SYNERGIE SKIN
CLEAN SCIENCE

CLINICAL DATA – ACETYL HEXAPEPTIDE-49

A disturbing skin condition

A great part of the population suffers from sensitive skin, which is a disturbing skin condition that can detrimentally affect the quality of life.

- Prickling, burning, tingling, pain or itching, and occasionally, erythema and flush.
- Barrier function is compromised, worsening these symptoms.
- Can be induced by environmental factors (e.g. Pollution, UV radiation, dryness, heat), lifestyle factors (e.g. cosmetics, soap), psychological factors (e.g. stress) or hormonal factors.

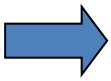
Sensitive skin may worsen and give rise to inflammatory and/or pruritic chronic skin disorders such as atopic dermatitis (AD), dry skin, and acne, (barrier impairment and increased vascular reactivity).

Itch and pain

During inflammation and after tissue damage various products are released, which are collectively referred to as the inflammatory soup.

Activates specific type of primary sensory neurons releasing peptide and neurotransmitters.

They act on mast and immune cells, keratinocytes, neurons, and vascular smooth muscle.



- Skin peripheral sensitisation.
- Inflammation (redness, warmth, swelling, and hypersensitivity, worsening neurogenic inflammation.

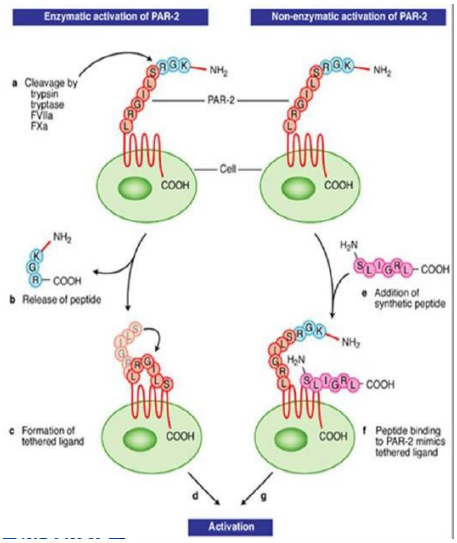
Acute or chronic skin inflammation lowers the threshold for pruritic stimuli causing peripheral itch sensitisation.

PAR-2 is involved in itch and neurogenic inflammation

PAR-2:

- Is a key receptor involved in neurogenic inflammation and itch.
- Is abundantly expressed: keratinocytes, endothelial cells, fibroblasts, sensory neurons, and inflammatory cells.
- Is activated by proteases (from plants, mites, or human inflammatory cells e.g. trypsin, tryptase) and non-enzymatic compounds.

Certain proteases act as signalling molecules by cleaving the members of the family of G-protein coupled PARs within the extracellular N-terminal domains to expose tethered ligands that bind to and activate the cleaved receptors.



Acute or chronic skin inflammation lowers the threshold for pruritic stimuli causing peripheral itch sensitisation.

PAR-2 in sensitive skin

During cutaneous inflammation, the released PAR-2 endogenous activators activate PAR-2 amplifying the inflammation via the up-regulation of inflammatory mediators.

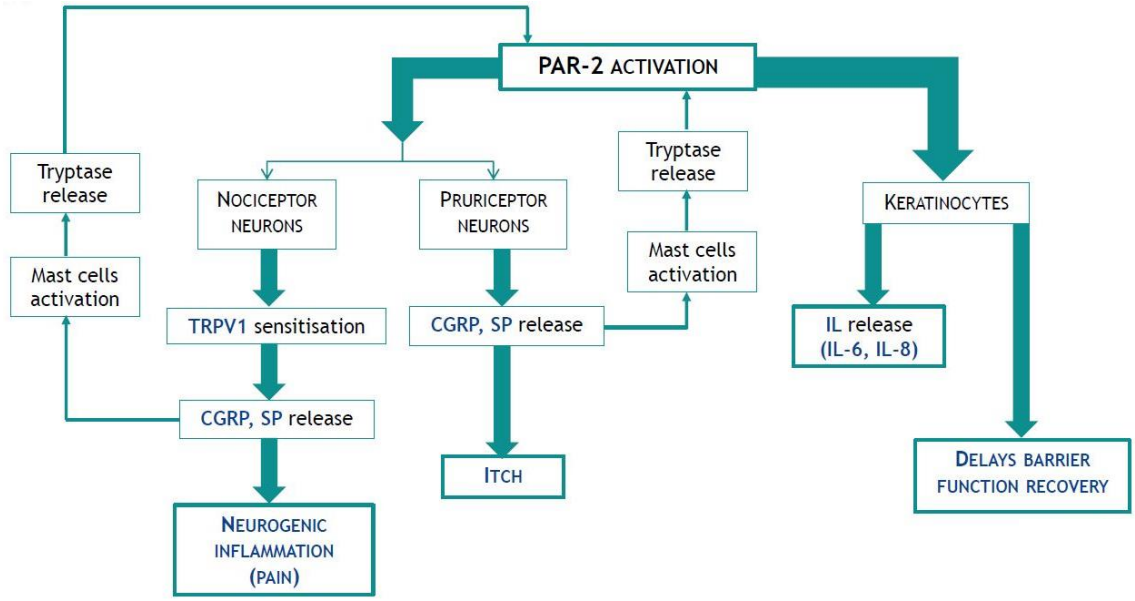
In AD patients:

- Itch could not be suppressed by anti-histaminic treatments
- PAR-2, tryptase, and trypsin are up-regulated on lesional skin.
- PAR-2 agonists administration induce pruritus.

