

ORIGINAL ARTICLE

Development of a new diaper dermatitis-like reconstructed skin equivalent for testing children atopic dermatitis relieving cosmetics

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Abstract

Background: Diaper dermatitis (DD) is the most common acute inflammatory skin disease. It has a serious effect on children's and families' quality of life. We aimed to screen and evaluate the efficacy of different formulas for relieving the diaper dermatitis symptoms by developing a kind of diaper dermatitis-like reconstructed human skin equivalent in vitro.

Materials and Method: We developed the human skin equivalent for diaper dermatitis with 0.2% Sodium lauryl sulfate (SLS). The diaper dermatitis-like human skin equivalent was characterized by high level of inflammation, such as overexpression of interleukin-1 α (IL-1 α), and impaired skin barrier. Four formulas with potential of anti-inflammation and promotion of skin barrier function were topically applied on the diaper dermatitis-like human skin equivalent surface. The afterward protection efficacy was evaluated by endpoints of IL-1 α , tissue viability, and skin barrier function.

Results: The chemical irritant induced high release of IL-1 α , impaired tissue viability, and skin barrier function. The cream prepared with potential of anti-inflammation and skin protection could effectively decrease and relieve the impact of irritant with decreased level of IL-1 α and the higher tissue viability than the placebo exposure.

Conclusion: The results showed that diaper dermatitis-like human skin equivalent induced by SLS can mimic the skin irritation response of the diaper rash.

KEYWORDS

anti-inflammation, Diaper dermatitis, reconstructed human skin equivalent in vitro, skin barrier

1 | INTRODUCTION

Diaper dermatitis is a common cutaneous disorder characterized with red and spotty, and is sometimes itchy in the diaper area of the infant. The condition is one type of acute nonimmunologic skin inflammation. There were mainly three types of diaper dermatitis, including diaper dermatitis caused by candidiasis, irritant contact dermatitis, and chaffing dermatitis.¹ Chaffing is the most widespread form of mild diaper dermatitis commonly affecting most infants at some point in time. It happens in areas where frictional coefficient

from the nappy is high, including the inner surface of the buttocks, thighs, and the surface of the genital areas.¹ Irritant contact diaper dermatitis (ICDD) is commonly found in the gluteal crease, buttocks, perianal, and the public areas. It can also include the lower abdominal area as well as the upper thigh area. Irritant contact diaper dermatitis can range in severity from a mild case, with mild localized erythema and minimal scaling, to a moderate case, with increased erythema as well as papules noted to the affected area, and finally to a more severe case where the skin in the diaper area is noted to have papules, pustules, and skin breakdown with open areas.²

The causes of diaper dermatitis are multiple factors (physical, chemical, enzymatic, and microbial factors). The main reason is relative to the features of prolonged occlusion of the nappy area such as urine or feces, friction on the skin, and the presence of a high skin pH.³

The skin of diaper area was exposed to water and irritation which resulted in over-hydration. Elevated skin hydration predisposed compromised integrity of skin and skin damage, including increasing its permeability to irritants, increasing its frictional rate, and promoting microbial growth. Elevated pH increases the activity of fecal enzymes that can attack the skin, and increases the permeability of skin. Urine and feces are also detrimental to skin integrity via disruption of the SC lipids and ceramides leading to increased penetration and epidermal inflammation.⁴⁻⁷ In addition to irritants exposed to skin in diaper area, micro-organisms such as candidal colonization in the perineal area is one contributing factor in the causation of nappy rash. It was reported that diaper rash was very prevalent in the group of infancy aged 9 months to 2 years.⁸ The incidence of diaper dermatitis in infancy population was 7% and 35%.⁹⁻¹¹ Pain and deteriorate caused by diaper dermatitis for infants have been a big troublesome for their parents. So, protection of skin in the diaper area has always been a challenge.¹²

Although a competent skin barrier of the full-term infant was reported, with epidermis and stratum corneum,¹³ the cell junction between keratinocytes was not perfect, and that full maturation of the skin barrier might not be complete until after a year of age.¹⁴ The key to prevent the diaper dermatitis is to keep baby skin from prolonged contact with irritants such as feces and urine. Once it happened, to decrease inflammation, repair the damage to the skin and prevent reoccurrence would be the overall objective.¹⁵ To achieve the goal, the superabsorbent disposable diapers and the skincare routines for babies in intensive care and special care have been advised. The former could keep the skin dry to reduce the incidence of diaper dermatitis.¹⁶ Skin care with barrier creams, especially increased application of water-in-oil emollient led to a significant improvement in skin condition reflected by less visible dryness, redness, and skin surface damage by coating the surface of the skin and/or by supplying lipids that can penetrate the intercellular spaces of the stratum corneum to prevent entry of moisture and irritants into the skin and protect from nappy rash.¹⁷⁻¹⁹ With being the back bone of prevention and treatment for diaper dermatitis, several skin care products and topical compounds have been available in the market, however, there is a lack of clear evidence to prove the efficacy of one product over another, making the selection of an appropriate cream being difficult for families and practitioners.^{9,11,20}

