Preventing epidermal jet lag by resetting the circadian clock

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Abstract

The 2017 Nobel Price for Physiology/Medicine was awarded for the decoding of the circadian rhythm. This important internal clock is not only relevant for the entire organism but also the skin. Especially the epidermis has its own daily biorhythm. Modern lifestyle ignoring the natural day/night cycle brings the clock out of sync and produces epidermal jetlag. Even daily skin care can interfere with the epidermal clock. Here we show that a cosmetic active protecting epidermal cells from sunlight induced DNA-damage and reactive oxygen species is capable to reset the cellular circadian clock and maintain the daily regeneration cycle of the skin. The result is an improved skin barrier and reduced skin reddening during the day.

Introduction

The skin's biorhythm is strongly dictated by hormones released from the so-called suprachiasmatic nucleus in the brain, which influences parameters like skin hydration, barrier strength, cell turnover and DNA-repair capabilities [1]. The suprachiasmatic nucleus is triggered by light stimulus of the eyes and energy, for example in form of a breakfast. However, it is reported that peripheral cells like skin cells also have their own biorhythm which is regulated by light. Certain molecules like opsins translate the light/dark signals into differentiated gene expression of positive and negative circadian regulator genes [2], which in turn steer the expression of important genes involved in all cellular processes. Positive (or daylight) regulator genes are *ARNTL* (formerly known as *BMAL1*) and *CLOCK* while their counterparts *CRY1/2* and *PER1/2* are repressor genes of *ARNTL* and *CLOCK* that reset the circadian rhythm for the night time.
How does the circadian rhythem interfere with skin ageing? In total, there are nine factors that contribute to tissue ageing: genomic instability, epigenetic alterations, mitochondrial dysfunction, loss of proteostasis (the maintenance of the cells' protein portfolio), cellular senescence, deregulated nutrition sensing, altered intercellular communication, telomere attrition and stem cell exhaustion [3].
There is increasing evidence to show that the correct circadian control of the key factors in the above-mentioned processes is essential to prevent premature ageing. Any interference with circadian control can lead to disturbance of these finely balanced biochemical processes. Due to ageing, the amplitude of oscillation of the daytime and night-time gene expression is reduced and a phase shift may occur [4]. As we enter old age, we may experience the effect of this in the form of having trouble sleeping, being tired during the day or being in a low-key mood. At present, it is still unclear whether ageing influences the circadian clock or if the circadian clock influences ageing. However, it is apparent that a life with a defined day and night rhythm could be worth striving for if we want to continue to stay young-looking even at an advanced age.

To maintain its functionality, the skin permanently sustains homeostatic processes of assembly and disassembly. These processes are true mainly for the dermis, while the epidermis continuously renews itself within each 3-4 week period. The basal layer of keratinocytes consists of stem cells that divide to create daughters that are pushed outward to serve as one of the tightest tissues our body can produce: the stratum spinosum of the epidermis. The further fate of the cells is to differentiate into granulocytes, which provide the stratum corneum with the molecules required to build an effective skin barrier, mainly lipids and natural moisturisation factor (NMF). Their cell envelopes later on serve as corneocytes, which are shed from the skin [5].

What may seem to be like an assembly line process can also be described as a recurrent mechanism that occurs on a day-to-day basis. Here, the epidermal clock plays an essential role (Figure 1) [6].

In the morning, the cycle starts with the activation of the circadian clock genes and the skin barrier starts to recover from the stress it was exposed to the day before. The biggest threat in daytime is exposure to the sun and consequently to UV radiation [1]. To counteract this, light-induced and ROS-mediated DNA damage is steadily repaired during daytime [7], which increases the inflammatory status of the skin and produces skin reddening. Nevertheless, in the afternoon, the skin barrier reaches its maximum resistance to wind and weather and also skin hydration reaches its maximum as the cells of the stratum granulosum reach final differentiation [8]. At night, the skin can recover and it starts to create a new layer of the epidermis by means of stem cell division in the stratum basale. Metabolically-induced ROS are efficiently scavenged and ROS-induced DNA damage is repaired by carefully clock-set repair enzymes [9]. The most common oxidative DNA lesion is 8-oxoguanine, which has a major potential to create permanent mutation in the next cell division

cycle [10]. Hence enzymes such as 8-oxoguanin DNA glycosylase (OGG1) are upregulated and reach their maximum concentration during the night [11]. Having the lowest level of skin hydration and weakest skin barrier at that time [12] can cause itching but normally this has no effect on us because we are asleep. Decreased epidermal stress is visible in the form of reduced skin redness in the morning and the cycle restarts. Thanks to this mechanism, we have the highest skin protection capacity when we need it most and the weakest when we neither need it nor are aware of it. And to achieve this, the avoidance of epidermal jetlag is crucial.