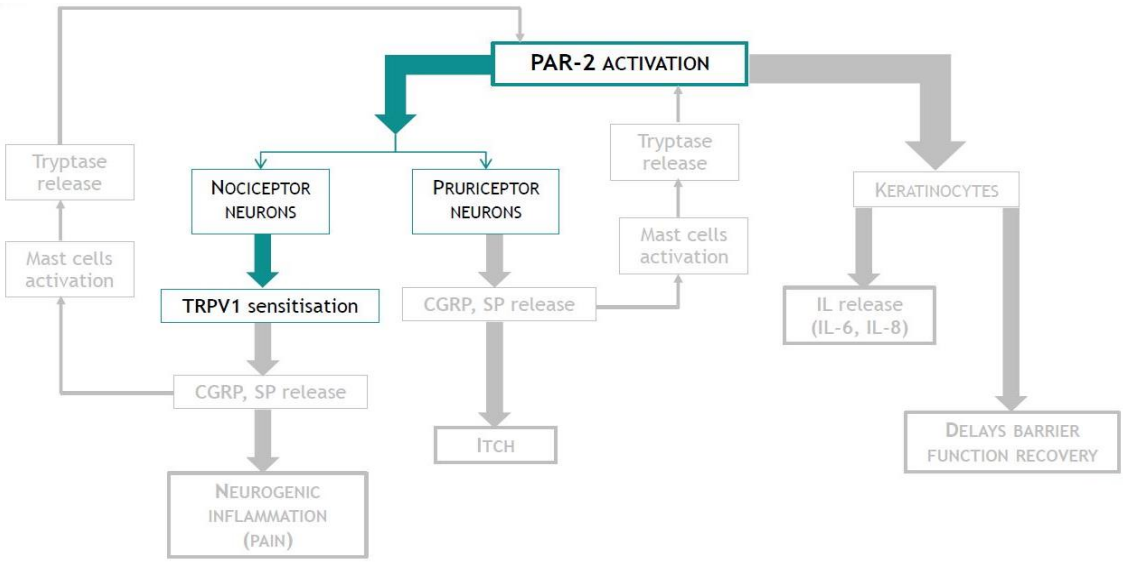
PAR-2 is a key receptor for proteases inducing itch.

Inhibition of PAR-2 activity could be a novel approach for attenuating inflammation and relieving itch.

PAR-2 activation cycle



PAR-2 Activation cycle

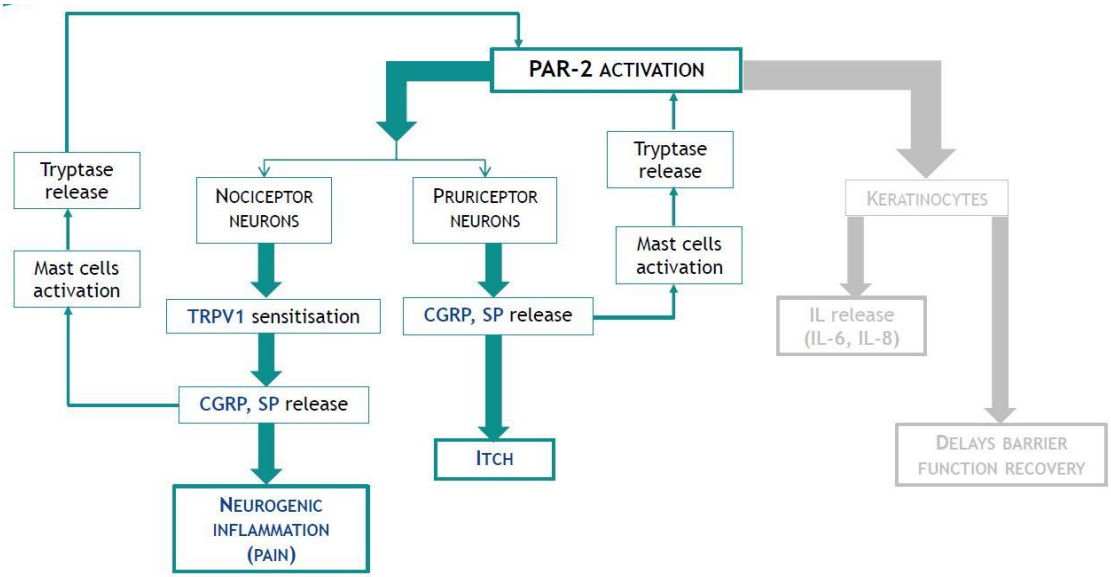


PAR-2 induces TRPV1 sensitisation

PAR-2 activation increases neurons excitability and may sensitise their response to agonists (e.g. proteases) of other receptors involved in inflammatory processes such as TRPV1:

- Is a non-selective plasma-membrane cationic and heat–sensitive ion-channel that functions as a molecular thermometer at the cell surface.
- Is mainly expressed on sensory C-fibres, a subtype of primary afferent neurons.
- Mediates responses to stimuli (heat, protons, and chemical irritants, such as capsaicin, which causes burning, pain or pruritus).
- Over-activation is involved in the neurogenic inflammation enhancing CCGRP and SP release – mast cells are stimulated leading to further release of proteases increasing even more PAR-2 activation.

