

Skin Care Function of Lactoferrin Was Characterized Using Recombinant Human Epidermal Model

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Abstract: The effect of lactoferrin on skin was simulated using a recombinant human epidermal model. The anti-inflammatory and soothing effect of lactoferrin was verified using IL-1 α and TSLP Elisa assay. The effects of lactoferrin on the expression of related genes and proteins were detected using qPCR and immunofluorescence staining. The results showed that lactoferrin can effectively enhance the Transepidermal Electrical Resistance (TEER) and inhibit the secretion of inflammatory cytokine IL-1 α and TSLP. In addition, it was confirmed using qPCR that lactoferrin had high expression levels on AQP3, FLG, IVL, CLDN1 and HAS1 genes. Immunofluorescence staining confirmed that lactoferrin had high fluorescence intensity and expression in AQP3, Filaggrin and Involucrin. The results showed that lactoferrin improved the skin barrier at higher than 1.5 mg/mL. At the same time, it can have anti-inflammatory and moisturizing effects. This study provides a strong basis for the application of lactoferrin in cosmetics and daily chemical products.

Keywords: lactoferrin; epidermal model; skin care; AQP3; Filaggrin



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1. Introduction

Lactoferrin (LF) is an 80 kDa iron-binding glycoprotein [1], produced by epithelial cells and neutrophil precursors [2]. LF exists in the milk of most mammals and many other tissues and their secretions [3]. The structures of LF from different sources have great similarities. Bovine lactoferrin (bLF) and human lactoferrin (hLF) are composed of 689 and 691 amino acids, respectively, with a homology of 69% [4]. Among them, bLF is formed by folding two basically symmetrical and highly homologous leaf-like structure polypeptide chains. And hLF is higher in human milk, tear film, upper respiratory tract fluid, seminal plasma and cervical mucus suppositories. It is found in low amounts in earwax, saliva, small intestine, vaginal fluid and amniotic fluid. It does not exist in the alveoli [5] and skin [6], but exists in the sweat secreted by normal human skin [7]. LF has a high affinity with iron ions, and natural LF is usually only partially saturated with 15–20% of iron [8]. LF has anti-inflammatory [9], antibacterial [10], anti-tumor [11,12], antiviral [13], whitening, repairing skin damage caused by inflammation, immunomodulation and osteogenic activity [14] and other biological activities.

LF is considered to be a protein with great development potential. It has a wide range of application prospects in the fields of food [15], medicine [16] and cosmetics [17]. The skin is the largest organ of the human body and a physical, chemical and immune barrier to isolate harmful substances [18,19]. The efficacy of LF on the skin is particularly prominent, and it has the potential to be used as a new raw material for skin care products. There have been many studies on the effect of LF on the skin. In terms of anti-inflammatory activity, many studies have pointed out that LF plays an effective anti-inflammatory effect in local inflammation sites (including the respiratory and gastrointestinal tract) [20]. Farid et al. [21] found that LF has the ability to reduce liver inflammation by using LF in a Wistar rat model administered with carbon tetrachloride (CCl₄). This illustrates the direct anti-inflammatory effect of LF.

In addition, when the skin produces wounds, inflammation is also crucial to the effect of wound healing. Long-term or excessive inflammation can lead to chronic wounds that cannot be healed. Takayama et al. [22] found that LF can promote skin wound healing by enhancing the initial inflammation stage and neutralizing excessive immune responses. At the same time, on the third day of the wound, LF increased the production of key repair inflammatory mediators IL-8, IL-6, macrophage inflammatory protein 1 α (MIP-1 α) and tumor necrosis factor α (TNF- α) [23]. The study by Ushasree et al. [24] also confirmed that bLF at 2.5 mg/mL stimulated human limbal epithelial wound healing, and this stimulation was mediated by upregulation of platelet-derived growth factor (PDGF) or IL-6. The contents of IL-1 α [25] and thymic stromal lymphopoietin protein (TSLP) [26] were used as corresponding indexes in a human skin anti-inflammatory test. IL-1 secretion is an important expression of KC cells in response to external stimuli and self-injury, and KC cells mainly contain IL-1 α . IL-1 α plays a key role in the overall inflammatory response of cells and skin [27]. Thymic stromal lymphopoietin (TSLP) produced by KC cells plays a crucial role in promoting allergen sensitization that occurs in the skin [28].

