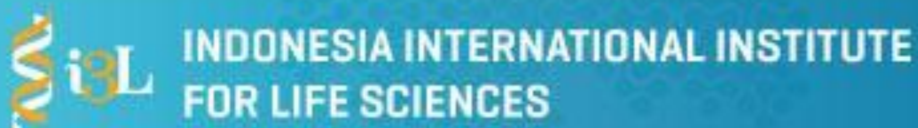

Study report : **PAR-03-2200704**
Quotation No : **YAY-2205-00034**

TITLE

In vitro study of the effect of moisturizer 0921-F dan 0921-G on gene expression related to inflammation, nerve sedation and skin barrier

Study Sponsor : PT. Paragon Innovation and Technology
Laboratory : Skinovation Centre, Indonesia International Institute for Life Sciences
Principal Investigator : apt. Pietradewi Hartrianti, Ph.D

The investigators, data collector, author and everyone involved in this study hereby certify the validity of the data presented in the report and has come to an agreement to the conclusions written at the end of the report.

The Head of Laboratory and Laboratory Coordinator also certifies that a quality control has been performed at each stage of the method described in a specific protocol. This control leads to accurate investigations to the tested product and to guarantee the reliability of analyzed data in accordance with this standard procedure. The present report constitutes a precise description of the performing of experimentation, processing of data and detailed procedure that have been used.

Any information concerning products to study such as patents, formulae, raw materials, manufacturing procedures etc. directly or indirectly provided by the sponsor of the study, will be regarded as confidential, will remain his property and will not be disclosed without his express consent.

Date : September 21 2022

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1. Introduction

Within the last few decades, sensitive skin has become a concern in 71% of the population (Chen et al., 2020). This condition is usually characterized by unpleasant feelings to the skin, including stinging, burning, and itching sensations and is usually triggered by environmental factors such as sun exposure and pollution or the presence of irritating substances in cosmetic products (e.g., AHA, alcohol, fragrances) (Diehl, 2018; Duarte et al., 2017). Although the pathophysiology of sensitive skin is not yet well understood, sensitive skin is currently thought to be the result of the decrease in stratum corneum thickness, which is the outer layer of the skin, and/or the dysfunction of neural activities in the skin. These two conditions will further trigger a series of inflammation which will worsen sensitive skin symptoms (Talagas & Misery, 2019). Currently, various treatments have been developed to treat sensitive skin. One of the well-known treatments is the use of emollient cream or moisturizer. Moisturizer is mentioned to be able to support skin barrier enhancement while reducing skin inflammation in subjects with higher skin sensitivity, which leads to the promising potential of moisturizer development that is targeted for sensitive skin (Baldwin, Santoro, Lachmann, & Teissedre, 2019).

This study aims to evaluate a sensitive skin moisturizer (Moisturizer-0921-F) along with its base and active pharmaceutical ingredients (API) for their capability in improving skin barriers without eliciting neurological and skin inflammation.

2. Material and Methods

2.1. In vitro model

- Model : HaCaT cell line (immortalized human keratinocytes cells).
- Culture medium : Dulbecco's Modified Eagle Medium (DMEM)
  Containing:
  - 2 mM L-glutamine
  - Pyridoxine hydrochloride

  Supplemented with:
  - 3.7 gr/L sodium bicarbonate
  - 1% Penicillin-Streptomycin (Pen-Strep)
  - 10% fetal bovine serum (FBS)
- Culture condition : 37°C, 5% CO_2.

2.2. Test Compound (Groups of study or experimental design)

Sponsor	Product Reference	Reception Date of The Study	Aspect	Product Storage	Study Date
PT Paragon Technology and Innovation	Moisturizer-0921-F	30/9/21	Cream	In a jar, protected from light	8/10/2021 to 13/3/2022
	Moisturizer-0921-G		Cream	In a jar, protected from light	
	Raw Mat-0921-A		Powder	In a plastic clip, protected from light	
	Raw Mat-0921-F		Liquid	In a transparent bottle, protected from light	
	Raw Mat-0921-G		Liquid	In a transparent bottle, protected from light	
	Raw Mat-0921-H		Liquid	In a transparent bottle, protected from light	

	Raw Mat-0921-P		Powder	In a plastic clip, protected from light	
	Raw Mat-0921-R		Liquid	In a transparent bottle, protected from light	
	Raw Mat-0921-T		Liquid	In a transparent bottle, protected from light	
	Raw Mat-0921-O		Liquid	In a transparent bottle, protected from light	

2.3. Method

2.3.1. Method Origin

The effect of the moisturizer was analyzed through a gene expression analysis by observing the expression of the skin barrier, inflammation, and nerve sedation-related genes following a cell cytotoxicity study. These analyses were conducted based on the methods described by Kim and colleagues in 2018, with the title of “Skin Protective Effect of Epigallocatechin Gallate”, which was modified and suited for this study's objectives.

