Establishment of an in vitro model to study the effect of psychological stress on human skin stem cells

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Abstract (Maximum of 250 words)

Introduction: As the effect of psychological stress on skin stem cells is largely unexplored, we aimed to develop an *in vitro* model using skin stem cells and a psychological stress inducer along with an appropriate end point, which can be used for efficacy screening to identify modulators of chronic psychological stress in the skin.

Methods: The assay was established in human epidermal progenitor/stem cells (HEPSCs), treated with different stress inducers for 6 hours or for 2 and 4 days to simulate and compare acute and chronic psychological stress, respectively. Supernatants were harvested at each time point and analyzed for IL-1β, IL-8 and IL-6 by ELISA to assess potential biomarkers of the stress response. In addition, RNA was isolated from the cells to quantify NF-κB gene expression by qPCR. In a last step, using this model, four plant extracts were tested for their potential to protect HEPSCs from psychological stress.

Results: Whereas the secretion of IL-8 and IL-1β was less pronounced and consistent, IL-6 was identified as a reliable biomarker for psychological stress in this model. Chronic stress induced by treatment with norepinephrine or the CRF1 agonist stressin I led to a significant increase of IL-6 secretion and NF-κB expression after 4 days of treatment. Using this model, two plant extracts with protective potential against psychological stress were identified.

Conclusion: The established *in vitro* model can be used for efficacy screening of molecules to identify modulators of chronic psychological stress in the skin, which is important for the development of a new generation of neurocosmetic active ingredients.

Keywords: epidermal stem cells, psychological stress, norepinephrine, corticotropin releasing factor (CRF), neurocosmetics

Introduction.

Even though anecdotal evidence for the impact of psychological stress on the skin exists, the molecular mechanisms of how stress leads to a response in the skin are still not fully clear. Besides mechanical, chemical, and physical stresses, psychological stress has been shown to play a role in skin function with consequences such as delayed wound healing, disrupted barrier function and stratum corneum integrity, and compromised epidermal immunity [1-4]. The link between stress and skin health is further supported by the fact that many skin diseases, e.g. atopic dermatitis, psoriasis, and acne, as well as skin conditions, e.g. dry or oily skin, are strongly influenced by psychological stress [5-8]. Studies have not only confirmed a connection between high levels of psychological stress and diverse skin conditions but also observed that psychiatric treatment can have positive effects on dermatologic diseases affected by stress [9]. One of the major stress response systems in vertebrates is the hypothalamus-pituitary-adrenal (HPA) axis that connects the central nervous system with the endocrine system and regulates the secretion of glucocorticoids, such as cortisol (Figure 1). Psychological stress increases endogenous glucocorticoid levels and activates the autonomous nervous system which allows the host to respond to various situations. The response to stress differs for acute and chronic stress, where especially chronic stress is linked to increased susceptibility to infections, and allergic and inflammatory diseases and is discussed as a factor contributing to skin aging [7].