PAR-2 Activation cycle

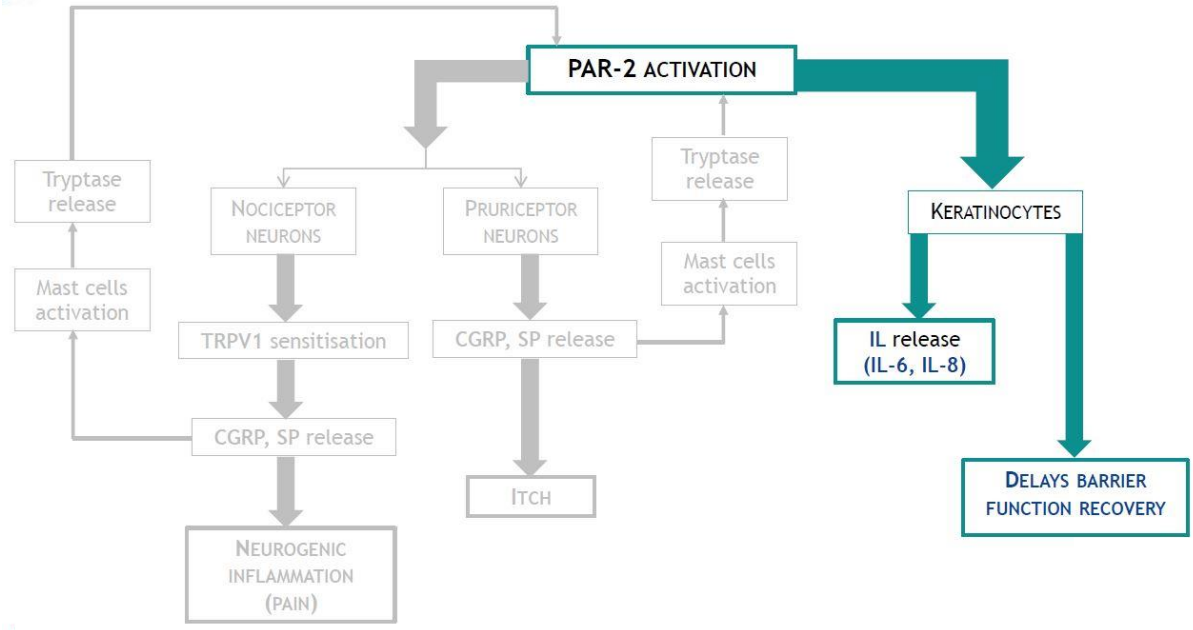


CGRP and SP in neurogenic inflammation and itch

SP and CGRP:

- Released from the peripheral terminal induce vasodilatation and plasma extravasation, leading to oedema.
- Are also released due to UV radiation, as keratinocytes are sensitised inducing PAR-2 expression – further inflammation and itch.
- Are further released by PAR-2 activation in pruriceptor neurons, mediating itch.
    - CGRP potentiates SP release and inhibits its degradation by neutral endopeptidase.
    - SP stimulates the release of proteases, activating PAR-2 and exacerbating the neurogenic inflammation.

PAR-2 Activation Cycle



PAR-2 Activation on keratinocytes

- Stimulation of PAR-2 on keratinocytes increases the secretion of inflammatory cytokines (IL-6 and IL-8), mediating inflammation.
- Skin chronic inflammatory disorders present an impaired barrier function.
    - Topical treatment with chemicals (e.g. detergents), mechanical injury e.g. scratching can remove extracellular lipids disrupting the skin barrier permeability.
    - PAR-2 is involved in the maintenance of epidermal permeability barrier homeostasis.
    - PAR-2 activation inhibits Lamellar Bodies (LB) secretion and delays barrier recovery.
    - Acute barrier disruption raised the ambient pH or normal SC activating serine proteases in the outer epidermis.

Serine proteases degrade the key lipid processing enzymes required for normal permeability barrier homeostasis and activate PAR-2.

Sensitive skin without signs

- Acetyl hexapeptide – 49 is a new hexapeptide for cosmetic products especially designed for sensitive skin, which was identified by a combinatorial chemistry approach.
- The combinatorial peptide library was screened for PAR-2 activity inhibition in a keratinocyte cell model based on fluorescence detection of calcium mobilisation.

Acetyl hexapeptide – 49 diminishes PAR-2 activity and as a result CGRP, IL-6 and IL-8 release from skin cells, attenuating neurogenic inflammation and itch.

Sensitive skin without signs

Acetyl hexapeptide – 49:

- Decreases the nagging pain sensations, discomfort feelings, redness and itching intensity and warming up sensation.
- Mitigates the stinging and burning feeling providing an immediate soothing effect, and relieves inflammation due to various factors such as cosmetic allergens.
- Improves scaling, smoothness, suppleness and increases skin hydration.
- Helps to recover the skin barrier function by stimulating cell proliferation, and restoring the damaged tissue integrity, helping the re-epithelisation and repair of damaged skin.

Acetyl hexapeptide – 49 diminishes PAR-2 induced release of pro-inflammatory mediators, attenuates neurogenic inflammation and itch related to sensitive skin besides helping to restore the barrier function.

