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"Skin fatigue is caused by stress hormones deteriorating the stratum corneum barrier"

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1. Introduction

Recent studies have indicated that stress hormones, particularly cortisol and adrenaline, have a profound impact on skin health. Cortisol, known as the "stress hormone," is released during times of psychological stress and can lead to various skin issues, including increased sebum production and inflammation. Adrenaline, while primarily associated with the fight-or-flight response, also has implications for skin conditions.

Acute stress has been shown to exacerbate inflammatory skin diseases such as psoriasis and eczema. Previous studies have demonstrated that psychological stress can impair the skin's barrier function, increasing moisture loss and causing dry skin [1]. Another previous study also found that exposure to stress hormones can delay recovery after skin barrier breakdown, leading to rough and dry skin [2].

Moreover, chronic stress has been linked to premature aging of the skin. Previous studies have shown that chronic stress reduces the skin's ability to repair itself, exacerbating signs of aging such as wrinkles and fine lines. This is especially important in today's lifestyles where chronic stress is prevalent [3].

In our study, we aim to delve deeper into the mechanisms through which stress hormones influence keratinocytes in the epidermis. Keratinocytes are the predominant cell type in the epidermis and play a crucial role in maintaining the skin's structural integrity and defense mechanisms. By utilizing a 3-dimensional cultured tissue model, we can closely observe the interactions between stress hormones and keratinocytes under controlled conditions.

Previous research has suggested that cortisol can affect keratinocyte proliferation and differentiation. For example, elevated cortisol levels have been shown to inhibit keratinocyte proliferation, thinning the epidermis and making it more susceptible to environmental damage [4]. Additionally, cortisol has been found to downregulate the expression of certain proteins essential for skin barrier function, such as filaggrin and loricrin.

Adrenaline's role in skin health, while less studied, is equally important. Adrenaline can induce vasoconstriction, reducing blood flow to the skin and potentially impairing nutrient and oxygen delivery. This can result in pallor and a dull complexion, characteristic of "skin fatigue."

Understanding the interplay between psychological fatigue and skin roughness opens new avenues for therapeutic interventions. By identifying key molecular pathways influenced by stress hormones, we can develop strategies to mitigate their adverse effects on the skin. This could include topical treatments that counteract the effects of cortisol and adrenaline or lifestyle modifications aimed at reducing stress levels.

In conclusion, the relationship between psychological fatigue and skin roughness is complex and multifaceted. Our study seeks to shed light on this connection by focusing on the impact of stress hormones on keratinocytes within a 3-dimensional cultured tissue model. Through this research, we hope to contribute to the broader understanding of how psychological stress can manifest physically, particularly in terms of skin health.

2. Materials and Methods

Cell line

We used Normal Human Epidermal Keratinocyte (NHEK) (KURABO, Japan). These were cultured with HuMedia-KG2 (KURABO, Japan) at 37℃, 5.0% CO_2. The three-dimensional skin model used was the LabCyte EPI-MODEL 6D purchased from J-TEC in Japan.

Adrenaline and cortisol stimulation for NHEK

NHEKs were seeded onto a 24-well plate at 1.0×10^5 cells/well. And adrenaline was added to 100 μM or cortisol to 1 μM. After 24 hours, the cells were harvested using ISOGEN II (Nippon Gene, Japan).

Cortisol stimulation for 3-dimentional skin model

After 24 hours of pre-incubation of LabCyte EPI-MODEL 6D, cortisol was added to the culture medium at 0.3 or 3 μM and cultured for 8 days. At the same time, the epidermis side was repeatedly exposed to purified water or plant extract for 3 hours. After incubation, the tissue was fixed in formalin, sections were prepared, and then subjected to immunostaining. Total RNA was also extracted using ISOGEN II.

RT-qPCR

NHEKs were seeded onto 24-well plates in HuMedia-KG2. The cells were collected with 0.5 mL of ISOGEN II reagent (Nipon gene, Japan). The recovered total RNA was subjected to reverse transcription reaction using the prescribed kit (PrimeScript RT reagent Kit with gDNA Eraser, TAKARA BIO, Japan), and cDNA was synthesized. All gene expression levels were measured by using Thermal Cycler Dice Real Time System Single with SYBR Premix Ex TaqTM2(TAKARA BIO, Japan). The test results were compared with the expression of each gene in each test section when the expression of the GAPDH gene as a housekeeping.

