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Immediate comfort provided by an original mechanism: how TRPM8 regulates the activation of TRPA1 and TRPV1

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1. Introduction

Sensitive or reactive skin is a common condition that affects many people. Approximately 50% of individuals report having reactive skin, although this patient-reported prevalence varies across countries [1]. Sensitive skin refers to a sensory reaction triggered by contactors and/or environmental factors, usually without visible clinical manifestation [2]. A consensus definition was proposed as: “A syndrome defined by the occurrence of unpleasant sensations (stinging, burning, pain, pruritus, and tingling sensations) in response to stimuli that normally should not provoke such sensations. These unpleasant sensations cannot be explained by lesions attributable to any skin disease. The skin can appear normal or be accompanied by erythema” [1].

Itch is defined as an unpleasant sensation that evokes a desire to scratch [3]. This trivial definition, about 200 years old, has not been modified in spite of advancing knowledge. The sensation occurs when pruritogens activate receptors on cutaneous sensory C-fibers. There are two types of receptors that transmit itch: G protein-coupled receptors (GPCRs) and downstream transient receptor potential channels (TRP). TRP channels are a superfamily of cation permeable channels that are involved in a variety of sensory modalities.
TRPA1 (Transient Receptor Potential Ankyrin 1) and TRPV1 (Transient Receptor Potential Vanilloid 1) are involved in nociception and in the perception of strong to moderate itch sensations [4]. These receptors are expressed in sensory nerves as well as in keratinocytes [5]. TRPA1 is implicated in thermal sensation (below 17°C) and can be activated by AITC (allyl isothiocyanate). TRPV1 is responsible for high temperature (>43°C) and can be activated by the natural agonist capsaicin [6].
Both TRPA1 and TRPV1 activation lead to the release of neuropeptides, including SP (Substance P) and CGRP (calcitonin gene-related peptide) that can induce neurogenic inflammation. Moreover, pro-inflammatory mediators produced by keratinocytes such as cytokines can stimulate TRPA1 and TRPV1 which can in turn also induce the production of pro-inflammatory neurotransmitters [6, 7].
TRPM8 (Transient receptor potential cation channel subfamily M member 8), activated by menthol, has also been shown to play a major physiological role in inflammation and itch [8]. Numerous studies examining the role of TRPM8 in inflammatory conditions, such as chronic neuropathic pain, noxious cold, and colitis, have demonstrated an upregulation of TRPM8 expression.One mechanism by which the upregulation of channel expression affects inflammatory conditions is through mediating the release of inflammatory cytokines. Evidence suggests cytokine regulation through TRPM8 occurs through its interactions with the Nuclear Factor -κB (NF-κB).

Moreover, TRPM8 can also mediate inflammation through crosstalk with other TRP channels. Numerous studies have exhibited how TRPM8 activation suppresses the release of TRPV1-mediated inflammatory neuropeptide, CGRP. So, TRPM8 may serve an anti-inflammatory function to balance the pro-inflammatory responses of TRPV1 and TRPA1, mediating chemosensory deactivation and inflammatory neuropeptide release [8, 9].
By activating TRPM8, inflammatory responses induced by TRPV1 and/or TRPA1 activation can be mediated, limiting CGRP release and then promoting skin soothing.

Cosmetic products could help to relieve and improve sensitive skin condition, especially with immediate effects. In this context, natural extracts containing bioactive molecules known for their soothing properties such as triterpens are of great interest. Adaptogen plants and mushrooms which are a rich source of highly effective biomolecules may be a solution for the development of new immediate soothing active ingredients.
We have studied the soothing effect of *Ganoderma lucidum* extract (GLE) to improve the symptoms associated with sensitive skin, focusing on an innovative pathway based on TRPM8 / TRPA1 / TRPV1 interactions.

2. Materials and Methods

GLE

Ganoderma lucidum Karst, a basidiomycete mushroom, native to Asia, known as “Ling Zhi” in China and “Reishi" in Japan and ‘Yeongji’ in Korean, has been used in Traditional Chinese Medicine for thousands of years. GLE is an hydroalcoholic extract of *Ganoderma lucidum* mushrooms diluted in propanediol. This extract is rich in ganoderic acids.