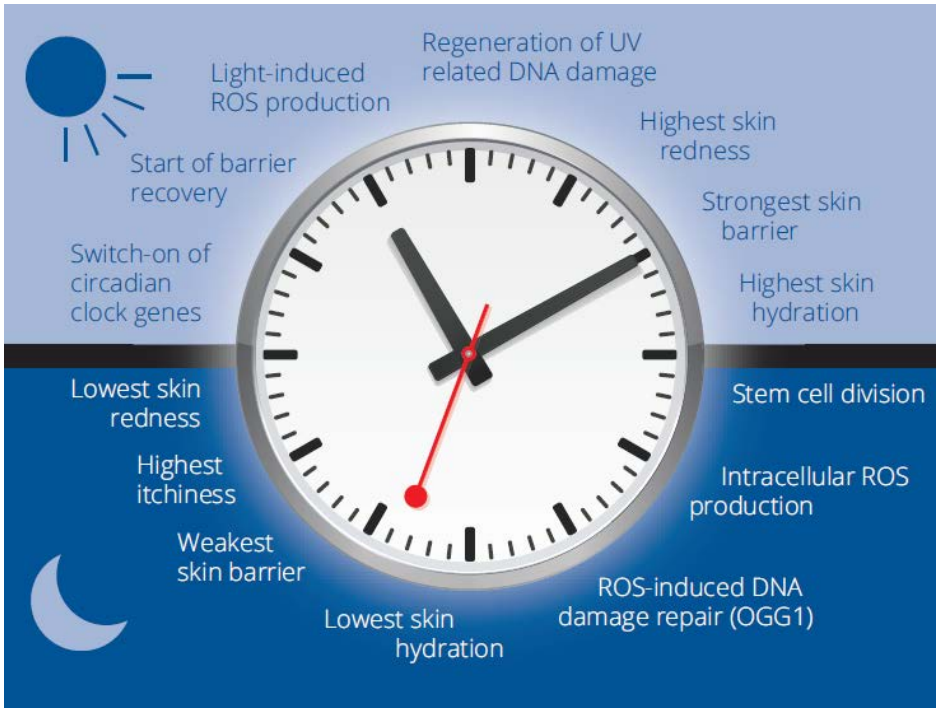


Figure 1: The circadian cycle of the epidermis. During daytime, the epidermal cells are exposed to numerous deleterious factors of which UV light is the most harmful. The cells need to recover and undertake repairs to damage during the day and especially at night, resulting in disturbance of the skin barrier function and low moisture content particularly during the latter. The timing of the epidermal clock leads to full recovery of the skin barrier during daytime.

Materials and Methods

Cosmetic active ingredient CELLIGENT® (INCI: Helianthus Annuus Seed Oil, Ethyl Ferulate, Rosmarinus Officinalis Leaf Extract, Tocopherol). Formulation in in-vitro study: Aqua, Caprylic / Capric Triglyceride, Glycerin, Glyceryl Stearate Citrate, Cetearyl Alcohol, Sucrose Stearate, Phenoxyethanol, Caprylyl Glycol, Sodium Hydroxide, Xanthan Gum, Carbomer, Parfum, with or without 3 % CELLIGENT®.

Gene expression analysis of circadian clock genes *CLOCK* and *ARNTL* in day/night synchronized human primary keratinocytes after UV light irradiation including *OGG1*.

Primary human keratinocytes from a 37-year-old female donor were cultivated in a 12 h light/dark environment. A synchronized induction of the circadian rhythm was achieved by means of supplementation with 100 nM dexamethasone for 20 minutes. Afterwards, the cells were irradiated with 15 mJ/cm^2 UVB. The simulated day/night cycle was maintained over 2 days and samples were taken after 6, 18, 30 and 42 hours. Gene expression was determined using quantitative reverse transcriptase real time PCR (qRT-PCR). Statistical analyisis by unpaired Student's t-test.

3D epidermal skin model irradiated with UVB to induce DNA damage.

The study was carried out using an epidermal 3D skin model made from human keratinocytes. After topical application of test compounds for 30 minutes, the skin was irradiated at 1.5 J/cm^2 with a Mega-RAY solar UV simulator lamp (UVA and UVB). The DNA damage caused was made optically manifest after 1, 4, 24 and 48

hours with the help of a specific antibody against thymine dimers (TT dimers [13]). Statistical analysis by unpaired Student's t-test.

Epidermal stem cells irradiated with full sunlight.

Human epidermal stem cells were isolated from foreskin. The cells were cultivated for 3 days with and without 0.00004 % active ingredient and exposed to a full sunlight-simulating lamp (Suntest CPS+, Atlas MTT) at 1.5 J/cm^2. Subsequently, the cells were seeded to form a 3D epidermal skin model. After formation of a confluent cell layer, cultures were exposed to the air phase to induce 3D growth and generation of a stratum corneum.

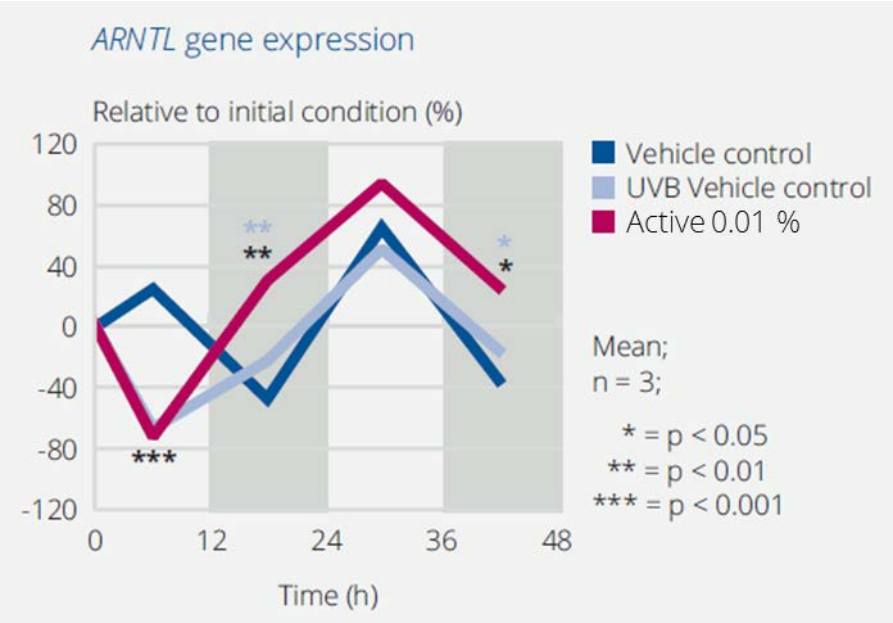
In-vivo study showing the effect of a formulation with 3 % of the cosmetic active or placebo in the course of the day.

