IFSCC 2025 full paper (IFSCC2025-682)

"Establishment and Application of an In Vitro Retinol Damage Model"

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1. Introduction

As the first barrier of the body against the external environment, the skin is exposed to environmental stressors like ultraviolet light and chemicals. These factors can induce acute skin damage and inflammatory responses [1]. Retinol (ROL), a vitamin A derivative widely used in cosmetic formulations, has demonstrated efficacy in improving wrinkles, fine lines, irregular pigmentation, and treating acne [2, 3]. By activating nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [4, 5], ROL regulates multiple target genes involved in cell growth and differentiation, thereby influencing the structure and function of the skin. However, when applied improperly, ROL acts as a dermal stressor, inducing dose-dependent adverse reactions that typically include erythema, pruritus, burning sensations, dryness, desquamation, and a condition known as "retinoid dermatitis" at the application site [6].

In the development and evaluation of repair products for retinol-induced damage, validating their efficacy through models represents an indispensable step. Current research on retinol-induced skin damage primarily relies on clinical studies and animal experiments for model selection. Although human trials provide direct clinical data, their widespread application is constrained by high costs, potential risks, and ethical considerations. Animal models also face challenges due to biological disparities with human skin and concerns for animal welfare (adhering to the 3R principles: replacement, reduction, and refinement) [7]. Therefore, this study employs a 3D epidermal model to establish an *in vitro* model of retinol-induced damage. In this research, an *in vitro* retinol damage model was established by simulating clinical skin damage caused by improper retinol use, with histological and biological characterization as evaluation metrics. This model provides a scientific basis for developing novel therapeutic strategies and serves as a valuable tool for screening cosmetics/drugs with skin damage repair potential.

2. Materials and Methods

Materials:

Retinol was purchased from Sigma-Aldrich Chemical Co(USA); DMEM medium, fetal bovine serum, and Goat-anti-rabbit IgG Alexa Fluor 488 secondary antibody were purchased from Invitrogen (USA); Human IL-8 ELISA Kit was purchased from Multisciences (Lianke) Biotech, Co., Ltd (China); Human monocyte chemotactic protein 1/monocyte chemotactic and activating factor (MCP-1/MCAF) ELISA kit was purchased from Wuhan Huamei Biotechnology Co., Ltd.

(China); Anti-Filaggrin antibody, Anti-Loricrin antibody, Anti-Involucrin antibody, Anti-Occludin antibody, and Anti-Ki67 antibody were purchased from Abcam Inc(UK); AVIDIN/BIOTIN blocking reagent was purchased from Life Technologies (USA); Mounting Medium, antifading (with DAPI) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (China).

Methods:

Reconstructed human epidermis model

The 3D epidermal model were derived from using normal human foreskin keratinocytes. Briefly, keratinocytes were seeded in polycarbonate cell culture insert for one days, followed by air-liquid interface culture for another 7 days.

Establishment and application of Retinol damage Model

To simulate retinol-induced skin damage using the 3D epidermal model, this study investigated the effects of concentration, treatment duration, and application frequency via three independent experiments. The specific experimental design is as follows:

(1) Concentration gradient experiment: The 3D epidermal models were divided into a negative control group (solvent control) and experimental groups (0.3% and 0.03% retinol). All groups were cultured at 37°C and 5% CO_2 for 48 h.

(2) Time gradient experiment: With the retinol concentration fixed at 0.3%, models were treated for 48 h and 72 h under identical conditions, using solvent as the control.

(3) Treatment frequency experiment: Using 0.3% retinol, a second treatment was applied 24 h after the first, with samples collected after 48 h of cumulative treatment. The control group underwent synchronous treatment.

(4) Damage repair efficacy evaluation: According to the optimal damage conditions, after retinol treatment twice, the repair cream was evenly applied to the surface of the epidermal model. After a total of 48 h of treatment, the residual cream on the surface was washed off with PBS.

After the process, collect the skin tissue and the culture supernatant. The tissue was immediately fixed in 10% Neutral Formalin. They were then either embedded in paraffin for histological analysis or embedded in an OCT compound and frozen at -80°C for immunohistochemical analysis.