Acetyl hexapeptide – 49 efficacy

In vitro efficacy:

- Inhibition of PAR-2 activity
- Determination of CGRP release
- Inhibition of PAR-2 evoked IL-6 and IL-8 release
- Photoprotection test
- Proliferation assay
- Cicatrisation assay
- Efficacy against cosmetic allergens

In vivo efficacy

- Stinging induced by capsaicin
- Stinging induced by lactic acid
- Skin hydration assay

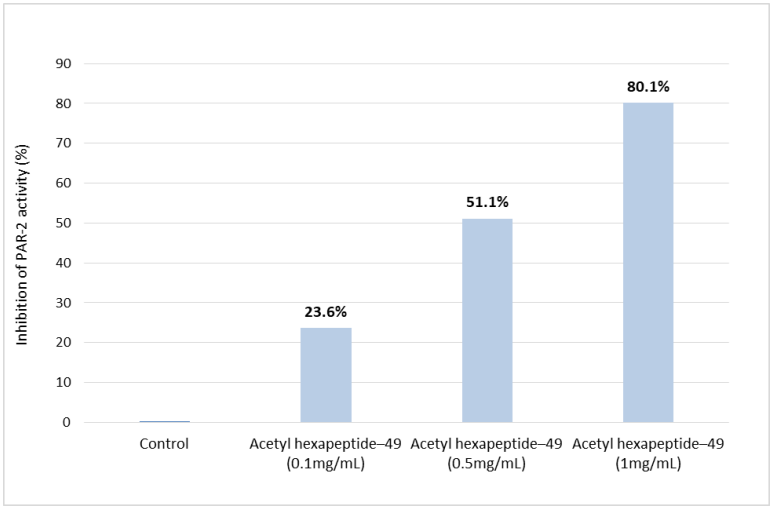
Inhibition of PAR-2 Activity

Human keratinocytes were incubated with Acetyl hexapeptide – 49 or vehicle for 1 h. After incubation, a mixture with the Fluo-4 NW indicator and Probenecid were added to

the wells, and then were incubated for 30 min at 37°C. Afterwards PAR-2 was activated by treatment with PAR-2 agonist I.

PAR-2 activation was evaluated by fluorescence with a FLUOstar Galaxy microplate fluorimeter (λ_{exc} = 500nm, λ_{em} = 520nm). Non-treated cells (vehicle) activated with a PAR-2 agonist were used as a control.

Acetyl hexapeptide – 49 presented a significant dose-response dependent inhibition of PAR-2 activity reaching reductions of 23.6, 51.1, and 80.1% when the cells were treated with 0.1, 0.5 and 1mg/mL.

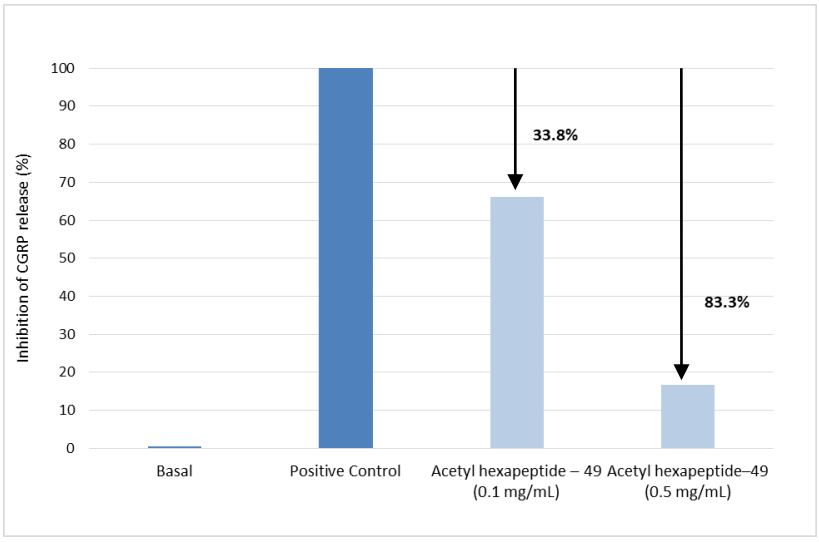


Acetyl hexapeptide – 49 inhibits PAR-2 activity, reaching a decrease of 80.1% at 1mg/mL.

Determination of CGRP release

DRG neurons were incubated with Acetyl hexapeptide – 49 in HBSS for 15 min at 37°C. Then, neurons were sensitised with PAR-2 agonist for 15 min at 37°C. CGRP release was induced by adding capsaicin for 5 min at 37°C. Supernatants were harvested and the amount of released CGRP determined by a double-antibody sandwich technique using the Rat CGRP EA kit.

Absorbance was read at 405nm with a microplate spectrophotometer. The intensity of the yellow colour formed is proportional to the CGRP present in the well. PAR-2 agonist I and capsaicin were mixed and used as a positive control.



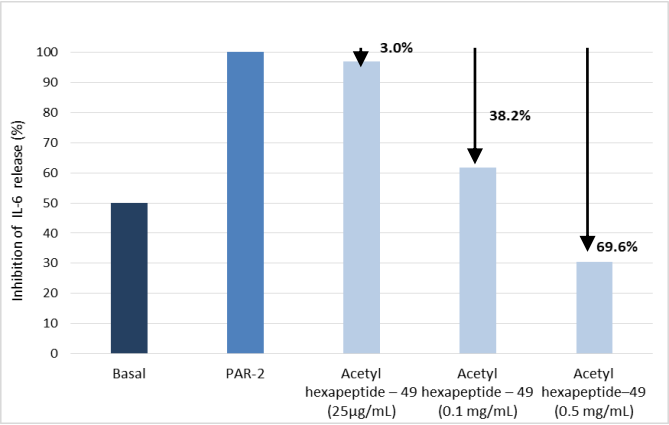
CGRP release was significantly inhibited by Acetyl hexapeptide – 49 reaching decreases of 33.8 and 83.3% at 0.1 and 0.5 mg/mL respectively.

