Title： Application Of reconstructed epidermis model in the evaluation of cosmetic soothing efficacy

Qiang Wu[1] ;Zhe Feng[1];Yaling Song[2];Shaomin Wei[3];Xin Yang*

Proya Cosmetics Co.,Ltd, hangzhou city, China

Xin Yang,Proya Cosmetics Co.,Ltd, hangzhou city, China, +8618500465159,yangxin@proya.com

Abstract (Maximum of 250 words) should provide:

Background: The traditional efficacy test methods of cell biology is limited by the characteristics of the cell experiment itself, so it is difficult to assess the formula of the cosmetics products, benefits from the omni-directional simulation of organ structure, the reconstructed skin model technology has developed by leaps and bounds in recent years. It can well reproduce the effects of the product on human skin, however its scientificity and stability still need to be compared with traditional cell biology experiments.

Methods: In this paper,three skin care formulas with anti-inflammatory effect were used as test samples,The SDS-induced soothing effect evaluation model of reconstructed epidermis was established.The levels of inflammatory factors in epidermis equivalent and medium matrix were identified by molecular biological methods ELISA,and compared with the classical RAW264.7 macrophage inflammation model induced by LPS.

Results: The three skin care formulas with anti-inflammatory effect can significantly reduce the expression of NO, iNOS, IL-1β, TNF-α, and IL-10 mRNA in the LPS-induced cell anti-inflammatory model. In the SDS-induced reconstructed skin model detected by ELISA, they can significantly reduce the release of IL-1α and IL-8 inflammatory factors.

Conclusion: The three samples exhibited a certain correlation on different soothing efficacy testing platforms., which provides a scientific basis for the application of reconstructed skin model in the soothing efficacy evaluation of cosmetic products.

Keywords: inflammatory factors, reconstructed skin model,RT-QPCR,ELISA,RAW 264.7 macrophages.

Introduction.

In recent years, consumers pay more and more attention to the efficacy of cosmetics, and the efficacy evaluation of the traditional cosmetics industry is facing new challenges[1].With the introduction of a large number of emerging technologies such as molecular biology, cell biology, and in vitro organ culture, in vitro detection has been gradually applied by the industry due to its advantages of short time-consuming, low cost and easy access to experimental materials. The traditional cell biology method is limited by the characteristics of cell experiments, Second, there are also clear differences in the growth and culture of skin cells in a three-dimensional environment. Studies have pointed out that the types and densities of cells contained in different layers of skin tissue are different, so in vitro cell experiments are not sufficient to accurately characterize the exact structure of human skin. [2-4].

The reconstructed skin model that appeared in recent years is constructed in vitro by utilizing the structure of normal human skin cells, to obtain a complete three-dimensional anatomical structure, which can highly simulate human skin. The reconstructed skin model of the skin makes it very similar to the structure of normal human skin, which can replace the normal human skin to detect the effect of active substances or other stimuli on the expression of relevant essential proteins in the skin, and better reproduce the effect of cosmetics on human skin[5,6].Therefore, it can replace human skin to complete the safety detection and efficacy evaluation of cosmetic raw materials, and the efficacy evaluation of finished products, and is currently widely used in the cosmetics industry.

Reconstructed skin models have become the development trend of the cosmetic industry in the evaluation of cosmetic efficacy.It is not known whether the reconstructed skin models respond similarly to real skin.. This problem directly affects the design and application of in vitro detection methods for cosmetic efficacy evaluation[7]. Cosmetic anti-inflammatory efficacy test is carried out at two levels of cell and reconstructed skin model, and multi-dimensional data is integrated to comprehensively evaluate the validity and consistency of in vitro test data. To realize multi-dimensional unified scientific logic and ensure the validity of reconstructed skin model and the stability of the evaluation, further providing a scientific basis for the application of the reconstructed skin model in the evaluation of cosmetic soothing efficacy[8, 9].

