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Effectiveness for Sensitive skin: From novel scientific global concept to performance on 3D innervated epidermis

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1. Introduction

Sensitive skin is a common and challenging condition for skin research and care, characterized by a hyper-reactive state of the skin, primarily on the face. It is associated by subjective symptoms such as discomfort, burning sensation, stinging, itching and tightness, especially when exposed to physical, chemical, or psychological stimuli. Objective signs, such as redness, may or may not be present.

For a long time sensitive skin was not considered, mostly because its clinical conditions were not necessarily well defined and as it was not well understood. It should be remembered that sensitive skin is primarily a very uncomfortable condition, characterized by perceptions that often cannot be monitored (signs initially felt before being visible) [1].

For a while, sensitive skin was partially understood, only correlated with a weakened skin barrier, and with a lack of lipids. It is now established that the phenomenon is much more complex. Last decade's discoveries put forth how sensitive skin, beyond the admitted skin barrier's defect, has multifactorial origins, including keratinocytes inflammation and hyper-reactivity of nerve fibers (also called neurogenic inflammation) [2]. This becomes two vicious cycles that lead to the self-maintenance of sensitive skin (figure 1).

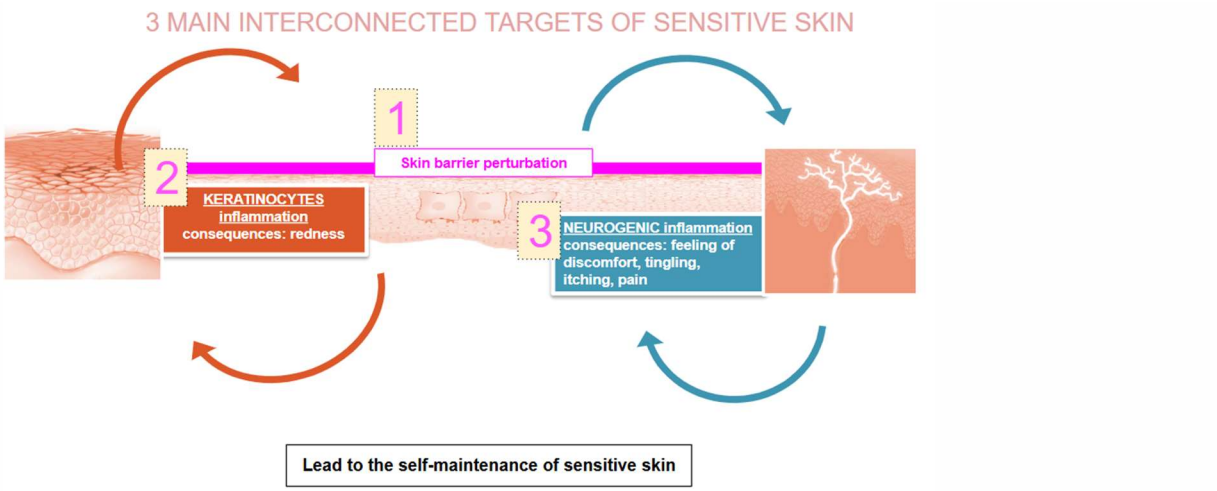


Figure 1: Self-maintenance of sensitive skin is driven by the interplay of 3 main interconnected targets

We aimed to elucidate a novel global biological approach for sensitive skin by first identifying its multifactorial origins. Subsequently, we selected and evaluated the effects of targeted active ingredients of natural origin using 2 innovative 3D *in vitro* models. First, mimicking sensitive skin with a barrier defect, and second, with neurogenic inflammation involving sensitive nerves implication.

2. Materials and Methods

2.1. Biological targets

Last decade's discoveries put forth how sensitive skin, has multifactorial origins, including 3 interconnected biological targets:

- Compromised barrier function

The weakening of the epidermis increases the skin's permeability, leading to dehydration and an increased penetration of irritating agents. This lowers the skin's tolerance threshold, making it more sensitive to normally well-tolerated stimuli.

