The innovative approach to develop cosmetics for sensitive skin: the relation between preservatives and inflammatory cytokines

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Abstract

Background: Although preservatives are essential ingredients to maintain the quality of cosmetic products, these are considered to be one of the causes of skin irritation. In the IFSCC 2020 Yokohama Congress, we reported that several preservatives increase the levels of inflammatory cytokines in our skins. On the other hand, many ingredients which have the same effect as preservatives are used in most cosmetics for sensitive skin. However, there are no reports investigating the expression of inflammatory cytokines induced by these ingredients. Thus, we examined the inflammatory cytokines induced by preservatives commonly used in cosmetics worldwide and their alternatives, and how they affect the formation of stratum corneum.

Methods: Normal human epidermal keratinocytes (NHEK) and a reconstructed human epidermis (RHE) were treated with preservatives and their alternatives. The gene expressions were examined by quantitative PCR, and productions were done by enzyme-linked immunosorbent assay (ELISA). Furthermore, we evaluated the formation of stratum corneum by transepidermal water loss (TEWL). As for the evaluation of preservative efficacy, we used our original method of inoculating the product with bacterium and culturing them to observe their log reduction.

Results: The expression levels of inflammatory cytokines, interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α), were different depending on the type of preservatives and their alternatives. Furthermore, we found that anti-inflammatory cytokine, Interleukin-37 (IL-37), was also affected by them, and some of them decelerated the formation of stratum corneum.

Conclusion: This study provides evidence that preservatives and their alternatives should be selected carefully, even though the alternatives are not defined as preservatives.

Keywords: Preservatives, Sensitive skin, Inflammatory cytokines, Anti-inflammatory cytokines

Introduction.

We have often seen cosmetics for sensitive skin as consumer's awareness of sensitive skin and the interest of hypoallergenic cosmetics are increasing worldwide in recent years. Although preservatives are essential ingredients to maintain the quality of cosmetic, they are considered to be one of the causes of skin irritation. As the data to support this fact, in the IFSCC 2020 Yokohama Congress, we reported that several preservatives, such as phenoxyethanol, Ethylhexylglycerin, and 1,2-Hexanediol, increase the expression levels of inflammatory cytokines by activating transcription factors such as nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK). We mainly examined 5 preservatives and their aids, Methylparaben (MET), Pentylene Glycol (PEN), 1,2-Hexanediol (HEX), Ethylhexylglycerin (ETH), and Phenoxyethanol (PHE) in our previous study. However, it is the fact that not only these preservatives, but also a wider variety of preservatives and their alternatives are used in cosmetics recently following regulations of each country, trends, uses and properties. As one of the current trends, natural cosmetics and cosmetics for sensitive skin, which appeals parabens-free or no skin irritation, are gaining consumer's attention. Although these cosmetics often seem not to contain preservatives, they use ingredients which have antibacterial effects and are not listed as preservative on the positive list of cosmetic standards. They may be attractive to consumers, nevertheless, there are no reports investigating the expression of inflammatory cytokines induced by these alternatives. It is known that inflammatory cytokines induce not only the skin inflammatory response but also aging [1]. In addition, it is considered that suppression of inflammation is important in brightening since keratinocytes during inflammation produce melanocyte-stimulating factors such as endothelin-1 and prostaglandin, which lead to the formation of spots [2]. One of the representative examples of the cause of skin inflammation induced by

inflammatory cytokines is Ultraviolet rays. For example, several studies have indicated that ultraviolet rays can cause skin photodamage by stimulating reactive oxygen species (ROS) generation, activating downstream signaling and inflammatory cytokine formation [3]. The inflammatory response is mainly mediated by cytokines which are induced via MAPK and NF-κB pathways [4-6]. On the other hand, it has been reported that IL-37, as an anti-inflammatory cytokine, suppresses the release of inflammatory cytokines and plays a role as an inflammatory inhibitor within an organism. IL-37 was discovered as an anti-inflammatory and immunosuppressive cytokine of the IL-1 family [7]. IL-37 binds to IL-18R and recruits IL-1R8 to form the IL-37/IL-1R8/IL-18Rα complex which regulates signal transduction. IL-37 inhibits the increase of pro-inflammatory cytokines such as IL-6 [8]. In addition, human β-defensins (hBDs) elevated the expression and secretion of IL-37 via caspase-1, caspase-4, Smad3, CCR6, and the MAPK and NF-κB pathways in human keratinocytes [9]. However, the function of IL-37 in human skin has not been fully elucidated yet.