2.3.2. Method Principle

The study utilized monolayer HaCaT cell culture for the analysis of the gene expression upon cell treatment with the moisturizer samples. Prior to gene expression analysis, a cytotoxicity test was conducted to determine the suitable concentration of the moisturizer product, base, and API to be incorporated into the gene expression study, in which the proper treatment concentration was determined based on two parameters: significance test through statistical analysis and the guideline provided by ISO-10993-5 for the in vitro cytotoxicity test, where cell viability <70% is considered cytotoxic. After determination of the sample's concentration, analysis of gene expression related to skin inflammation (*IL1A, IL1B, CALCB, CXCL8*), neurological inflammation (*TRPV1, TNFa),* and skin barrier integrity (*CERS3, SMPD1, FLG, HAS2*) was performed through qRT-PCR study. This method measures the relative expression of the related genes upon cell treatment compared to control (untreated). The gene expression analysis was conducted in four biological replicates, with each biological replicate consisting of two technical replicates.

2.3.3. Description of The Method

2.3.3.1. HaCaT Cell Culture

The HaCaT cells were obtained as a gift given by Professor Ng Kee Woei from the School of Materials Science and Engineering, Nanyang Technological University. HaCaT cells were maintained in a T25 flask and cultured in a standard two-dimensional model. The cells were grown in Dulbecco's modified eagle medium (DMEM) containing L-glutamine and pyridoxine hydrochloride, supplemented with 3.7 g/L sodium bicarbonate, 1% penicillin-streptomycin, and 10% fetal bovine serum. The cells were maintained in a humidified atmosphere at 37°C in the 5% CO_2 incubator.

2.3.3.2. Cytotoxicity Test

The cytotoxicity test was performed following the MTS assay protocol (CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay, G3582). Prior to the MTS assay, the cells were seeded to a 96-well plate with the density of 0.01 x 10^6 and grown until they reached approximately 80% confluence.

Along with the cell seeding process, treatment media were made by preparing five different concentrations of product, base/vehicle, and API **(Table 2.3.3.2.1).** To determine the cytotoxic concentration of the tested moisturizer towards

HaCaT cell lines and also to determine an optimum amount of the moisturizer to be used, the amount of cream applied to the skin must equal the amount applied to the cell culture. The standard amount of any cream to be applied to one's face is 1 gram. This number is based on a clinical trial done by Kaewsanit et al. (2021) whereby the application of a topical product on a face is equal to 2 FTUs, or 1 gram. According to Long & Finlay (1991), two FTUs can cover ≅572 cm^2 of skin. Because the treatment will be done in a 24-well plate for the gene-expression analysis, and a 96-well plate for the MTS assay, the amount of cream has to be scaled down accordingly.

$$\frac{amount\ of\ cream\ on\ skin}{amount\ of\ cream\ on\ 24-well\ plate} = \frac{572}{1.9}$$

Figure 2.3.3.2.1. The formula to convert the range of concentration to be tested for their cytotoxicity so that it is equal to the surface area of a 24-well plate.

Using the formula in **figure 2.3.3.2.1.** the aforementioned range is converted to a range equal to the surface area of a 24-well plate and is illustrated in **table 2.3.3.2.1.** This study will also evaluate the expression level of just the base of the moisturizer and the active pharmaceutical ingredient (APIs) of the moisturizer.

The stock media was prepared with the highest treatment media concentration. The samples were weighed and added with DMEM only, in which the solutions were heated at 60-80°C and vortexed until the samples were completely dissolved. The solutions were centrifuged at 10,000 rpm for 10 minutes and then sonicated for approximately 6 hours until the solutions were completely homogenized. Serial dilution was conducted to achieve the desired concentrations as listed in **table 2.3.3.2.1**. The treatment media was sterilized through a filtration process with a 0.22 µm polyethersulfone (PES) syringe filter and the sterilized media were transferred to a sterile 1.5 mL microcentrifuge tube.