Immunohistochemistry

the cells were washed twice with PBS and shaken in PBS containing 0.5% Triton-X100 at room temperature for 1 hour, and then shaken in Blocking solution (1% BSA/0.1% Tween20/PBS) for 1 hour. Primary antibodies (anti-occludin, anti-transglutaminase 1) were diluted 200 folds in blocking solution and allowed to stand for overnight at 4℃. After removing the supernatant,

the cells were washed 5 times with PBS containing 0.5% Triton-X100 for 5 minutes, and then permeabilized by adding secondary antibody (Alexa 488 or 546 labeled) and shading at room temperature for 2 hours. After removing the supernatant, the cells were washed 5 times with PBS containing 0.5% Triton-X100 for 5 minutes. Then, 1,000 folds diluted Hoechst 33342 with PBS was added to each sample. We observed by BZ-X810 (KEYENCE, Japan).

3. Results

3.1. Both of adrenaline and cortisol decrease mRNA expression level of filaggrin in NHEK

Normal human epidermal keratinocytes were cultured for 24 hours with 100 μM adrenaline and 1 μM cortisol, which are stress hormones. Real-time PCR showed that filaggrin expression was decreased in the adrenaline exposure group (Figure 1a). Likewise, filaggrin expression was decreased in the cortisol exposure group (Figure 1b).

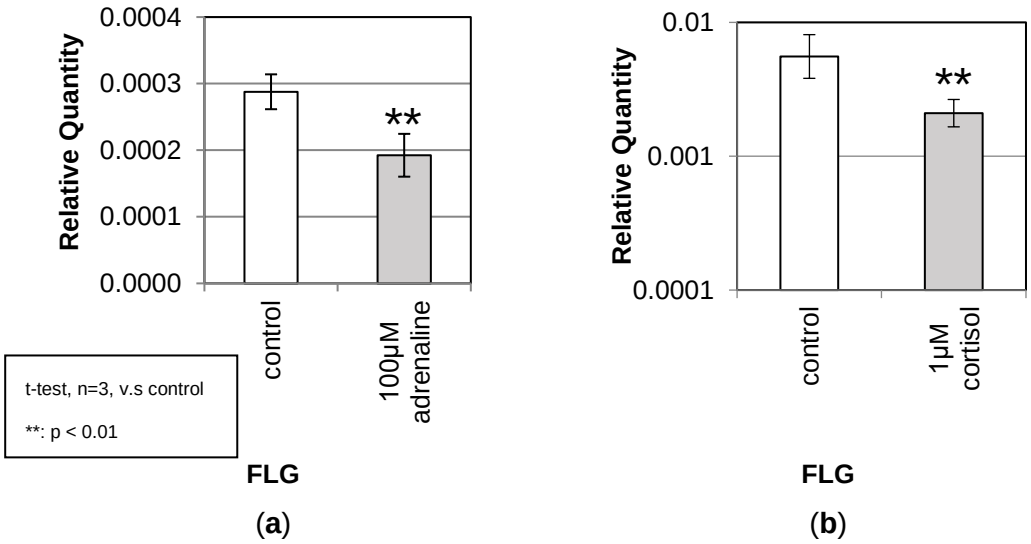

Figure 1. *Normal human epidermal keratinocytes (NHEKs) were seeded on a 24-well plate and stimulated with 100 μM adrenaline or 1 μM cortisol. After stimulation, the cells were cultured for 24 hours. Total RNA was collected and the expression level of FLG was plotted relative to that of GAPDH as a housekeeping gene by RT-qPCR.*

3.2. Adrenaline decrease mRNA expression level of genes associated with tight junction in NHEK

Next, the expression levels of tight junction-related proteins were compared by real-time qPCR when 100 μM adrenaline was applied to normal human epidermal keratinocytes. It was confirmed that the expression levels of CLDN1, OCLN and DKK1 decreased (Figures 2a, b, and d). On the other hand, adrenaline exposure did not affect the expression levels of TGM1 in normal human epidermal keratinocytes (Figure 2c). Similarly, when the expression levels of tight junction-related proteins were compared by real-time qPCR when 1 μM cortisol was applied to normal human epidermal keratinocytes, it was confirmed that the expression levels of CLDN1 decreased, and the expression levels of TGM1 were significantly reduced (Figures 2e,f).