If we consider about the primary objective of cosmetics, cosmetics should be used for the purpose of keeping the skin healthy. It is important to know the action mechanism of the inflammatory reaction caused by preservatives and their alternatives to develop cosmetics which anyone can use. Primary skin irritation tests have been conducted regarding the safety of raw components used in preservatives and cosmetics, and while this data is abundant, there are no studies which investigate the expression of inflammatory cytokines induced by preservatives and their alternatives comprehensively except for our previous study.

In this study, we investigated the expression of inflammatory cytokines, IL-6, IL-8 and TNF-α, when NHEK cells and RHE models were treated with 10 preservatives and their alternatives which are often used worldwide: Potassium Sorbate (POT), Caprylyl Glycol (CAP), Sodium Benzoate (SOD), Ethylparaben (ETP), Butylparaben (BUT), and Propylparaben (PRO), Bisabolol (BIS), Butylene Glycol (BUG), Benzyl Alcohol (BEN), and Glyceryl Caprylate (GLY). Moreover, in order to reveal further mechanism and consequences happened on the skin, we evaluated the expression of IL-37, and how the preservatives and their alternatives affect the formation of stratum corneum.

Materials and Methods.

1. Preservatives and their alternatives

Methylparaben, Ethylparaben, Butylparaben, Propylparaben, Ethylhexylglycerin, Bisabolol, Phenoxyethanol, 1,2-Hexanediol, Sodium Benzoate, Potassium Sorbate, Butylene Glycol, Benzyl Alcohol were purchased from FUJIFILM Wako Pure Chemical Corporation. Pentylene Glycol was purchased from Santa Cruz Biotechnology. Caprylyl Glycol was purchased from Kanto Chemical Co, Inc. Glyceryl Caprylate was purchased from Taiyo International, Inc.

2. Cell culture

NHEK cells (Kurabo, Japan) were cultured in HuMedia-KG2 (Kurabo, Japan) containing 0.5 ml insulin, 0.5 ml hEGF, 0.5 ml Hydrocortisone, 2 ml BPE (Bovine Pituitary Etract), 0.5 ml Gentamicin/ amphotericin. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO2.

3. A RHE model for gene and protein assays

Each cup of RHE model (LabCyte EPI-MODEL) was placed into a 24-well plate, and assay medium was added under the cup. After incubating (37°C, 5% CO_2) for 1 day, each preservative and its alternatives were added to the top of the RHE surface. After 18 hours treating preservatives, the RHE model was subjected to real-time qRT-PCR, and the medium was subjected to ELISA assay. Each preservative and its alternative except for GLY were dissolved in water. GLY was dissolved in mineral oil. This is because GLY dissolved in water had cytotoxicity despite the concentration generally used in cosmetics. On the other hand, GLY dissolved in oil did not show any cytotoxicity. In addition, to dissolve ETP, BUT, PRO in water, we used 20% of BUG and 4% of Ethanol.

4. An immature RHE model for the stratum corneum formation process

Each cup of immature RHE model (LabCyte EPI-MODEL 6D), which is the early stage of the stratum corneum formation, was placed into a 24-well plate, and assay medium was added under the cup. After incubating (37°C, 5% CO_2) for a day, each preservative and its

alternative were added to each medium. TEWL was measured by using Tewitro TW24 (Courage+Khazaka electronic GmbH, Köln, Germany) after 0, 1, 2- and 3-days treating preservatives.

5. RNA isolation and quantitative real-time RT-PCR

Total RNA was extracted from cells using ReliaPrep RNA cell miniprep system (Promega, USA), reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen S.A., France). The cDNA was subjected to real-time quantitative PCR analysis using SYBR Premix Ex Taq (TaKaRa, Japan) by Light Cycler 96 (Roche, Switzerland).

6. ELISA assay

IL-6, IL-8 and TNF-α protein concentrations were measured using human IL-6, IL-8 and TNF-α ELISA kit (Invitrogen, USA) by varioskan flash (Thermo scientific, USA).