2. Materials and Methods

2.1. Cell Culture

Normal human epidermal keratinocyte cells (KC) were purchased from Guangdong Biocell Co., Ltd. (Guangdong, China) and cultured in EpiLife (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) medium containing 60 μ M Ca²⁺ and HKGS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) in a 5% CO₂ incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C. The medium was replaced every two days, and the cells were used at 70% to 80% confluence.

2.2. Cell Viability Assay

Cell counting kit-8 (Beyotime Biotechnology, Shanghai, China) was used to test the viability of KC cells. Normal human epidermal keratinocytes (6000 cells/well) were seeded in 96-well plates (Nunc, Thermo Fisher Scientific, Waltham, MA, USA). After being stimulated with different concentrations (0.5 mg/mL, 1 mg/mL, 1.5 mg/mL, 2 mg/mL, 5 mg/mL, 10 mg/mL, diluted in the EpiLife medium) of LF (INGERDIA NUTRITIONAL, St, Pol, France) for 24 h, KC cells were incubated at 37 °C for 4 h, and 10 μ L/100 μ L CCK-8 was added. Optical density (OD) results were measured by reading absorbance at 450 nm with a microplate reader (Molecular Devices, San Francisco, CA, USA). For calculating the cell viability ratio, the calculation formula is as follows:

$$Viability(\%) = \left(\frac{OD \text{ treatment group}}{OD \text{ blank control group}} \right) \times 100$$

2.3. Construction of RHE Models and LF Topical Treatment

The RHE model was established using a complete EpiLife growth medium and Cell Culture Inserts (REF: MEPI500CA, Thermo Fisher Scientific, Waltham, MA, USA). The complete EpiLife growth medium was prepared by adding HKGS (REF: S0015, Thermo Fisher Scientific, Waltham, MA, USA), 140 μ M CaCl₂ (C7902-500G, Sigma-Aldrich, Burlington, MA, USA), 50 μ g/mL ascorbic acid (A4544-25G, Sigma-Aldrich, Burlington, MA, USA), and 10 ng/mL keratinocyte growth factor (104-07, PrimeGene, Shanghai, China). All non-sterile reagents require a 0.22 μ m filter (Millipore Express PES Membrane Filter Unit, 0.22 μ m, Merck Millipore Ltd., Co., Cork, Ireland). According to the standard protocol, Collagen type I (REF: 354236, Corning, USA) was diluted in D-PBS (Thermo Fisher Scientific, Waltham, MA, USA) by 1:100 precoated in the insert and left at room temperature for 1 h. Cells were seeded in precoated inserts with a 0.5 mL growth medium in both lower and upper compartments at a density of 7.5×10^4 cells/cm² for 4 days. Then, the inserts were transferred to supporting racks, and the medium was changed to CnT-Prime 3D barrier medium (CnT-PR-3D, Cellntec, Bern, Switzerland) for 14 days of air-liquid culture (0.75 mL

CnT-Prime 3D in lower compartments). After 14 days of liquid phase culture, a testable epidermal model was obtained. Medium was changed to EpiLife medium supplemented with stimulation cocktail (50 ng/mL rHuIL-4, 50 ng/mL rHuIL-13, 10 ng/mL rHuTNF- α , 10 ng/mL poly I:C, R&D, Minneapolis, MN, USA) [29]. And the RHE was treated for 24 h with samples prepared in the medium at different concentrations as described in Section 2.2. NC refers to the negative control. Hydrocortisone at 50 μ g/mL was set as a positive control (PC1) for the anti-inflammatory experiment. DHA (100 μ M) was set as a positive control (PC2) in qPCR experiments [30]. The culture medium was collected the next day for ELSIA testing. Tissues were collected, half for RNA extraction and half for immunohistochemical staining.

2.4. Quantitative Real-Time PCR Analysis

Total RNA was extracted from tissues following the protocol recommended by TRIZOL reagent (Life Technologies, Carlsbad, CA, USA). The concentration and quality of RNA was measured using Qubit 3.0 (Thermo Fisher Scientific, Waltham, MA, USA). This RNA was used for subsequent cDNA synthesis using the PrimeScript RT kit (Thermo Fisher Scientific, Waltham, MA, USA). Changes in mRNA levels were measured using the Light Cycler 96 system (Roche) and SYBR Premix Ex Taq II (Takara Biotechnology, Dalian, China) following the manufacturer's recommended protocol. All primers for AQP3, FLG, IVL, CLDN1, HAS1 and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed in Table 1. The relevant data were analyzed using the delta cycle threshold method, and the relative expression levels of each gene were normalized to the Ct of GAPDH and calculated according to the $2^{-\Delta\Delta C_T}$ method.