TRPM8 activation

After 19 days of culture, co-cultures of neurons obtained from human induced pluripotent stem cells (iPS) and keratinocytes were treated or not with the TRPM8 receptor inhibitor, M8-B, during 1 h. Then, the medium was removed and the cells were incubated in the presence or absence of M8-B inhibitor and with the calcium-specific fluorescent probe for 30 min. The cells were then washed with PBS and incubated in medium without phenol red and containing or not the TRPM8 inhibitor.
Immediately afterwards, the cultures were transferred to the motorized stage of the inverted microscope and were observed in fluorescence. Images were taken every 300 ms with a software-controlled camera (baseline evaluation). After 20 seconds of observation, the neurons were activated by GLE (0.01% or 0.005%) or menthol (0.016%). The images were recorded for 2 min.
The increase in the average fluorescence level of the sensory neurons was measured and compared to the negative control conditions (treated with the control medium). Data were expressed as mean ± SEM (standard error of the mean). All experimental conditions were performed in 6 replicates.

Interactions between the TRPM8 and TRPV1 and TRPA1 signalling pathways

After 17 days of culture, co-cultures of neurons and keratinocytes were treated or not with the TRPM8 receptor inhibitor, M8-B, during 4 h. Then, tested ingredients were added (menthol at 0.016% or GLE at 0.01% or 0.005%) with or without reference inhibitors (capsazepine for TRPV1 or HC030031 for TRPA1).
After 24h of pre-incubation, the calcium-specific fluorescent probe was added for 30 min. Then cells were washed with PBS and incubated in medium without phenol red and containing or not the TRPM8 inhibitor with menthol (0.016%), or GLE (0.01% or 0.005%) and with or without reference inhibitors (capsazepine for TRPV1 or HC030031 for TRPA1).
Immediately afterwards, the cultures were transferred to the motorised stage of the inverted microscope and were observed in fluorescence. Images were taken every 300 ms with a

software-controlled camera (baseline evaluation). After 20 seconds of observation, the neurons were activated by capsaicin (TRPV1 activator) or AITC (TRPA1 activator).
The images were recorded for 2 min. The increase in the average fluorescence level of the sensory neurons was measured and compared to the negative control conditions (treated with the control medium).
After 24h of incubation, the culture medium was removed and stored at -80°C. CGRP neuropeptide release was measured by ELISA method. The quantities of neuropeptide detected were compared with those obtained in the control condition.
Data were expressed as mean ± SEM. All experimental conditions were performed in six replicates.

NF-kB activation
Keratinocytes were seeded in 48-well plates. After 3 days of incubation, GLE 0.01% or 0.005%, or NF-κB inhibitor III (positive reference), were added to the culture medium. After 24h, the medium was replaced by an inflammatory stress, IL-1α at 50 ng/mL for 30min. Then, cells were fixed using paraformaldehyde (PFA) 4%. NF-κB translocation was evaluated by immunofluorescence (three replicates, four pictures/well).

***In vivo* immediate soothing effect after capsaicin stress**
The study was performed as a monocentric, double blinded, placebo controlled, randomised study. 22 women aged between 23 and 57 years old (mean 43 y.o.), with a phototype between I to III, were recruited for this study. Subjects have declared presenting sensitive and reactive skin and their skin presented redness 30 minutes after the application of a solution of capsaicin 0.075%.
For each subject, 4 zones were randomly defined on their forearms:
- 3 treated zones:
  - capsaicin without product (NTC, not treated control);
  - capsaicin + GLE 1% formula;
  - capsaicin + placebo formula;
- one control area: no capsaicin stress and no product application.

A solution of capsaicin 0.075% was applied under occlusive patch on the defined zones for 30 minutes, inducing skin redness. Then on each treated zone, subjects received one single standardised application of each product (T0) for 1 h. Skin redness (a* parameter) was measured and image acquisition was performed with C-Cube® (T0, T1h).
Means and SEM were calculated for each group at T0 and T1h. Percentages of variation on the means were also calculated at T1h versus T0 for active and placebo groups.

***In vivo* immediate soothing effect after lactic acid stress**
The study was performed as a monocentric, double blinded, parallel groups placebo controlled randomised study. 43 women aged between 19 and 70 years old (mean 49 y.o.), declaring sensitive and reactive skin on the face, were recruited for this study. Only the positive responders to an acid lactic stinging test were included. The subjects' sensations were evaluated according to the following scale: 0: no stinging, 1: mild stinging sensation, 2: moderate stinging sensation, 3: severe stinging sensation. A score was assessed at each time of kinetics for both nasolabial folds
For the stinging test, 10% lactic acid solution was applied to both nasolabial folds. After 1-2 minutes, the subjects were asked concerning the stinging sensations felt on each nasolabial fold. Then subjects received one single standardised application of placebo or formula with 1% of GLE. They were asked concerning the stinging sensations felt on each nasolabial fold immediately 5 minutes (T5min) after product application.
Means and SEM (standard error of the mean) were calculated for each group (active, placebo) at T0 and T5min. Percentages of variation on the means were also calculated at T5min versus T0 for active and placebo groups.

