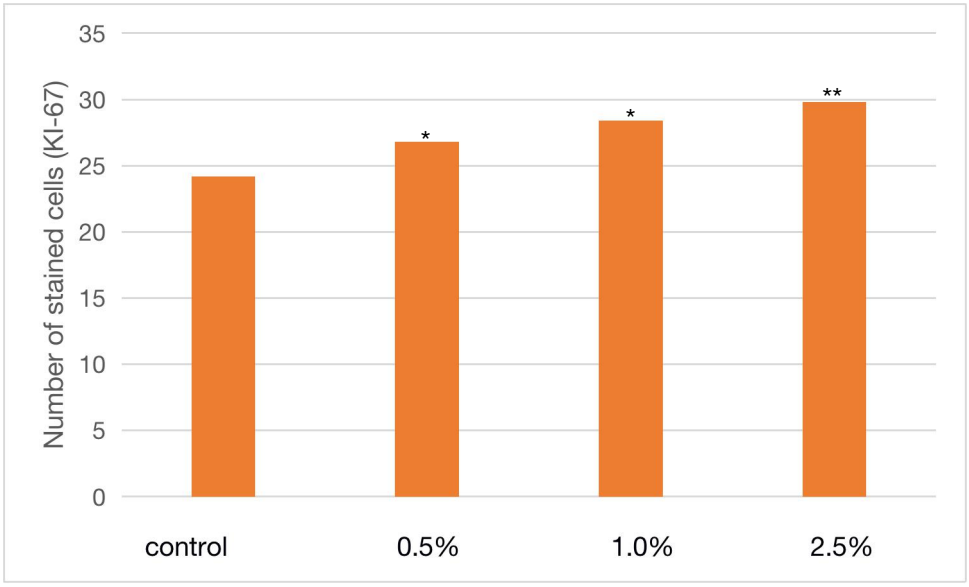


Figure 3: KI-67 expression on reconstructed epidermis. Immunohistochemical staining of **KI-67** (brown) in treated and treated reconstructed epidermis. Automatic quantification of **KI-67** expression per area of epidermis. Images analyzed per condition: n = 9. Statistical analysis: t-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3. Evaluation of the innovative active ingredient complex's effect on the immune system of the skin

The obtained results show that the Interleukin 1-α (IL1-α) release is stimulated in response to Lipopolysaccharids (+57%) compared to negative control. The co-culture

treatment by the innovative active ingredient complex at the concentrations of (0.5%, 1.0% and 2.5%) significantly decreased the IL1-α release by 21%, 27% and 32% respectively (Fig 4).