- Keratinocytes inflammation

Keratinocytes, acting as sentinels of the skin, react to the penetration of irritants by releasing pro-inflammatory mediators (cytokines, prostaglandins, leukotrienes), thus initiating the inflammatory cascade. These signals spread inflammation throughout the skin's structures. In the epidermis, the mediators further weaken the already impaired skin barrier, increasing its permeability and allowing greater penetration of irritants, which perpetuates the production of

mediators and continuously weakens the barrier function. In the dermis, these mediators communicate with fibroblasts, which in turn react by releasing more inflammatory molecules (especially PGE2) and MMPs. MMPs weaken the blood vessel membranes by degrading the extracellular matrix, making them more vulnerable to the action of PGE2, which are permeabilizing and vasodilating agents.

- Neurogenic inflammation

Recent studies suggest that sensitive skin is closely linked to a hyper-activation of the TRPV1 receptor, leading to neurogenic inflammation, which is now considered the reference marker for this condition. Skin sensitivity is not the result of an increased density of nerve fibers but rather their hyper-reactivity. The activation of these nerve fibers releases pro-inflammatory mediators (Substance P, CGRP, Acetylcholine) responsible for cutaneous neurogenic inflammation and unpleasant skin sensations such as pain, itching, heat, and tingling. Substance P is the primary agent triggering this inflammation.

These mediators transmit information about the aggression by binding to specific receptors on the surface of keratinocytes, thereby increasing the inflammation level of these cells. This leads to an over-excitation of keratinocytes inflammation, creating a neurogenic vicious cycle.

2.2. Active ingredients selection

To select the best active ingredients for these 3 specific biological targets, we compared over seventy ingredients according to specific criteria such as phytochemical composition, efficacy tests, stability in formulation (oil-in-water emulsion) and current regulations.

Our work allowed us to select 3 key active ingredients of natural origin (figure 2):

- Compromised barrier function: *Ophiopogon japonicus* root extract strengthens the cohesion of the epidermis.
  In fact, it significantly increases the synthesis of claudin-1 and the synthesis of ZO-1, 2 major proteins of tight junctions that play important roles in intercellular cohesion and homeostasis of the barrier function (study realized by Western blot on normal human keratinocytes).
- Keratinocytes inflammation: Biosaccharide gum-2 limits the release of keratinocyte-derived inflammatory mediators.
  In fact, it significantly decreases the release of PGE2 and therefore highlights its power to reduce inflammatory reactions (study realized by ELISA on normal human

keratinocytes stressed by PMA (Phorbol 12-myristate 13-acetate at 1µg/ml) after 24 hours of treatment).

- Neurogenic inflammation: *Laminaria ochroleuca* extract limits the release of neurogenic inflammatory mediators.
  In fact, it significantly decreases the release of neurotransmitters (Substance P, CGRP and Acetylcholine) and therefore reduce neurogenic suractivation (study realized by ELISA on primary cultures of nerve cells after 24 hours of treatment).