3. Results

3.1. GLE activates TRPM8 receptor

We have evaluated the cytoplasmic calcium mobilization of human sensory neurons in coculture with keratinocytes.

Activation of sensory neurons by menthol at 0.016% (1mM) resulted in a significant increase in calcium mobilization (+92%, $p<0.05$ vs control condition). When neurons were pre-incubated in the presence of M8-B, menthol did not increase cytoplasmic calcium mobilization (100% inhibition).

GLE at 0.005% and 0.01% resulted in a significant dose-dependent increase in cytoplasmic calcium mobilization (176%, $p<0.01$ and 232%, $p<0.001$). When neurons were pre-incubated in the presence of the inhibitor M8-B, calcium mobilization was significantly inhibited: inhibition of 70% ($p<0.01$) and 88% ($p<0.01$) with 0.01% and 0.005% respectively (Table 5, Figure 10). To conclude, GLE activates the TRPM8 channel *in vitro*.

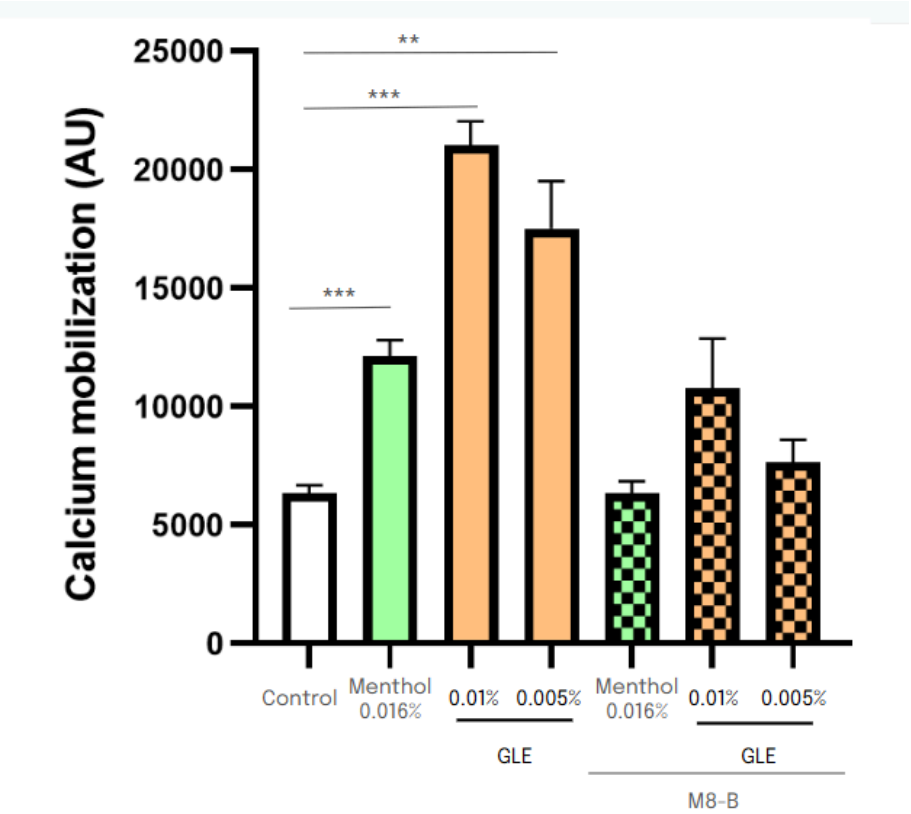

Figure 1. GLE activates TRPM8 receptor on a co-culture of human sensory neurons and keratinocytes. ** $p<0.01$, *** $p<0.001$.

3.2. Interactions between the TRPM8 and TRPV1 signalling pathways

As observed in Figure 2, treatment with capsaicin resulted in a significant increase in CGRP neuropeptide release from sensory neurons (+68%; $p<0.01$ vs solvent control).

Pre-incubation of capsazepine for 24h significantly inhibited the release of CGRP due to capsaicin activation of neurons (-50%, $p<0.001$ vs capsaicin control).

Pre-incubation of menthol for 24h significantly inhibited the release of neuropeptide CGRP due to capsaicin activation of neurons (-22%, $p<0.05$ vs capsaicin control).