Histological staining

Paraffin-embedded formalin-fixed samples were cut into 5μm sections. After dewaxing and rehydration, sections were stained with hematoxylin and eosin for routine histological analysis.

Inflammatory Cytokine Assay

The supernatants were collected, and levels of MCP-1 and IL-8 were quantified using commercial ELISA kits, according to the procedures recommended by the manufacturer.

Immunofluorescence Staining

The OCT-embedded blocks were sectioned into 5-μm-thick slices using a cryostat. Sections were blocked with 5% BSA at room temp for 1 h, incubated with primary antibodies (Ki67, FLG, LOR, OCLN) at 4°C overnight, then with secondary antibodies for 1 h. Slices were mounted with DAPI-containing medium for nuclear counterstaining, and photos were taken under a fluorescence microscope.

Statistical Analysis

Fluorescence data were quantitatively analyzed using Image J software to obtain the mean value of average fluorescence intensity. Images were processed with GraphPad Prism, and results were presented as mean ± SD. Statistical differences between groups were evaluated

using Student's T-test.Statistically significant differences are indicated by asterisks as follows: *p<0.05, **p<0.01, and ***p<0.001. All experiments were repeated at least three times.

3. Results

Screening of Critical Conditions for Retinol Damage Model

Concentration-Dependent Effects

Fig. 1a shows that 0.03% retinol had no significant impact on the 3D epidermal model's tissue structure. In contrast, 0.3% retinol led to epidermal thickening, mild stratum corneum separation, and impaired granular layer differentiation. ELISA analysis (*Fig. 1b*) revealed that 0.03% retinol had similar MCP-1 and IL-8 release to the NC group, while 0.3% retinol significantly increased MCP-1 release (*p<0.05).

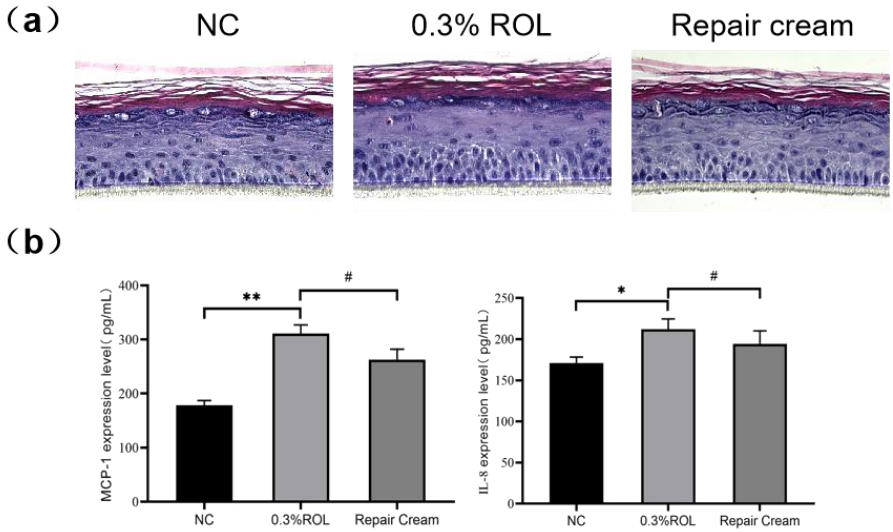

Figure 1. H&E staining (a) and inflammatory cytokine expression (b) in the 3D epidermal model treated with vehicle or different concentrations of ROL.

Based on histological and inflammatory profiles, 0.3% retinol was selected as the optimal concentration for establishing the *in vitro* retinol damage model.

Duration-Dependent Effects

As depicted in *Fig. 2a*, 48 h treatment with 0.3% retinol caused epidermal thickening, mild stratum corneum separation, and impaired granular layer differentiation. Paradoxically, 72 h treatment reduced epidermal thickness and exacerbated SC separation. ELISA (*Fig. 2b*) indicated an MCP-1 increase at 48 h (*p<0.05) and a decrease in both MCP-1 and IL-8 at 72 h. These results suggest an inflammatory peak at 48 h, with potential excessive damage occurring at 72 h.