In this study, skin hydration, transepidermal water loss (TEWL) and skin redness were assessed. All test parameters were initially measured before application of any formulation at day 0 in the morning (between 8:00 and 10:00) and in the evening (between 20:00 and 22:00) for baseline determination. Subsequent measurements on days 28, 56 and 84 were performed prior to the application of the corresponding formulation. Half of the subjects (44 female aged 35 – 65 years with Caucasian, healthy skin) applied placebo while the other half applied the identical formulation containing 3 % of the active ingredient.
Skin hydration was measured with a Corneometer® CM 825 at three different positions on the forehead. TEWL was assessed with a Tewameter® TM300 in the central region of the forehead. For determination of skin redness, measurements with a Mexameter® MX 18 in the malar region were performed. Statistical analysis by unpaired Student's t-test.

Results

The active ingredient recalibrates circadian clock genes after UV stress (*in-vitro* study)

While *ARNTL* exhibited pronounced circadian regulation, the *CLOCK* gene was not significantly regulated in all tested conditions (Figure 2, dark blue lines, a daytime peak was expected). Without an external trigger, the active ingredient did not significantly influence the expression of circadian genes, demonstrating its good compatibility with epidermal skin cells (not shown).
Irradiation of the keratinocytes with UVB light extensively affected expression of the *ARNTL* and *CLOCK* genes after 6 hours in the daylight phase and disrupted the circadian cycle. Gene expression recovered after 18 hours in the dark phase and then reached normal levels in the vehicle control experiment. In contrast, cells treated with 0.01 % of the active ingredient upregulated gene expression faster, which after 30 hours reached a maximum in the daylight phase of treatment day 2, with values for *CLOCK* and *ARNTL* roughly twice that in the vehicle control. Importantly, there was no effect on the periodicity of gene expression.
As a result, the positive regulation of the circadian clock genes should result in a positive regulation of downstream genes, which are switched on due to CLOCK/ARNTL activity. An important gene among these is the DNA repair gene *OGG1*, coded for 8-oxoguanine DNA glycosylase. This enzyme repairs DNA damage due to internal oxidative stress but also induced by UVB irradiation. Indeed, incubation with just 0.001 % active ingredient resulted in recovery of the suppressed gene expression of *OGG1* after 18 hours so that here there was even outperformance of the vehicle control after 42 hours (Figure 3). The peak was reached in the dark, the time of most effective oxidative DNA damage repair. This indicates an increased DNA repair capability after UVB irradiation in active ingredient-treated cells.



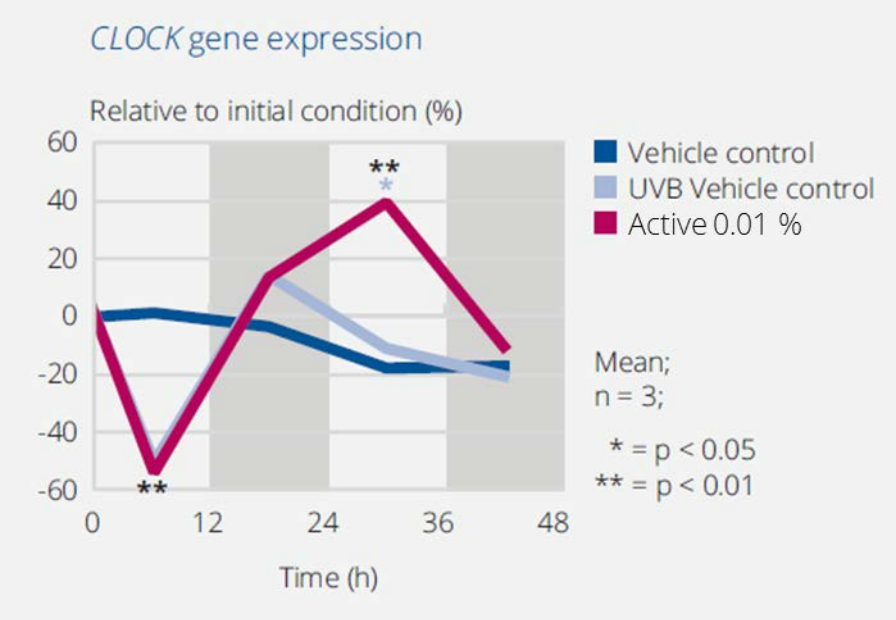


Figure 2: *ARNTL* and *CLOCK* gene expression fall after exposure to UVB radiation. The expression of both daylight-active genes was heavily affected after UVB irradiation but was upregulated to a level almost twice that in vehicle control 30 hours after UVB irradiation in the presence of 0.01 % active ingredient. Unpaired Student's t-test. The statistical values in black are the result of with vehicle control while the blue value is the result of comparison with the UVB-irradiated vehicle control.

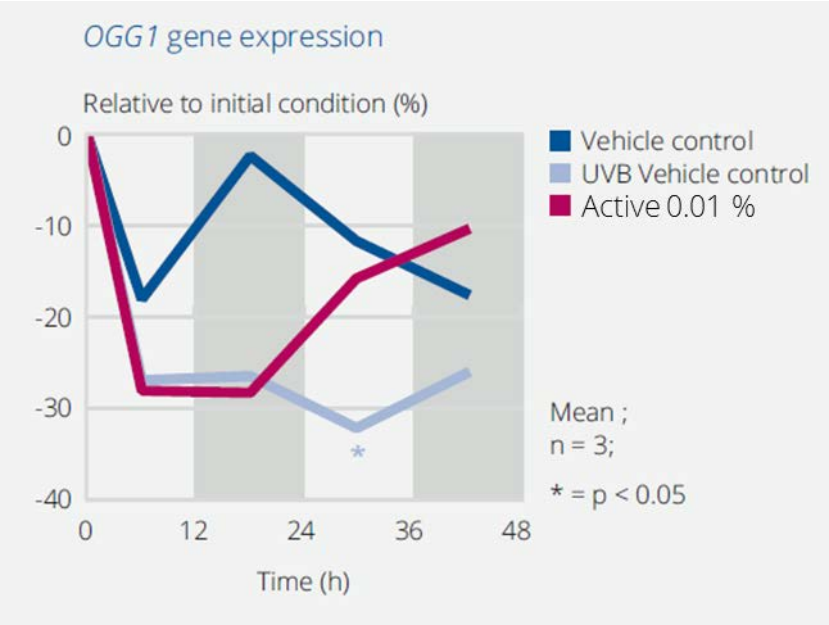


Figure 3: The active ingredient restores DNA repair after UVB irradiation. From 18 hours after irradiation, the gene expression of the 8-oxoguanine repair gene *OGG1* recovered back to normal levels in the presence of the active ingredient while the *OGG1* expression in vehicle control remained significantly reduced. Unpaired Student's t-test. The statistical value in blue is the result of comparison with the UVB-irradiated vehicle control.