Treatment of the co-culture with the inhibitor M8-B prior to pre-incubation with menthol, menthol lost its inhibitory effect. These results confirmed that menthol's inhibitory effect on

the TRPV1 (capsaicin) signaling pathway is due to an action via the TRPM8 signaling pathway.
Pre-incubation of GLE at 0.01% or 0.005% for 24h significantly inhibited CGRP release due to capsaicin activation of neurons (-55%, p<0.001 and -47%, p<0.001 vs capsaicin control). Treatment of the co-culture with the M8-B inhibitor prior to pre-incubation of GLE also results in a loss of the active ingredient's inhibitory effect against the action of capsaicin (135% inhibition at 0.01%, p<0.001; 116% inhibition at 0.005%, p<0.001 - Figure 2).
Same results were observed on calcium mobilisation after capsaicin treatment (data not shown).

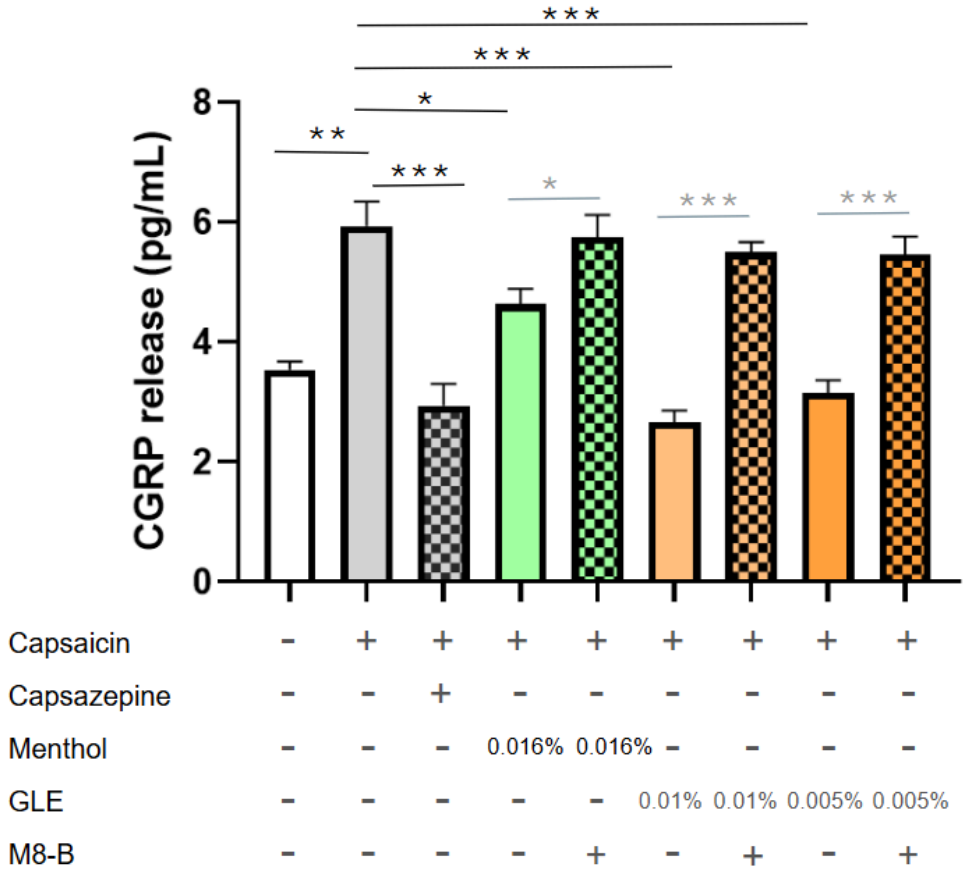

Figure 2. Evaluation of TRPM8/TRPV1 signalling pathway activity in a co-culture of keratinocytes and sensory neurons by analysis of CGRP release. * p<0.05, ** p<0.01, *** p<0.001.

3..3. Interactions between the TRPM8 and TRPA1 signalling pathways

Treatment of sensory neurons with AITC resulted in a significant increase in cytoplasmic calcium mobilization (+94%, p<0.001 vs solvent control). Pre-incubation of HC030031 (AITC inhibitor) for 24h significantly inhibited calcium mobilization due to AITC activation of neurons (-45%, p<0.001 vs AITC control).
Pre-incubation of menthol for 24h significantly inhibited calcium mobilization due to AITC activation of neurons (-59%, p<0.001 vs AITC control). Treatment of the co-culture with the inhibitor M8-B prior to pre-incubation with menthol induced the loss of menthol inhibitory effect. These results confirmed that menthol's inhibitory effect on AITC is indeed due to an action via the TRPM8 signaling pathway.
Pre-incubation of GLE at 0.01% or 0.005% for 24h significantly inhibited calcium mobilization due to activation of neurons by AITC (-50%, p<0.001 and -55%, p<0.001 respectively vs AITC control). As observed in Figure 3, treatment of the co-culture with the M8-B inhibitor prior to pre-incubation of GLE at 0.01% or 0.005% for 24h also resulted in a loss of the active

ingredient's inhibitory effect against the action of AITC (104% inhibition at 0.01%, p<0.001; 114% inhibition at 0.005%, p<0.001 - Figure 2). So, GLE efficacy is due to an action via the TRPM8 signaling pathway.

