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## ***“Soothing the skin and favorizing well-being feelings : acting on the skin-brain axis with a topic application.”***

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### **1. Introduction**

In contemporary society, psychological stress has emerged as a pervasive factor affecting overall health. Among other disorders on human health, increasing evidence highlights the profound impact of chronic stress on dermatological conditions and skin diseases [1]. Stress is described to impair skin barrier function, promote inflammation, and exacerbate a range of skin disorders such as acne vulgaris, atopic dermatitis, psoriasis, and even accelerate the aging process [2]. Psychological stress acts through a complex network of neuroendocrine and immune pathways collectively referred to as the “brain-skin axis.” This bidirectional communication system allows the central nervous system to influence cutaneous homeostasis and immune responses through the release of stress mediators such as neuropeptides, neurotransmitters and hormones [3].

A central effector of the stress response is the hypothalamic-pituitary-adrenal (HPA) axis, which ends in the skin with the chronic release of cortisol, a glucocorticoid hormone traditionally regarded for its anti-inflammatory and immunosuppressive properties. In the skin, local analogs of the HPA axis also exist, allowing the cutaneous tissue to synthesize cortisol in response to stress-related signals. Under acute stress, cortisol may help to maintain homeostasis by dampening excessive inflammatory responses. However, under conditions of chronic psychological stress, the role of cortisol becomes paradoxical.

Prolonged exposure of the skin to elevated cortisol levels can lead to glucocorticoid receptor desensitization and functional impairment of anti-inflammatory signaling. Consequently, cortisol loses its protective role and contributes to a pro-inflammatory state [4]. Moreover, chronic stress promotes the release of neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) from sensory nerve endings in the skin [5,6]. These neuropeptides trigger

mast cell degranulation, vasodilation, and the recruitment of immune cells, leading to a phenomenon known as neurogenic inflammation [7-9].

The interplay between dysregulated cortisol signaling and neurogenic inflammation results in a feed-forward loop that exacerbates skin inflammation, contributing to the pathophysiology of stress-exacerbated dermatological conditions such as psoriasis, atopic dermatitis and acne.

Targeting the neuronal component with functional antagonist could therefore help reduce neurogenic inflammation and improve comfort and symptoms associated with psychological stress and favoring the emergence of well-being feelings.  $\beta$ -Endorphin is an endogenous opioid peptide derived from proopiomelanocortin (POMC) that acts primarily through mu-opioid receptors (MOR), which are expressed in cutaneous cells, including keratinocytes, immune cells, and sensory neurons [10,11]. Activation of MOR by  $\beta$ -endorphin inhibits the release of pro-inflammatory neuropeptides such as substance P and CGRP, thereby reducing neurogenic inflammation [12,13].

Sensory neurons with receptors located in the epidermis layers can be targeted by neurocosmetics to modulate responses to stress and inflammation. The aim of this study was thus to evaluate the potential of a mixture of ingredients produced by Silsey to act on sensory neurons, inhibit neurogenic inflammation and thus soothe the deleterious sensations linked with chronic stress exposure.

## 2. Materials and Methods

### 2.1 Generation of hiPS and human sensory neuron differentiation in monolayer

Human primary skin fibroblasts were dedifferentiated using a lentivirus encoding for KLF4, OCT4, SOX2 and c-MYC. Obtained hiPS colonies were picked about 4 to 6 weeks after infection based on their morphology and expanded in mTESR1 medium in BD Matrigel® coated dishes. hiPS cells were plated in 6 wells culture plates coated with a thin layer of BD Matrigel® and cultured at 37°C in DMEM-F12 medium supplemented with 0.1  $\mu$ M of retinoic acid, 1  $\mu$ g/mL of EPO, 10% of KSR, 1% of P/S and a cocktail of inhibitors for 10 days. At day 8 cells were plated at a density of 17000 cells by wells, on 96 wells plates coated with a thin layer of BD Matrigel®. At day 10, medium was replaced by DMEM-F12 supplemented with 10ng/mL of NT-3, 10ng/mL of GDNF, 10ng/mL of BDNF, 10ng/mL of NGF and 1% P/S. Medium was changed every 2 or 3 days.