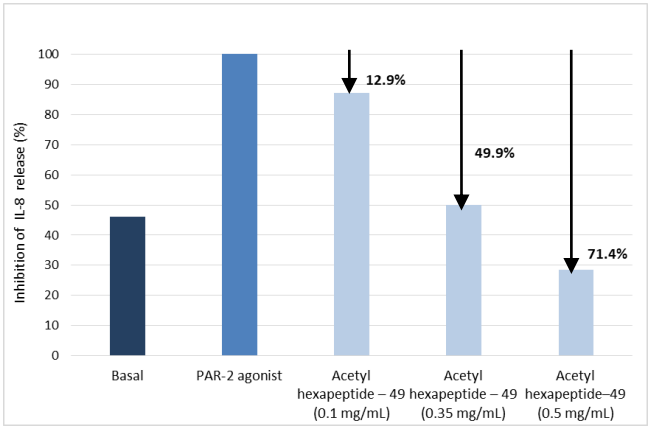
Acetyl hexapeptide – 49 is a suitable candidate to attenuate neurogenic inflammation and skin itch.

Inhibition of PAR-2 evoked IL-6 and IL-8 release

HEKa were seeded in well plates pre-coated with a collagen matrix. After 48 h, medium was removed and cells incubated with different treatments in the presence of PAR-2 agonist for 48 h at 37°C. Supernatants were harvested and the cytokines determined by ELISA test.

Results were normalised by the Crystal Violet assay. This value is proportional to the number cells in each well.



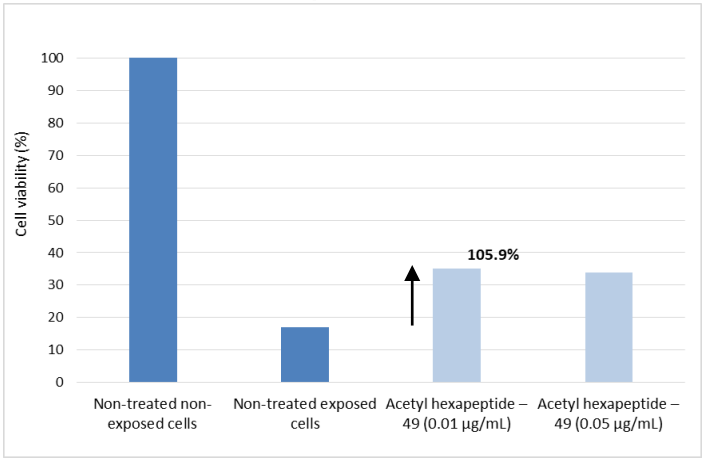


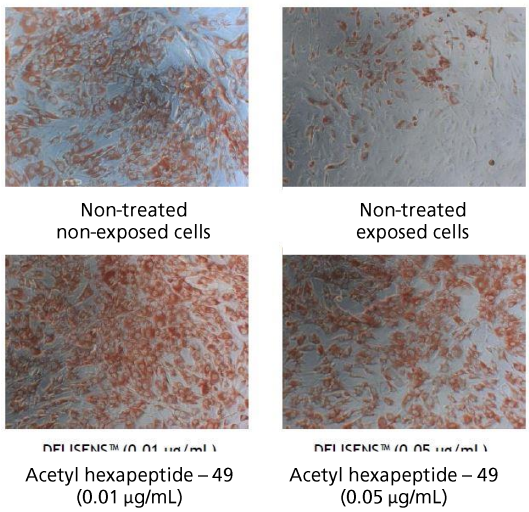
Acetyl hexapeptide – 49 inhibited PAR-2 mediated IL-6 and IL-8 release by 69.9 and 71.4% respectively in HEKa treated with 0.5mg/mL.

Acetyl hexapeptide – 49 is an excellent peptide for relieving inflammatory conditions.

Photoprotection test

Pre-incubated HDFa with 0.01 and 0.05µg/mL Acetyl hexapeptide – 49 were irradiated with stimulated solar light (~36 J/cm^2) for 150min.





Cell viability was determined after 24 hours by Neutral Red Uptake (NRU), measuring the optical density of the NR extracts at 540 nm in a spectrophotometer. Non-treated non-exposed cells and non-treated exposed cells were used as controls.

Acetyl hexapeptide – 49 increased cell viability by 105.9% in cells treated with 0.01µg/mL respect to non-treated irradiated cells.

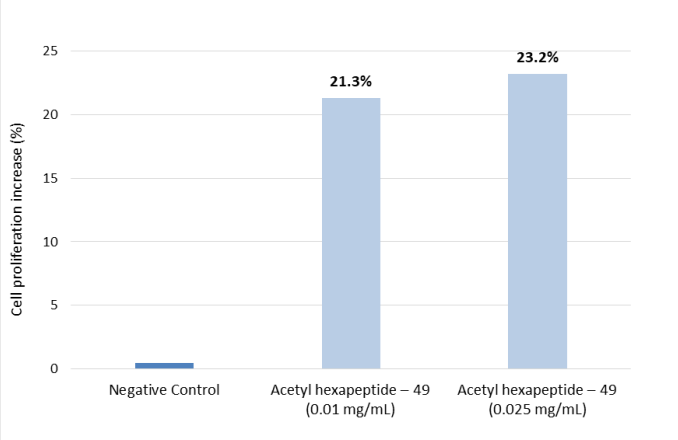
Acetyl hexapeptide – 49 showed a significant protective effect on HDFa against UV-induced damage.