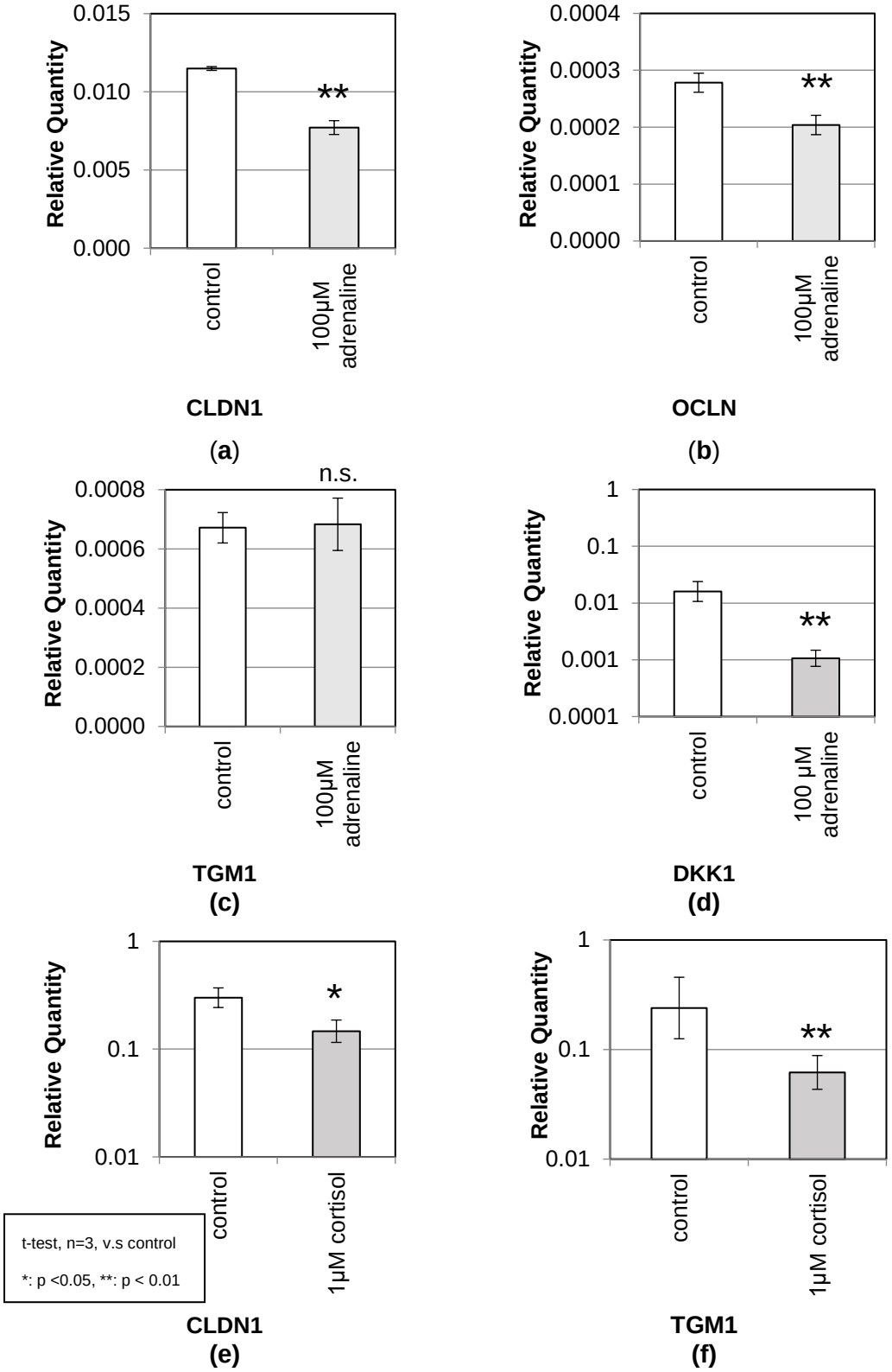

Figure 2. *Normal human epidermal keratinocytes were seeded on a 24-well plate and stimulated with 100 μM adrenaline or 1 μM cortisol. After stimulation, the cells were cultured for 24 hours, and in the case of cortisol, for 96 hours. Total RNA was collected and the expression level of CLDN1, OCLN, TGM1, DKK1 was plotted relative to that of GAPDH as a housekeeping gene by RT-qPCR.*

3.3. Cortisol makes a thinner stratum corneum in LabCyte EPI-MODEL 6D

LabCyte EPI-MODEL 6D was cultured for several days with 0.3 μM cortisol added to examine the effects on stratum corneum formation. After culturing, LabCyte EPI-MODEL 6D was stained with HE, and as shown in Figure 3, the thickness of the stratum corneum decreased.