After reaching 80% confluency, the cells were treated with 168.42 µL of treatment media and incubated for 48 hours in the standard cell culture condition. The MTS assay began by replacing the old treatment media with 100 µL DMEM only. 15 µL of MTS reagent was added to each well and the cells were incubated for 3 hours at 37°C in a 5% CO_2 incubator. To measure the cell viability, the absorbance was measured with a 96-well spectrophotometer at 490 nm. The cell viability was calculated with the formula shown in **figure 2.3.3.2.2**.

Table 2.3.3.2.1. The treatment media concentration of the moisturizer product, base, and API for cytotoxicity test.

Samples	Concentration (mg/mL)					Treatment Volume (µL)
	1	2	3	4	5	
Product	4.32	3.32	2.32	1.32	0.32	168.42

Base	3.92	3.02	2.11	1.20	0.29	
API	0.40	0.30	0.21	0.12	0.03	

$$Cell\ viability\ (\%) = \frac{Absorbance\ of\ samples - absorbance\ of\ blank}{Absorbance\ of\ control - absorbance\ of\ blank}\ x\ 100\%$$

Figure 2.3.3.2.2. The formula used to calculate the cell viability percentage based on the absorbance measurement.

Table 2.3.3.2.2. The layout of the 96-well plate for the MTS assay of the moisturizer. C1-C5: is the range of concentration listed in **table 2.3.3.2.1. (-)** Control: Cells + DMEM; Blank: DMEM

	1	2	3	4	5	6	7	8	9	10	11	12
A	C1	C1	C1	C1	C1	C1	C1	C1	C1			
B	C2	C2	C2	C2	C2	C2	C2	C2	C2			
C	C3	C3	C3	C3	C3	C3	C3	C3	C3			
D	C4	C4	C4	C4	C4	C4	C4	C4	C4			
E	C5	C5	C5	C5	C5	C5	C5	C5	C5			
F												
G												
H												

Legend: Product (columns 1–3, rows A–E); Base (columns 4–6, rows A–E); API (columns 7–9, rows A–E); (-) Control (columns 1–3, row F); Blank (columns 1–3, row G)

2.3.3.3. Gene Expression Analysis

Prior to the cell treatment media preparation, the HaCaT cells were seeded to a 24-well plate with a cell density of 0.05 x 10^6 cells per well with 500 µL complete media. The cells were cultured in a standard condition until it reached approximately 80% cell confluency.

Based on the MTS assay results, concentration of product, base, and API of 2.32 mg/mL, 2.11 mg/ml, and 0.21 mg/mL, respectively, were used for the gene expression analysis as they maintained cell viability of >70% and they create no significance difference in cell viability compared to the control group. The treatment media was prepared by weighing the necessary amount of samples and mixed with DMEM only by heating at 60-80°C and vortexing until the samples were completely dissolved. The solutions proceeded to the centrifugation process at 4,500 rpm for 30 minutes and continued with sonication for approximately 6 hours for complete homogenization. The treatment solutions were sterilized through a filtration process with a 0.22 µm syringe filter and transferred to a new sterile 15 mL centrifuge tube.

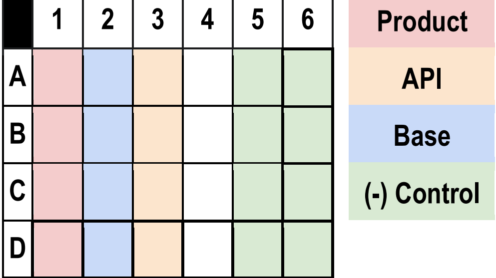
The cell treatment process was conducted by treating the seeded cells with 1 ml of the prepared treatment media. The cells were treated for 48 hours in a standard condition. After 48 hours, the gene expression analysis

began by extracting the cellular RNA with GENEzol™ TriRNA Pure Kit (GeneAid, GZX100) according to the manufacturer's protocol. The quality and quantity of the extracted RNA were determined with NanoDrop™ Lite Spectrophotometer (Thermo Scientific™) and the RNA integrity was validated with agarose gel electrophoresis. The extracted RNA was converted to cDNA through the cDNA synthesis process using RevertAid First Strand cDNA Synthesis Kit (Thermo ScientificTM, K1622) according to the manufacturer's protocol.