Proliferation assay

The cell proliferation of human keratinocytes, which had been previously pre-incubated with Acetyl hexapeptide – 49, was evaluated by a fluorescence-based cell viability method after 24h. Live cells were distinguished by the presence of intracellular esterase activity, determined by the enzymatic conversion of the non-fluorescent cell-permeant calcein-AM to the intensely fluorescent calcein.

Fluorescence was read at λ_{exc} = 485nm and λ_{em} = 530nm in a microtiter plate (Genios, Tecan). Non-treated cells (only medium) were used as the control.

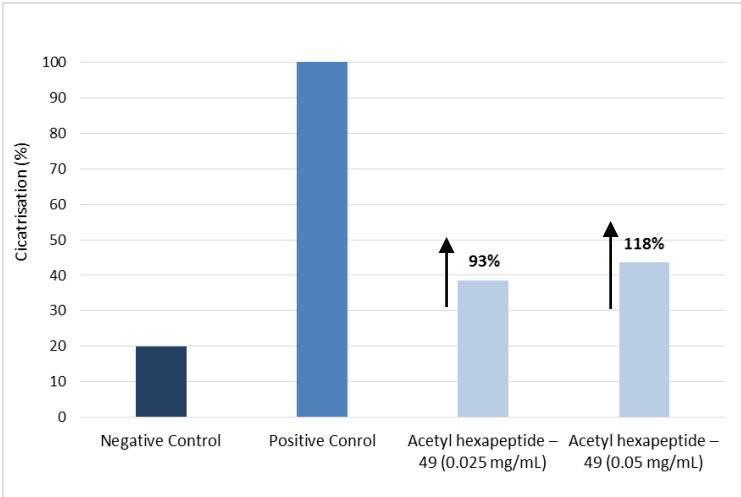
Acetyl hexapeptide – 49 significantly stimulated human keratinocytes cell growth, increasing cell proliferation by 21.3% and 23.2% when the cells were treated with 0.01 and 0.025 mg/mL respectively.



Acetyl hexapeptide – 49 presents a significant stimulatory effect on human keratinocyte cell proliferation.

Cicatrisation assay

Human keratinocytes were incubated for 48 h after seeded. A cell-free area was induced by scraping the monolayer with a pipette tip. Fresh medium was added with Acetyl hexapeptide – 49. Cells were allowed to migrate into the wound space for 48h.

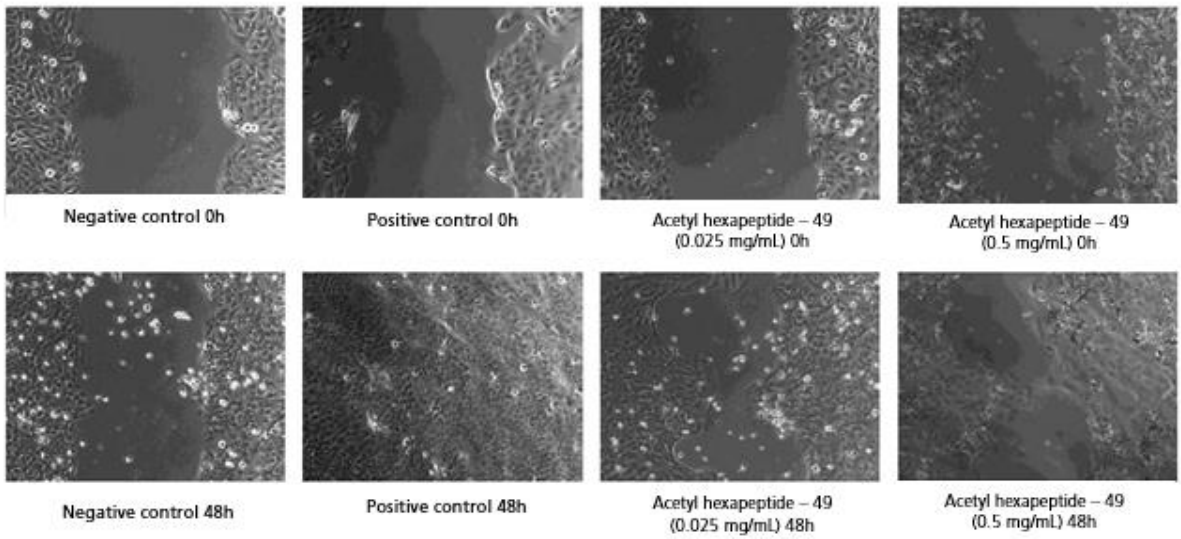


Non-treated cells and cells treated with DMEM and FBS were used as negative and positive controls, respectively. Photographs were taken at the initial time and after 48h, measures from free-cell area obtained from the photographs were processed and the percentage of cicatrisation was calculated. Results are the mean of two different photographs.

Acetyl hexapeptide – 49 significantly accelerated cicatrisation by 93 and 118% after 48 h when the cells were treated with 0.025 and 0.5 mg/mL, respectively.

Cicatrisation assay

Acetyl hexapeptide – 49 showed to be an efficient cicatrisation inducer in human keratinocytes at the tested concentrations.