To screen effective formulations targeting diaper dermatitis, there is a need for skin models that mimic the pathologic features and pharmacologic intervention of diaper dermatitis. In this study, we constructed the three-dimensional skin equivalent which was characteristic of impaired skin barrier function, acute inflammation caused by contact irritant. In addition, we also evaluated the efficacy of one commercial available product and our developed formulas with such diaper dermatitis-like reconstructed skin equivalent in vitro.

2 | MATERIALS AND METHODS

2.1 | Culture of keratinocytes

Normal human keratinocytes were cultured from the foreskin of healthy children (less than 12 years old) donors undergoing medical surgery. The skin tissue was collected with informed consent form and institutional approval. Primary cells were isolated as previously described by Rheinwald and Green.¹ The primary keratinocytes culture medium was Keratinocyte-SFM medium (Gibco/BRL, Gaithersburg, Md.). Cultures were incubated in a humidified and 5% CO₂ atmosphere at 37°C and culture medium was changed every 2 days. Keratinocytes were digested with trypsin-EDTA when reaching over 70% confluence and passaged in Keratinocyte-SFM medium to passage 7 for the following preparation of reconstructed skin equivalents.

2.2 | Preparation of diaper dermatitis-like reconstructed skin equivalents

Keratinocytes were seeded onto the polycarbonate membrane with a density of $5 \times 10^5/\text{cm}^2$, and followed by culture in Keratinocyte-SFM medium for 48 h. The cultures were then elevated to the air-liquid interface with differentiation medium, which contained DMEM with hydrocortisone (0.4 µg/mL), insulin (5 µg/mL), ascorbic acid 2-phosphate (1 mM), antibiotics, and bovine pituitary gland extracts (BPE, 25 µg/mL). On the 10th day of normal air-liquid interface culture, 0.2% SLS (Sigma, St Louis, MO, U.S.A.) was applied (diaper dermatitis-like reconstructed skin equivalents) or not (normal reconstructed skin equivalents) onto skin equivalent surface, and followed by culture for another 24 h to make diaper dermatitis-like and normal reconstructed skin equivalents, respectively.

2.3 | Application of skin care formulas

In order to verify the applicability of diaper dermatitis-like reconstructed skin equivalents for screening of skin care formulas, anti-irritation actives and skin barrier promotion agents were included into various formulas, and their effects on anti-inflammation and barrier promotion were evaluated in vitro. Each formula composed of anti-irritation actives and skin barrier promotion agents was exposed topically for 24 h before the adding of 0.2% SLS. Finally, skin equivalents and the underlying medium for different groups, including blank control, negative control (0.2% SLS only), and experimental groups (formulas and 0.2% SLS), were harvested to test tissue viability, ET₅₀, histology, and Interleukin-1α (IL-1α).

2.4 | Tissue viability

Tissue viability was assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) test, according to a validated test protocol. By measuring changes in absorbance reading of