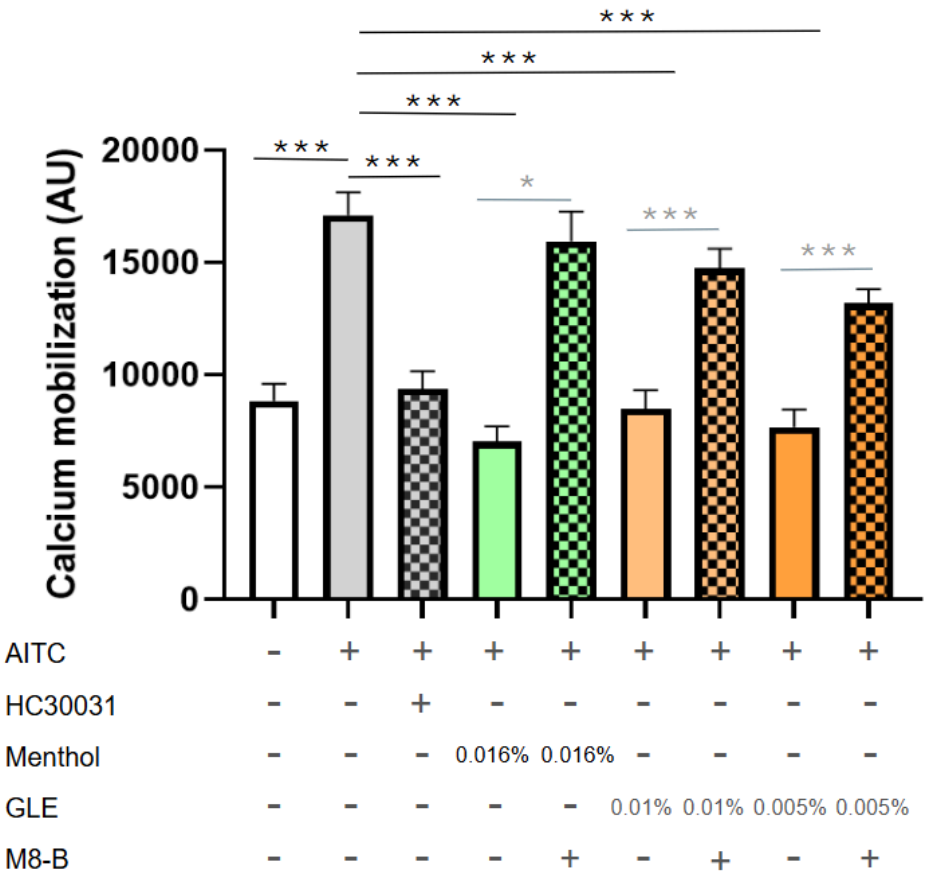

Figure 3. Evaluation of TRPM8/TRPA1 signalling pathway activity in a co-culture of keratinocytes and sensory neurons by analysis of cytoplasmic calcium mobilisation. *** p<0.001.

Same results were observed on CGRP release after AITC treatment (data not shown).

3.4. NF-κB activation

As expected, IL-1α induced a significant increase in NF-κB translocation (+706% vs untreated cells, p<0.01) in keratinocytes. NF-κB inhibitor III significantly protected keratinocytes from NF-κB translocation by 105% (p<0.05). GLE also allowed a significant protection of keratinocytes from NF-κB translocation whatever the concentration tested (88% protection, p<0.05, at 0.01%, and 90% protection, p<0.05, at 0.05%).
By protecting keratinocytes from NF-κB translocation, GLE protected cells from NF-κB activation and thus limited the inflammatory state.