7. The test of preservative efficacy

The culture of *Pseudomonas aeruginosa*, *Burkholderia cepacian*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter cloacae* and *Candida albicans* at second passage strain were used to prepare. The inoculum density was obtained by dilution of each culture to 1×10^8 cfu/mL, and they were mixed in equal amount. Each test formulation (20 g) was inoculated with 0.2 mL (1/100 amount) of the mixed bacterium suspension and stored at 22.5 ℃± 2.5 ℃. After 2 days, 7 days, and 14 days of storage, the required amount of microbiological test was collected from them. If it fell below the detection limit, the test ended at that point. To evaluate the number of surviving bacteria in each solution, the following microbiological challenge tests, diluted smear test and enrichment test, were conducted.

[Diluted smear test]

Each solution (1 g) was evenly mixed in 9 mL of SCDLP medium. For immiscible test formulations, there were premixed with 1 g of T20 / S80 surfactant and mixed evenly in 8 mL of SCDLP medium. Each mixture (0.1 mL) was dispensed onto SCDLP agar medium and smeared evenly. After cultivating at 32 ℃ for 3 days or more, the detected colonies were measured, and calculated the number of surviving bacteria per 1 g of test formulations.

[Enrichment test]

The mixed solution prepared by the diluted smear test were incubated at 32 ℃ for 3 days or more. Only when the bacteria were detected on the plate as a result of the diluted smear test, the culture solution was drawn and cultured at 32 ℃ for 24 hours or more. If bacteria were detected, it was judged as enriched (+). Theoretically, the number of viable bacteria remaining was 1 to 99 cfu / g, but for convenience, it was regarded as 99 cfu / g. If no bacteria were detected, it was judged as enriched (-). Similarly, although the number of viable bacteria remaining was less than 1 cfu / g, it was considered to be 1 cfu / g for convenience.

To evaluate the storage efficacy of the test formulations, we calculated the initial number of bacteria per 1 g of each formulation from the number of bacteria in the mixed bacteria culture solution, and the logarithmic reduction value from the number of surviving bacteria at each test day from the following equation.

Log reduction = Log (the number of inoculums in 1 g of formulation) - Log (the number of surviving bacteria in 1 g of formulation on each test day)

As an evaluation standard, a test formulation in which the number of bacteria decreased by 2.5 Log or more on the 7th day or the number of bacteria decreased by 3.5 Log or more on the 14th day was judged to have enough antimicrobial effect as a cosmetic product.

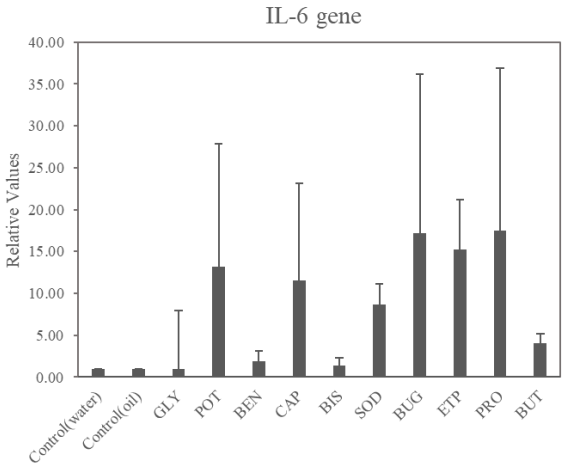
Results.

1. The expressions of inflammatory cytokine genes in a RHE model

We measured cell viability by using MTT assay when a RHE model was treated by 10 preservatives and their alternatives, which are frequently used worldwide (data not shown). Taking into account the general concentrations used in the products, we decided their concentrations which is over 90% cell viability. To examine the effects of 10 preservatives and their alternatives on the gene expressions of inflammatory cytokines, IL-6, IL-8 and TNF-α, by real-time PCR, the cells were treated with them for 18 hours. As a result, mRNA expressions were different depending on preservatives and their alternatives (Fig.1). ETP significantly increased the expression of IL-8 gene.