### 2.2 Generation and treatment of 2 dimension co-culture of sensory neurons and keratinocytes

Human sensory neurons were co-cultured with adult human keratinocytes from day 14 in 96-well plates. The co-culture was subjected to a 10-days chronical exposure of cortisol 0.1  $\mu$ M (Sigma Aldrich ; ref : H-0888) in presence or not of the ingredients mixture or the reference molecule  $\beta$ -endorphin (Bachem; ref: H-2700). On the last day, an additional acute stress with capsaicin (Sigma Aldrich ; ref : M-2028) was applied during 30 minutes. Cells were fixed with a paraformaldehyde solution just after caspaicin exposure.

## 2.3 Generation and treatment of 3 dimension coculture of sensory neuron and reconstructed epidermis

Human sensory neurons were seeded under the membranes serving as support for the reconstruction of epidermis (reconstructed epidermis provided by StratiCELL). After 6 days of co-culture, cells were treated or not with the ingredients mixture or the reference molecule  $\beta$ -endorphin and 24-hours later were subjected to a 30-minutes capsaicin exposure. Finally, a final 24-hour incubation was carried out in the absence of capsaicin before the cells were fixed with a paraformaldehyde solution.

## 2.4 Measurement of neuropeptide CGRP release

The amount of neuropeptide CGRP released by sensory neurons in co-culture were determined by ELISA following the instructions of provider (Abbexa ; ref : abx257902).

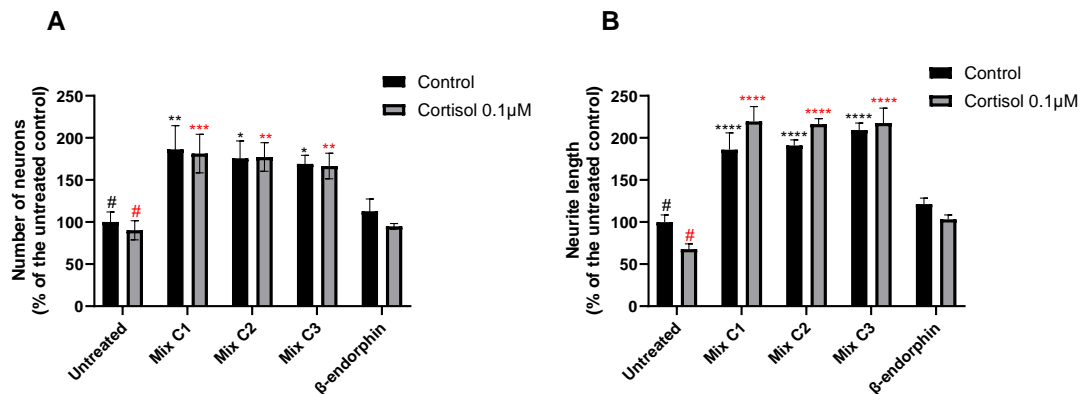
## 2.5 Neurons survival, neurite length, MOR and GR expression measurement analyses

Human sensory neurons were stained with an anti- $\beta$ -tubulin (Sigma Aldrich; ref : T8660) antibody, whereas MOR (Biotechne; ref : NB100-1618) and GR (Sigma Aldrich ; ref : HPA004248) expression were detected by specific antibodies. Fixed cells were incubated with primary antibodies then with fluorescence-labeled secondary antibodies (Fisher Scientific ; ref : A11001, A11011 and A21105). The nuclei were stained with Hoechst's solution (Sigma Aldrich ; ref : H-33258). Per well, 20 photographs were taken with an automatic microscope (InCell 2200; GE Healthcare) at x20 magnification. A count of the neurons was done, the length of their neurites was also determined. The MOR and GR expression were colocalized with  $\beta$ -tubulin staining to determine the expression rate of these two receptors in the sensory neurons. Statistical analysis was performed using a One-way ANOVA test followed by a Dunnett's test.