Materials and Methods

Chemicals and Reagents

Dulbecco's Modified Eagle Mediumfetal (DMEM) culture medium and bovine serum (FBS) were purchased from GIBCO BRL (USA). The RAW264.7 cells were obtained from the BeNa Culture Collection (BNCC,Beijing,China), Human epidermal growth medium and Skinovo-Ep human constructed epidermis model were purchased from Regenovo Biotechnology Co.,Ltd.(Hangzhou,China); The LPSs (Esche richia coli 055:B5) and Dexamethasone (≥98%) were from Sigma. Cell Counting Kit-8(CCK-8) was from Amyjet Scientific(Wuhan China).Sodium dodecyl sulfate(SDS) was from Sinopharm Chemical Reagent Co.,Ltd. (shanghai,China).Total Nitric Oxide Assay kit (Beyotime Institute of Biotechnology, Shanghai,China)Human(IL-1α, IL-8 and PGE2)ELISA Kit were purchased from Fine Test (WuHan,China).The three basic skin care formulas with anti-inflammatory efficacy were selected as test samples.

Cell culture

Murine macrophage RAW264.7 cells were purchased from the BeNa Culture Collection (Beijing, China) and cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin at 37℃ in 5% CO_2.The cells were passaged every 24 hours.

Reconstructed human reconstructed epidermis culture

In vitro Reconstructed Human Epidermis was purchased by Skinovo-Ep (Regenovo Biotechnology Co.,Ltd,Hangzhou,China). The model was made of human keratinocytes as seed cells and induced to differentiate after 3D printing in vitro. And its structure and function are very close to the human epidermis. The tissue cultures were maintained in the incubator at 37 °C, 5% CO_2 and saturated humidity only for 24 h using the special medium. The medium was changed after 24 h and the treatments started after overnight incubation.

Cell viability detection

Sample processing method:Dilute the three samples with water to 40%, ultrasonically, and heat (40°C) for 1 h to make the emulsion evenly dispersed; then dilute the treated samples with culture medium to 4%, ultrasonicate, and heat (40°C) After treatment for 1h,the active ingredients were dissolved into the medium effectively,and then filtered and sterilized for use.

The RAW264.7 cells (1.2×10^4 per well in a 96-well plate) were washed with phosphate-buffered saline (PBS), and cell viability was detected using a Cell Counting Kit-8 according to the manufacturer's protocol. Briefly, RAW264.7 cells were treated at 37°C with different concentrations of sample for 24 h and then incubated at 37°C with 10 μl CCK-8 working solution for 1 h. The absorbance was detected using a Thermo Fisher Scientific Co., Ltd (Shanghai, China) at 450 nm. All experiments were repeated in triplicate,and the data are presented as mean ± standard deviation (SD).

Nitric oxide detection

RAW264.7 cells were treated at 37°C with LPS (1μg/ml) and different concentrations of sample treatment for 24h. NO production in the cell culture medium was determined using a Total Nitric Oxide Assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. The absorbance at 540 nm was measured using a QuantStudio 3 (Thermo Fisher Scientific Co., Ltd, Shanghai, China). Each experiment was repeated in triplicate for all sample concentrations.

Reverse transcription polymerase chain reaction (RT-PCR)

Intracellular total RNA was extracted from RAW264.7 cells (3×10^5 cells per well in a 6-well plate) using TaKaRa MiniBEST Universal RNA Extraction Kit, according to the manufacturer's instructions.The concentration and integrity of the RNA were measured at a 260/280 nm ratio.Then a PrimeScript II 1st Stand cDNA Synthesis Kit was used to synthesise cDNA, and TB Green® Fast qPCR Mix was used for fluorescence quantitative PCR operation.The PCR primers were designed using Primer 5.0 software, and the primer sequences have been shown in Table1.The GAPDH gene was used as invariant housekeeping gene internal control.The thermocycling conditions were as follows: 95°C for 3 min, followed by 45 cycles of 95°C for 15 sec, 55°C for 15 sec, 72°C for 15 sec, the last was 95°C for 15 sec, 60°C for 1 min, 95°C for 1sec.The relative gene expression was quantified by the comparative $2^{-\Delta\Delta CT}$ method.

Table 1. Sequence information on the primers used for RT-PCR.