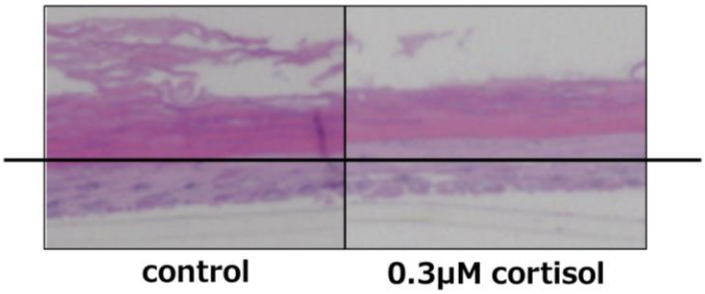

Figure 3. *After adding 0.3 μM cortisol to the culture media side and culturing the three-dimensional skin model for a total of 8 days, it was fixed with formalin and HE staining was performed. A tendency for the stratum corneum, which was stained pink, to become thinner was confirmed.*

3.4. Cortisol reduces the transglutaminase 1 in LabCyte EPI-MODEL 6D

Subsequently, LabCyte EPI-MODEL 6D was cultured for 8 days with 3 μM cortisol added and then fixed and immunostained (Figure 4). No significant changes were observed in Occludin, but a decrease in signal intensity of transglutaminase 1 was observed.

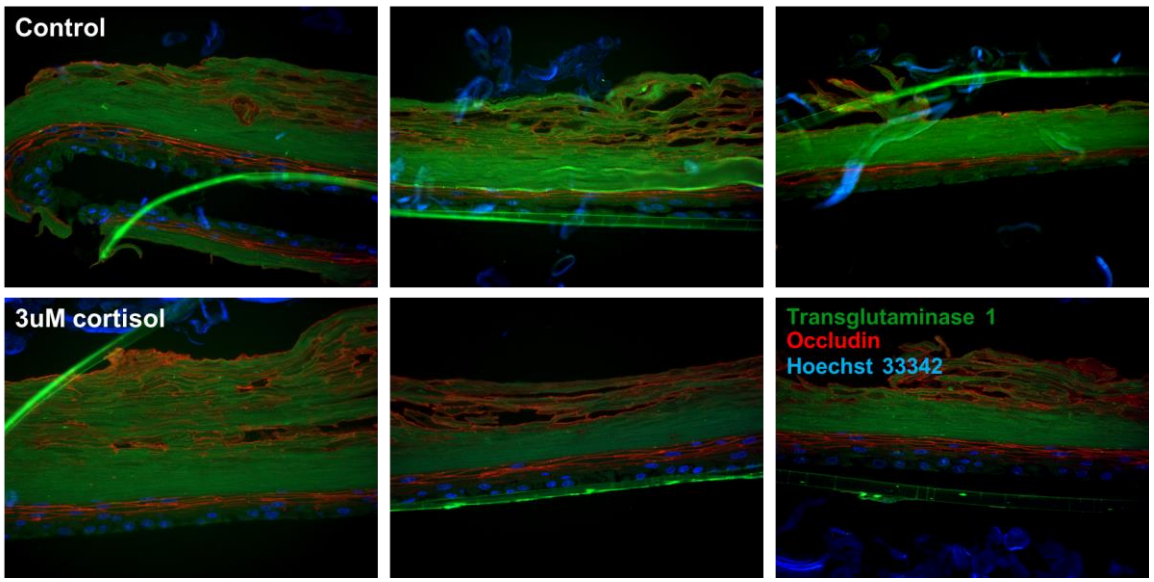

Figure 4. *LabCyte EPI-MODEL 6D was cultured with or without 3 μM cortisol for 8 days. Sections of the 3D model were created after fixation in formalin. They were stained with anti-transglutaminase 1, occludin antibody for immunohistochemistry and Hoechst 33342 for DNA*

staining. Green shows trans transglutaminase 1. Red shows occludin. Blue shows cellular nuclear.