# 3. Results

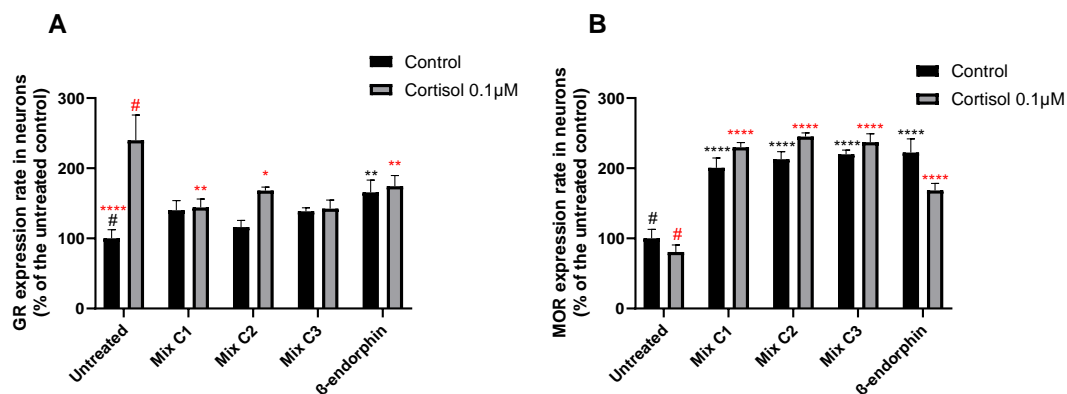
## 1.1. Mixture of ingredients protects neurons in a two dimension co-culture system

The ability of the mixture of ingredients to protect sensory neurons from chronic cortisol exposure was determined. The co-culture was treated during 10 days with cortisol 0.1  $\mu$ M in presence of the mixture of ingredients or the reference compound  $\beta$ -endorphin at 1  $\mu$ M. At the end of this treatment, the number of neurons (Figure 1A) and the length of the neurites (Figure 1B) were assessed and compared to untreated cells. Sensory neurons maintained in co-culture with keratinocytes saw their number decrease over the course of the culture period. In the absence of cortisol, the ingredient mixture protected sensory neurons, resulting in an increase of at least 69% in their number compared to the untreated control while  $\beta$ -endorphin had only a modest effect (+13%). Interestingly chronic cortisol exposure led to a slight decrease in neuronal survival (-10%) but the mixture of ingredient was able to protect neurons from this effect. In the same way, the ingredient mixture promoted neurite growth of sensory neurons inducing an increase of at least 86% which was higher than the effect observed in the presence of  $\beta$ -endorphin (+21%). Cortisol induced a decrease in neurite length (-32%) but the mixture of ingredient was able, at the three tested concentrations, not only to fully protect neurite length but to stimulate it.



**Figure 1. Neuroprotective effect of the mixture of ingredients. Evaluation of neuronal survival (A) and neurite length (B) of human sensory neurons co-cultured with keratinocytes and treated during 10 days with cortisol at 0.1  $\mu$ M in presence or not of the mixture of ingredient at 3 concentrations (C1>C3) or  $\beta$ -endorphin at 1  $\mu$ M.**

### 1.2. Mixture of ingredient protects neurons demonstrated by an activity on corticoid and endorphin signaling pathways in a two dimension co-culture system



**Figure 2. Effect of the mixture of ingredients on GR (A) and MOR (B) expression in neurons. Human sensory neurons co-cultured with keratinocytes were treated during 10 days with cortisol at 0.1  $\mu$ M in presence or not of the mixture of ingredient at 3 concentrations (C1>C3) or  $\beta$ -endorphin at 1  $\mu$ M.**

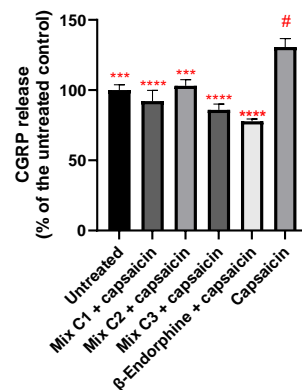
To investigate the effect of the mixture of ingredient on cortisol signaling pathway and on endorphin signaling pathway, which is known to inhibit cortisol-induced stress, these receptors were colocalized in neurons. The ingredient (Figure 2A) mix showed, just like  $\beta$ -endorphine, a tendency to increase the expression of glucocorticoid receptors in absence of cortisol. Interestingly, in presence of chronic cortisol exposure, an important increase of GR expression is observed (+140%) and this effect is partially and significantly inhibited by the mixture of ingredient (minimum -72%) with an efficacy comparable or superior to that observed with  $\beta$ -endorphine (-66%).