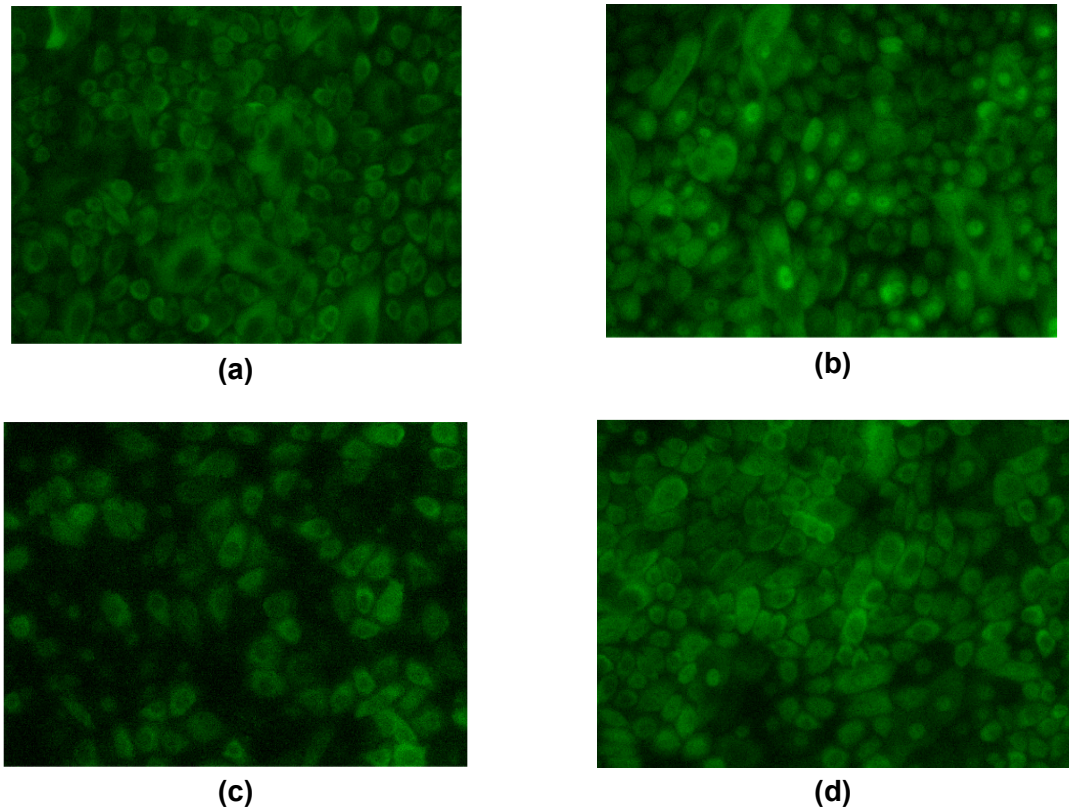

Figure 4. Effect of GLE on NF-κB translocation induced by IL-1α effect on keratinocytes. (a) control cells; (b) IL-1α 50 ng/mL; (c) NF-κB inhibitor III (positive reference); (d) GLE 0.01%

3.5. In vivo immediate soothing effect after capsaicin stress

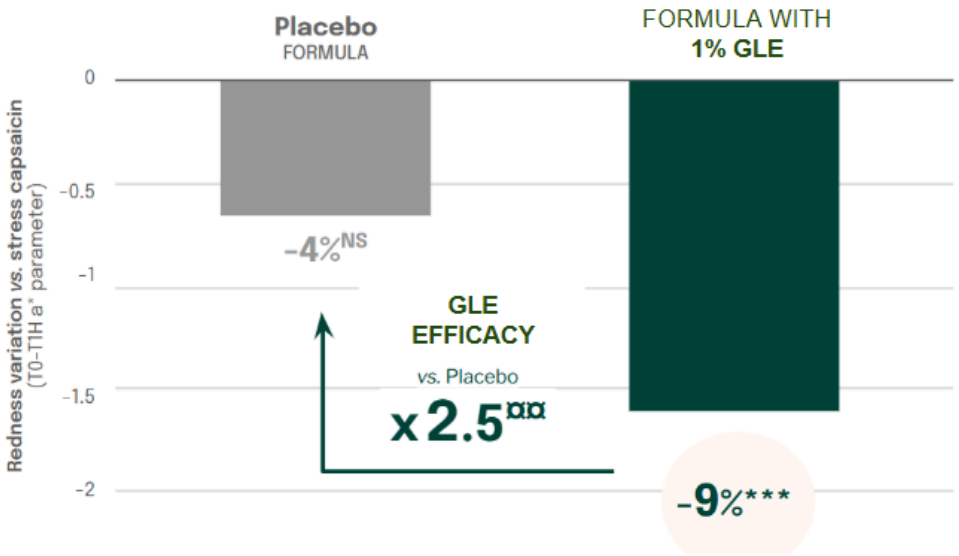

Figure 5. Variation of redness (a* parameter) 1h after capsaicin stress between placebo and GLE-treated group. ^{NS} non significant vs capsaicin treated area,***p<0.01 vs capsaicin treated area, ¤¤ p<0.01 vs Placebo.