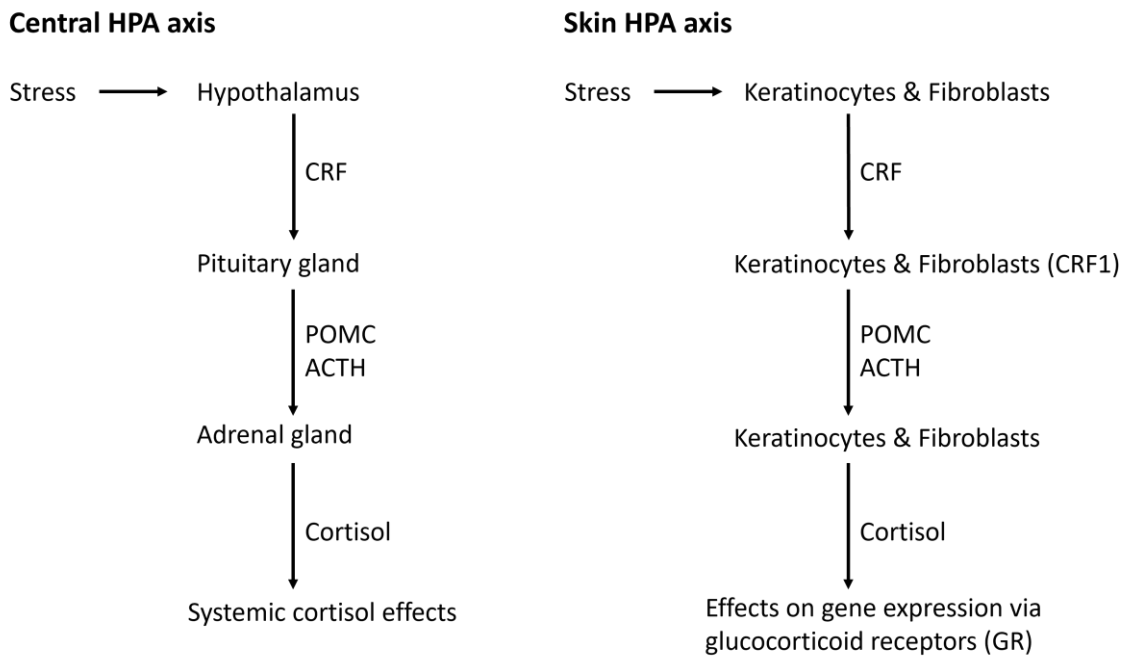

Figure 1: The central HPA axis and the elements of the HPA axis expressed in skin cells.

The skin, however, is not only a target of stress mediators but also a local source of these factors. Interestingly, the human skin itself expresses major elements of the HPA axis including corticotropin releasing factor (CRF), also called corticotropin releasing hormone (CRH), CRF receptors and glucocorticoids [10]. Out of the two CRF receptor types (CRF1, CRF2), human epidermis preferentially expresses CRF1 [11], a G-protein coupled receptor which responds to binding of CRF by activation of different pathways and thereby regulation of the expression of genes, such as POMC, interleukins, involucrin, or cytokeratin 14, in epidermal keratinocytes [12]. POMC is the precursor of adrenocorticotropic hormone (ACTH), alpha-melanocyte-stimulating hormone (α-MSH) and β-endorphin, which themselves bind their corresponding receptors to initiate further responses [13]. This complex interaction of several pathways leads to phenotypic effects, such as the stimulation of steroidogenesis, differentiation and melanogenesis, the inhibition of cell division and the regulation of the immune response [12] (Figure 1). Stress also involves the sympathetic nervous system and the release of catecholamine neurotransmitters, such as epinephrine and norepinephrine [14]. These neurotransmitters as well as the adrenergic receptors they bind to are also present in the skin. Epinephrine may have positive effects on epidermal proliferation and differentiation, but can also impair wound healing and may contribute to skin aging

together with norepinephrine and cortisol by compromising genome integrity [7]. Epinephrine and norepinephrine are produced in human keratinocytes [15] and CRF has been shown to stimulate the immune response regulator nuclear factor-kappa B (NF-κB) and the production of the cytokine IL-6 in epidermal keratinocytes [16, 17].

The effect of psychological stress on stem cells of the skin, however, is largely unexplored. Therefore, we aimed to develop an *in vitro* model using skin stem cells and a psychological stress inducer along with an appropriate end point, which can be used for efficacy screening to identify modulators of chronic stress in the skin. For this, we tested human epidermal progenitor/stem cells (HEPSCs) with different psychological stress inducers for different periods to simulate and compare acute and chronic stress. As readouts the release of several interleukins and the gene expression for NF-kB were analyzed. After identification of the most robust biomarker and the most effective stressor, the experiments were repeated to test for reproducibility. In a last step, the newly established model for chronic psychological stress effects was used to test the protective effect of four plant stem cell extracts on HEPSCs.

Materials and Methods.

Materials

Corticotropin releasing factor (CRF) and CRF1 agonist stressin I were purchased from Tocris Bioscience, UK, whereas norepinephrine and hydrocortisone were purchased from Sigma-Aldrich, MO, US. For plant stem cell extracts, cells derived from callus cultures of four different plants were cultivated in a wave reactor (25° C, in the dark, set to 9° and 20 rpm/min, with an air flow of 5-10 L/h). The suspensions were homogenized by Ultra-Turrax® T25 (IKA, Germany) followed by two rounds over a microfluidizer (Microfluidics Corporation, MA, US). Subsequently they were centrifuged at 4700 rpm for 20 min (Multifuge 1 S-R, Heraeus, Germany) and filtrated using a paper filter followed by sterile filtration (Steritop bottletop filter, 0.2 µm, Millipore, MA, US).