Table 1. Primer sequence information.

Primer Sequences	Primer Name
GGGGAGATGCTCCACATCC	AQP3-F
AAAGGCCAGGTTGATGGTGAG	AQP3-R
TGAAGCCTATGACACCACTGA	FLG-F
TCCCCTACGCTTTCTTGTCTCT	FLG-R
TCCTCCAGTCAATACCCATCAG	IVL-F
CAGCAGTCATGTGCTTTTCCT	IVL-R
CCTCCTGGGAGTGATAGCAAT	CLDN1-F
GGCAACTAAAATAGCCAGACCT	CLDN1-R
GAGCCTCTTCGCGTACCTG	HAS1-F
CCTCCTGGTAGGCGGAGAT	HAS1-R
GGAGCGAGATCCCTCCAAAAT	GAPDH-F
GGCTGTTGTCATACTTCTCATGG	GAPDH-R

2.5. Immunohistofluorescence Assay

After 24 h of sample addition, RHE was fixed with PBS and 4% paraformaldehyde and cooled for 20 min. The treated RHE slices were blocked with a 5% bovine serum albumin (BSA) buffer for 60 min. The RHE models were incubated with primary antibody (FLG 1:500, AQP3 1:1000, IVL 1:500, Abcam, Cambridge, UK) overnight at 4 °C, followed by Alexa Fluor 488-conjugated secondary goat anti-rabbit IgG (1:1000; Invitrogen, Carlsbad, CA, USA) for 2 h in the dark at 37 °C. The nuclei were stained using incubation for 10 min at room temperature with 4,6-diamidino-2-phenylindole (DAPI, 1:1000, Thermo Fisher Scientific, Waltham, MA, USA). Immunohistofluorescent images were visualized using a fluorescence microscope (EVOS FL Auto, Life Technology, Carlsbad, CA, USA) and analyzed using the EVOS Viewer Imaging Software 1.7 (Thermo Fisher Scientific, Waltham, MA, USA).

2.6. TSLP and IL-1 α Using ELISA

The culture medium of RHE was collected and the pro-inflammatory mediators TSLP (REF: 88-7497-88, Thermo Fisher Scientific, Waltham, MA, USA) and IL-1 α (DY200, R&D,

Minneapolis, MN, USA) were measured using ELISA kit. Hydrocortisone at 50 µg/mL was set as the positive reference group. These tests were performed strictly according to the manufacturer's instructions.

2.7. Statistical Analyses

All values are reported as mean SD. Mean values were calculated from data from at least three independent replicate experiments. The data were analyzed using Duncan test. A *p* value of less than 0.05 was considered statistically significant. All statistical analyses were performed with SPSS 25.0 (IBM Corporation, Armonk, NY, USA).

3. Results

3.1. Effect of LF on Cell Viability

As shown in Figure 1, the samples have no toxic effect on the KC cells in the concentration range of 5–50 mg/mL; the sample at the concentration without a cytotoxic effect will be selected to process the RHE model and the relevant efficacy evaluation will be carried out.

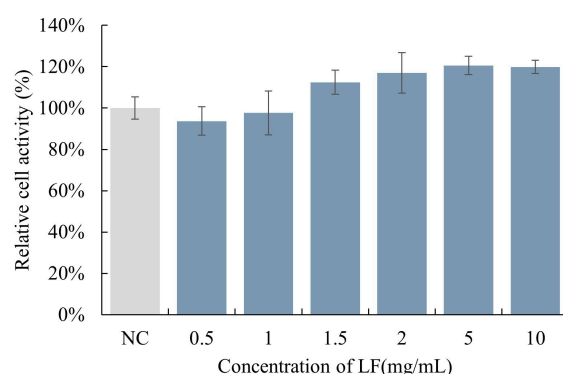


Figure 1. Effect of samples on KC cell activity.