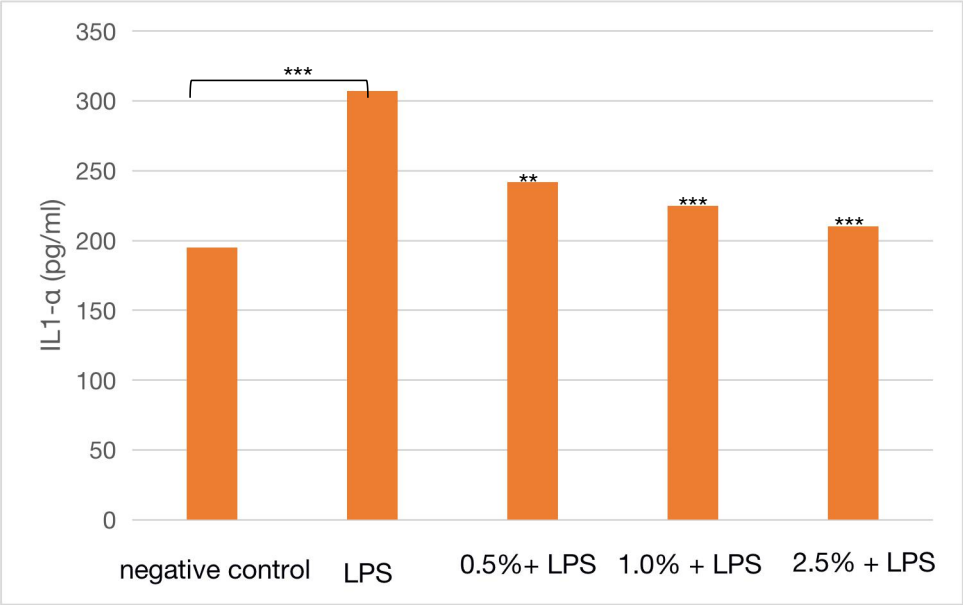


Figure 4： The concentration of IL1-α detected by Elisa assay. Statistical analysis: t-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Interleukin-6 (IL-6) is a cytokine involved in the acute phase of inflammation. On the last day of culture, supernatants from the different conditions (treated or not) were collected and interleukin-6 was measured by Elisa. Fig 5 shows that Interleukin 6 (IL-6) release is stimulated in response to LPS, compared to untreated control. The treatment by the innovative active ingredient complex at the concentrations of (0.5%, 1.0% and 2.5%) significantly decreased the IL6 release by 20%, 25% and 30% respectively.

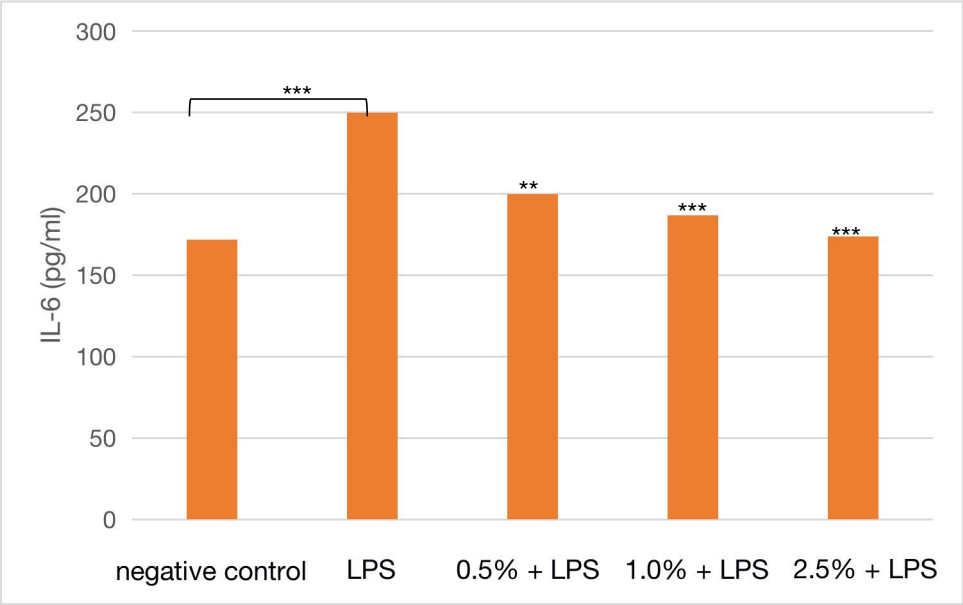


Figure 5: The concentration of IL-6 detected by Elisa . Statistical analysis: t-test. * p <0.05; ** p <0.01; *** p <0.001.

Tumor Necrosis Factor (TNFα) release is stimulated in response to Lipopolysaccharids (+39%) compared to negative control, while the treatment by the innovative active ingredient complex at the concentrations of (0.5%; 1.0% and 2.5%) significantly decreased the TNF release by 19%, 26% and 30% respectively(Fig 6).