Interestingly, treatment with either  $\beta$ -endorphine or mixture of ingredient resulted in a significant increase in MOR expression (Figure 2B). The effect was similar in the presence of the

mix, regardless of concentration (+110% on average), compared to  $\beta$ -endorphine. Once again, this positive effect on MOR expression was preserved in presence of chronic cortisol treatment. Indeed, cortisol induced a decrease of MOR expression (-20%) but the mixture of ingredient fully protected neurons and induced overexpression of MOR (+137% on average with the mix versus +68% for  $\beta$ -endorphine compared to the cortisol control).

### 1.3. Mixture of ingredient is able to inhibit markers of neuro-inflammation in the two dimension co-culture system

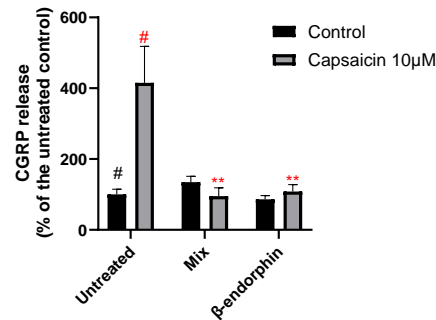
The neuropeptide CGRP is one of the key effectors of neuro-inflammation. The ability of the mixture of ingredient to inhibit its release was thus analyzed (Figure 3). Indeed, at the three tested concentrations the Mix was able to completely inhibit CGRP release although the effect was slightly less pronounced than that observed for  $\beta$ -endorphin .



**Figure 3. Inhibitory effect of the mixture of ingredients on CGRP release.** Human sensory neurons co-cultured with keratinocytes were subjected to a 30-minutes capsaicin stress at the end of the culture in presence or not of the mixture of ingredient at 3 concentrations (C1>C3) or  $\beta$ -endorphin at 1 $\mu$ M.

### 1.4. Final product containing the mixture of ingredient confirmed its neuroprotective effect and its ability to inhibit neurogenic inflammation markers

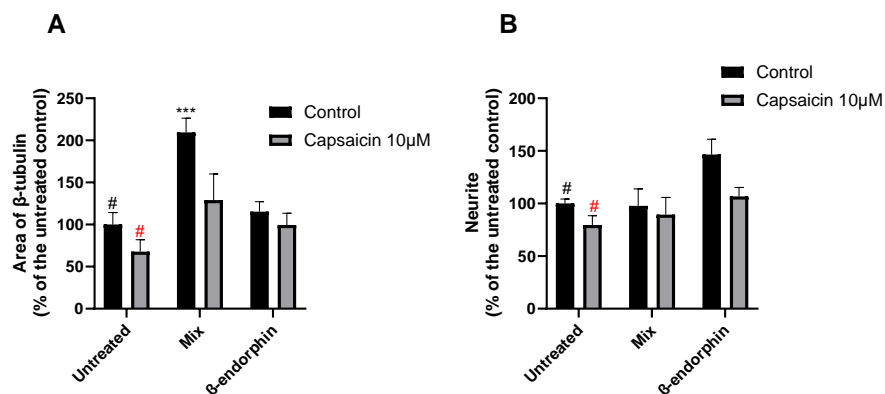
The three dimension co-culture model of sensory neurons and reconstructed epidermis allows for the evaluation of the efficacy of finished products. A formulation containing the same ingredient mix was evaluated (Figure 4). The formulation was applied topically to the epidermis, while capsaicin was added to the neuron culture medium to trigger the release of CGRP.



**Figure 4. Inhibitory effect of the formulated mixture of ingredients on CGRP release.** Human sensory neurons co-cultured with reconstructed epidermis were subjected to a 30-minutes capsaicin stress in presence or not of the mixture of ingredient or β-endorphin at 0.2%. Capsaicin was added in the culture medium while the formulated mixture of ingredient or β-endorphin were applied topically on the epidermis.