Cell culture and treatment

The assay was established in pooled human epidermal progenitor/stem cells (HEPSCs), isolated from three healthy adult donors. Experiments were performed using an early passage of cells (4^{th} passage). Cells were expanded, counted (Luna II, Logos biosystems, South

Korea) and seeded in 6-well plates at 4000 cells/cm^2 and cultured in serum-free cell culture medium (CB-TAK-GM, Curio Biotech, Switzerland) without hydrocortisone in an active humidified cell culture incubator (Memmert, Germany) at 37 °C and 5 % CO_2. The cells were grown for 2 days, and the medium was replaced with medium containing or not (control) the stress inducers and/or the test compounds at different concentrations. The incubation time was chosen to simulate acute (6 hours) or chronic stress (2 and 4 days). Supernatants were harvested at each time point and frozen for analysis. For 4 days of induction, the supernatants of day 2 and day 4 were pooled, as the medium was replaced after 2 days.

Cytotoxicity Assay

The four plant stem cell extracts were diluted to a range of concentrations (0.016%-2.0%) and cells were grown either in presence or absence of these test samples for 2 days in triplicates. Cells were incubated with WST-8 solution (Cell Counting Kit 8, MedChemExpress, NJ, USA) and optical density (OD) was recorded in a multimode plate reader (Hidex Sense, Hidex, Germany).

Interleukin measurement

Supernatants were analyzed for IL-1β, IL-8 and IL-6 by commercially available ELISAs (human IL-1b, human IL-8, human IL-6, R&D systems, MN, US) in duplicates (first tests) or triplicates (later tests with statistics) according to the manufacturer's protocol. Results were presented as percentage compared to untreated control with mean or mean +/- standard deviation.

Gene expression

For gene expression analysis of NF-kB, after 4 days of treatment with 100 nM stressin, 0.5 µM or 1 µM norepinephrine, cells were lysed and RNA was isolated using the Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories, CA, US) and quantified (Nanodrop, Thermo Fisher Scientific, MA, US). Reverse transcription was performed using Bio-Rad MyCycler Thermal Cycler (Bio-Rad Laboratories, CA, US) and gene expression was performed using SYBR qPCR (Bio-Rad Laboratories, CA, US) and Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, CA, US). Cyclophilin was used as housekeeping

gene. The results were expressed as fold change in percentage compared to the untreated control.

Statistics

For all experiments performed in triplicates statistical analysis was performed using unpaired T tests.

Results.

To develop a model of psychological stress in human skin stem cells, different inducers of psychological stress were tested for their potential to induce a stress response. For this, human epidermal progenitor/stem cells (HEPSCs) were incubated with different stress inducers for 6 hours or 2 and 4 days to simulate acute or chronic stress, respectively. As stress inducers CRF at 1 µM, stressin I, a potent and selective CRF1 agonist [18], at concentrations of 25 nM, 50 nM, or 100 nM, hydrocortisone at 75 ng/ml, 250 ng/ml, or 500 ng/ml and norepinephrine at 1 µM, 1.5 µM, or 2 µM were used. Supernatants were analyzed for interleukins IL-1β, IL-6, and IL-8 by ELISA to assess potential biomarkers of the stress response. In this context, IL-6 was identified as a reliable biomarker in this model. IL-6 secretion was induced when HPEKs were incubated for 6 hours with different concentrations of the CRF1 agonist stressin I or norepinephrine. CRF did not influence IL-6 secretion, whereas hydrocortisone downregulated IL-6 levels in this setup (Figure 2). Under chronic stress conditions, cells showed enhanced IL-6 secretion after treatment with 100 nM stressin I or different concentrations of norepinephrine or 1 µM CRF for 2 days. After 4 days, cells responded with increased IL-6 levels for almost all compounds and concentrations tested with the strongest response observed for norepinephrine and CRF (Figure 2).