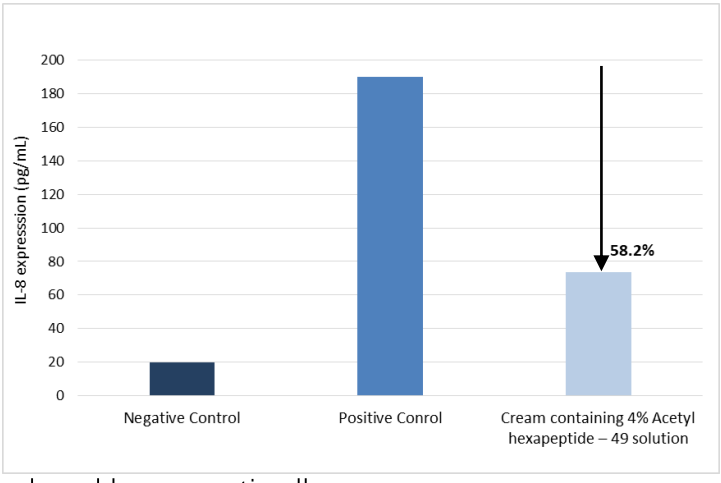
Efficacy against cosmetic allergens

A cream containing 4% Acetyl hexapeptide – 49 and a placebo cream were formulated with sensitising agents: hexyl cinnamal and farnesol. Each cream was applied directly over the surface of the epithelium on an *in vitro* Reconstructed Human Epidermis (RHE).

After the application, the tissues were incubated on growth medium for 24 h. The product was removed and the epithelia were rinsed with PBS. The media was kept, and IL-8 concentration was determined by an ELISA test.

Negative controls were performed with PBS, and the placebo cream was considered a positive control.

The reduction of IL-8 expression was of 58.2% when RHE was treated with the cream containing Acetyl hexapeptide – 49 for 24 h with respect to the placebo cream containing hexyl cinnamal and farnesol.



Acetyl hexapeptide – 49 highly counteracts the release of cytokines induced by cosmetic allergens.

Stinging induced by capsaicin

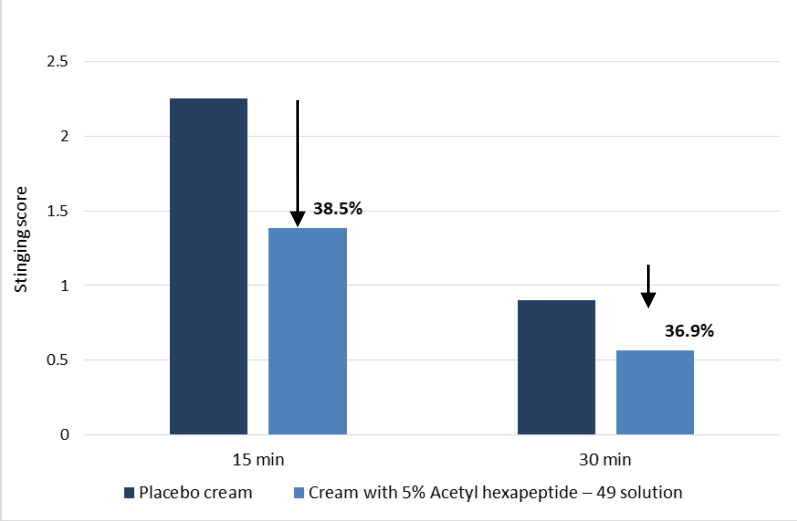
A panel of 12 volunteers (aged from 25 to 42) pre-selected as capsaicin stingers applied a cream with 5% Acetyl hexapeptide – 49 solution on a forearm and a placebo on the

other for 7 days twice daily. A stimulus with 0.03% capsicum solution was applied on each forearm after 7 days of treatment.

The volunteers scored the tolerance from 0 to 3 (null-intense) after 15 and 30 minutes of capsaicin application.

The stinging sensation in the areas treated with Acetyl hexapeptide – 49 was significantly reduced an average of 38.5% with respect to placebo cream after 15 minutes of capsaicin application.

Acetyl hexapeptide – 49 is able to mitigate the stinging and burning effect of capsaicin on the skin.

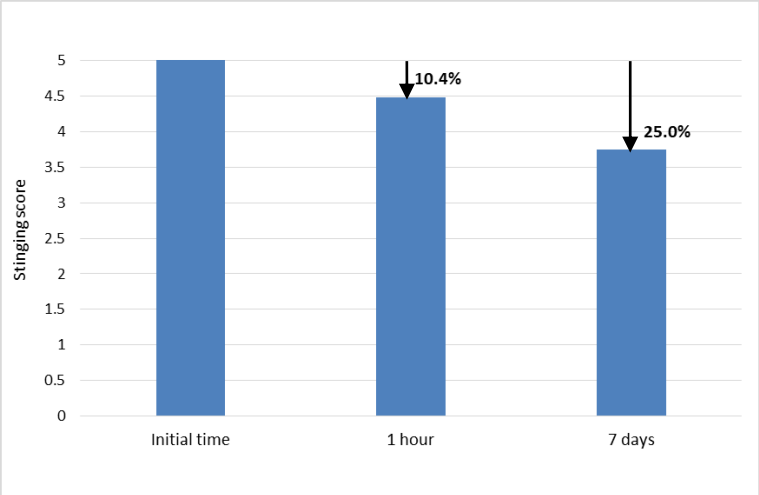


Stinging induced by Lactic acid

A panel of 25 volunteers (aged from 24 to 67) pre-selected for their sensitivity to lactic acid applied a cream containing 2% Acetyl hexapeptide – 49 solution on the face once a day for 7 days. A 10% lactic acid solution was applied to one side of the nose on the naso-labial fold after 1 h and after 7 days of treatment with Acetyl hexapeptide – 49, and the itching effect was evaluated.