The active ingredient prevents DNA damage (*in-vitro* study)

The active ingredient clearly inhibited the formation of DNA-damage as shown in Figure 4. No damage occurred to DNA after UV irradiation. In contrast, DNA damage (black dots) was induced in the untreated control sample and in the case of skin treated with the negative control (caprylic/capric triglyceride).
The results of quantitative evaluation are shown in Figure 5. Measurement values are based on the number of cells which were stained black with an antibody against TT-dimers. The high proportion of UV damage after irradiation can clearly be identified in the untreated and negative control experiments. The reduction in UV damage over time can be attributed to the cells activating their DNA repair mechanism. The active ingredient either directly prevented DNA damage or helped to immediately repair the damage when it occurred.

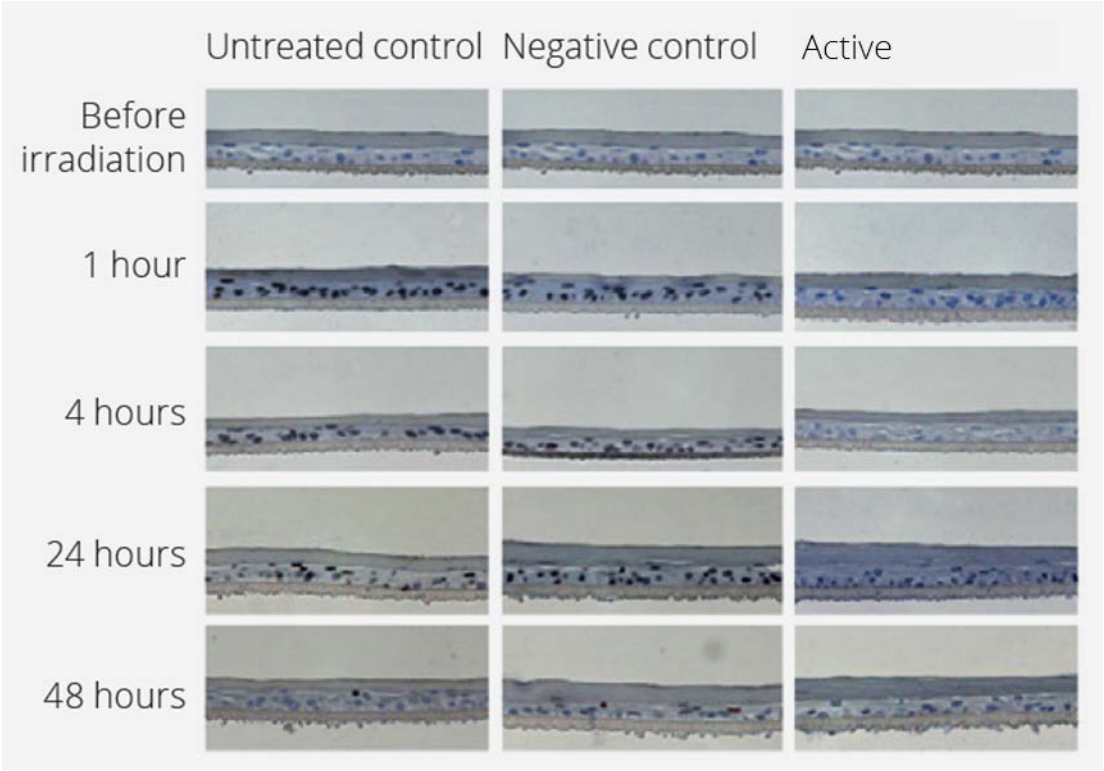


Figure 4: **The active ingredient protects the epidermis against DNA damage**. Immunohistochemical staining to show DNA damage to epidermal 3D skin models. Nuclei of healthy cells are shown in blue. DNA damage is identified by the black staining. Negative control: caprylic/capric triglyceride.