When we conducted the same experiment with 5 preservatives, Methylparaben (MET), Pentylene Glycol (PEN), 1,2-Hexanediol (HEX), Ethylhexylglycerin (ETH) and Phenoxyethanol (PHE), in our previous study, any gene expressions were not increased significantly (data not shown).

a



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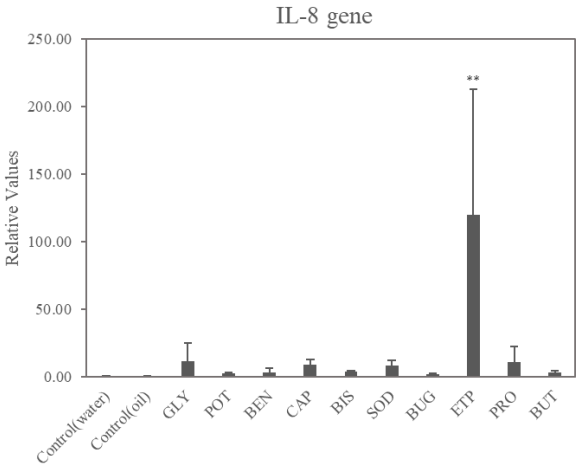

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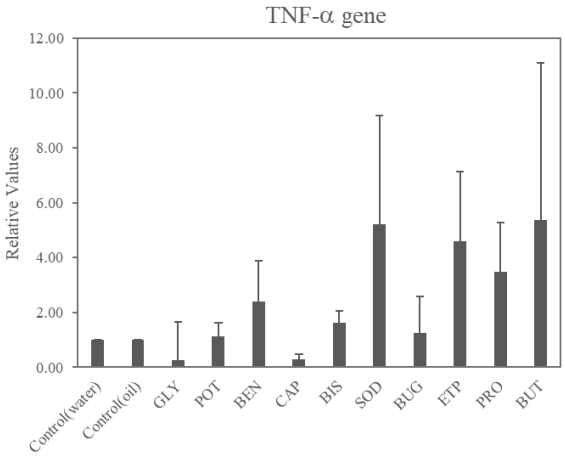

Fig.1 mRNA expression level of inflammatory cytokines IL-6 (a), IL-8 (b) and TNF-α (c) by 0.2% GLY, 0.3% POT, 0.3% BEN, 0.25% CAP, 0.5% BIS, 0.4% SOD, 20% BUG, 0.2% ETP, 0.1% PRO and 0.01% BUT for 18 hours treatment in a RHE model. GAPDH mRNA was used as an internal control. Dunnett tests was conducted with all preservative except for GLY vs control (water), t-tests were conducted with GLY vs control (oil), N=3, $**p < 0.01$, Values are mean ± SD

2. The expressions of inflammatory cytokine proteins in a RHE model

We also investigated the expressions of inflammatory cytokine proteins, IL-6, IL-8 and TNF-α with 10 preservatives and their alternatives by ELISA. As a result, the protein expression level of IL-8 was significantly increased with 18 hours of CAP treatment (Fig.2). In addition, PHE, MET, ETH and HEX significantly raised the protein expression level of IL-8 in our previous study (data not shown). However, we could not observe the same significant increase between gene and protein. These results indicated that other factors are involving. As one of the possibilities, we focused on anti-inflammatory cytokine, IL-37.

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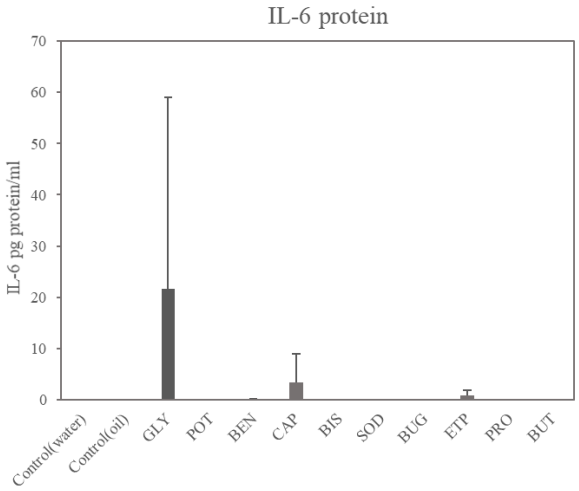