The gene expression was analyzed using qRT-PCR, in which the kit was sourced from QuantiNova® SYBR® Green RT-PCR Kit (QIAGEN®). Non-template control was incorporated in the study to validate the absence of primer-dimer and reagent contamination. The fold change was calculated using the Pfaffl method (2001), in which the fold change and relative gene expression were calculated with the consideration of the primer efficiency value. The specificity of the PCR product was validated through the melting curve analysis and agarose gel electrophoresis.

Table 2.3.3.3.1. The layout of the 24-well plate for the RT-PCR of the moisturizer. **(-)** Control: Cells + DMEM; Blank: DMEM.

	1	2	3	4	5	6
A						
B						
C						
D						



2.3.4. Materials and Equipment

The materials and equipment used in this study were listed in **table 2.3.4.1**.

Table 2.3.4.1. The list of materials and equipment utilized and incorporated in the study.

No.	Materials	Equipment
1	T25 flask	Biosafety Cabinet
2	24 well plate	Pipette Gun
3	96 well plate	Micropipette

4	1000 µL micropipette tips	Inverted microscope
5	200 µL micropipette tips	Binocular microscope
6	10 µL micropipette tips	Tally Counter
7	25 mL serological pipette	Hemocytometer
8	10 mL serological pipette	Cell incubator
9	5 mL serological pipette	Centrifuge
10	50 mL centrifuge tube	Ice Box
11	15 mL centrifuge tube	Gel Electrophoresis Apparatus (Tank, PSU, Gel Casting Tray)
12	1.5 mL microcentrifuge tube	Syngene G:Box
13	0.2 mL PCR tube	SimpliAmp™ Thermal Cycler
14	10 mL syringe	Rotor-Gene Q Thermal Cycler
15	3 mL syringe	Analytical Balance
16	1 mL syringe	Bandelin Sonorex Sonicator Bath
17	0.22 µm polyethersulfone syringe filter	NanoDrop™ Lite Spectrophotometer (Thermo Scientific™)
18	Dulbecco's Modified Eagle Medium	Multimode Microplate Reader: Infinite® 200 PRO NanoQuant
19	Fetal bovine serum	
20	Sodium bicarbonate	
21	Penicillin-Streptomycin	
22	Trypsin-EDTA	
23	CellTiter 96® AQueous One Solution Reagent (Promega)	
24	GENEzol™ TriRNA Pure Kit (GeneAid, GZX100)	

25	RevertAid First Strand cDNA Synthesis Kit (Thermo ScientificTM, K1622)	
26	QuantiNova SYBR Green PCR Kit (QIAGEN)	
27	Absolute ethanol	
28	RNase-free Water	
29	1 kb DNA ladder	
30	GeneRuler 50 bp DNA Ladder	
31	Loading dye	
32	Agarose powder	
33	TAE buffer	
34	SYBR Safe gel stain	
35	Trypan blue	
36	Aluminum foil	
37	Cling wrap	
38	Moisturizer Product	
39	Moisturizer Base	
	Cetearyl Alcohol, Cetearyl Glucoside, Water, Glucose	
	Glyceryl Stearate, PEG-100 Stearate	
	Dicaprylyl Carbonate	
	Shea Butter (Butyrospermum parkii)	
	Cetearyl Alcohol	

		Dimethicone	
		Squalane	
		Dimethicone, Polysilicone-11, Butyrospermum Parkii (Shea) Butter	
		Hydroxyethyl Acrylate/Sodium Acryloyldimethyl Taurate Copolymer	
		Butylene Glycol	
		Glycerin	
		Xanthan Gum	
		Disodium EDTA, Water	
		Chlorphenesin	
		Caprylhydroxamic Acid (and) 1,2-Hexanediol (and) Propanediol	
40	Moisturizer API		
		Allantoin	
		Bifida Ferment Lysate	
		Butylene Glycol (and) Propanediol (and) Mirabilis Jalapa Extract	
		Bisabolol	
		Water, Phragmites Kharka Extract, Poria Cocos Extract, Citric Acid, Sodium Citrate, Sodium Benzoate	
		Saccharide Isomerate, Aqua, Citric Acid, Sodium Citrate	

		Tocopheryl Acetate	

2.4. Data Management, Calculation, and Statistical Analysis

The results were processed with GraphPad Prism 8.0.1 (GraphPad Software, San Diego, California USA), in which the results of the cytotoxicity test and the gene expression analysis were described as mean ± standard deviation (SD) and mean ± standard error of the mean (SEM), respectively. The distribution of the data was analyzed using the Shapiro-Wilk test. For the significance test, the normally distributed data was processed with one-way ANOVA. Meanwhile, the Kruskal-Wallis test was applied for the data that were abnormally distributed. The post-hoc test was further conducted to analyze the significant correlation between each sample, in which Dunnett's multiple comparison test was applied for the results of one-way ANOVA, while Dunn's multiple comparison test method was conducted for the Kruskal-Wallis test results. The confidence interval of the study was 95%, in which the p value below 0.05 was considered as statistically significant.