While having no significant effect in absence of capsaicin stress, the formulated version of the mixture of ingredient confirmed its ability to totally inhibit the release of neuropeptide CGRP (-320% compared to the capsaicin control).

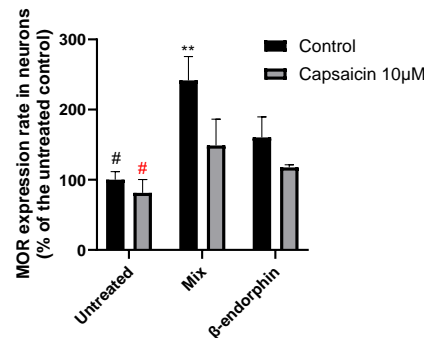
In this three dimension model, the mixture of ingredient was also confirmed to have neuroprotective properties (Figure 5A). It increased neurons survival (+110%) with a higher efficacy than β-endorphin (+15%). The mixture of ingredient also protected neurons from capsaicin stress (+61% of survival compared to the capsaicin control). Nevertheless the formulated mixture of ingredient had no effect on neurite length (Figure 5B).



**Figure 5. Neuroprotective effect of the mixture of ingredients. Evaluation of neuronal survival (A) and neurite length (B).** Human sensory neurons co-cultured with reconstructed epidermis were subjected to a 30-minutes capsaicin stress in presence or not of the mixture of ingredient or β-endorphin at 0.2% before a 24-hours incubation post-stress. Capsaicin was added in the culture medium while the formulated mixture of ingredient or β-endorphin were applied topically on the epidermis.

The expression rate of MOR was also measured in this 3D system (Figure 6). Once again the results obtained with the 2D co-culture were confirmed in this 3D co-culture system with an increase of MOR expression both in absence (+142% compared to the untreated control) and

in presence (+68%) compared to the capsaicin control of capsaicin stress, showing an effect greater than that observed with  $\beta$ -endorphine.



**Figure 6.** Effect of the mixture of ingredients on MOR expression by neurons. Human sensory neurons co-cultured with reconstructed epidermis were subjected to a 30-minutes capsaicin stress in presence or not of the mixture of ingredient or  $\beta$ -endorphin at 0.2% before a 24-hours incubation post -stress. Capsaicin was added in the culture medium while the formulated mixture of ingredient or  $\beta$ -endorphin were applied topically on the epidermis

#### 4. Discussion

In this study, we demonstrated the ability of a mixture of ingredients developed by Sisley on CGRP to positively impact biological phenomenon implicated in psychological stress was assessed.

Chronic cortisol stress was used to mimic psychological stress in a two dimension co-culture system. The mixture of ingredients protected the neuronal network (improving neurons survival and promoting neurite length) from cortisol-induced impairment. It also increased the expression of  $\beta$ -endorphin receptors (MOR) while decreasing the expression of glucocorticoid receptors (GR) suggesting that the mixture of ingredient inhibits cortisol signaling pathways while favorizing endorphin detection. Finally it inhibited the release of neuropeptide CGRP, a well-known marker involved in neurogenic inflammation. These results, obtained in 2D model, were confirmed in a 3D model in which a formulation containing the mixture of ingredient was applied topically while capsaicin was added in the culture medium to induce the release of CGRP. In this model, the final product thus demonstrated neuprotective effect (enhanced survival of neurons) as well as inhibitory effect on CGRP release and an increase in MOR expression rate. These results confirm the potential of this mixture of ingredient to soothe neurogenic inflammation and reduce negative sensations. Furthermore its ability to upregulate the expression of MOR suggests a modulatory action on this signaling pathway and can favorize the perception of more positive sensations associated with well-being.

#### 5. Conclusion

The ingredients mixture, developed by Sisley, inhibited the release of neuropeptides involved in irritation, negative sensations and inflammation. It thus demonstrates a strong potential to protect the skin from various stress factors and to promote the detection of more positive sensations. Therefore, our results pave the way for a new field in cosmetics: neurocosmetics, dedicated, beyond skin's physiology, to whole body well-being.

## 6. References

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