3.5. Cortisol reduces the transglutaminase 1 in LabCyte EPI-MODEL 6D

LabCyte EPI-MODEL 6D was cultured with 3 μM cortisol added and then *Citrus tachibana* leaf extract was added from the stratum corneum side. After culturing, Labcyte EPI-MODEL 6D was immunostained with anti-Occludin antibody and anti-transglutaminase 1 antibody, and it was confirmed that the signal intensity of transglutaminase 1 improved with *Citrus tachibana* leaf extract (Figure 5).

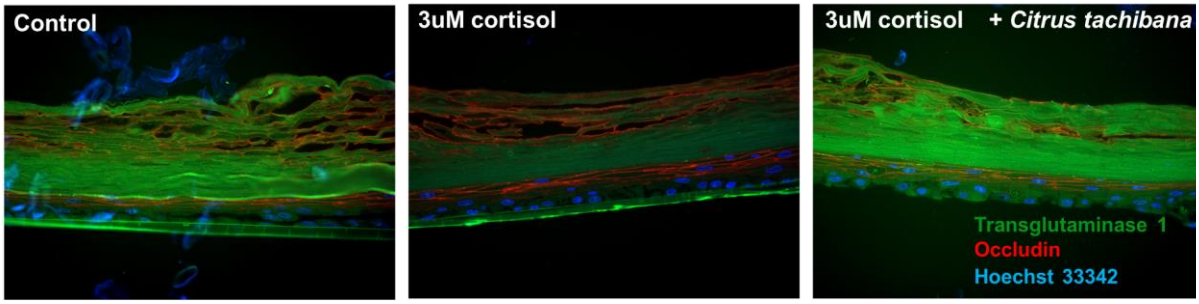

Figure 5. *LabCyte EPI-MODEL 6D was cultured with or without 3 μM cortiso and 2.0% Citrus tachibana extract for 8 days. Sections of the 3D model were created after fixation in formalin. They were stained with anti-transglutaminase 1, occludin antibody for immunohistochemistry and Hoechst 33342 for DNA staining. Green shows transglutaminase 1. Red shows occludin. Blue shows cellular nuclear*

3.6. Polymethoxyflavonoids normalize stratum corneum formation by increasing gene expression in epidermal keratinocytes.

Finally, *Citrus tachibana* leaf extract was fractionated into single components and added to normal human epidermal keratinocytes for 24-hour culture. When expression levels of genes such as filaggrin and transglutaminase 1 were compared using real-time qPCR (Table 1), several polymethoxyflavonoids were identified (Figure 6).

Table 1. Comparison of polymethoxyflavonoids and NMF-related gene expression in normal human epidermal keratinocytes

Sample	PADI1	PADI3	CASP14	TGM1
Control	100.0	100.0	100.0	100.0
5,6, 7,3' ,4' -pentamethoxyflavanone	127.5	132.9	115.7	136.6
Citromitin	77.4	49.3	90.8	89.5
Nobiletin	130.1	141.4	97.9	180.3
Tangeretin	109.4	87.7	185.3	200.0