3. Result

3.1. MTS Assay

The treatment of HaCaT cells with the moisturizer revealed no significant changes in cell viability in all tested concentrations, suggesting that the moisturizer is safe at all these tested concentrations. Meanwhile, 3.92 and 3.02 mg/ml of the base were shown to cause a significant decrease in cell viability, which indicates their toxicity potential. The API, on the other hand, caused significantly higher cell viability, especially in the lower concentrations range, suggesting that the API might have proliferative capabilities (**Figure 3.1.1.)**.

Based on these results, 2.32 mg/ml of moisturizer, which equals 2.11 mg/ml of base and 0.21 mg/ml of API, was chosen as the concentration to be tested in the gene expression analysis. These concentrations were chosen as they are the highest concentrations that do not cause cytotoxic effects.

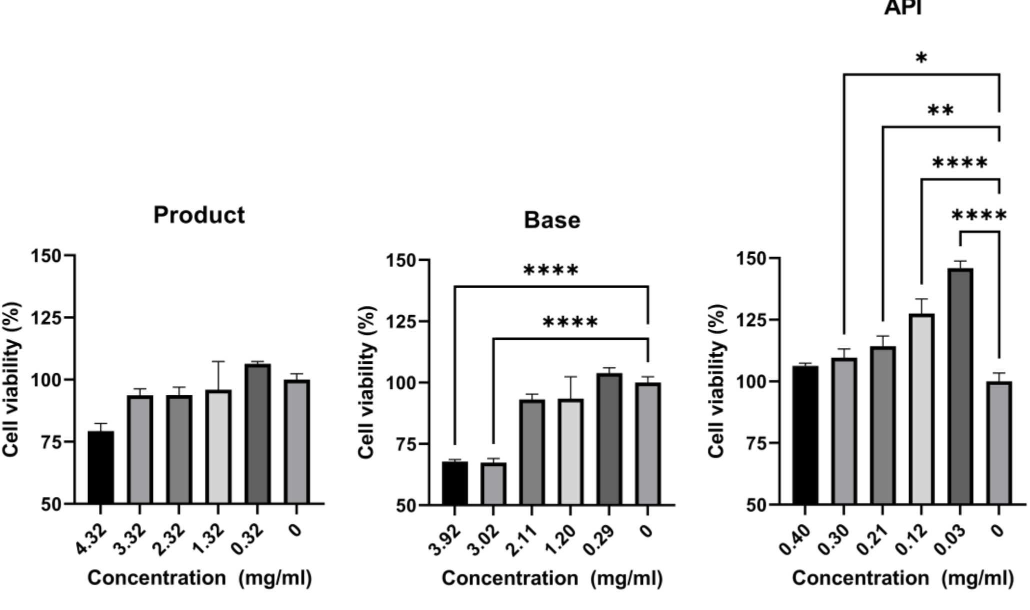


Figure 3.1.1. The MTS assay result for the product, base, and API of the moisturizer at varying concentrations (n=3).

3.2. qPCR

3.2.1. Inflammatory Potential

The gene expression analysis of *IL1A* (IL-1a)*, IL1B* (IL-1b)*, CALCB* (CGRP)*,* and *CXCL8* (IL-8) as displayed in **figure 3.2.1.1** demonstrated that the moisturizer, be it the product, base, or API

caused no significant difference in terms of their gene expression. There was a steep increase in the relative expression of IL-1a to the negative control, which was 3.375 fold higher. However, the statistical analysis revealed that this difference was not significant (P-value > 0.99). The relative expression of IL-1b among the treatment groups was more or less the same, denoted by the lack of significant difference in terms of relative expression among the treatment groups. For IL-8, the base and the API of the moisturizer reduced the expression of IL-8 by 0.5 fold, albeit not statistically significant. This was not the case when the base and the API were mixed together (product), as the product did not cause any significant increase in terms of IL-8 gene expression. For CGRP, the expression of *CALCB* was relatively unchanged, denoted by the lack of significance in terms of relative expression increase or decrease among the treatment groups relative to the negative control.