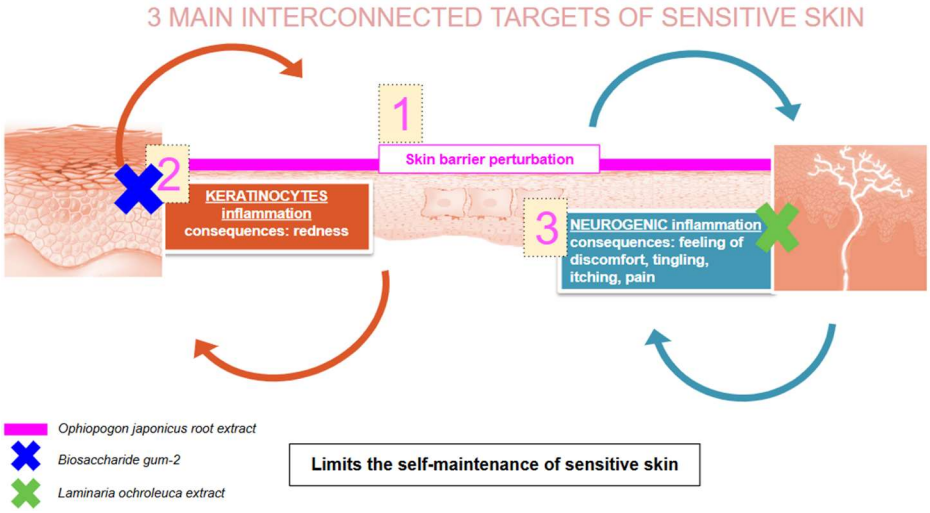


Figure 2: Complementary active ingredients help reduce the self-maintenance of sensitive skin

2.3. Experimental methods

A/ Targeting barrier integrity and inflammatory process : the immature RHE model

Our work was inspired from the OCDE n°439 (skin irritation *in vitro*) protocol using Reconstructed Human Epidermis (RHE) as a biological model. We essentially adapted the culture time of the RHE (10 days of culture instead of 17 days of maturation as mentioned in the OCDE protocol). The lower maturation can be considered as a model of “sensitive skin” because it is well known and described in scientific papers that one of the parameters of sensitive skin is a thinner barrier / finer *stratum corneum*. As shown below (Figure 3), the first step was the comparison between RHE cultivated for 10 days (called “sensitive” or “immature” skin) versus normally cultivated RHE (17 days = called “normal” or “mature” skin”). At the end of the culture, cryosectioning of RHE was realised using a cryostat Leica CM1860UV. Microscopic observations were done using a Nikon Eclipse TiE microscope (G: 10x) equipped with a CCD camera.

In order to compare the RHE cultivated for 10 or 17 days, the effect of SDS (Sodium Dodecyl

Sulphate; the positive control of the OCDE method) was used at different concentrations on the RHE. The effect of the SDS on the viability of the RHE was evaluated. This whole protocol is summarized in the figure 3 below.

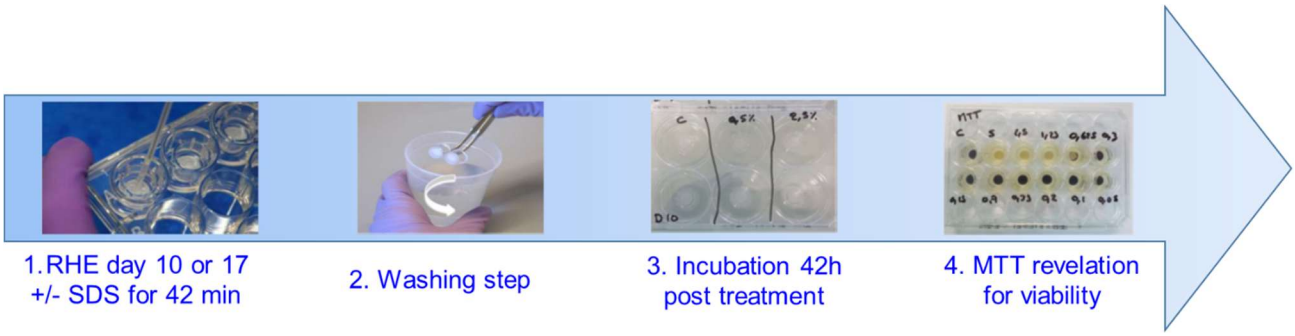


Figure 3: The above diagram summarizes the applied protocol for the the immature RHE model

For each RHE, the IC50 was calculated (IC50: Concentration of SDS giving 50% of viability). Immature RHE, at day 10, was used to test a product (PROD-19-041) applied for 3 days before testing different concentrations of SDS. SDS is used to mimick skin barrier perturbation. Il-1 α quantification in the supernatant was used as endpoint for inflammation induced by SDS barrier alteration.