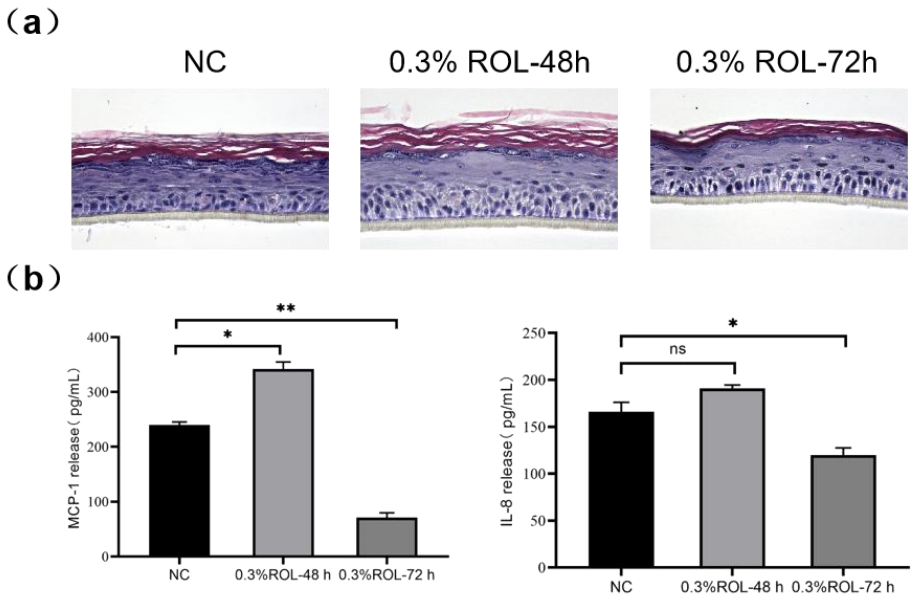

Figure 2. H&E staining (a) and inflammatory cytokine expression (b) in the 3D epidermal model treated with vehicle or ROL for different durations.

Based on histological and inflammatory profiles, 48 h was determined as the optimal treatment duration for establishing the *in vitro* retinol damage model.

Frequency-Dependent Effects

Fig. 3a shows that a single 0.3% retinol treatment thickened the epidermis with relatively clear granular layer differentiation. In contrast, two treatments with 0.3% retinol-induced notable morphological alterations: epidermal thickening, aggravated stratum corneum separation, and decreased keratohyalin granules. ELISA (*Fig. 3b*) showed a significant increase in MCP-1 after one treatment (*p<0.05) and significant increases in both MCP-1 and IL-8 after two treatments (*p<0.05).