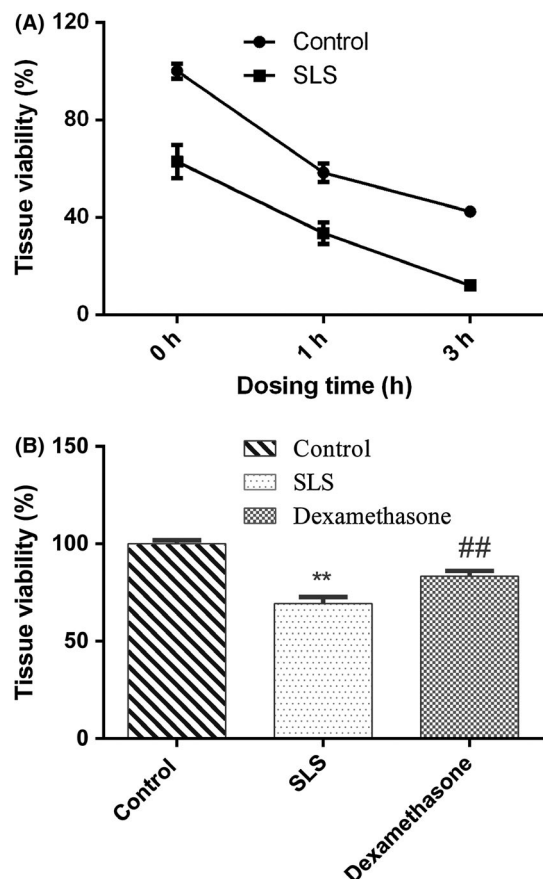


FIGURE 1 A, Verification of diaper dermatitis-like reconstructed skin equivalents by ET_{50} . SLS served as the irritant. B, Verification of diaper dermatitis-like reconstructed skin equivalents by tissue viability test. SLS served as the negative control group, and dexamethasone served as the positive control group (** $P < 0.01$, compared with blank control; ## $P < 0.01$, compared with the negative control)

formazan accumulation in the living reconstructed skin equivalents, tissue viability can be estimated. The tissue viability of the blank control served as the reference, of which were set to 100%.

2.5 | Determination of ET_{50}

After the final incubation, Triton X-100 with a concentration of 0.3%(V/V) was slowly added onto the skin equivalents surface, and followed by exposure of 0 h, 1 h, and 3 h in a humidified, 5% CO_2 atmosphere at 37°C. Then, tissue viability of each skin equivalent was assessed by the MTT test. The tissue viability result was profiled to increasing exposure time and the ET_{50} was calculated as the exposure time at which reduces the tissue viability to 50%.

2.6 | Histological analysis

Skin equivalents were fixed in 4% (v/v) formalin solution, dehydrated and then embedded in paraffin. Six-micrometer-thick sections were cut and stained with hematoxylin and eosin (H&E) following the conventional staining procedure. At least three random photographs

were taken of each skin equivalent and compared for histological change.

2.7 | Interleukin-1 α measurement by ELISA

Underlying medium of each skin equivalent was collected and stored at -20 °C until test. The content of IL-1 α was analyzed by specific enzyme-linked immunosorbent assay (ELISA) Kit (Abcam) with specific instructions. Optical density ($\lambda=450$ nm) was measured by enzyme-linked immune detector (Biotech Epoch, America).

2.8 | Statistical analysis

Results are expressed as mean $\pm$ SD. Statistical comparisons were performed by one-way analysis of variance (ANOVA) with repeated measures. Differences were considered as statistically significant at $P < 0.05$.

3 | RESULTS

3.1 | Verification of diaper dermatitis-like reconstructed skin equivalents

In order to verify the applicability of the diaper dermatitis-like reconstructed skin equivalents, inflammatory cytokine, tissue viability, and skin barrier function were used as key endpoints. Dexamethasone was used to evaluate the response of the diaper dermatitis-like reconstructed skin equivalents to such well-known anti-inflammation medicine in vitro. Tissue viability and ET_{50} results revealed that skin equivalents induced by 0.2% SLS resulted in significant decrease of tissue viability and skin barrier (ET_{50}) as compared to the normal skin equivalents without SLS application (Figure 1a). Skin tissue viability treated with anti-inflammatory agent dexamethasone, on the other hand, was increased significantly compared with the skin equivalents with only SLS application (Figure 1b). The application of dexamethasone assisted of the protection of tissue viability which may be assaulted by the irritation nature of SLS. The results proved that SLS application resulted in diaper dermatitis-like skin equivalents and dexamethasone, with properly skin tissue protection response, can serve as positive control.

3.2 | In vitro anti-diaper dermatitis efficacy evaluation for skin care formulas

We further examined the morphology of negative control which was irritated by 0.2% SLS, by H&E staining, to validate whether the reconstruction and induction of skin equivalents meet the characteristics of diaper dermatitis (Figure 3). A large numbers of injured cells were characterized with dark cytoplasm and pyknotic nucleus in the viable layers of the negative control. SLS irritation resulted in impaired tissue integrity, which was consistent to the decreased tissue viability as compared with the blank control (decreased by 43%, $P < 0.01$). These results proved successful skin equivalents reconstruction and induction by SLS, mimicking the model of diaper dermatitis.

The formula NO.3 and NO.4 exhibited significant promotion of tissue viability compared with the negative control. However, the formula NO.1 and NO.2 exhibited no effect of protection of tissue viability (Figure 2). The morphology of each group (Figure 3) was consistent with the results of tissue viability. As in the case of the formula NO.4, its application improved the tissue viability with the basis of SLS irritation, together with increased tissue integrity shown in the morphology results.