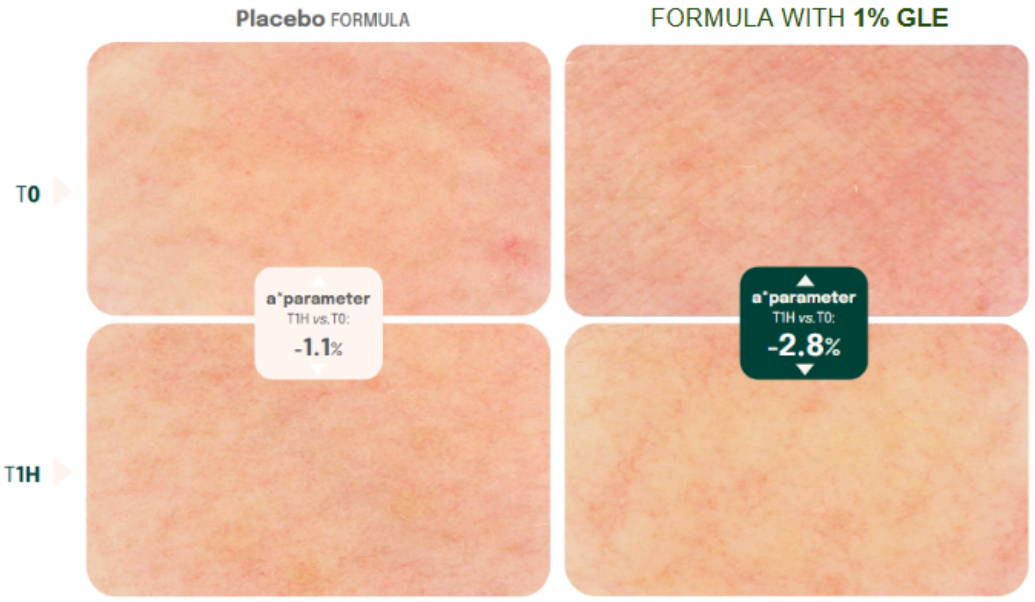

Figure 6. Illustration of skin redness observed with C-Cube® on volunteer n°31.

1 hour after product application on capsaicin stressed forearms, GLE formula induced a significant decrease of the skin a* redness and erythema index in comparison with baseline ($p<0.001$ for both parameters) and placebo ($p<0.01$ for both parameters).
So GLE formula was able to soothe skin under capsaicin stress.

3.6. In vivo immediate soothing effect after lactic acid stress

5 minutes after stinging stress (T5min), the application of GLE formula significantly decreased stinging sensation grade compared to baseline (T0) and to placebo group.
This result evidences a feeling of an immediate soothing effect of the GLE formula after lactic acid stress.

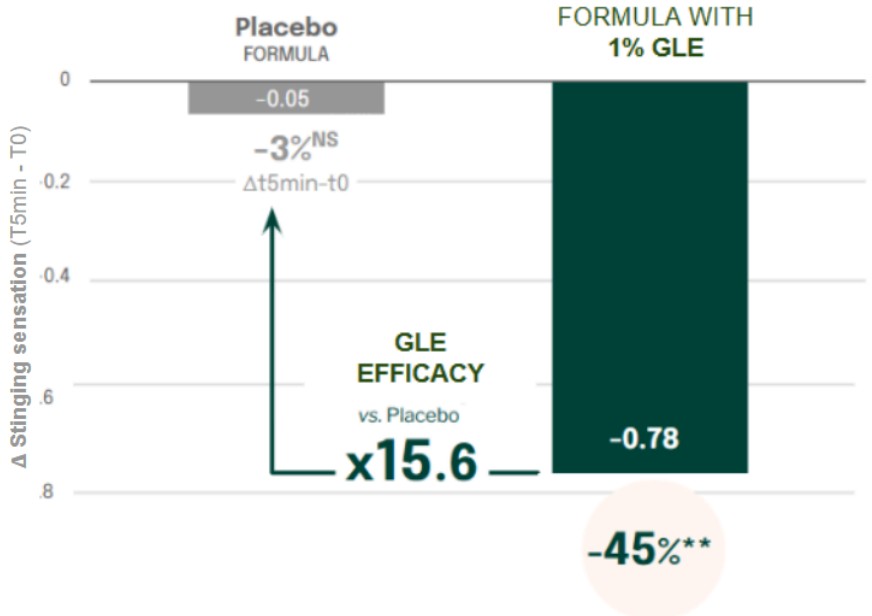

Figure 7. (T5min - T0) variation of stinging sensation grade for placebo and GLE groups. ^{NS} not significant, ** $p<0.01$ vs acid lactic treated area , ¤¤ $p<0.01$ vs Placebo.