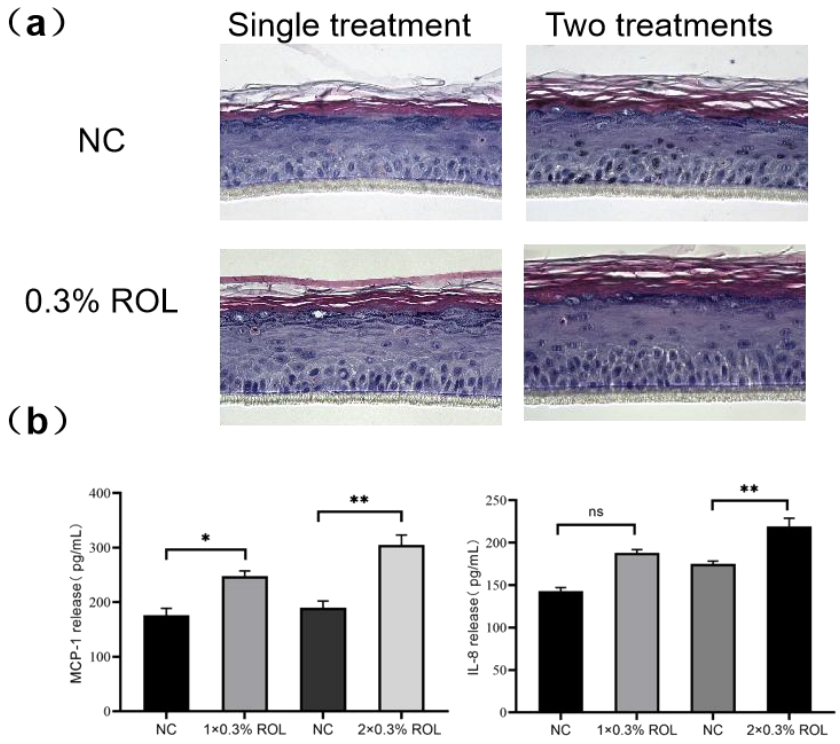

Figure 3. H&E staining (a) and inflammatory cytokine expression (b) in the 3D epidermal model treated with vehicle or ROL for different treatment frequencies.

Based on histological and inflammatory profiles, the secretion of inflammatory factors increased significantly (*p<0.05) after two retinol treatments, with obvious histological morphological changes that stopped short of excessive damage rendering the epidermis irreparable. Therefore, two treatments with 0.3% retinol were selected to mimic frequent clinical retinol misuse, which induces cumulative irritation.

Characterization of the Retinol Damage Model

Retinol treatment significantly increased the number of proliferative epidermal cells, as assessed by Ki67 immunoreactivity (*Fig. 4*). *Fig. 4* also illustrates the effects of retinol on barrier protein expression in the 3D epidermal model. Compared with the vehicle control, treatment with 0.3% retinol led to significant reductions in the relative fluorescence intensities of filaggrin (FLG), loricrin (LOR), and occludin (OCLN) (*p<0.001 for all).