Gene	Primer Sequence (Forward/Reverse 5′ -3′)
IL-1β	Forward:TGCCACCTTTTGACAGTGATG
	Reverse:TGATACTGCCTGCCTGAAGC
iNOS	Forward:GTTCTCAGCCCAACAATACAAGA
	Reverse:GTGGACGGGTCGATGTCAC
IL-10	Forward:CTGAAGACCCTCAGGATGCG
	Reverse:TGGCCTTGTAGACACCTTGG
TNF-α	Forward:GCACCACCATCAAGGACTCA
	Reverse:TGCACCTCAGGGAAGAATCTG
GAPDH	Forward:CCCTTCATTGACCTCAACTACAT
	Reverse:ACGATACCAAAGTTGTCATGGAT

Enzyme-linked immunosorbent assay (ELISA) analysis

The Skinovo-Epi epidermal model was randomly divided into a blank control group, SDS group and sampleA-C group, with 6 models in each group. Add 10 μL of 0.1% SDS to the upper surface of the model in the SDS group, add 10 μL 0.2% SDS to the upper surface of the sample group and add an equal volume mixture of the sample.

After adding samples according to the experimental groups, the epidermal models were incubated in a 37°C incubator for 24 h, and then the culture medium of each group was taken for the detection of inflammatory mediator release. IL-1α, IL-8 and PGE2 inflammatory mediator production in the cell culture medium were determined using a Fine Test ELISA Kit, according to the manufacturer's protocol.

Statistical analysis

All data were presented as mean ± standard deviation, The above experiments were repeated 3 times.Statistical analyses were performed using Prism 8.3 software (GraphPad Software, Inc., La Jolla, CA, USA). The results were analyzed by one-way analysis of variance followed by a post-hoc Student's test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of Three kinds of cosmetics on the viability of RAW264.7 cells

First, we examined the effect of 3 kinds of cosmetics on the viability of RAW264.7 cells. According to the above experimental results, we screened the sample concentration with a cell survival rate of more than 90% as the safe concentration. In order to facilitate the comparison of subsequent experiments, the three sample concentrations were selected as 0.125%.

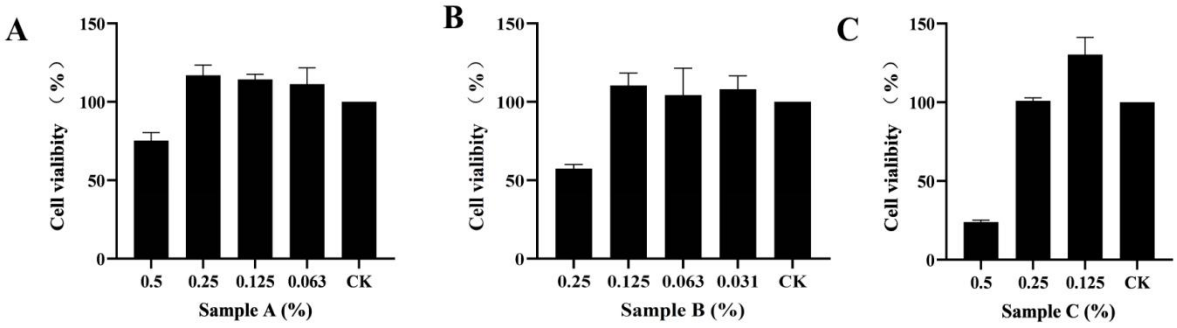

Figure1. Effect of Three kinds of cosmetics on the viability of RAW264.7 cells

(A) The viability of sample A in cells was detected by Cell Counting Kit-8 assay. (B) The viability of sample B in cells was detected by Cell Counting Kit-8 assay. (C) The viability of sample B in cells was detected by Cell Counting Kit-8 assay. Data are presented as mean ± standard deviation of three independent experiments. The cell activity of untreated group (control group) was set as 100%, and the calculated value of each concentration of sample was the relative cell activity to the control group. CK:control check group

Three kinds of cosmetics inhibited the production of LPS-induced mRNA expressions of TNF-α, iNOS, IL-1β and IL-10 , secretion of Nitric oxide cytokines in the RAW264.7 cells

At the same time, in order to evaluate the effect of cosmetics on proinflammatory mediators, gene expressions of TNF-α, iNOS, IL-1β and IL-10 were determined.Compared with the blank group, the concentrations of secreted NO cytokines, TNF-α mRNA ,iNOS mRNA ,IL-1β mRNA and IL-10 mRNA were increased sharply after LPS alone stimulation ($P < 0.05$).Figure 2 showed that three kinds of cosmetics could markedly reduce the mRNAs expressions of TNF-α, iNOS, IL-1β and IL-10 ($P < 0.05$) compared with the LPS alone-treated group.Besides, the three kinds of cosmetics have the ability to significantly inhibit NO production upregulated by LPS.These data indicated that three kinds of cosmetics decreased the expression level of LPS-induced pro-inflammatory mediators.