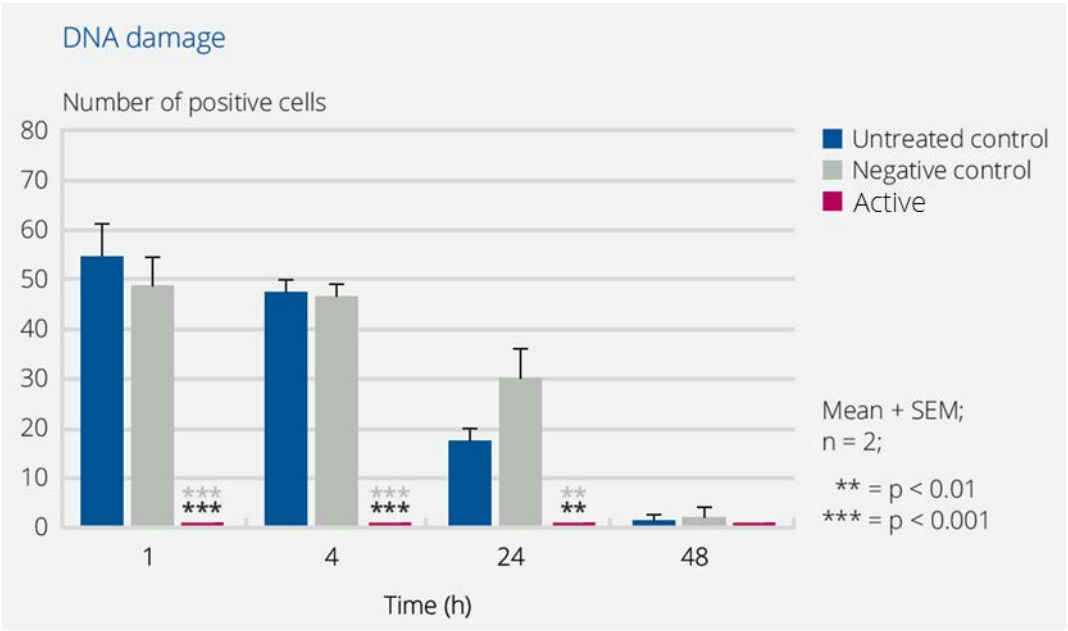


Figure 5: The active prevents UV-induced thymine dimer generation. On exposure to 1.5 J/cm^2, the active ingredient protected all epidermal cells from DNA damage while untreated and negative control-treated tissue showed pronounced TT dimer generation. Unpaired Student's t-test. The statistical values in black are the result of comparison with untreated control while the grey value is the result of comparison with negative control.

The active ingredient protects epidermal stem cells against UV-related performance drop-down (*in-vitro* study)

To test the potential of the active ingredient to protect epidermal stem cells against full sunlight and maintain their capacity to rebuild a fully functional epidermis. The UV fraction of full sunlight damages the DNA of cells and prevents proper cell division and performance. The result would be a thinner epidermis after prolonged UV exposure [14].

The active ingredient was able to protect the epidermal stem cells from full sunshine-induced cell damage. Irradiation of cells in the presence of the active did not markedly reduce the epidermal thickness as compared with the non-irradiated untreated control sample (Figure 6). Interestingly, the skin barrier seemed to be more compact as in the untreated control, whereas in both samples, some irregularities on the top of the stratum corneum were apparent. Without sunlight stress, the epidermis of active ingredient-treated cells appeared to be even thicker than the untreated control with a very pronounced skin barrier.

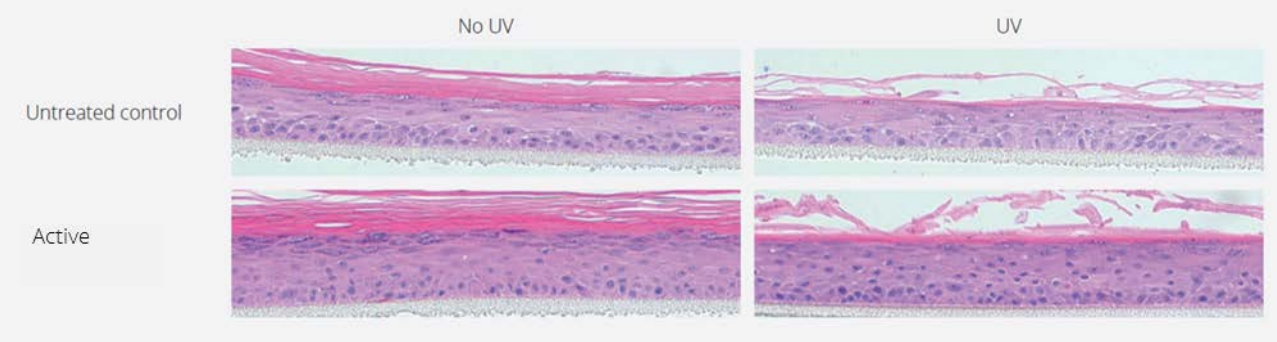


Figure 6: Development of a 3D epidermal skin model after full sunlight exposure. Epidermal stem cells were incubated with active ingredient for 3 days and irradiated with a full sunlight source. After seeding the cells, a 3D epidermal skin model was generated and investigated for developmental deficits. Active-treated cells clearly showed a better capacity to form a 3D epidermal skin in both samples, with or without irradiation.

The active ingredient preserves the circadian rhythm, strengthens the skin barrier and reduces light stress symptoms (*in-vivo* study)

This study was performed to investigate the skin's baseline circadian rhythm and on skin treated with cosmetic formulations. Cosmetic formulations interact with the environment and can generate an extrinsic ROS burden on the skin barrier. Light stress during the day can lead to skin reddening in the evening due to pre-stages of sunburn or other stress factors. Our objective was to determine whether application of a cosmetic formulation with active ingredient influences basic skin parameters and how these relate to the skin's own circadian rhythm.

Circadian changes at baseline:

Before treatment, the skin parameters hydration, TEWL and redness were assessed in the morning and in the evening. We observed a circadian regulation of skin moisture, which was low in the morning and significantly higher in the evening (Figure 7). On the other hand, TEWL was high in the morning and low in the evening; however there was no significant difference. These findings are in agreement with the data from the literature [15].

Skin redness developed during the day and was significantly higher in the evening than in the morning. We postulate that this is more likely to be an extrinsic process evoked by sunlight-induced inflammatory reactions rather than an intrinsically-induced process dependent e.g. on blood pressure [16]. To support skin function, maintenance of the circadian rhythm of skin hydration and TEWL needs to be preserved while skin redness, as an extrinsic process, should be reduced to promote a non-inflamm'ageing environment.