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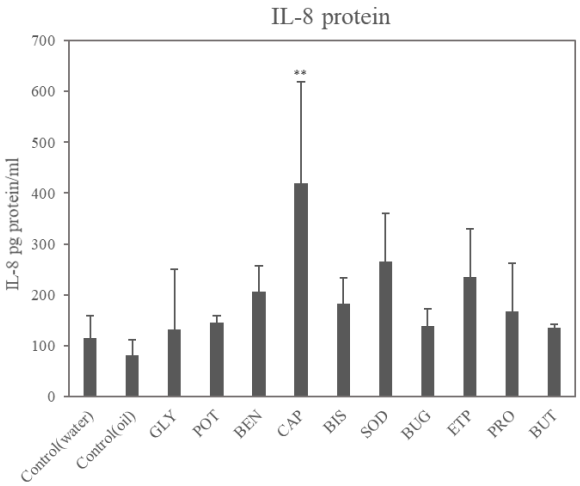

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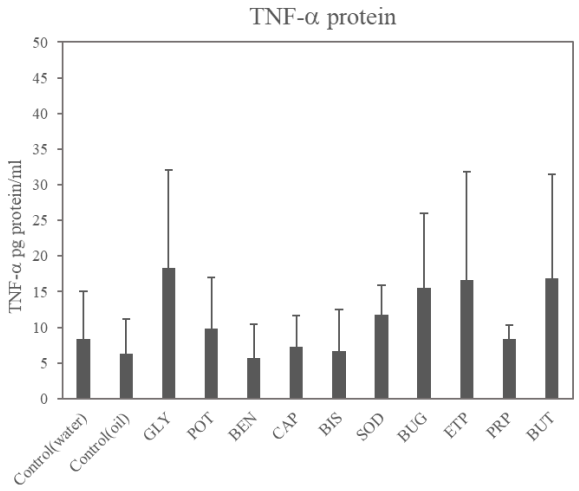

Fig.2 Protein expression level of inflammatory cytokines IL-6 (a), IL-8 (b) and TNF-α (c) by 0.2% GLY, 0.3% POT, 0.3% BEN, 0.25% CAP, 0.5% BIS, 0.4% SOD, 20% BUG, 0.2% ETP, 0.1% PRO and 0.01% BUT for 18 hours treatment in a RHE model. Dunnett tests were conducted with all preservative except for GLY vs control (water), t-tests were conducted with GLY vs control (oil), N=3, $**p < 0.01$, versas control,Values are mean ± SD

3. The expression of anti-inflammatory cytokine gene in a RHE model

We evaluated the gene expression of IL-37 by using the same samples which we examined inflammatory cytokines in a RHE model. As a result, CAP, SOD, ETP, BUT, PRO, BIS, BUG, and GLY significantly decreased the gene expression of IL-37 (Fig.3). This result suggests that the decrease of anti-inflammatory cytokines might involve in the increase of inflammatory cytokines induced by preservatives and their alternatives.

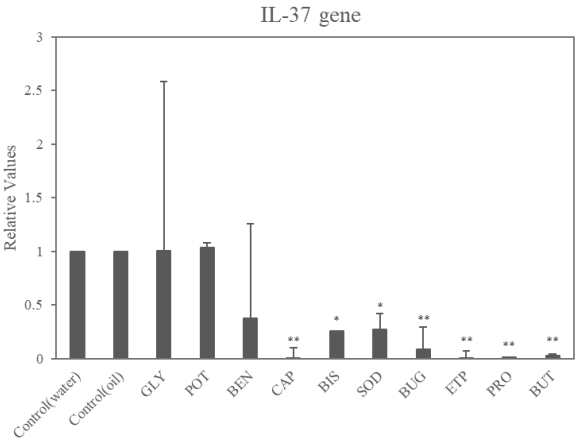

Fig.3 mRNA expression level of anti-inflammatory cytokine, IL-37, by 0.2% GLY, 0.3% POT, 0.3% BEN, 0.25% CAP, 0.5% BIS, 0.4% SOD, 20% BUG, 0.2% ETP, 0.1% PRO and 0.01% BUT for 18 hours treatment in a RHE model. GAPDH mRNA was used as an internal control. A Dunnett test was conducted with all preservative except for GLY vs control (water), a t-test was conducted with GLY vs control (oil), N=3, $*p < 0.05$, $**p < 0.01$, Values are mean ± SD