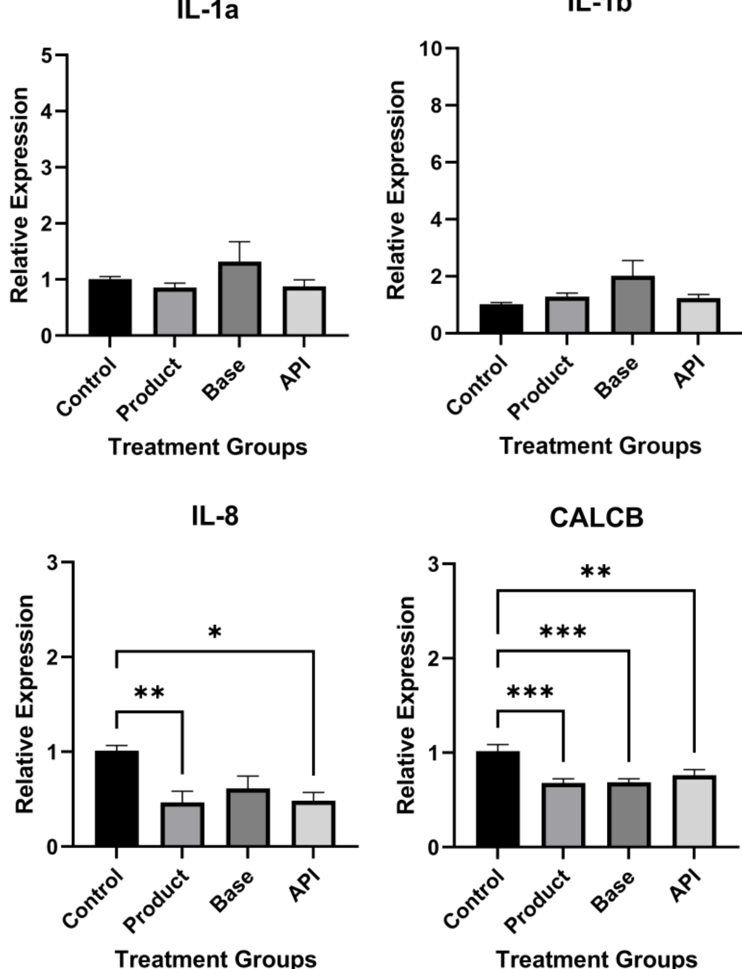


Figure 3.2.1.1 The relative expression of genes to monitor for the inflammatory potential of the moisturizer (n=8).

3.2.2. Nerve sedation

Gene expression analysis of the nociceptors TRPV1 and inflammatory cytokine TNFa revealed no significant changes upon treatment with the moisturizer, base, and API (**Figure 3.2.2.1)**.