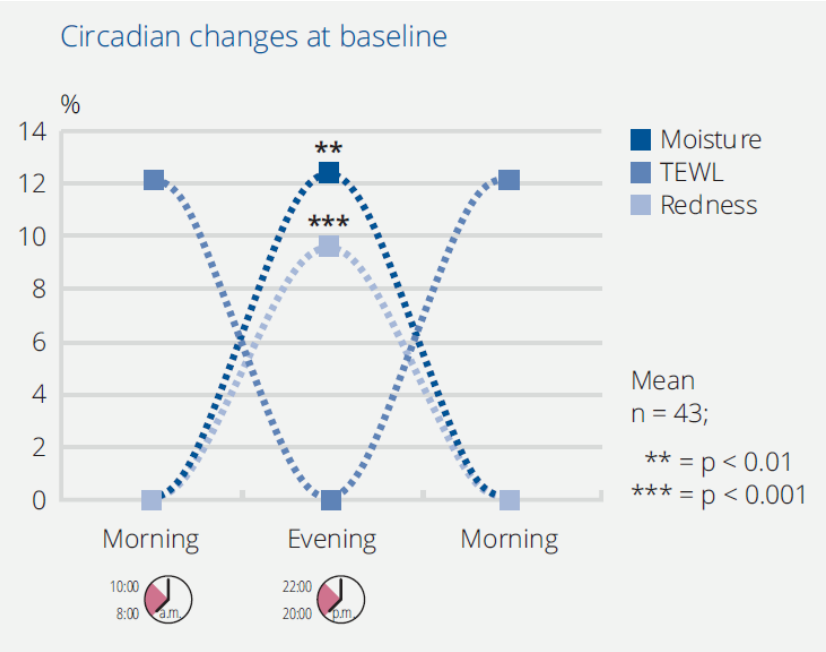


Figure 7: Circadian changes to skin parameters. Skin hydration, TEWL and redness were assessed in the morning and in the evening on the same day before application of cosmetic formulations by the entire subject panel. A pronounced variability of these parameters could be observed. Please note that the measured values do not represent physiological maximal or minimal values as may be suggested here. Unpaired Student's t-test. The statistical values in black are the result of comparison of the evening value with the morning value.

Development of test parameters throughout the study:

Skin hydration: As expected, skin hydration with placebo and 3 % active ingredient were significantly improved at every measurement time point ($p < 0.001$) by up to 40 % over baseline status (not shown). The direct comparison of the active with placebo revealed a superior moisture supplementation by 4 % in the morning on day 56 (Figure 8). In contrast, a significant improvement of moisture supply by 11 % was achieved in the evening on day 56, already at the threshold of significance at day 28 while the positive effect was maintained also after 84 days (not shown).

Barrier strength was significantly improved as the TEWL values dropped by up to 26 % in the evening on day 56 compared to placebo (Figure 9). The morning value was almost the same. Application of active ingredient improved the barrier strength in the same way during the day and the night. While placebo did not significantly improve TEWL in the morning measurement compared with baseline status, it actually significantly worsened in the evening measurement by up to 15 % (not shown). The positive effect of the active over placebo was maintained also after 84 days (not shown).

Skin redness during the evening measurement was significantly reduced over baseline (day 28) and placebo (days 28, 56, Figure 10). Interestingly, placebo redness values were increased by up to a significant 14 % over baseline ($p < 0.001$, not shown). Also the morning redness in subjects applying the active ingredient decreased compared to placebo, however not significantly. The positive effect was also maintained after 84 days (not shown).

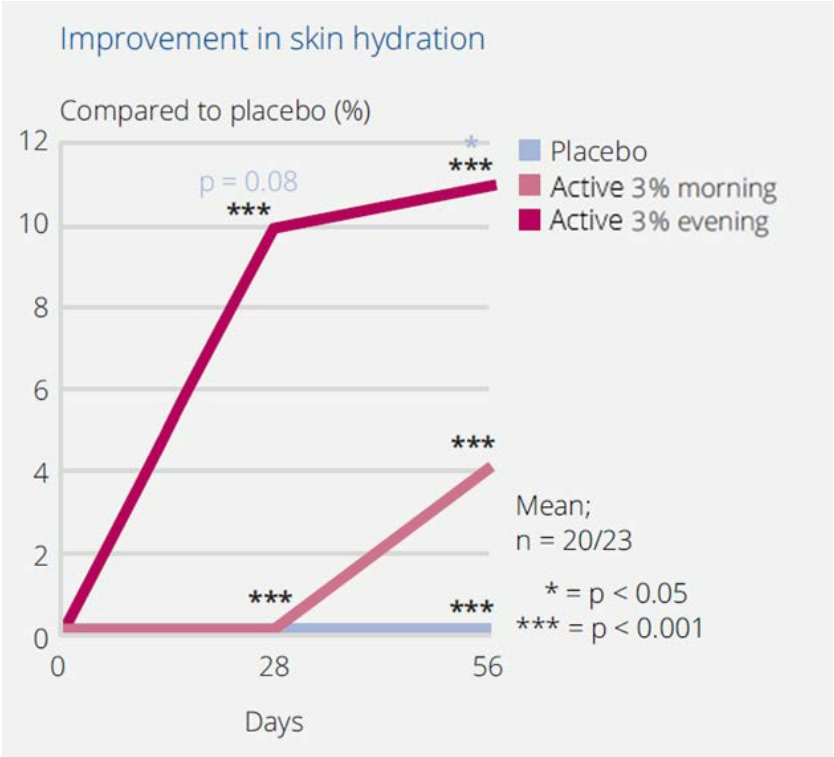


Figure 8: Improvement in skin hydration. In direct comparison with placebo, the active ingredient was most effective in providing extra moisture in the evening. Unpaired Student's t-test. The statistical values in black are the result of comparison with the baseline condition while the blue values are the result of comparison with placebo.