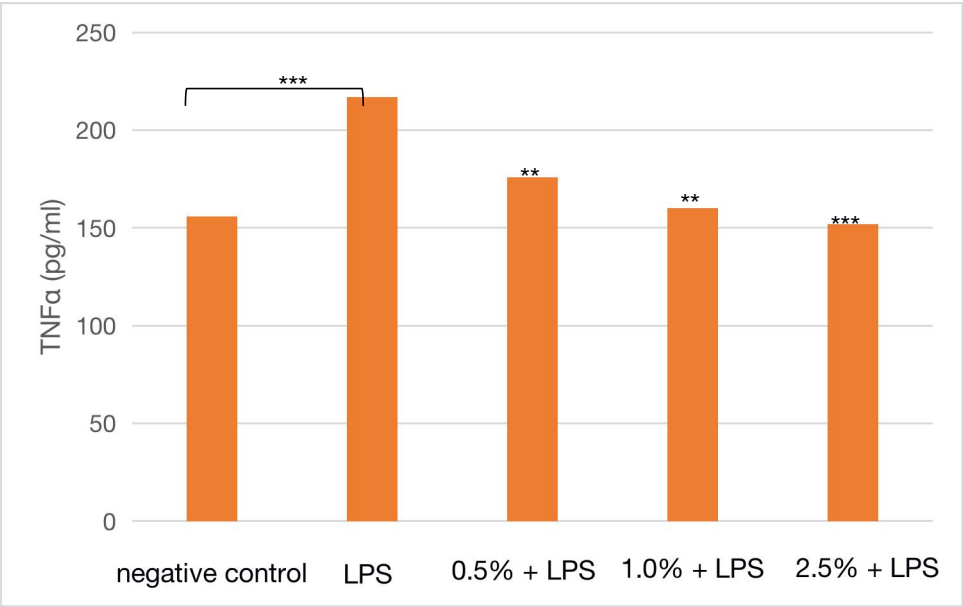


Figure 6: The concentration of TNF-α detected by Elisa . Statistical analysis: t-test. * p <0.05; ** p <0.01; *** p <0.001.

4. Effect of the innovative active ingredient complex on the extracelluar matrix in dermis

The total glycosaminoglycans were extracted and purifed by FPLC, then the total were detected by radioactivity. The obtained results show that the innovative active ingredient complex at the concentrations of 0.5%, 1% and 2.5% significantly increases the rate of glycosaminoglycans of the human fibroblasts in culture respectively by 16%; 27% and 35%.

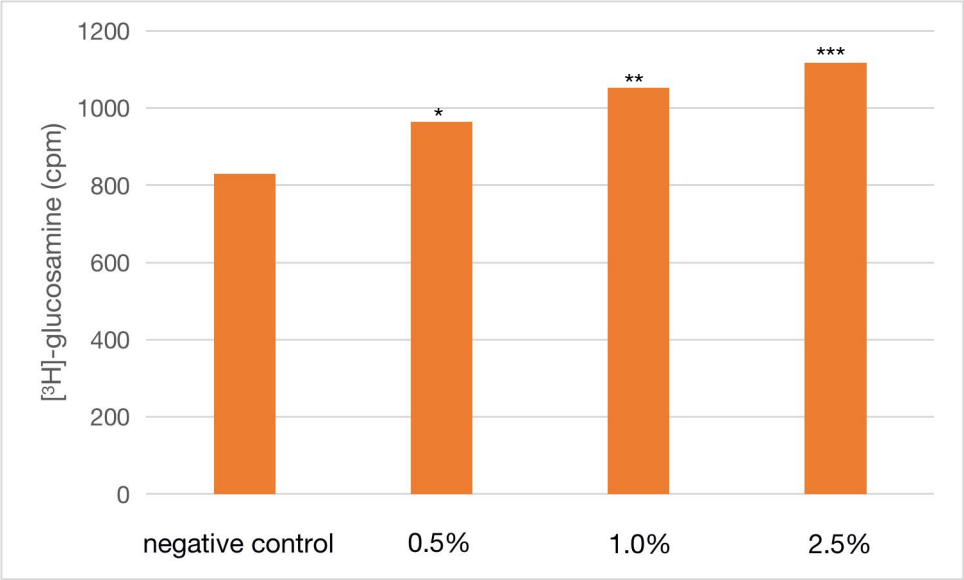


Figure 7： The glycosaminoglycans rate in fibroblast after with or without treatments. Statistical analysis: t-test. * p <0.05; ** p <0.01; *** p <0.001.

The obtained results show that treatment by the innovative active ingredient complex at the concentrations of 0.5%, 1% and 2.5% significantly increases the rate of the human fibroblasts collagens by 21%, 28% and 33% respectively(Fig 8).