3.3 | Inhibitory of IL-1 α over expression by skin care formulas

In order to evaluate the effect of each formulas on the anti-inflammatory cytokine in vitro, we analyzed IL-1 α content in the culture medium for each tissue by ELISA. As shown in Figure 4, SLS induced significantly higher IL-1 α release as compared with the normal skin equivalent. The formula NO.1 to NO.3 exhibited no effect on the inhibition of IL-1 α secretion in diaper dermatitis-like skin equivalents. The formula NO.4 significantly decreased the IL-1 α content. The results were consistent with the results observed in tissue viability and morphology study. High inflammatory cytokines always injured the living cells, and led to release more inflammatory factors further. According to the detailed information of these formulas, the formula NO.1 and NO.2 shared the same base and the same actives of skin barrier promotion, but higher concentration of actives was prepared in the formula NO.1. This composition difference may explain the result variation observed between the formula NO.1 and NO.2 in tissue viability, morphology, and anti-inflammation test. The formula NO.4, which contains the highest level of anti-inflammatory actives, exhibited the best anti-inflammation results in this run of test.

3.4 | Efficacy evaluation of skin barrier promotion for skin care formulas

Dysfunction of skin barrier always happened together with the diaper dermatitis. Irritants and environment stress played important roles in compromising the integrity of skin, including increasing its

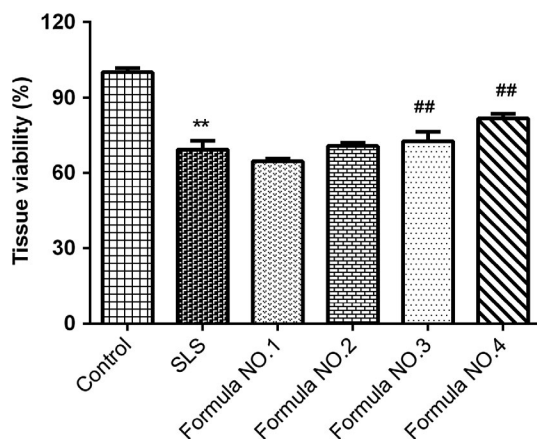


FIGURE 2 Tissue viability under different treatments. (** $P < 0.01$, compared with blank control; # $P < 0.05$, compared with SLS group; ## $P < 0.01$, compared with SLS group)

permeability to irritants, disruption of the SC lipids and ceramides leading to increased penetration and epidermal inflammation.^{4-6,21,22} We evaluated the effects of different formulas on skin barrier recovery by monitoring the variation of tissue viability after exposure to 1% Triton X-100 for 0 hour, 1 hour, and 3 hours with diaper dermatitis-like skin equivalents. The tissue viability result was profiled to increasing exposure time and the ET₅₀ was calculated as the exposure time at which reduce the tissue viability to 50%. ET₅₀ was positively correlated with the skin barrier function. The results presented in Figure 5 implied that the formulas can improve the skin barrier function as compared with the negative control. Among them, the formula NO.1 significantly improved the skin barrier of diaper dermatitis-like skin equivalents as compared with the negative control, the formula NO.3 and NO.4 also significantly improved the tissue viability and decreased the SLS damage at 3-hour exposure (Figure 6). The result illustrated that actives in the formula NO.1 decreased the damage of Triton X-100 to the stratum corneum. This result could be attributed to the use of high percent of ZnO, which effectively keep environment stress away from the stratum corneum and then inhibited further irritation. The formula NO.1 was composed of actives widely used skin conditioning agents in cosmetics, especially, N-palmitoyl hydroxyproline cetacean wax ester which played a key role as a kind of ceramide derivative. Although these two actives were also added into the formula NO.3, it did not present the same trend of ET₅₀ profile. This may be due to the additional concentration and the dosage effect. In the formula NO.3, higher content of N-palmitoyl hydroxyproline cetacean wax ester was used. In the formula NO.1 and NO.4, allantoin was the only active targeting the skin barrier function recovery. So, appropriate concentration and actives combination strategy in formulation design were responsible for the final efficacy of cosmetic formulas.

4 | DISCUSSION

Diaper dermatitis (DD) or napkin dermatitis is defined as an ecematization of the areas covered by diaper. It is most likely irritant contact dermatitis, a nonimmunologic reaction triggered by irritants present in the area covered by diaper.²³ Although this condition is relatively common, it can cause considerable pain and stress for infants and can be troublesome for their caregivers.¹² As a result of infants' inability to control urination and defecation, proper hygiene and protection of skin in the diaper area has always been a challenge. A study in the early 1990s demonstrated that regular application of a water-in-oil emollient was associated with significantly less dermatitis in neonates, changing established perceptions regarding the prevention of IDD.¹⁸

A subsequent study showed that when the guidelines were integrated into the skincare routines for babies in intensive care and special care, increased use of emollients, and reduced use of soap led to a significant improvement in skin condition reflected by less visible dryness, redness, and skin surface damage.¹⁹ Once DD has developed, the effective strategy on treatment was to facilitate the repair