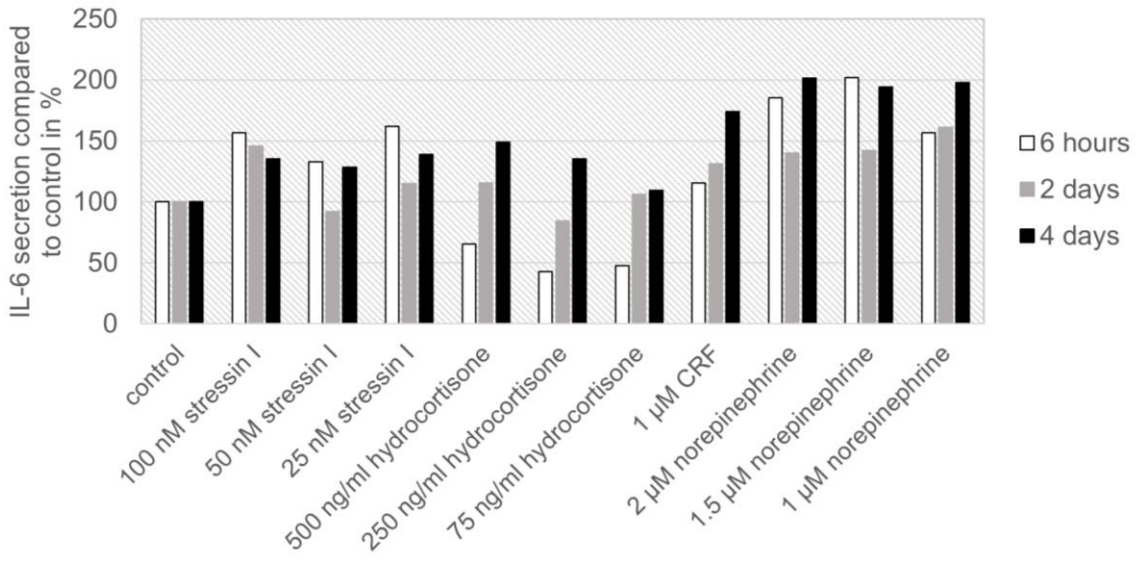

Figure 2: Release of IL-6 from HPEKs treated with different stress inducers for 6 hours to simulate acute stress or for 2 or 4 days to simulate chronic stress.

The secretion of IL-8 and IL-1β was less pronounced and consistent. IL-8 was induced slightly with all three concentrations of stressin I and two concentrations of norepinephrine after 6 hours, whereas 75 ng/ml hydrocortisone reduced the release of IL-8. After 2 days of treatment, however, none of the treatments led to an increase of IL-8 and after 4 days only a slight increase in IL-8 levels was induced by 1 µM norepinephrine (Figure 3). IL-1β was not induced by any of the conditions tested except for a slight increase of 30% for the treatment with 1 µM norepinephrine for 2 and 4 days. The treatment with 2 µM norepinephrine, however, led to a reduction of IL-1β (Figure 4).

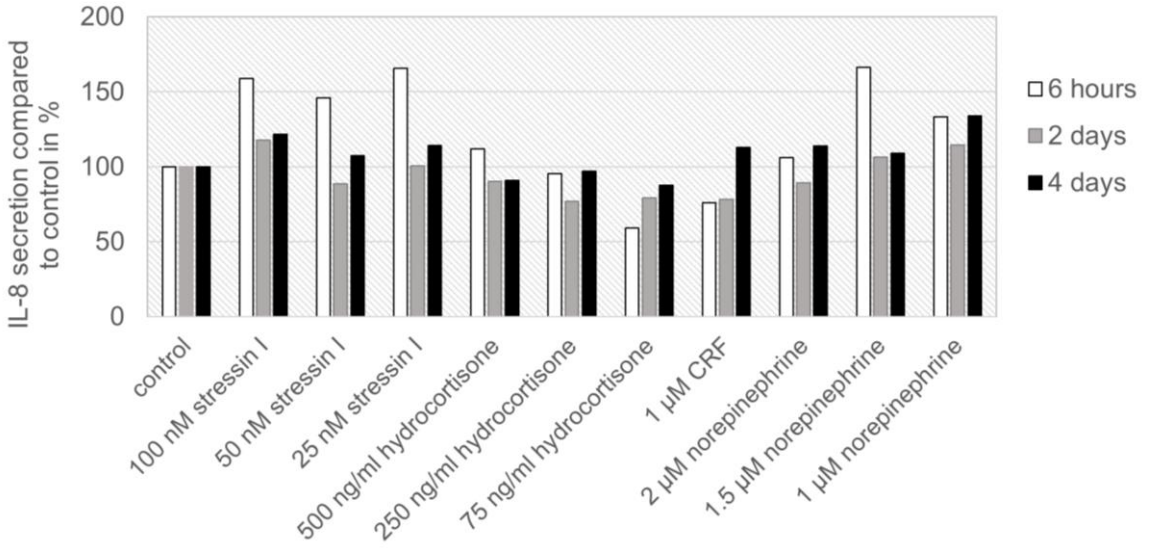

Figure 3: Release of IL-8 from HPEKs treated with different stress inducers for 6 hours to simulate acute stress or for 2 or 4 days to simulate chronic stress.