4. The expressions of inflammatory cytokine proteins in NHEK cells

To examine whether 15 preservatives and their alternatives, which we have investigated, have the same effect on the protein expression level of inflammatory cytokines, IL-6, IL-8 and TNF-α, in NHEK cells or not, cells were treated with each preservative and its alternative for 18 hours. We decided the preservative concentrations in 70% cell viability. As a consequence, IL-6 was significantly increased by GLY, PHE and ETH. In addition, IL-8 was significantly increased by GLY and ETH, and TNF-α was significantly raised by SOD (Fig.4). Taking together the all results of the expressions of inflammatory cytokines in NHEK cells and RHE models, which we conducted present and previous study, POT, BUG, BEN, BIS, BUT, PRO and PEN did not significantly increase any inflammatory cytokines (Table 1).

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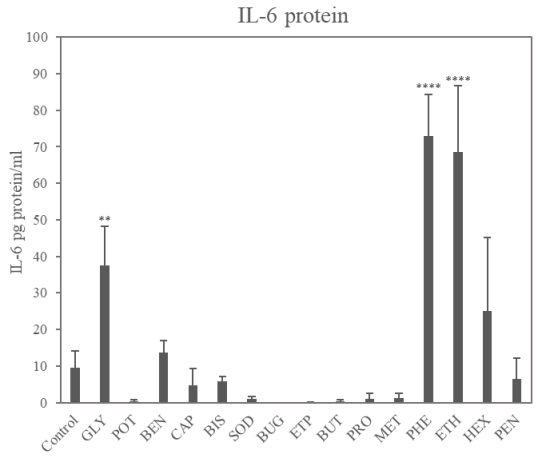

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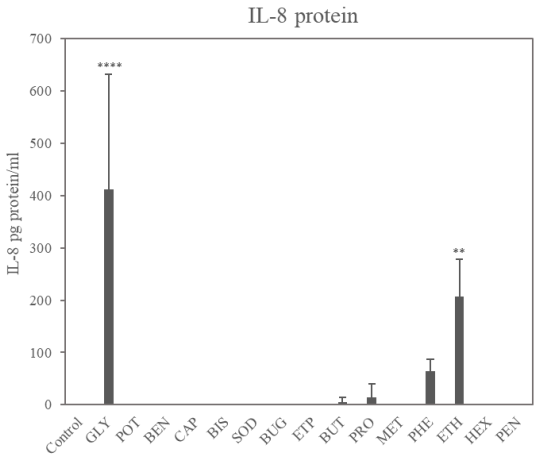

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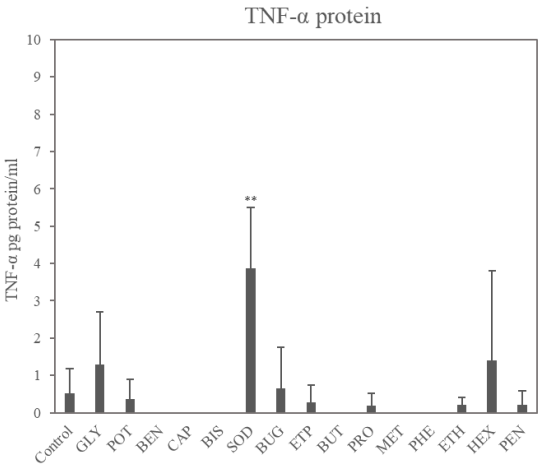

Fig.4 Protein expression level of inflammatory cytokines IL-6 (a), IL-8 (b) and TNF-α (c) by 0.2% GLY, 0.3% POT, 0.3% BEN, 0.25% CAP, 0.5% BIS, 0.4% SOD, 20% BUG, 0.2% ETP, 0.01% BUT, 0.1% PRO, 0.2% MET, 0.5% PHE, 0.05% ETH, 1% HEX and 5% PEN for 18 hours treatment in NHEK cells. GAPDH mRNA was used as an internal control. N=3, $**p < 0.01$, $****p < 0.0001$, vs control, Dunnett, Values are mean ± SD

Table 1. The expressions of inflammatory cytokines which had significant increse by each preservative and its alternative for 18 hours treatment in NHEK cells and RHE models, *$p < 0.05$,**$p < 0.01$, ****$p < 0.0001$, vs control, Dunnett