B/ Targeting neuroinflammatory process: the innervated immature RHE model

The aim of this study is to investigate the potential for neuroinflammation modulation. We used a model based on immature RHE to mimic sensitive skin. We completed this model by a hiPS neuron culture used to innervate immature RHE, mimicking sensitive neurons. RHE innervated model was incubated with topical application of products to screen formulas able to sooth sensitive skin neuroinflammation.

Capsaicin was used as a stress signal, irritant for neuron cells. Capsazepin, agonist of Capsaicin, was used as a molecule control. Quantification of the neurotransmetter CGRP in the supernatant was used as endpoint to evaluate neuronal irritation (ELISA Abbexa ; ref : abx257902 ; lot : E2104198F). We also realized immunostaining of neurite on the immature innervated RHE using anti-β-tubulin (Sigma Aldrich ; ref : T8660 ; lot : 034M4790V) counterstained with a secondary fluorescent antibody (Fisher Scientific ; ref : A11001 ; lot : 2284614). For each condition, 20 pictures were taken (G : 20x, InCell 2200 ; GE Healthcare) to count and measure neurites length, allowing to control neuronal survival in the different tested conditions.

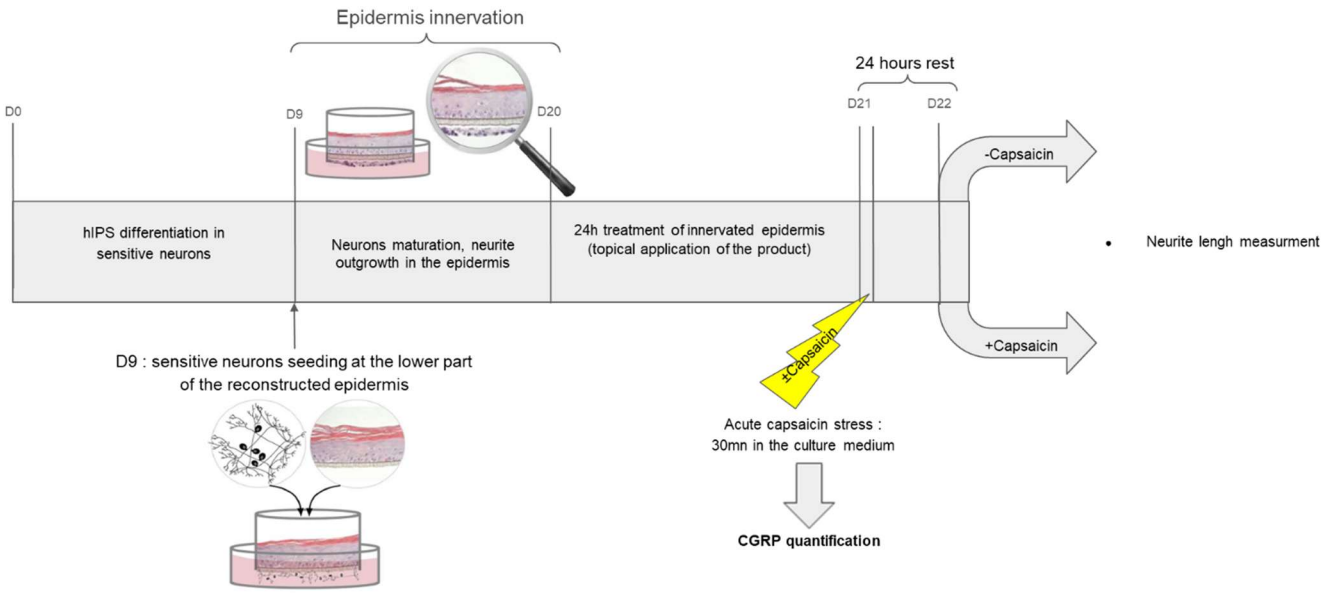
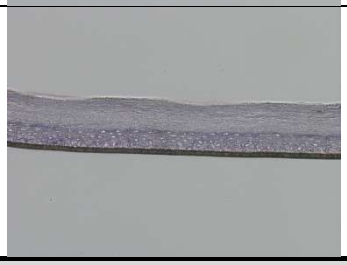