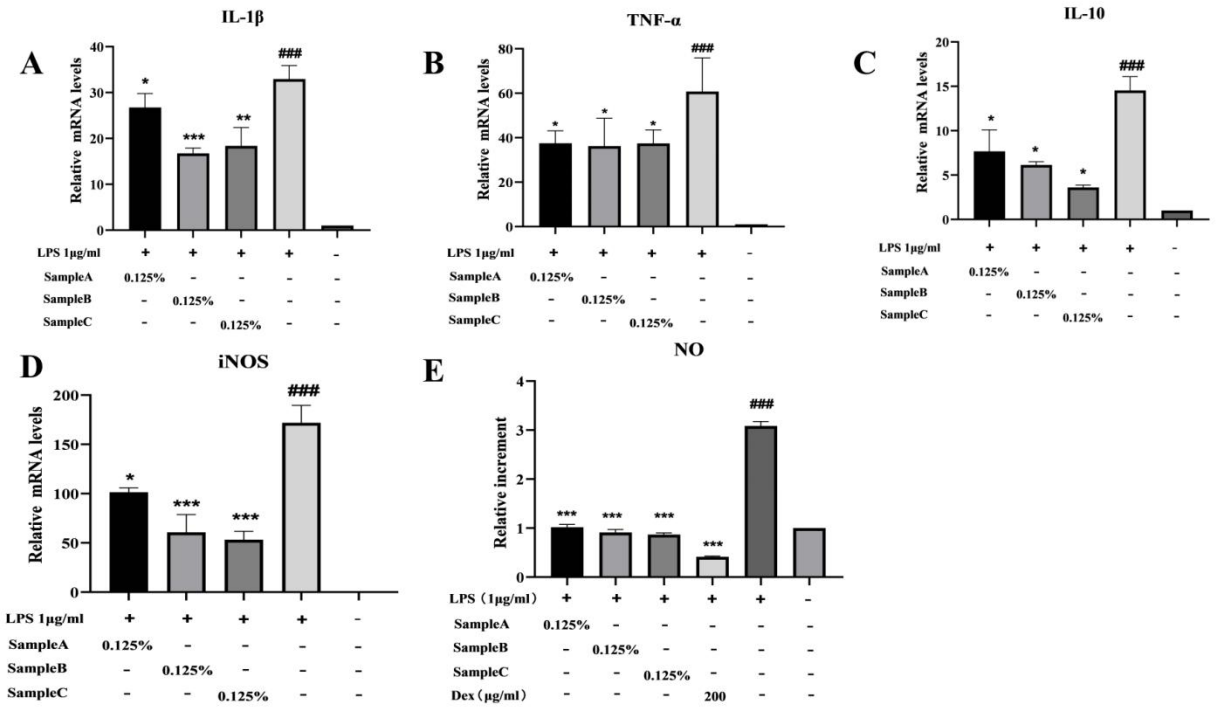

Figure2. Effect of Three kinds of cosmetics on TNF-α, iNOS, IL-1β and IL-10 mRNA expression in LPS-induced RAW264.7 cells. The mRNA expressions of TNF-α, iNOS, IL-1β and IL-10 were detected by qRT-PCR analysis. (A)IL-1β mRNA expression level. (B) TNF-α mRNA expression level. (C) IL-10 mRNA expression level. (D)iNOS mRNA expression level. (E) NO in the cell culture supernatants were detected by total nitric oxide assay. GAPDH was used as the internal reference gene. ###:$P < 0.001$ compared with control group; *:$P \leq 0.05$ compared with LPS alone； *:$P \leq 0.01$compared with LPS alone.