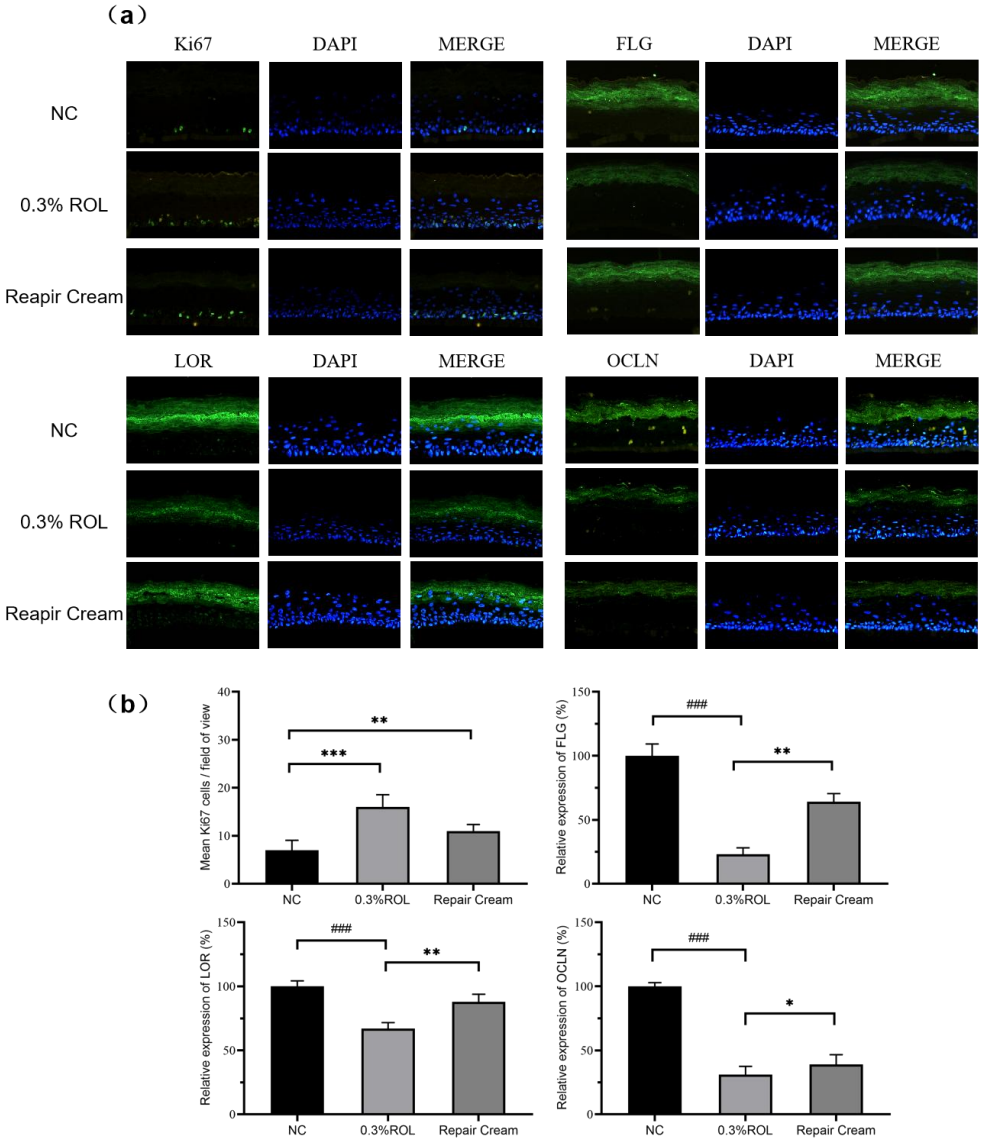

Figure 4. Immunofluorescence staining (a) and quantification of Ki67, FLG, LOR and OCLN expression (b) in the 3D epidermal model treated with vehicle or ROL. Ki67, FLG, LOR and OCLN were stained in green fluorescence and the nucleus was counterstained in blue DAPI.

The above experimental results indicate that under the condition of two consecutive treatments with 0.3% retinol, the H&E results show thickening of the epidermis layer, separation of the stratum corneum, and unclear differentiation of granular layer cells. The proliferation of epidermal basal layer cells and the expression of skin barrier related proteins decrease, which is consistent with the clinical simulation of retinol-induced damage reactions. This indicates that the *in vitro* retinol damage model has been successfully established.

Effects of the Repair Cream on the Retinol Damage Model

As shown in *Fig. 4* and *Fig. 5,* the repair cream restored tissue structure in retinol damaged 3D epidermal models: the stratum corneum appeared more compact, and granular layer differentiation was clearer compared with the retinol damage group. Biochemically, the cream mitigated retinol-induced reductions in barrier proteins and decreased the release of pro-inflammatory cytokines MCP-1 and IL-8 (*p<0.05). These findings suggest the cream alleviates retinol irritation by suppressing inflammation and reinforcing the epidermal barrier.

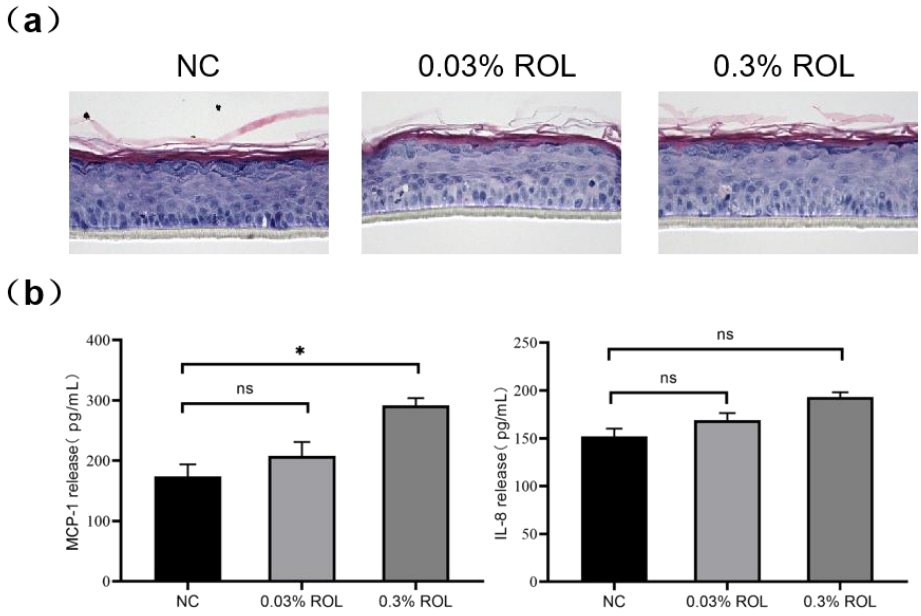

Figure 5. H&E staining (a) and inflammatory cytokine expression (b) in the 3D epidermal model treated with vehicle, ROL, or Repair Cream.