The volunteers scored their tingling feeling from 0 to 3 (non-intense to dull) in presence of a dermatologist every minute for 5 min. If the sum of the scores at 3 and 5 mins was superior to 3, the volunteers were considered stingers.

The stinging feeling decreased a mean of 10.4% and 25.0% only 1h after the first application and at 7 days, and the number of non-stingers was 16 and 32%, respectively.



Most of the volunteers felt an immediate soothing effect and a decrease in the nagging pain sensations, discomfort feelings, redness, itching intensity and warming up sensation.

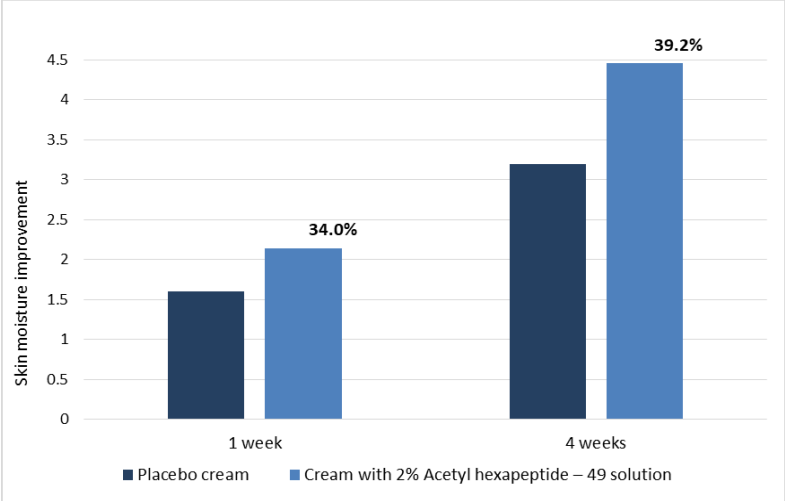
Acetyl hexapeptide – 49 provides immediate and long-term relief of the stinging sensation induced by lactic acid.

Skin hydration assay

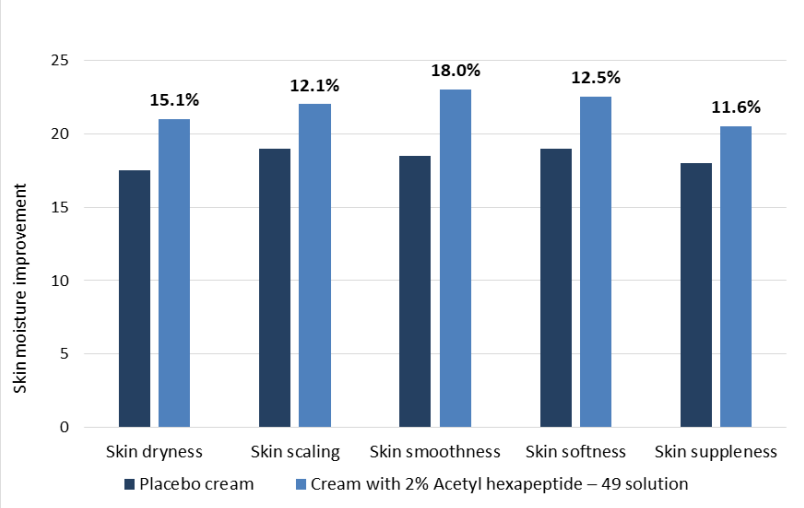
A panel of 20 volunteers (aged from 18 to 55) with sensitive skin and itch sensation on their legs applied a cream containing 2% Acetyl hexapeptide – 49 solution on the left leg and a placebo cream on the other twice a day for 4 weeks.

Measurements were taken before the first application and after 1 and 4 weeks with a corneometer. A trained specialist performed the evaluation of dryness, scaling, smoothness, softness, and suppleness before and after 4 weeks.

The hydration of the skin of the legs treated with Acetyl hexapeptide – 49 solution significantly increased an average of 34.0 and 39.2% more than that with the placebo cream, after 1 and 4 weeks respectively.



Skin hydration assay



Acetyl hexapeptide – 49 improved skin dryness, scaling, smoothness, softness and suppleness between 11.6 and 18.0% more than the placebo cream.

Acetyl hexapeptide – 49 enhances all the parameters evaluated in sensitive and dry skin.

Conclusions

- Diminishes PAR-2 activity and as a result of CGRP, IL-6 and IL-8 release from skin cells attenuating neurogenic inflammation and itch.
- Counteracts the release of cytokines induced by cosmetic allergens.
- Helps to recover the skin barrier function by stimulating cell proliferation, and restoring the damaged tissue integrity.

- Provides an immediate and long-term relieve of the stinging sensation induced by lactic acid by 10.4 and 25.0% only 1 h after the first application and after 1 week.
- Mitigates the stinging and burning feeling of capsaicin, as proved to significantly decrease the stinging sensation a mean of 38.5% respect to placebo cream after 15 min
- Enhances skin hydration and ameliorates skin dryness, scaling, smoothness, softness and suppleness in people suffering from very dry skin.