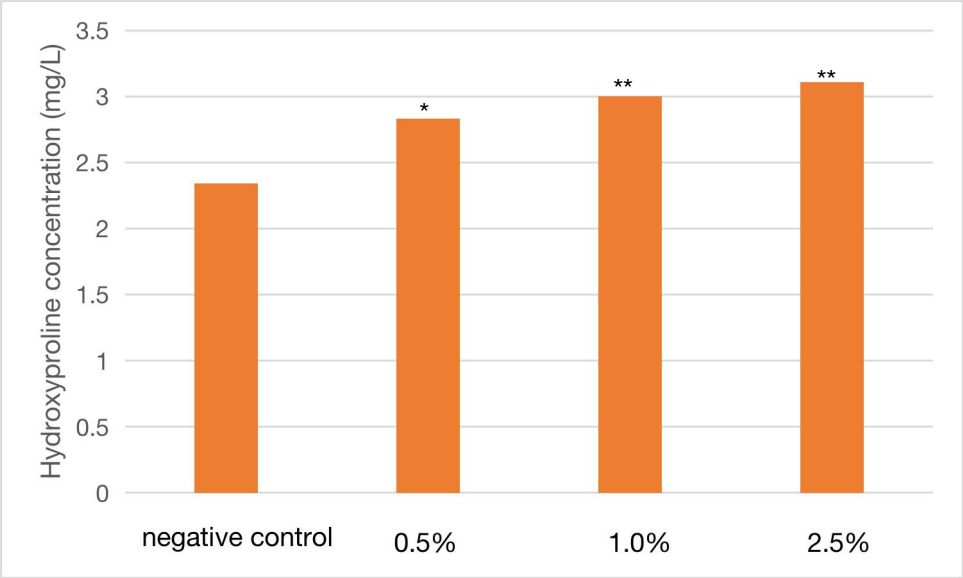


Figure 8: The Collagen in presence and absence of the innovative active ingredient complex. Statistical analysis: t-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

5. Clinical test

After 28 days application of product containing 0.5% innovative active ingredient complex, there is a significant decrease of the redness, imperfection and pigmentary spot on the face area of the volunteers' , compared to D0/T0 (Fig 9).

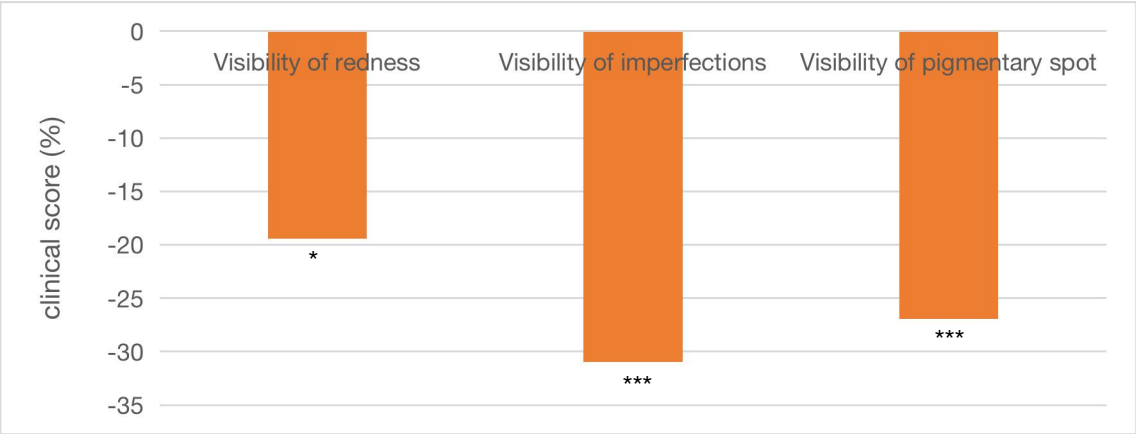


Figure 9： The clinical scoring on redness,imperfection and pigmentary spot after 28 days of using the innovative active ingredient complex. Statistical analysis: t-test. * p <0.05; ** p <0.01; *** p <0.001.

The results show a statistically significant improvement of the pH measurements on D28. And the product leaves a normal pH on the face after 28 days of application of the product in conditions of normal use (Fig 10).