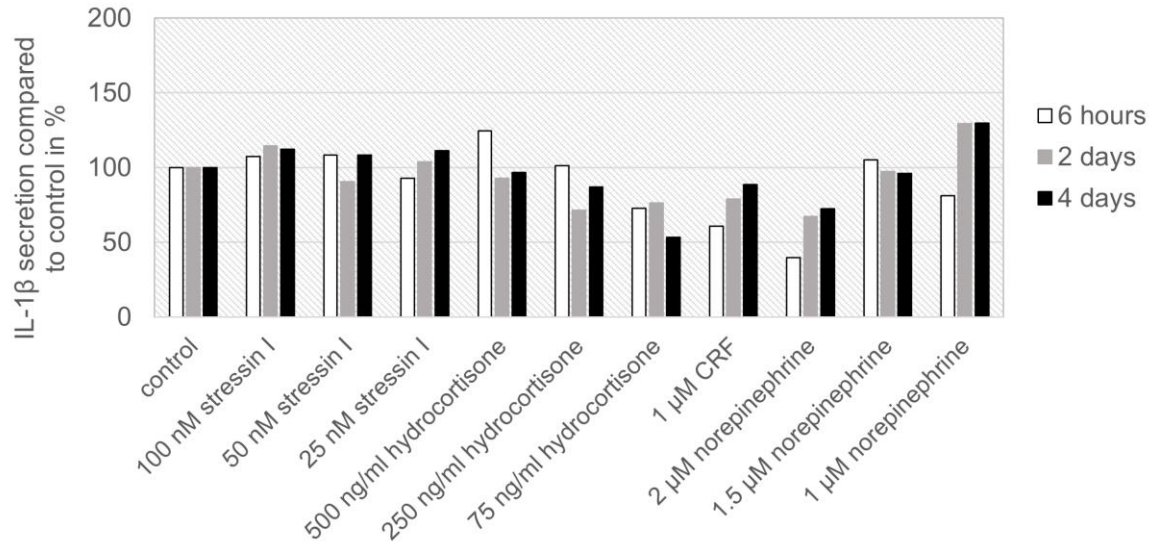

Figure 4: Release of IL-1β from HPEKs treated with different stress inducers for 6 hours to simulate acute stress or for 2 or 4 days to simulate chronic stress.

After identification of the most effective concentrations of each stressor and IL-6 as the most robust biomarker, the experiments were repeated to test for reproducibility. Due to the strong effect observed at the lowest concentration of norepinephrine (1 µM), 0.5 µM norepinephrine was tested in addition. Chronic stress induced by treatment with 0.5 µM norepinephrine, 1 µM norepinephrine or 100 nM stressin I led to a significant increase of IL-6 secretion compared to the control after 2 days of treatment by 37%, 54% or 51%, respectively. After 4

days of treatment the effect was even stronger showing an increase in IL-6 secretion by 163%, 153% and 150% by 100 nM stressin I, 0.5 µM norepinephrine and 1 µM norepinephrine, respectively. After 4 days of treatment, CRF also led to a significant increase of IL-6 levels, which was, however, less pronounced (Figure 5). Based on these results, 100 nM stressin I and 0.5 µM norepinephrine were chosen as the most effective stress inducers in this model.