3.2. Anti-Inflammatory Effect of LF on Inflammatory Factor Complex-Induced RHE Model

As shown in Figure 2A, there was no inflammatory response for all concentrations of the samples. Samples at 1.5 mg/mL significantly reduced IL-1α relative to the NC (Negative control) group (*p* < 0.05). Samples at 2, 5 and 10 mg/mL reduced IL-1α very significantly (*p* < 0.01). This indicates that the concentration of LF at 1.5 mg/mL or more can inhibit the release of IL-1α and TSLP, thus achieving anti-inflammatory effects. The effects of lactoferrin on IL-1α and TSLP expression are likely due to its concentration-dependent activation or inhibition of keratinocyte; high concentrations (above (include) 1.5 mg/mL) of lactoferrin can bind to and saturate its receptors on the cells, preventing their activation and subsequent production of pro-inflammatory cytokines like IL-1α. But lower concentrations (below 1.5 mg/mL) of lactoferrin may activate the transcription factor NF-κB, which regulates the expression of various genes involved in inflammation, including IL-1α.

3.3. TEER and qPCR Analysis

The results of the effect of samples on TEER of RHE were shown in Figure 3A. The TEER of RHE was significantly enhanced at sample concentrations up to 1.5 mg/mL, while concentrations at 2, 5 and 10 mg/mL were highly significant in enhancing the TEER of RHE, indicating that LF concentrations above 1.5 mg/mL could act as a skin barrier enhancer.

The expression of AQP3 was shown in Figure 3B. The results showed that the concentration of LF above 1 mg/mL could increase the expression of AQP3 extremely significantly. The expression of FLG was shown in Figure 3C. The LF concentration at 1 mg/mL significantly increased the expression of FLG. The expression of IVL is shown in Figure 3D. An LF concentration above 1.5 mg/mL significantly increased the expression of IVL. The expression of CLDN1 was shown in Figure 3E. The results showed that the concentration of

LF above 1.5 mg/mL could extremely significantly increase the expression of CLDN1. The expression of HAS1 was shown in Figure 3F. The results showed that a concentration of LF higher than 1.5 mg/mL could extremely significantly increase the expression of HAS1.

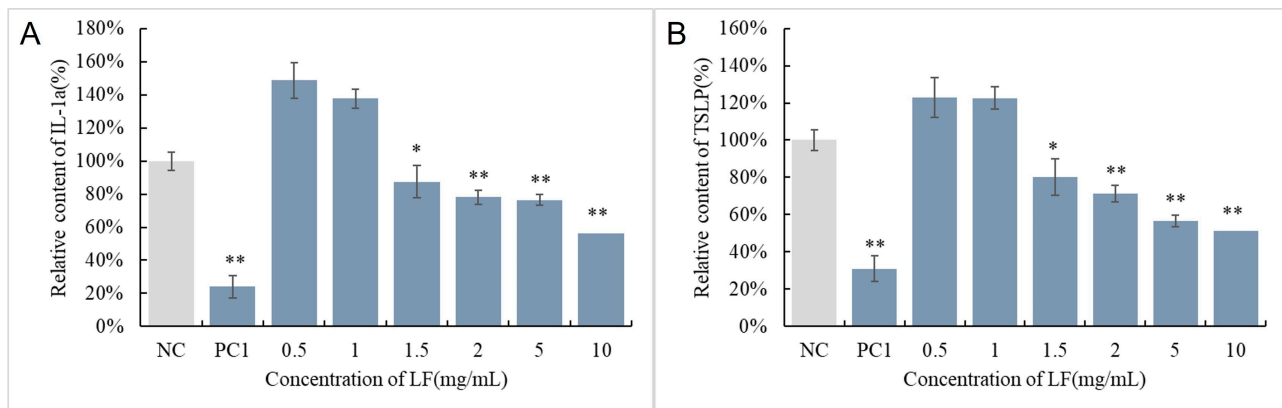


Figure 2. Effects of LF samples on IL-1α (A) and TSLP (B) stimulated by inflammatory in reconstructed human epidermis model. * Compared with the NC group, $p < 0.05$, ** compared with the NC group, $p < 0.01$.