Figure 4: The above diagram summarizes the applied protocol for the innervated RHE Model

3. Results

A/ The immature RHE model

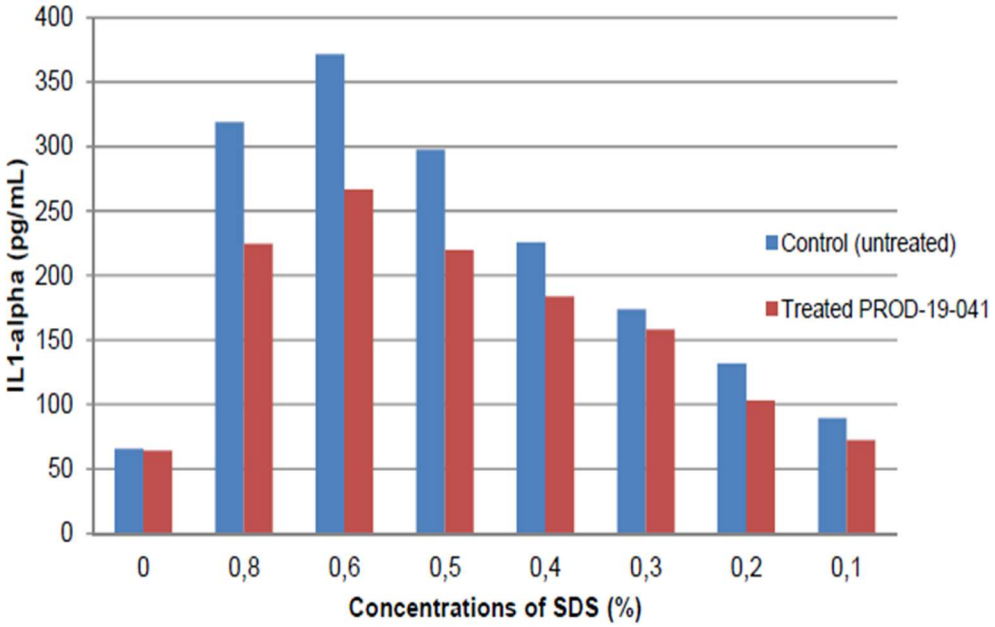
Table 1: The table below represents the obtained results (N= 6 independent assays) for the RHE viability

RHE (N=6)	IC50 (%)	Representative image
Immature (day 10)	0.258 ± 0.049	
Mature (day 17)	0.475 ± 0.142	
Ratio Mature/Immature	1.66 ± 0.07	

The above results show differences between the two different maturities of RHE. Images confirm the lack of differentiation and the *stratum corneum* fragility on the immature model. Therefore, immature RHE (day 10) can be considered as a relevant model to study sensitive skin.

Interleukin-1α:

Table 2: The graph below provides the results obtained for IL-1α quantification in the supernatant of untreated and treated immature RHE after the stress with SDS.



These results show that less Il-1α is secreted by treated (PROD-19-041) immature RHE versus the untreated case. This can be observed for all SDS concentrations tested.

B/ The innervated immature RHE model

Below are images of the RHE H/E stained at the end of the culture :