Three kinds of cosmetics inhibited the production of SDS-induced inflammatory factor expressions of IL-1α and IL-8 on the reconstructed epidermis model

To evaluate the effect of three cosmetics on inflammatory factors induced by SDS, ELISA Detection kit was used to examine the effect of three cosmetics on the production of IL-1α,IL-8 and PGE2 inflammatory factors in the reconstructed epidermis model. Compared with the blank group, the concentrations of secreted IL-1α, IL-8 and PGE2 inflammatory factors were increased significantly after SDS alone stimulation ($P < 0.05$). Figure 3 showed that three kinds of cosmetics could markedly reduce the mRNA expressions of IL-1α and IL-8 ($P < 0.05$) compared with the SDS alone-treated group. But three kinds of cosmetics did not down-regulate the

expression of PGE2 inflammatory factors. The data showed that Three kinds of cosmetics decreased the expression level of SDS-induced pro-inflammatory mediators.

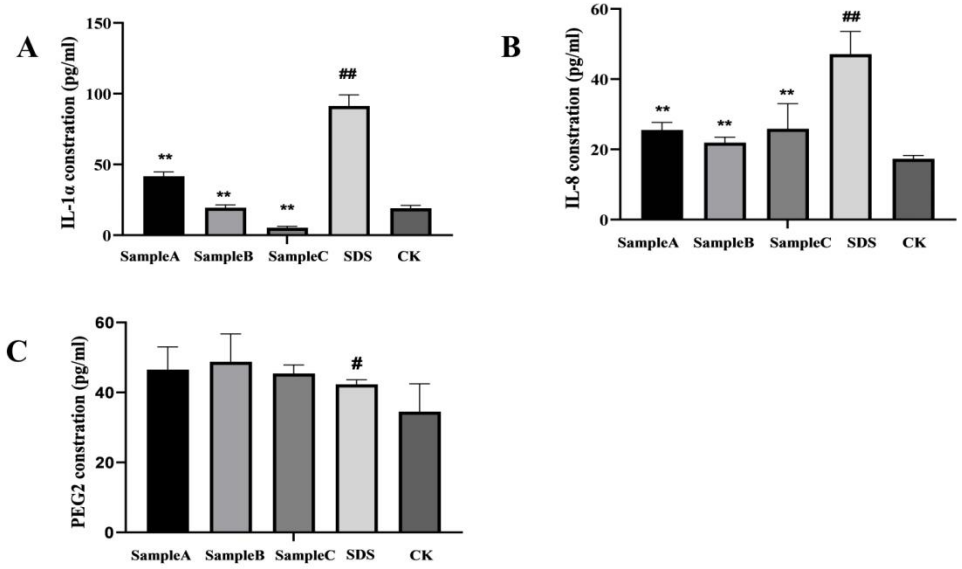

Figure3. The inflammatory factor expressions of IL-1α, IL-8 and PGE2 were detected by Elisa analysis respectively. (A) IL-1α inflammatory factor expression level. (B) IL-8 inflammatory factor expression level. (C)PGE2 inflammatory factor expression level. ###:$P < 0.001$ compared with control group. *:$P \leq 0.05$ compared with SDS alone. *:$P \leq 0.01$compared with SDS alone.

Discussion：

The skin is the largest organ of the human body. As the first line of defence of the human body, it plays an important role in protecting and resisting external stimuli. Following microbial infection of the skin, macrophages play an important role in the host immune defence system, they are activated by different stimuli, including bacterial lipopolysaccharide (LPS).We established an inflammatory model by using LPS-induced RAW264.7 cell model and verified 3 formulations with soothing efficacy cosmetics have soothing properties.

Compared with the monolayer cells, the reconstructed human skin model has a functional stratum corneum structure, which can be directly used for the smear test without being affected by the test substance formulation[10], and the tissue structure has been improved significantly, further demonstrate that artificial skin is an ideal model. Studies [11]have shown that lactate dehydrogenase (LDH), prostaglandin E2 (PGE2), and interleukin-1alpha (IL-1α) levels are significantly increased in human epidermal models treated with SDS. Surfactants with higher concentrations can cause skin barrier damage and induce inflammatory responses[12], and are often used in the inflammatory induction of epidermal models in vitro. We established an inflammatory model by using our SDS-induced reconstructed epidermis model and verified 3 formulations with soothing efficacy cosmetics have soothing properties. Upon SDS stimulation in this research, the production of proinflammatory mediators (as measured by IL-1α and IL-6) was increased significantly, compared with normal control cells, and treatment with the 3 formulations (its soothing efficacy cosmetics), however, inhibited the SDS-induced increases in the contents of the inflammatory factors. Besides,Smerigliode et al[13]. treated with pro-inflammatory mediators (PBS 1 X and LPS) and HT-based formulation on reconstructed human epidermis, then detected IL-1α and IL-8 inflammatory factors by using ELISA method, and observe the morphological structure as an indicator of anti-inflammatory evaluation.