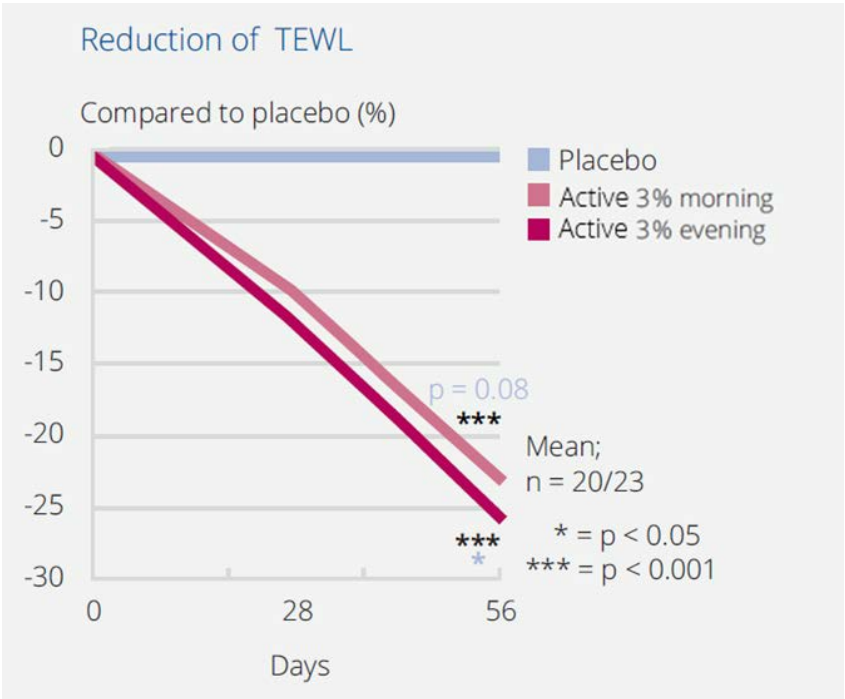


Figure 9: The active ingredient is an excellent barrier-strengthening agent. It reduced TEWL in the morning as well as in the evening. Unpaired Student's t-test. The statistical values in black are the result of comparison with baseline status while the blue values are the result of comparison with placebo.

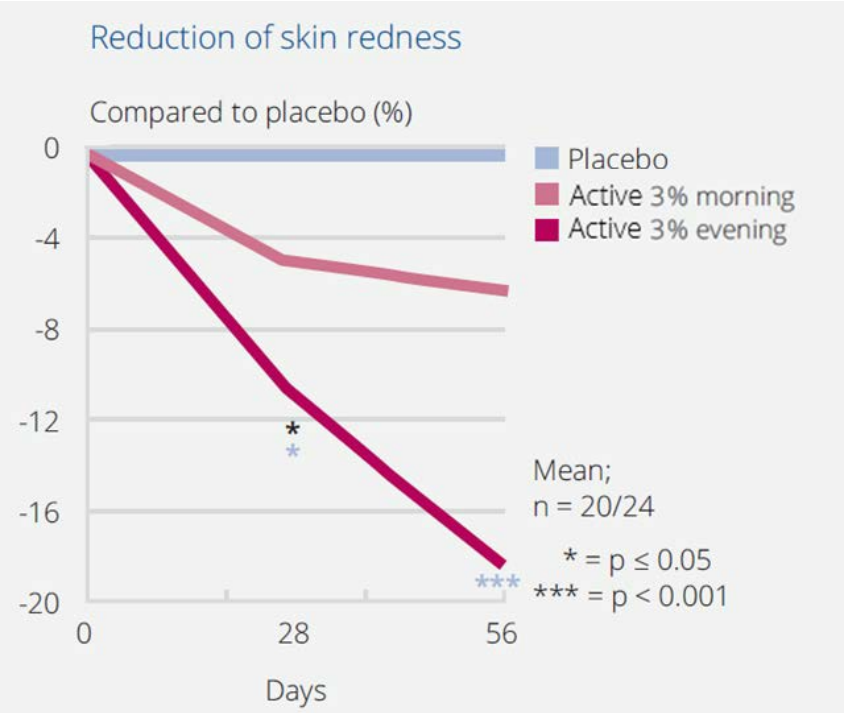


Figure 10: Skin redness continuously falls in the morning and evening measurements compared to placebo. The effect was significant over placebo and baseline in the evening. Unpaired Student's t-test. The statistical values in black are the result of comparison with baseline status while the blue values are the result of comparison with placebo.

Circadian changes during test formulation application:

The baseline measurements revealed a significant circadian variability only for skin hydration and skin redness (Figure 7). Both parameters were evaluated for their circadian development throughout the entire study duration of 84 days.

Skin hydration: Investigation of the circadian differences at day 28, 56 and 84 after test formulation application revealed a significant maintenance of the circadian skin moisture changes throughout the application of active ingredient (Figure 11). In contrast, the variation in skin moisture throughout application with placebo dropped dramatically to a non-significant level as expected for a suppressed circadian rhythm in aged skin. This indicates that the natural intrinsic circadian regulation of skin moisture was disturbed in the presence of the placebo formulation. On the other hand, the amplitude was preserved with the active ingredient-containing formulation, reflecting the behaviour of youthful skin.

Skin redness: The amplitude of skin redness increased during the study in the case of placebo application (Figure 12). At day 84, the amplitude doubled, which means that skin redness was even more pronounced in the evening compared to the morning value. In contrast, the active continuously reduced the amplitude to zero, which means the skin redness was identical in the morning and evening measurements at day 84. As skin redness is not an intrinsically circadian regulated process, we can assume that the activity of the active protected the skin during the daytime from deleterious light-induced inflammatory reactions.