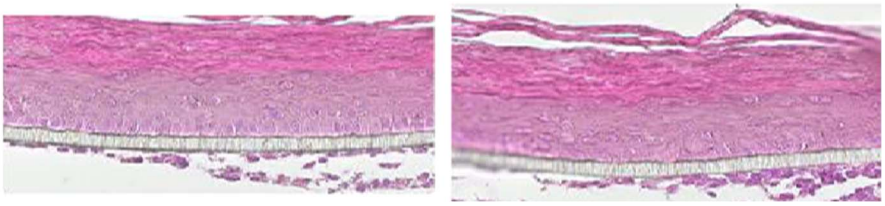
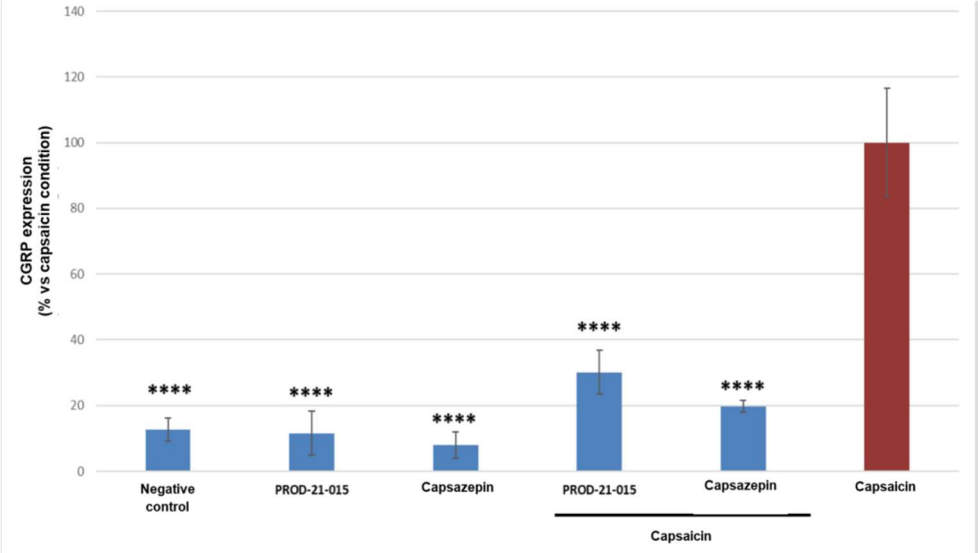


Figure 5: Representative images of innervated RHE H/E stained at the end of the culture

We can clearly observe the neurons on the bottom of the culture, confirming that the RHE is efficiently innervated.

a) CGRP quantification :

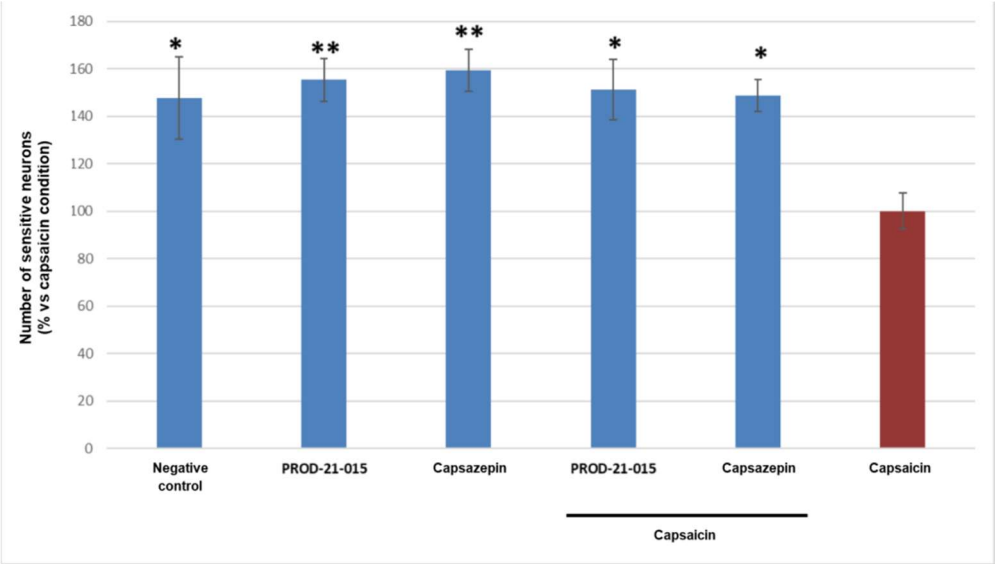
Table 3: The table below provides quantification of CGRP expression in the culture supernatant after 30mn stress with capsaicin followed by 24hours rest with the different treatments