	IL-6			IL-8			TNF-α		
	gene (RHE)	protein (RHE)	protein (NHEK)	gene (RHE)	protein (RHE)	protein (NHEK)	gene (RHE)	protein (RHE)	protein (NHEK)
Potassium Sorbate (POT)									
Caprylyl Glycol (CAP)					**				
Sodium Benzoate (SOD)									**
Bisabolol (BIS)									
Butylene Glycol (BUG)									
Benzyl Alcohol (BEN)									
Glyceryl Caprylate (GLY)			**			****			
Ethylparaben (ETP)				**					
Butylparaben (BUT)									
Propylparaben (PRO)									
Methylparaben (MET)					*				
Pentylene Glycol (PEN)									
1,2-Hexanediol (HEX)					*				
Ethylhexylglycerin (ETH)			****		*	**			
Phenoxyethanol (PHE)		*	****		*				

5. The antimicrobial efficacy

Another significant aspect of selecting and evaluating preservatives is the antimicrobial efficacy for bacteria. Therefore, we investigated the antimicrobial efficacy at the same concentration as when the expressions of inflammatory cytokines were evaluated in a RHE model. As a consequence, CAP, BUG, GLY, ETP, BUT, PRO, MET and PEN were able to pass our antimicrobial efficacy test, suggesting that they have enough efficacy of maintaining the cosmetic quality (Table 2).

Table 2. The antimicrobial efficacy of each preservative and its alternative was evaluated by our original method of inoculating the product with bacterium and culturing them to observe their log reduction.

	Concentration (%)	Log reduction			Result
		Day0	Day7	Day14	
Standard	-	0	2.5	3.5	-
Potassium Sorbate (POT)	0.3	0	0	0	Fail
Caprylyl Glycol (CAP)	0.25	0	3.8	6.1	Pass
Sodium Benzoate (SOD)	0.4	0	0	0	Fail
Bisabolol (BIS)	0.5	0	0	0	Fail
Butylene Glycol (BUG)	20	0	6.1	-	Pass
Benzyl Alcohol (BEN)	0.3	0	1.5	1.6	Fail
Glyceryl Caprylate (GLY)	0.2	0	6.1	-	Pass
Ethylparaben (ETP)	0.2	0	6.1	-	Pass
Butylparaben (BUT)	0.1	0	6.1	-	Pass
Propylparaben (PRO)	0.01	0	6.1	-	Pass
Methylparaben (MET)	0.2	0	4.1	6.1	Pass
Pentylene Glycol (PEN)	5.0	0	3.2	6.1	Pass
1,2-Hexanediol (HEX)	1.0	0	1.9	2.4	Fail
Ethylhexylglycerin (ETH)	0.05	0	0.8	0	Fail
Phenoxyethanol (PHE)	0.5	0	2.4	5.2	Fail

6. The formation of stratum corneum in an immature RHE model

As a consequence of increasing of inflammatory cytokines, we assumed that it might have some influence on the formation of stratum corneum. TEWL is a sensitive indicator of the integrity of the stratum corneum, and inversely proportional to skin barrier function. TEWL is relatively low in healthy skin. We selected 7 preservatives and their alternatives, SOD, BUG, GLY, MET, PEN, ETH and PHE, and observed how these preservatives affect the formation of stratum corneum by evaluating TEWL in an immature RHE model. We decided the concentrations of each preservative which over 90% cell viability by MTT assay. As a result, TEWL value was significantly higher in the PHE treatment than in the control on day 2. Furthermore, on day 3, TEWL values in PHE, MET, PEN, BUG and SOD treatments were also significantly higher. These results suggest that the formation of stratum corneum was disturbed by treating PHE, MET, PEN, BUG and SOD (Fig.5).