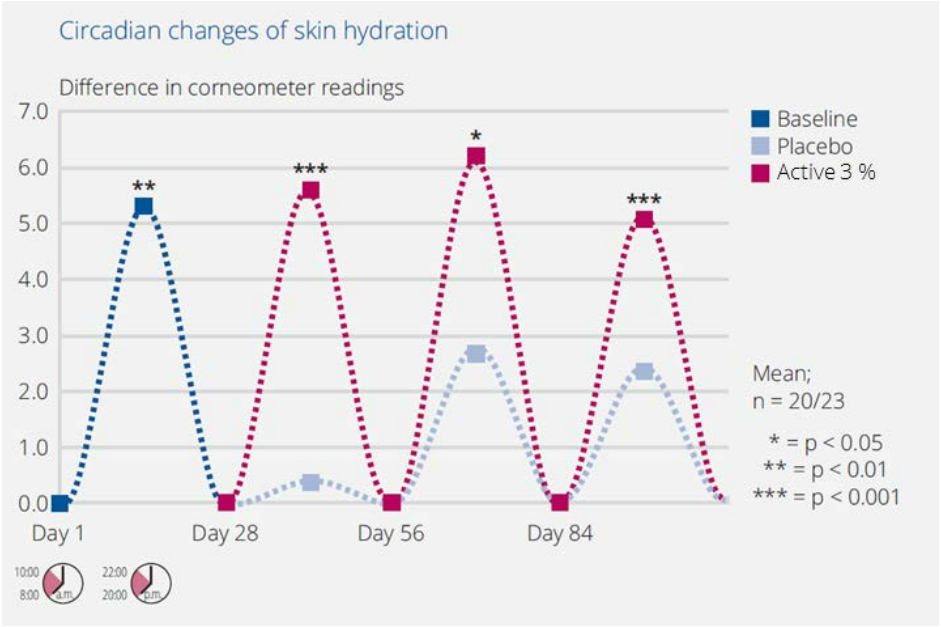


Figure 11: Circadian changes to skin hydration. The baseline values of the entire study population revealed a significant increase in skin moisture in the evening (blue line). Application of 3 % active ingredient preserved this periodicity while placebo interfered with this process. Unpaired Student's t-test. The statistical values in black are the result of comparison with baseline status (each measurement day in the morning).

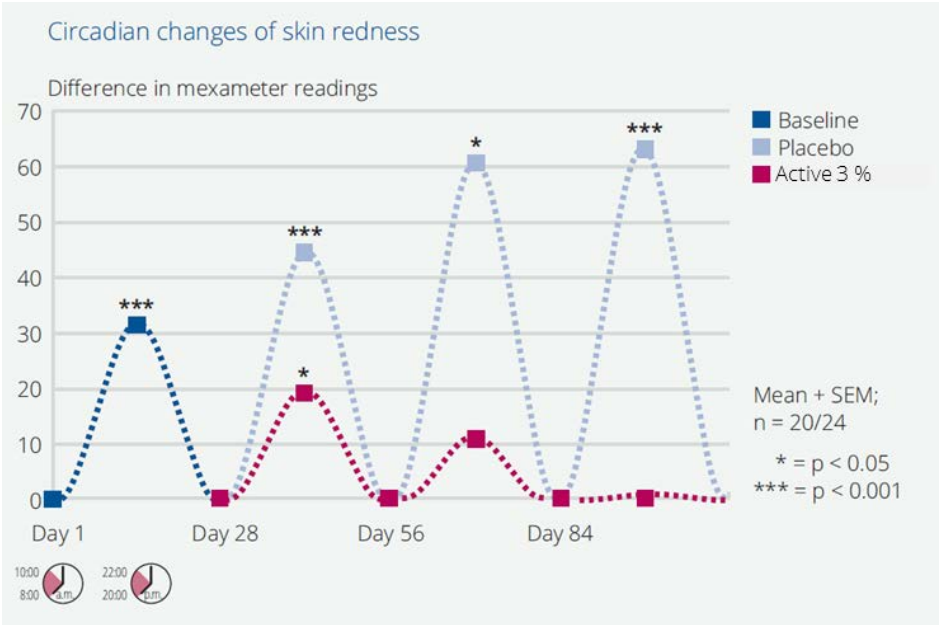


Figure 12: Circadian changes to skin redness. The facial skin redness was significantly higher in the evening at baseline (blue line). While placebo treatment led to a doubling of the amplitude, the active reduced skin reddening during the day up to no detectable difference compared to the morning value. Unpaired Student's t-test. The statistical values in black are the result of comparison with the baseline condition (each measurement day in the morning).

Discussion

UVA and UVB radiation can penetrate the skin and damage DNA either directly - e.g. through the formation of thymine dimers - or indirectly by means of the generation of reactive oxygen species (ROS). In secondary reactions, these ROS, in particular, cause a significant amount of 8-oxoguanine residues to accumulate on DNA.
The damage to DNA has to be repaired in order for cells to be fully functional and survive. However, severely damaged cells can no longer be repaired as the energy needed for regeneration cannot be made available in a short space of time, especially in the epidermis, which is not supplied with blood. Additionally, a disturbed epidermal circadian cycle prevents proper cell functionality. Cells that cannot regenerate vital cell functions die off. The consequence is a thinning of the epidermis over time and a reduced regenerative capacity over night.
The three active principles of the active ingredient act in concert. Ethyl ferulate, a natural antioxidant with UV-absorbing properties reduces the dose of DNA-damaging UV radiation that penetrates the skin and neutralizes dangerous ROS. Carnosic acid has a synergistic effect in that it combats free radicals – one of the main causes of DNA and cell damage. The active ingredient promotes the production of the 8-oxoguanine-repairing enzyme 8-oxoguanine DNA glycosylase (OGG1) and supports DNA repair. Taken together, these mechanisms also reduce skin reddening caused by sunlight exposure.
Moreover, the active ingredient is able to reinforce the skin's circadian rhythm. It reschedules the gene expression of the important positive regulators *CLOCK* and *ARNTL* (*BMAL1*) after UV irradiation while respecting the primal circadian rhythm, which ensures proper skin regeneration day by day.
The active ingredient is an ideal supplement to the daily cosmetic care when skin faces light stress and is prone to suffer from epidermal jetlag, the imbalance of the skin's biorhythm.

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