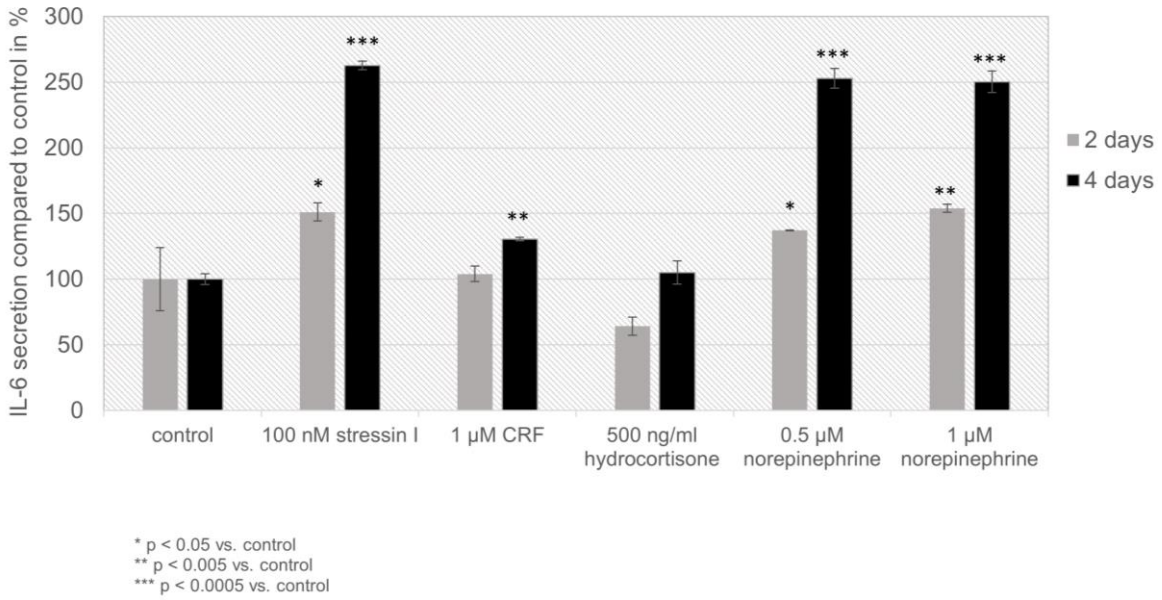

Figure 5: Release of IL-6 from HPEKs treated with different stress inducers for 4 days.

In addition, the gene expression of NF-κB was tested in this setup after treatment of cells for 4 days in a preliminary analysis. NF-κB expression was strongly upregulated by 118% and 191% by 100 nM stressin I and 0.5 µM norepinephrine, respectively (Figure 6).

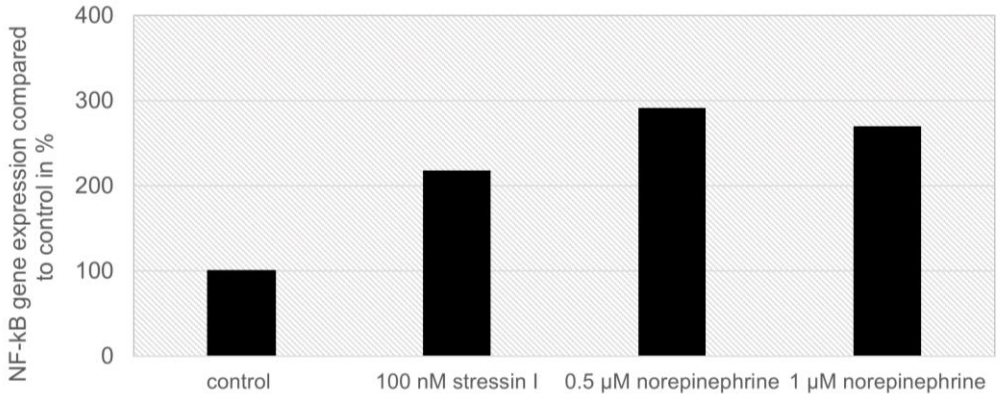

Figure 6: Gene expression of NF-κB in HPEKs after 4 days of treatment with the stress inducer stressin I or norepinephrine.

In a last step, the newly established *in vitro* model was used to test the protective effect against psychological stress of four plant stem cell extracts (PSCE-1-4) on HEPSCs. For this the HPEKs were treated with either 100 nm stressin I or 0.5 µM norepinephrine in the presence of the test compounds for 4 days. The concentrations of the plant stem cell extracts were chosen according to the results of a prior cytotoxicity test (data not shown). The study was performed as two independent experiments, in which two test compounds were analyzed per experiment. As demonstrated before, cells treated with stressin I or norepinephrine showed an upregulated release of IL-6 by 25-35% or 45-65%, respectively. This effect could be reduced by treatment with two of the extracts, PSCE-1 and PSCE-4, resulting in IL-6 levels similar to the ones of untreated control cells, whereas the other two test compounds did not show an effect or even slightly increased the IL-6 release (Figure 7-8). Thus, the plant stem cell extracts PSCE-1 and PSCE-4 have the potential to protect HPESCs from psychological stress.