3.4. Immunofluorescence Staining on RHE

The results of the immunofluorescence staining on RHE were shown in Figure 4. Figures 4A and 4B show the immunofluorescence staining results of AQP3 and Filaggrin, respectively. Water channel protein 3 (AQP3) has been shown not only to be highly permeable but also to act as an efficient transporter of glycerol. It is important for barrier repair and the moisturization of the epidermis. AQP3 brings circulating water into the epidermis, providing a short water circuit between the basal layer of epidermal cells and the stratum corneum, thus ensuring a constant water content in the epidermis [31]. Filaggrin, on the other hand, is a key protein required for the formation of the stratum corneum barrier, acting as a source of hydrophilic amino acids and their derivatives, maintaining skin moisture by binding water and being known as a natural moisturizing factor (NMF) [32]. The skin barrier it constitutes acts as a skin water lock. As seen in Figure 4A, AQP3 was expressed in close proximity to the basal and spiny layers. Compared to the NC group, the samples at 0.5 and 1 mg/mL showed a weaker expression of AQP3 and did not differ significantly. And from the quantitative results in Figure 4C, it was the sample concentration above 1.5 mg/mL that elevated the positive rate of AQP3 extremely significantly. The sample concentrations at 0.5 and 1 mg/mL significantly enhanced the fluorescence intensity, and sample concentrations above 1.5 mg/mL highly significantly enhanced the fluorescence intensity. This suggests that lactoferrin can somehow modulate AQP3. However, the specific mechanism needs to be further studied. In summary, sample concentrations above 1.5 mg/mL significantly enhanced skin hydration, resulting in moisturization.

When the epidermal barrier is altered, a more significant feature is the decrease in the synthesis of the envelope protein Filaggrin [33]. Therefore, Filaggrin can effectively reflect the epidermal barrier by showing the thickness, strength and integrity of the stratum corneum. The results of the Filaggrin immunofluorescence staining of samples on isolated skin are shown in Figure 4B. It can be seen that Filaggrin is expressed in the stratum corneum. The green fluorescence fraction was significantly stronger at sample concentrations higher than 1 mg/mL compared to the NC group. From the quantitative results, the sample concentration at 1 mg/mL significantly enhanced the positive rate and fluorescence intensity. A concentration higher than 1.5 mg/mL can highly and significantly enhance the positive rate and fluorescence intensity, and the stratum corneum is thicker and more intact. In conclusion, the sample concentration higher than 1.5 mg/mL can improve the skin stratum corneum barrier, thus achieving the effect of skin water locking. Uchida et al. [34] also found an increase in transepithelial electrical resistance (TER) after the addition of LF

to human keratinocytes (HaCaT). The increase in TER was accompanied by an increase in the expression of two differentiation marker proteins, namely Involucrin and Filaggrin. In addition, LF has been shown to promote the expression of Involucrin and Filaggrin in normal human epidermal keratin-forming cells (NHEK).