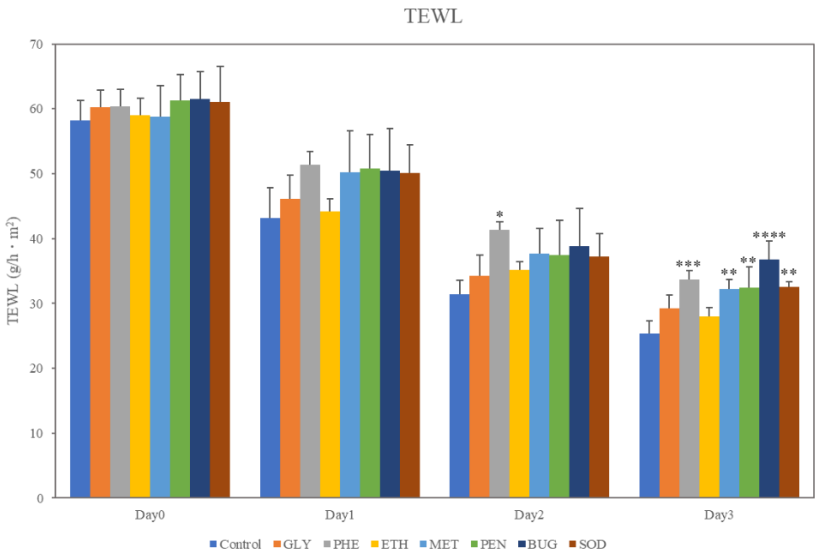

Fig.5 TEWL by 0.012% GLY, 0.4% PEN, 0.075% PHE, 2.5% BUG, 0.008% ETH, 0.15% SOD, and 0.025% MET for 3 days treatment in an immature RHE model, N=3, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, vs control, Dunnett,Values are mean ± SD

Discussion.

In this study, we demonstrated the effects of preservatives and their alternatives on NHEK cells and RHE models. In both cells and models, we confirmed that several preservatives and their alternatives increased inflammatory cytokines. On the other hand, POT, BUG, BEN, BIS, BUT, PRO and PEN did not significantly increase any inflammatory cytokines (Table 1). However, we were not able to treat a RHE model with normal concentrations of BUT and PRO because of cell toxicity even though other preservatives could evaluate with their concentrations which are normally used in cosmetics. Therefore, these are required further investigation, but except for this issue, POT, BUG, BEN, BIS and PEN resulted as a good option to be used in cosmetics for sensitive skin.

As for the different results between NHEK cells and RHE models, it might be affected by permeabilities of each preservative. A RHE model has cornified, granular, spinous, and basal layer, and it is more similar to human skin. These differences might affect results between NHEK cells and RHE models. However, further studies are required to address this issue.

We also confirmed that some preservatives increased only the gene expression of inflammatory cytokine, or the others increased only the protein expression. These differences

might be caused by several factors such as anti-inflammatory cytokine, IL-37. In this study, we only investigated its gene expression, but we need to do further studies.

Moreover, we found that PHE, MET, PEN, BUG and SOD disturbed the formation of stratum corneum in an immature RHE model. As one of the possibilities, inflammatory cytokines induced by preservatives might provoke a weakening of the barrier formation. Many cytokines have been reported to alter the expression of structurally important proteins involved in the formation of the cornified envelope, including loricrin (LOR) and filaggrin (FLG). For instance, TNFα inhibits FLG and LOR mRNA expression in calcium differentiated keratinocytes [10]. In addition, the expression of genes encoding key enzymes accountable for the synthesis of ceramides is inhibited by IL-6 treatment [11]. On the other hand, some cytokines reported to be important for the efficient repair of barrier activity [12, 13]. That is to say, the process of skin barrier formation is highly balanced by cytokines, and it is still unclear how cytokine balance is relevant for the protective function of the skin. Therefore, it is not surprising that inflammatory cytokines induced by preservatives might involve in weakening the barrier formation. However, it is the fact that PEN and BUG, which did not induce any significant increase of inflammatory cytokines, disturbed the formation of stratum corneum. To address this issue, we still require further investigations how these consequences were occurred by preservatives and their alternatives.

Conclusion.

The present study demonstrated that the expressions of inflammatory cytokines were different depending on the preservatives and their alternatives. In addition, some preservatives decreased the expression of IL-37, suggesting that the inflammatory response induced by preservatives might be affected by anti-inflammatory response. Furthermore, PHE MET, PEN, BUG and SOD caused abnormalities in the formation of the stratum corneum. These results indicate that preservatives and their alternatives should be selected carefully, even though the alternatives are not defined as preservatives. To develop the cosmetics that anyone can use safely, we will elucidate the inflammatory mechanisms of each preservative and further consequences that can be caused by inflammatory cytokines in the future.

Conflict of Interest Statement.

NONE.

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