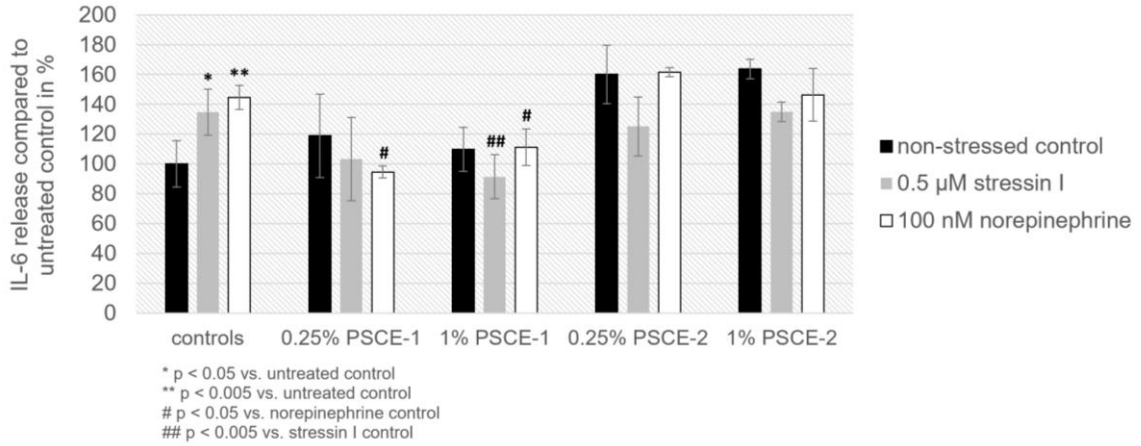

Figure 7: Release of IL-6 from stress-induced HPEKs after treatment with plant stem cell extracts PSCE-1 and PSCE-2.

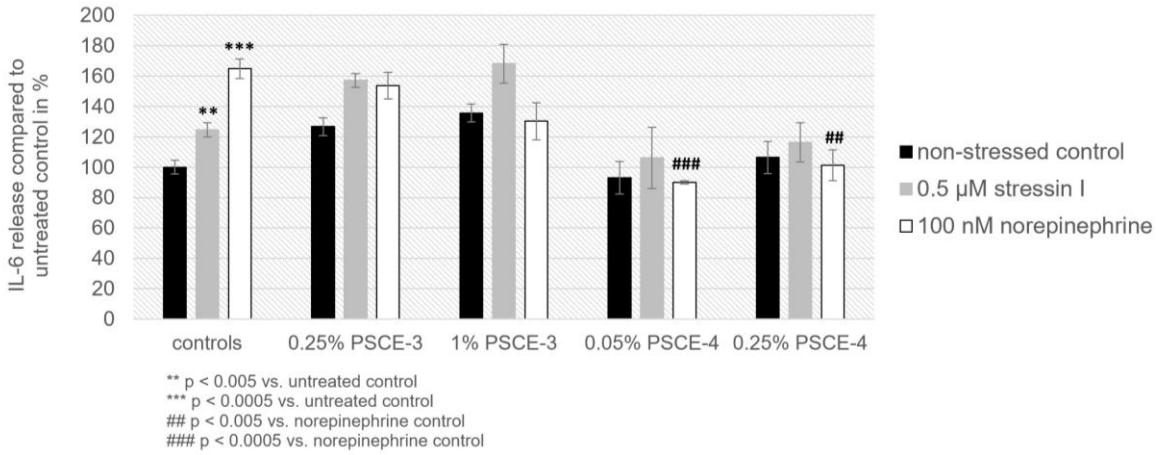

Figure 8: Release of IL-6 from stress-induced HPEKs after treatment with plant stem cell extracts PSCE-3 and PSCE-4.

Discussion.

This study presents the successful development of a new *in vitro* model to test the effect of chronic psychological stress on skin stem cells. Whereas the effect of stress on keratinocytes and fibroblasts are partially known, the function of stress on skin stem cells is largely unexplored. Thus, our model can help to elucidate the consequences of chronic psychological stress on the self-renewal capacity of the skin.

The effect of stress on the release of pro-inflammatory interleukins in the skin is complex. In this study, we observed an increase in IL-6 after chronic stress induction for 4 days for most stressors tested, whereas IL-8 levels did not or only slightly change and IL-1β release was even reduced under the same conditions. These results are in line with observations in HaCaT cells, where an increase of IL-6 and IL-11 but a decrease of IL-1β has been observed after treatment with CRF [16], suggesting that the response to psychological stress of keratinocytes and HPESCs may be similar.