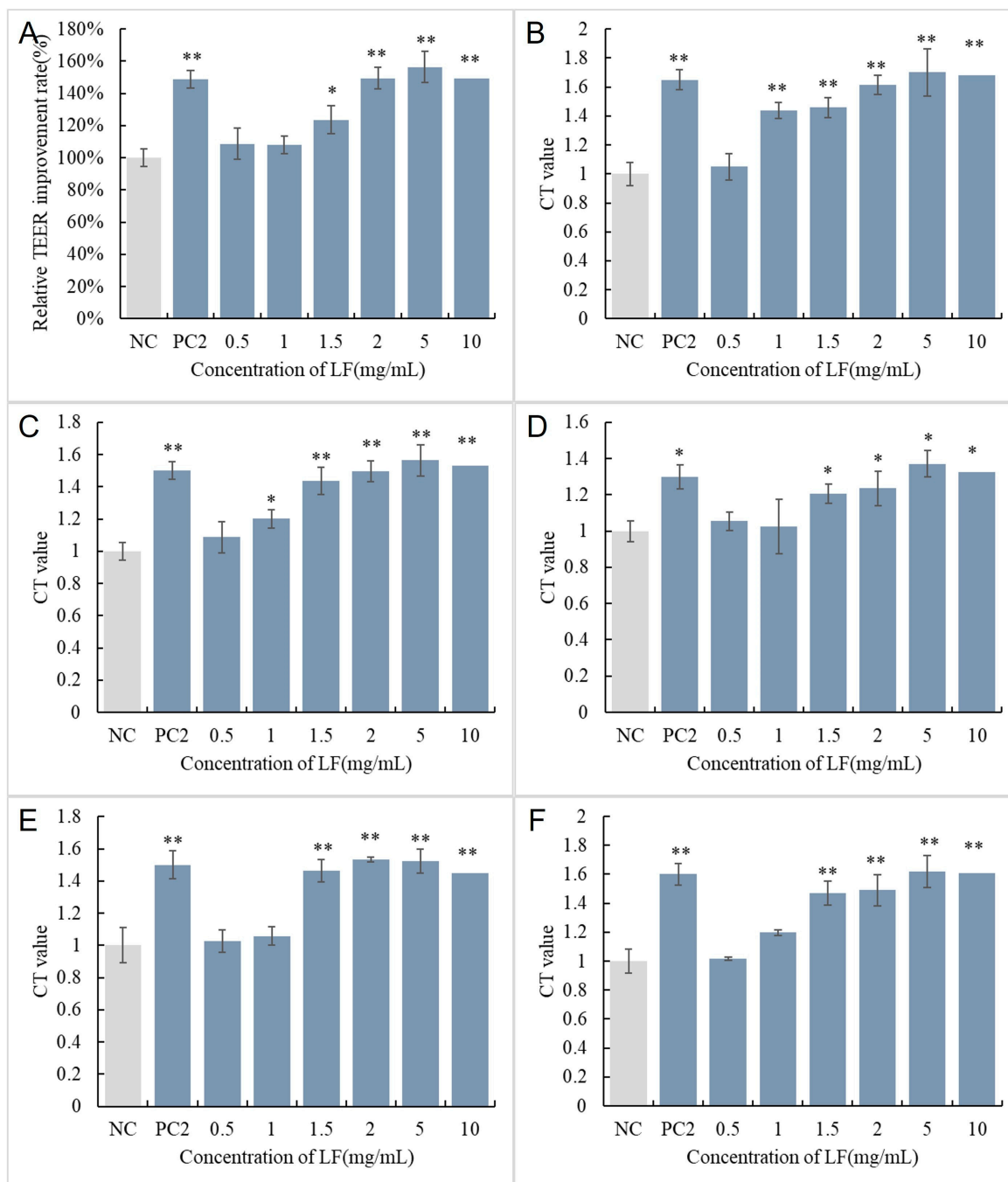


Figure 3. TEER and qPCR analysis results ((A), TEER; (B), AQP3; (C), FLG; (D), IVL; (E), CLDN1; (F), HAS1). * Compared with the NC group, $p < 0.05$, ** compared with the NC group, $p < 0.01$.