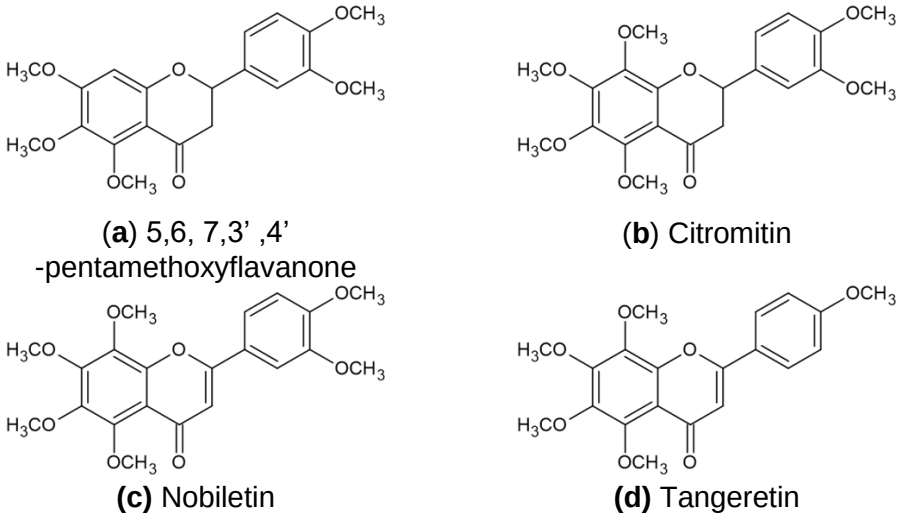

Figure 6. *Molecular skeleton of polymethoxyflavonoids.*

4. Discussion

Both of adrenaline and cortisol influence the natural moisturizing factors. Then adrenaline reduces DKK1 expression in keratinocytes, potentially increasing melanin production in pigment cells. Cortisol reduces the expression of TGM1 which is granular layer markers in NHEK. And cortisol makes a thinner stratum corneum in LabCyte EPI-MODEL 6D and decrease the expression of transglutaminase 1. These findings suggest that psychological stress leads to skin dryness and pigmentation. Finally, polymethoxyflavonoids extracted from *Citrus tachibana* improved the decrease of FLG caused by cortisol.

5. Conclusion

In summary, our study highlights the intricate relationship between psychological fatigue and skin roughness, emphasizing the role of stress hormones like cortisol and adrenaline. We demonstrated that these hormones negatively impact keratinocyte functions, leading to reduced expression of filaggrin and transglutaminase 1, which are essential for maintaining skin health. Additionally, the application of cortisol in 3D cultured tissue models resulted in a thinner stratum corneum (Figure 3).

Importantly, we found that extracts from *Citrus tachibana* leaves can counteract these detrimental effects by improving the expression levels of filaggrin and transglutaminase 1. This discovery opens potential avenues for developing therapeutic interventions to mitigate the adverse effects of psychological stress on skin health. By understanding and targeting the molecular pathways influenced by stress hormones, we can enhance skin resilience and overall well-being (Figure 7).

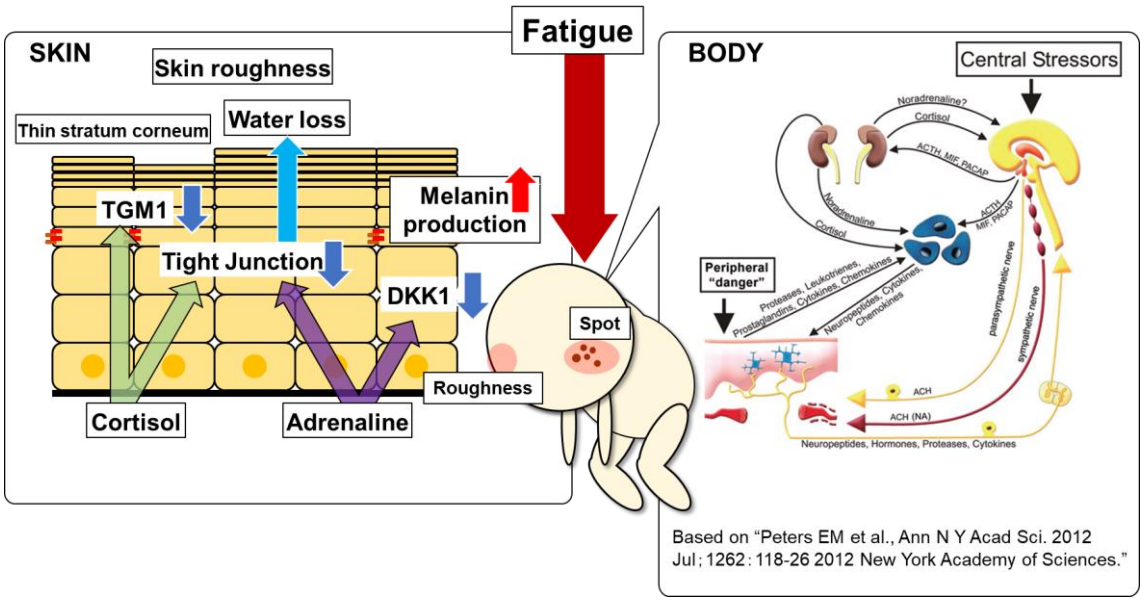

Figure 7. Conclusion for "Our thoughts on skin fatigue".

5. Reference

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