As we can see, capsazepin or PROD-21-015 do not modulate CGRP expression whereas capsaicin highly induce CGRP expression *vs* negative control. When capsaicin is applied, capsazepin or PROD-21-015 both have a strong and significant capacity to inhibate CGRP expression induced by 30mn capsaicin application *vs* capsaicin condition.

b) Neurites' length

Table 4: The graph below summarizes the measured length of neurites on the immature innervated RHE model in control condition and after 30mn of capsaicine stimulation :



It is clear that capsaicin treatment induces a significant reduction of neurites *vs* negative control, whereas Capsazepin or PROD-21-015 have no effect on neurite's number. Moreover,

both ingredients, when used in combination with capsaicin, have the ability to inhibit its irritant effect.

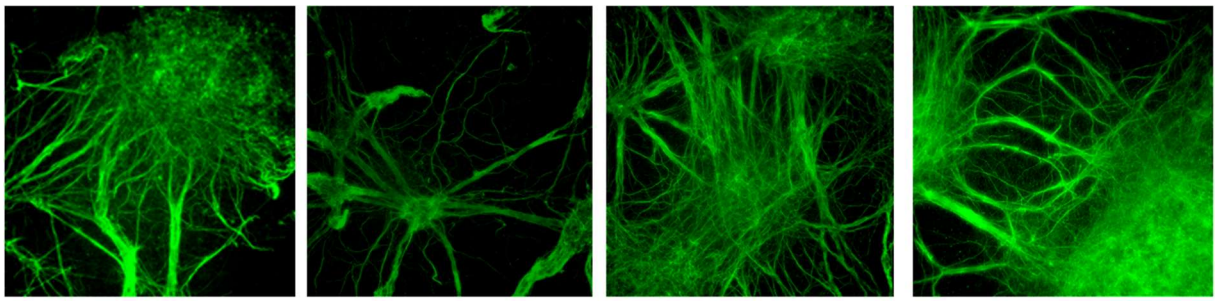


Figure 6: Representative images of β-tubulin staining in the different tested conditions: Capsaicin clearly reduces neuron's number and length while neurons are maintained in all the other conditions

Images clearly confirm the measured results : it is obvious that only capsazepin treatment reduces neurite outgrowth, when capsazepin and PROD-21-015 have the ability to inhibit capsaicin effect on neurons' length. Thus, we can confirm that both ingredients have a protective effect on neurons survival.

3. Results

The overall above results indicate that:

1. The differences observed between the two tested maturities of RHE confirm that immature RHE (day 10) is more fragile when exposed to SDS. Immature RHE (day 10) was therefore considered as a relevant model to study "sensitive skin".

The test product PROD-19-041 was then tested on the immature RHE model. Treatment of RHE with PROD-19-041 helped to reduce inflammation of immature RHE when exposed to an "inflammatory stress".

2. For the RHE-immature-innervated model, we confirm that capsaicin is able to induce CGRP expression and reduce neurons' number. It is the proof that TRPV1 receptors are activated.

However, both capsazepin and PROD-21-015 have the ability to reduce neuronal activation: these ingredients are potent candidates to protect neurons from exacerbated sensitivity.

4. Conclusion

Driven by a systemic review of sensitive skin knowledge, we integrated its diverse underlying causes. Our strategy was to target each identified biological pathway. The review highlighted the critical convergence of barrier defects, keratinocyte inflammation, and neurogenic inflammation as essential factors in the genesis and self-maintenance of sensitive skin.

Our extremely 2 innovative assays mimic skin sensitivity. In these 2 models, we demonstrated that we could significantly reduce biomarkers associated with skin's sensitivity, notably epidermal inflammation due to cutaneous barrier alteration and neuronal activation responsible for exacerbated sensitivity. Both models gave complementary results that allowed us to develop a global daily formula care dedicated to sensitive skin.

Acknowledgment :

We kindly thank Neuron Experts' team for their contribution to this work, developing the 3D innervated immature RHE model.

References

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