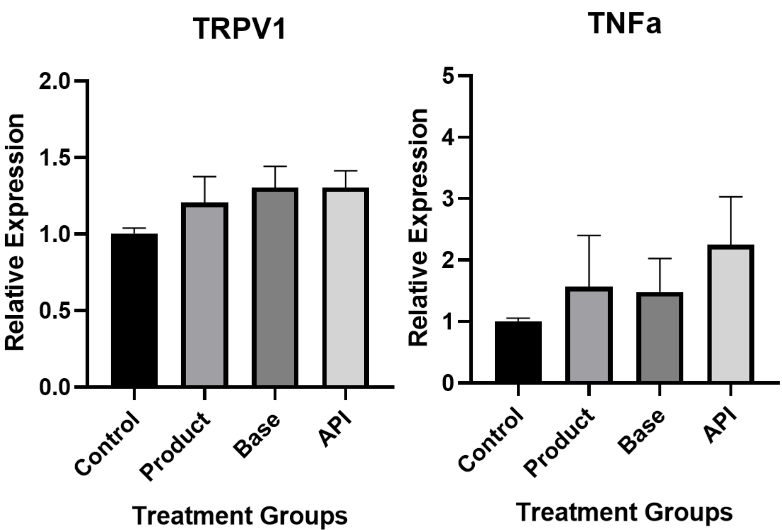
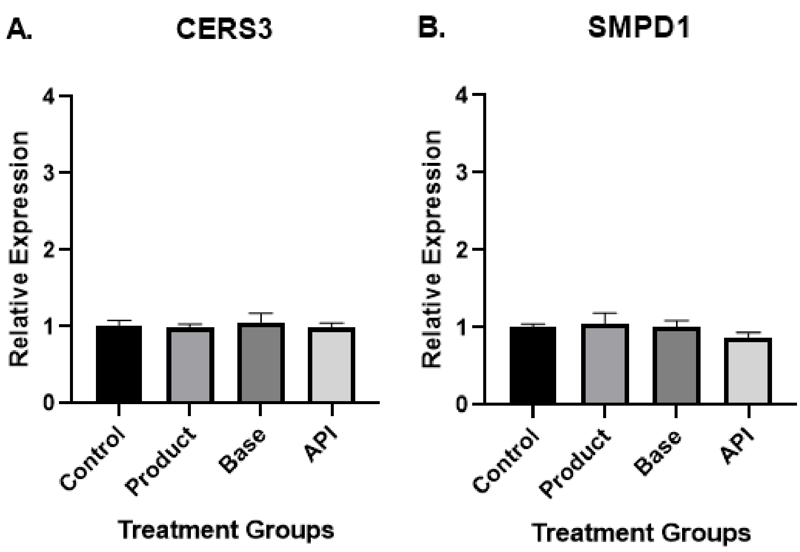


Figure 3.2.2.1 The relative expression of genes to monitor for the nerve sedation effect of the moisturizer (n=8).

3.2.3. Skin Barrier Integrity

The expression of several genes was assessed to determine the ability of the moisturizer in elevating the skin barrier integrity, specifically the genes that are related to ceramide biosynthesis (CERS3 and SMPD1), filaggrin biosynthesis (FLG), and hyaluronic acid biosynthesis (HAS2). The relative expression of the skin barrier-related genes was displayed in **figure 3.2.3.1**. Overall, it was shown that there were no significant differences in the relative expression of CERS3, SMPD1, and HAS2 upon the cell treatment with the moisturizer samples, which may indicate the absence of moisturizer formulation effect towards the ceramide and hyaluronic acid biosynthesis. However, the product and the base formulation of the moisturizer was proven to be able to significantly elevate the FLG gene expression by two-fold and three-fold, respectively. This result indicates the ability of the moisturizer to elevate the skin barrier integrity through the elevation of filaggrin protein production.



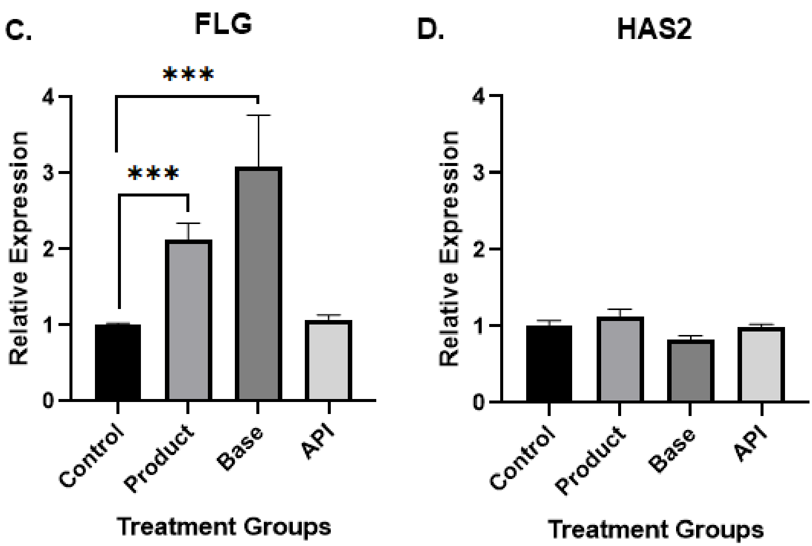


Figure 3.2.3.1 The relative expression of genes to monitor the moisturizer's ability in enhancing the skin barrier (n=8).

4. Conclusion

Considering that the moisturizer **does not cause any neurological and skin inflammation** as assessed through the qRT-PCR analysis, this sensitive skin moisturizer is deemed to be suitable for the usage by those with sensitive skin. Furthermore, the moisturizer product is also shown to be able to **elevate the skin barrier integrity through the elevation of the filaggrin gene expression**.

5. References

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