NF-κB is a transcription factor that is linked to stress and inflammation. It is stimulated by several pathways which all result in the translocation of NF-κB to the nucleus and subsequent activation of gene expression. In the skin, NF-κB is linked to inflammatory and immunomodulating signals, and the regulation of apoptosis, cytoskeletal changes, and differentiation [19]. CRF has been shown to stimulate NF-κB activity in mouse thymocytes [20] and human epidermal keratinocytes [17], whereas NF-κB activity was inhibited by CRF in melanocytes [21] and immortalized keratinocytes (HaCaT) [22]. In our model of HPEKs, NF-κB is induced by norepinephrine and CRF agonist stressin I, which is in line with the findings in human epidermal keratinocytes and emphasizes the inflammatory nature of chronic stress responses and the increased IL-6 secretion observed under the same conditions.

The study also highlighted the different reactions to acute and chronic stress. Acute short-term stress activates the sympathetic system and the HPA axis leading to rapid responses of the body and the innate immune system that are essential for the defense of the organism. These reactions are strictly controlled and reversible. The released cortisol levels are maintained within defined daily amounts to avoid harmful excessive cortisol excretion [9]. Under chronic stress, the excessive HPA activity leads to the loss of the daily cortisol rhythm and long-term stress also has different effects on the immune system generally suppressing immunoprotection, increasing susceptibility to infections and exacerbating allergic and inflammatory diseases [7, 9]. In our study, IL-6 secretion induced by hydrocortisone was reduced under acute stress conditions, whereas IL-6 levels were unchanged or even slightly increased for high hydrocortisone concentrations and a long-term treatment over 4 days. Also

considering the fact that only the lowest dose of hydrocortisone reduced IL-8 and IL-1β secretion, whereas higher concentrations did not have any effect, hydrocortisone showed clear time- and dose-specific effects on HPESCs. The observed effects emphasize the two actions of glucocorticoids and their dose-dependency [23, 24]. As hydrocortisone, however, was not able to induce a significant and reproducible release of IL-6, IL-8 or IL-1β, CRF1 agonist stressin I and norepinephrine were chosen as more reliable stress inducers in this model of chronic psychological stress.

Adjusting the treatment time with the stress inducers, our model allows to investigate differences between acute and chronic stress responses. To assess the effects of acute stress responses, however, the evaluation of additional markers besides IL-6 should be considered. Except for hydrocortisone, IL-6 secretion was induced under acute and chronic conditions for all other stress inducers. The secretion of IL-6 in the skin could therefore resemble the IL-6 secretion into the circulation that has been described to be increased in both acute and chronic stress situations [25]. In contrast, IL-8 response was mainly observed after acute stress in this study suggesting that IL-8 may be a candidate for a marker of acute stress. In urinary samples, however, IL-8 has been observed both under acute and chronic stress [26]. In general, the secretion of interleukins upon psychological stress in the skin is not well characterized and even study results on the detection of salivary inflammatory cytokines in response to acute stress, a topic that is addressed more often, are heterogenous [27], making it difficult to compare our findings.

With the screening of four plant stem cell extracts, we could demonstrate the potential of this model to identify modulators of psychological stress in the skin. The four plant stem cell extracts showed distinct effects on IL-6 release, which allowed to identify two extracts that significantly reduced IL-6 release and thus have a potential function in protecting the skin against psychological stress. Further, these results indicate that certain plant stem cells are able to protect skin stem cells, which may be due to their epigenetic factors and metabolites that help to maintain the self-renewal capacity of stem cells not only in plants but also across kingdom borders [28, 29].

Conclusion.

Overall, the established *in vitro* model clearly demonstrates the function of the CRF1 agonist stressin I and norepinephrine as stress inducers to simulate psychological stress in HEPSCs. IL-6, a well-known stress marker, and NF-κB, a transcription factor that is upregulated during stress and inflammation, have been identified as reliable biomarkers in this context. This reproducible model can be used for efficacy screening of molecules to identify modulators of psychological stress in the skin, as demonstrated by the screening of four plant cell extracts, where two of them were identified to significantly reduce the stress-induced release of IL-6. Thus, our *in vitro* model represents a valuable tool for the development of a new generation of neurocosmetic active ingredients.

Acknowledgments.

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Conflict of Interest Statement.

NONE

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