Thus, When using a skin model to evaluate the soothing and repairing efficacy of cosmetics, it can be used on epidermal or whole skin models stimulated by surfactants or microorganisms, and then the inflammatory mediators (PGE2) and inflammatory mediators (PGE2) and inflammatory mediators (PGE2) and inflammatory mediators (PGE2) and The secretion of inflammatory factors (such as IL-1a, IL-8 and TNF-α, etc.) and the expression of related genes were used to evaluate the anti-inflammatory ability of the formula.

Conclusion:

In this study, for three tested skin care formulas with anti-inflammatory properties, ELISA technique wa employed to detect the expression of inflammatory factors in reconstructed epidermis model. And compared with the classical methods of cell biology, the scientificity and

availibility of reconstructed epidermis model in the evaluation of soothing effect were verified. It provides a new method and idea for the efficacy test of skin care formula in vitro.

Acknowledgments:

The authors thank for Proya Cosmetics Co.,Ltd technical support.

Conflict of Interest Statement:

The authors declare no conflict of interest.

References:

[1] Tannenbaum J, Bennett B T (2015) Russell and Burch's 3Rs then and now:The need for clarity in definition and purpose[J]. Journal of the American Association for Laboratory Animal Science, 54 (2) :120-132.

[2] Ebara M, Kotsuchibashi Y, Uto K, et al (2014) Smart Hydrogels; Smart Hydrogels[C].: 9-65.

[3] Schroder J M(2010). The role of keratinocytes in defense against infection[J]. Curr Opin Infect Dis, 23(2): 106-110.

[4] Lee V, Singh G, TrasattiJ P, et al (2014) Design and Fabrication of Human Skin by Three-Dimensional Bioprinting [J].Tissue Engineering Part C: Methods, 20(6): 473-484.

[5] Jin Yan (2004) Principles and protocols of tissue engineering[M].Xi'an:The Fourth Military Medical University Press.

[6] Jin Yan (2014) Tissue engineering and regenerative medicine[M]. Beijing:People's Medical Publishing House (PMPH).

[7]Ng W L, WangS, Yeong W Y, et al (2016) Skin Bioprinting: Impending Reality or Fantasy[J]. Trends in Biotechnology.

[8] Schlotmann K, Kaeten M, Blacky A F, et al (2001) Cosmetic efficacy claims in vitro using a three -dimensional human skin modeI [J]. International Journal of Cosmetic Science, 23:309-318.

[9] Amano w, Nakajima S, Kunugi H, et al (2015) The Janus kinase inhibitor JTE-052 improves skin barrier function through suppressing signal transducer and activator of transcription 3 signaling [J].Journal of Allergy and Clinical Immunology, 136(3) :667-669 .

[10]Plaza C, Meyrignac C, Botto JM, Capallere C (2021 Jul) Characterization of a New Full-Thickness In Vitro Skin Model. Tissue Eng Part C Methods ;27(7):411-420.

[11]Ponec M, Kempenaar J(1995) Use of human skin recombinants as an in vitro model for testing the irritation potential of cutaneous irritants[J]. Skin Pharmacol;8(1-2):49-59.

[12]Walters RM, Gandolfi L, Mack MC, et al (2016) In Vitro Assessment of Skin Irritation Potential of Surfactant-based Formulations by Using a 3-D Skin Reconstructed Tissue Model and Cytokine Response[J]. Alternatives to Laboratory Animals ;44(6):523-532.

[13] Smeriglio A, Denaro M, Mastracci L, Grillo F, Cornara L, Shirooie S, Nabavi SM, Trombetta D (2019 Jun) Safety and efficacy of hydroxytyrosol-based formulation on skin inflammation: in vitro evaluation on reconstructed human epidermis model. Daru;27(1):283-293.