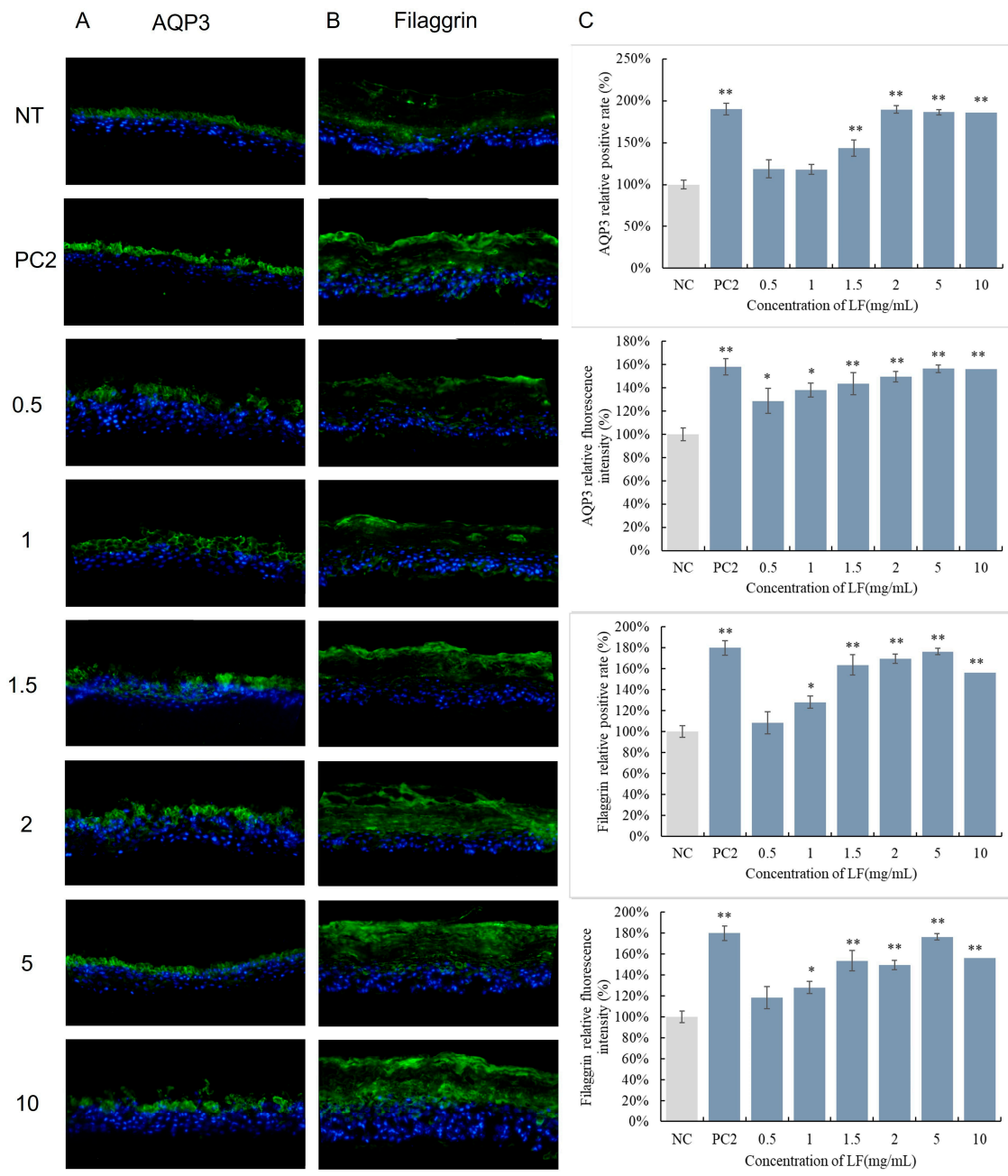


Figure 4. AQP3 (A) and Filaggrin (B) immunofluorescence staining on RHE, results of relative positive rate and relative fluorescence intensity (C). * Compared with the NC group, $p < 0.05$, ** compared with the NC group, $p < 0.01$.

4. Discussion

In this study, 3D skin modeling technology was used to investigate the moisturizing, anti-inflammatory and skin barrier improvement effects of LF in skin care. And the role of LF in skin care was discussed in depth. The results showed that the content of IL-1 α and TSLP could be significantly reduced when the content of LF was higher than 1.5 mg/mL, which indicated that LF could have an anti-inflammatory effect. This may be due to the ability of LF to bind to a variety of microorganisms and inhibit their growth and activity. In addition, lactoferrin can directly bind to inflammatory mediators such as IL-1 α and TSLP, reducing their biological activity and thus reducing the inflammatory response. At the same time, lactoferrin modulates the activity and function of immune cells, reducing their release of pro-inflammatory factors such as IL-1 α .

Meanwhile, the expression of AQP3, FLG, IVL, CLDN1, and HAS1 could be significantly enhanced. LF helps maintain skin moisture balance and enhance skin barrier function and elasticity by increasing the expression of AQP3. And by increasing the expression of FLG, it helps to enhance the skin barrier function and reduce skin sensitivity and inflammation. At the same time, increased IVL expression may indicate further support for skin health or the enhancement of specific skin functions. In addition, LF helps to enhance intercellular connectivity and barrier function by increasing CLDN1 expression. It may play a positive role in skin protection, wound healing and so on. LF may increase the synthesis of hyaluronic acid by increasing the expression of HAS1, thus enhancing the moisturizing, lubricating and repairing functions of skin. These findings provide new ideas and potential for the application of lactoferrin in skin care and medical fields. However, the specific mechanisms and clinical applications of these effects still need to be further studied and validated.

In addition, it can significantly increase the expression of AQP3 and Filaggrin. This result verifies that LF acts as a moisturizer and enhances the skin barrier from the perspective of protein expression. It provides a theoretical basis for the application of LF in skin care products and other cosmetics. It provides a new idea for the development of raw materials for skin care products. However, the results based on the RHE model still have some limitations and will need to be validated via clinical studies in the future.

5. Conclusions

It was experimentally verified that a concentration of LF higher than 1.5mg/mL can significantly reduce the content of IL-1a and TSLP. Meanwhile, it can significantly enhance TEER and significantly enhance the expression of AQP3, FLG, IVL, CLDN1, and HAS1. It also significantly enhanced the positive rate and fluorescence intensity of AQP3 and Filaggrin. Therefore, LF at concentrations higher than 1.5 mg/mL can provide skin care effects of moisturization, anti-inflammation, and can improve the skin barrier.

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Conflicts of Interest: The authors declare no conflicts of interest. Pigeon does not produce lactoferrin.

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