4. Discussion

The key functionalities of TRPM8 in sensing both innocuous and noxious cold (8-–26°C) have been shown to play a major physiological role in inflammation and itch. But TRPM8 can also mediate inflammation through crosstalk with other TRP channels [8, 9]. Indeed, TRPM8 activation can suppress TRPV1-mediated inflammatory neuropeptide CGRP release. In contrast with other TRP channels, TRPM8 activation could suppress itch rather than induce it.

First of all, we have studied the effect of GLE on TRPM8 activation. Menthol was used as a reference molecule for TRPM8 receptor activation. Both menthol and GLE induced an increase in cytoplasmic calcium mobilization of human sensory neurons in coculture with keratinocytes. In the presence of M8-B, the TRPM8 receptor inhibitor, calcium mobilization was significantly inhibited. So, GLE activated the TRPM8 channel.

Then, we evaluated the effects of menthol and GLE on TRPV1 and TRPA1 thanks to its activation of TRPM8. We have studied both calcium mobilization and CGRP (calcitonin gene-related peptide) release after capsaicin (TRPV1 activator) or AITC (TRPA1 activator) treatment.

Regarding menthol effects, interaction between the TRPA1 and TRPM8 signalling pathways (103% and 122% inhibition respectively for CGRP release and calcium mobilization) seems to be more important than between the TRPV1 and TRPM8 signalling pathways (55% and 100% inhibition respectively for CGRP release and calcium mobilization).

GLE significantly inhibited CGRP release as well as calcium mobilisation due to capsaicin or AITC activation of neurons (in coculture with keratinocytes). Treatment with the M8-B inhibitor resulted in a loss of the active ingredient's inhibitory effect against the action of capsaicin and AITC. These results demonstrate that GLE efficacy is due to an action via the TRPM8 signaling pathway. Contrary to menthol, GLE extract had the same effect on TRPA1 /TRPV1 and TRPM8 signalling pathways. We can therefore assume that GLE also had a direct action on TRPV1. This will have to be confirmed.

Thanks to the activation of the TRPM8 signaling pathway, GLE can reduce TRPV1 and TRPA1 activation, thus attenuating neurogenic inflammation by reducing neuropeptide release, limiting itch and pain and then promoting skin soothing. These results confirm that TRPM8 can mediate inflammation through crosstalk with other TRP channels.

Moreover, by protecting keratinocytes from NF-κB translocation, GLE protected cells from NF-κB activation and thus limited the inflammatory state. It would be interesting to study whether or not the effect on NF-κB translocation is linked to TRPM8 activation.

The soothing property of GLE has been confirmed by two clinical studies. A first *in vivo* study was conducted on 43 volunteers with lactic acid-reactive skin. Volunteers applied either placebo or GLE 1% formula to one nasogenian fold and self-assessed stinging sensation for 5 minutes after lactic acid application. As a result, GLE formula significantly decreased stinging sensation by 45% vs lactic acid treated area ($p<0.01$), as well as vs placebo.

Another clinical study was conducted on 22 volunteers having capsaicin-reactive skin. One hour after application to capsaicin-stressed forearms, GLE significantly decreased skin redness and erythema (-9% at 1 hour versus capsaicin-treated area, $p<0.001$), demonstrating its ability to soothe capsaicin-induced irritation, and reduce redness.

Moreover, GLE can strengthen skin barrier function by increasing filaggrin protein expression which can contribute to immediate hydration observed *in vivo* (data not shown).

5. Conclusion

Sensitive and reactive skin is a common condition that affects many people and characterised by a wide variety of symptoms such as redness, stinging or itching due to a strong inflammation response and/or a disturbed skin barrier function.
GLE immediately reduced stinging and redness *in vivo* provided by an original mechanism of action. GLE can regulate over-inflammation by regulating NF-κB activation and limiting cytokine production. Furthermore, it can regulate nociception sensations such as itching and stinging by regulating TRPA1 et TRPV1 activation thanks to TRPM8 activation. Then GLE can contribute to a soothing effect and can relieve unpleasant sensations. For the first time, we demonstrated that an active ingredient can regulate activation of TRPV1 and TRPA1, thanks to activation of TRPM8 channel, and then promote skin soothing. This innovative mechanism underscores GLE's potential as a breakthrough ingredient for immediate and comprehensive relief.

References

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