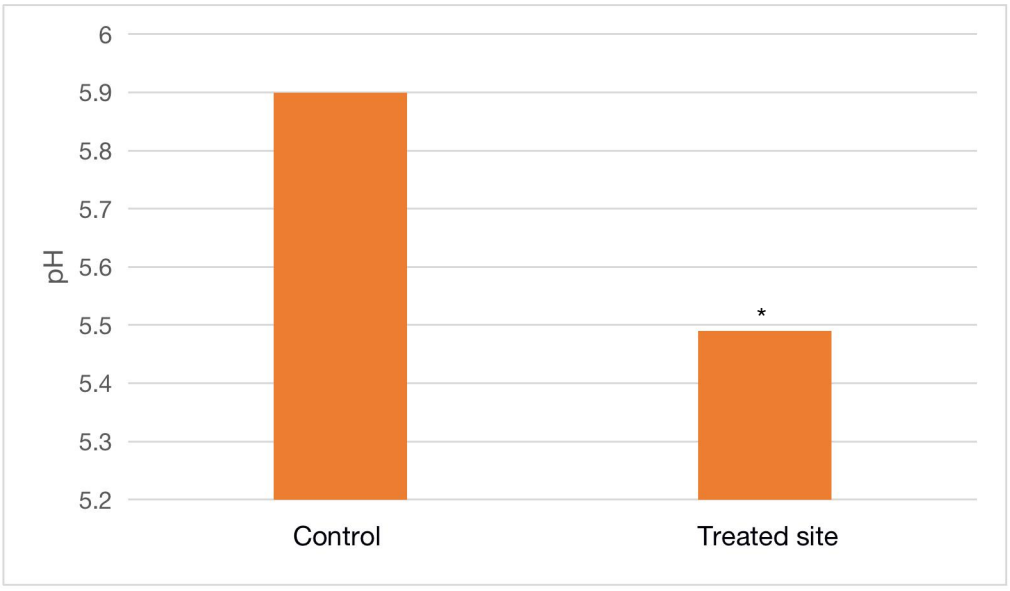


Figure 10： The pH after 28 days of using the innovative active ingredient complex. Statistical analysis: t-test. * p <0.05; ** p <0.01; *** p <0.001.

According to the self-assessment after 28 days, 58% of the subjects declared that their skin was soothed and they feel less sensation of irritation. 79% of the subjects declared their skin was more radiant and their imperfections were less apparent. 74% of the subjects declared their skin was more hydrated. 84% of the subjects declared that they

felt a sense of well-being and comfort. 79% of the subjects declared that their imperfections were less visible(Tab 1).

N°	Items	Subject satisfaction percentage
		D28
1	My skin is soothed, I feel less sensations of irritation	58%
2	My skin is more radiant	79%
3	My skin is more hydrated	74%
4	I feel a sensation of well-being and comfort	84%
5	My imperfections are less apparent	79%

Table 1：The Self-assessment after 28 days of using the innovative active ingredient complex.

Discussion

In the present study, the innovative active ingredient complex was evaluated the effects on: the epidermal barrier function, the immune system, and the extracellular matrix, as well as its soothing effect on the human body。

In the epidermis, the innovative active ingredient complex resulted in an increase in water retention and a decrease in the trans-epidermal passage of tritiated water. In the meanwhile, the complex induced an increase of the cellular proliferation, which represented a stimulation of the epidermis regeneration.

In the dermis, the results showed that the complex led to an increase in the rate of glycosaminoglycans and collagens of the human fibroblasts. When it comes to the effect on immune system, monocytes were cultured and the anti-inflammation effect were tested. It is clarified that the innovative active ingredient complex could significantly reduce the release of IL1-α, IL6 and TNFα induced by LPS.

Finally, the tests on volunteers for 28 days' application showed that the innovative active ingredient complex led to a statistically significant decrease of redness on the face area and 79% of the individuals stated that their imperfections were less apparent in their Self-assessment.

Conclusion

In this study, we used in vitro tests including reconstructed epidermis and cell culture to evaluate the effects of an innovative active ingredient complex on skin barrier function, immune system, and dermal matrix. Further more, clinical experiments have also revealed that this complex has a soothing effect on volunteers with skin imperfections and sensitivity. In conclusion, this research suggest that the complex could offer an innovative solution to those people whose skin are sensitive and with imperfections, assisting their skin to grow stronger, more uniform, and healthier.

Conflict of Interest Statement.

We have no conflicts of interest to declare.

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