4. Discussion

Although retinol has a significant therapeutic effect on skin conditions such as photoaging and acne, it also has potential irritancy. The development of reliable *in vitro* retinol-induced epidermal damage models is crucial for promoting targeted intervention measures. Our model employs 0.3% retinol—the maximum concentration permitted by the Scientific Committee on Consumer Safety (SCCS) for both leave-on and rinse-off cosmetic formulations, ensuring alignment with regulatory safety standards.

This study simulates clinical "retinol-induced damage" through three key mechanisms:

1. Epidermal structural disorder: The thickness of the epidermal layer, the degree of separation of the stratum corneum, and the differentiation of the granular layer are among the criteria for evaluating retinol-induced damage. H&E staining revealed epidermal thickening, stratum corneum separation, and reduced keratohyalin granules, which was consistent with the epidermal hyperplasia and keratohyalin granule loss observed in the reconstructed epidermal models treated with vitamin A derivatives in Hsia et al.'s study [7]. Epidermal thickening is at-

tributed to retinol activating nuclear retinoic acid receptors (RARs), driving keratinocyte proliferation—a phenomenon that matches the retinol-induced epidermal thickening reported in Kong et al.'s clinical studies [8].

2. Inflammatory response activation: It is well established that vitamin A can induce local skin irritation, a phenomenon speculated to be primarily mediated by the regulation of inflammatory mediators. Among the array of proinflammatory cytokines, the release of IL-8 and MCP-1 is recognized as a validated biomarker [9]. Inhibiting the production of these inflammatory mediators has been shown to significantly alleviate retinol-induced skin irritation in patch test models[10]. The significant elevation of pro-inflammatory cytokines MCP-1 and IL-8 in this retinol damage model aligned with cytokine upregulation observed in Kim et al.'s retinol-treated keratinocytes [9]. As a neutrophil chemotactic factor (IL-8) and monocyte chemotactic protein (MCP-1), their cooperative elevation reflects retinol-induced local immune activation, directly correlating with clinical symptoms of "retinoid dermatitis" such as erythema and pruritus.

3. Barrier function impairment: There exists a balance between the efficacy of retinoids and the adverse reactions of local application, which is reflected in the balanced expression of genes related to the cornified envelope and tight junctions (TJs). The CE, together with corneodesmosomes, provides structural integrity to the stratum corneum and is responsible for the physical epidermal barrier function [11]. TJs are complex cell-cell junctions that form a barrier in the stratum granulosum of mammalian skin [12]. Reduced expression of FLG, LOR, and OCLN in this retinol damage model confirmed retinol's disruptive effect on cornified envelope and tight junction proteins. Li et al. [13] demonstrated that retinoic acid changes the expression of barrier proteins and related tight junction proteins in the epidermis, manifested as downregulated expression of FLG, LOR, and CLDN1, as well as upregulated expression of CLDN2 and CLDN4. Cheong et al. [14] treated keratinocytes with retinoic acid and found that the expression of FLG and LOR decreased, and the mRNA results also confirmed this finding, further confirming that retinoic acid may cause damage to the skin barrier.

The validation of the repair cream's efficacy in this study demonstrates that the model can effectively evaluate barrier repair products, providing a standardized tool for cosmetic and drug development.

In conclusion, the *in vitro* retinol damage model established in this study demonstrates strong rationality and validity, offering a reliable platform for investigating the mechanisms of retinol-induced damage and exploring corresponding repair strategies.

5. Conclusion

This study established an *in vitro* retinol-induced epidermal damage model, which accurately recapitulates clinical retinoid-induced irritation responses through mechanisms such as epidermal hyperplasia, stratum corneum separation, degranulation, inflammatory activation, and downregulation of barrier proteins. This study provides a new platform for the *in vitro* screening of active substances for repairing retinol-induced damage and offers relevant scientific evidence for the repair of retinol-induced damage.

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