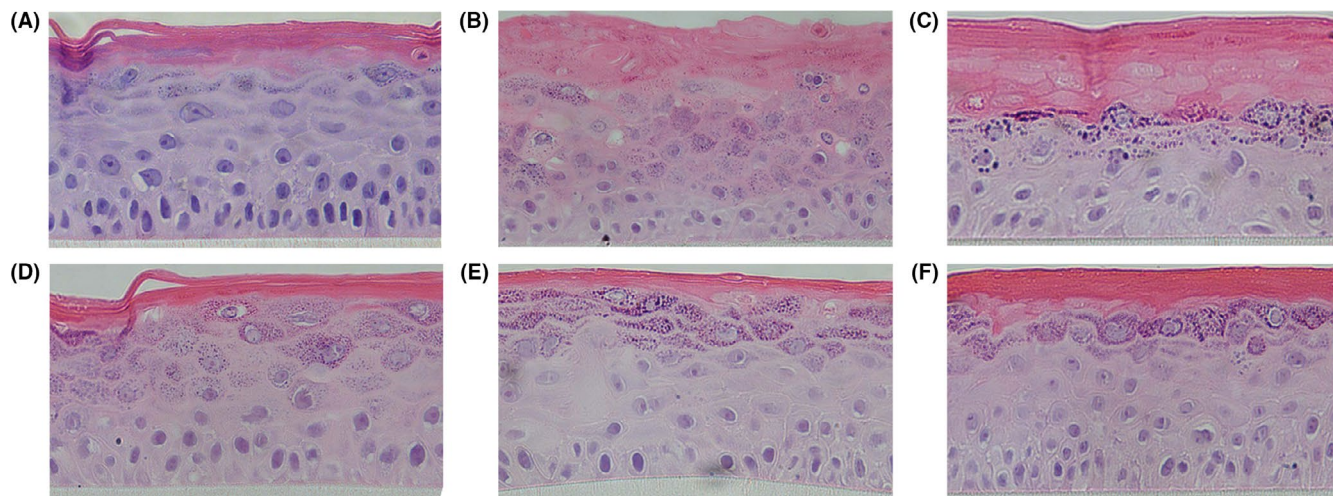


FIGURE 3 The morphology of skin equivalent treated with test sample after being induced by 0.2% SLS ($\times 20$ magnification). A, skin equivalents cultured in the absence of irritant (Blank control); B, skin equivalents induced by 0.2% SLS (Negative control); C, the formula NO.1; D, the formula NO.2; E, the formula NO.3; F, the formula NO.4

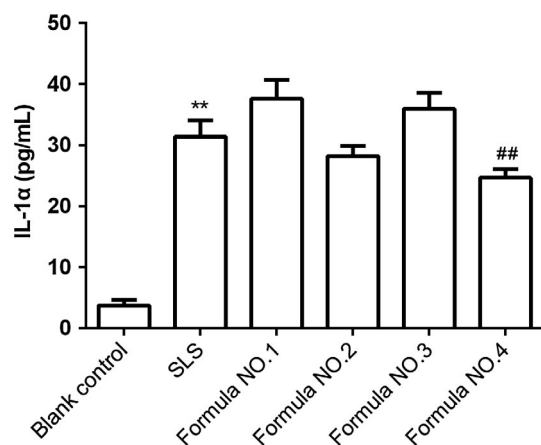


FIGURE 4 Interleukin 1 α (IL-1 α) secretion by skin equivalent s was assessed for each study groups. Results were expressed as the mean $\pm$ SD. (** $P < 0.01$, compared with blank control; ## $P < 0.01$, compared with SLS group)

of damaged skin and to prevent recurrence.³ It has been shown that the application of a barrier ointment at every diaper change is a valuable component of DD therapy.²⁴ Topical steroid therapy is also considered to be generally effective.²⁵

In this study, we designed four formulas targeting the diaper dermatitis by adding components with anti-inflammation, hydration improvement, skin barrier promotion and keeping away from irritants. Then, we assessed their efficacy by reconstructed skin equivalent in vitro. We generated and characterized the reconstructed skin equivalent by adding 0.2% SLS, and the skin equivalent obtained a characteristic diaper dermatitis-associated features of inflammation. Firstly, induction of skin equivalents with SLS altered the epidermal morphology and skin barrier function with lower tolerance to irritants. Secondly, keratinocytes of skin equivalents induced by SLS secreted higher IL-1 α level than normal skin equivalents. The characteristics were consistent with the report that significantly higher

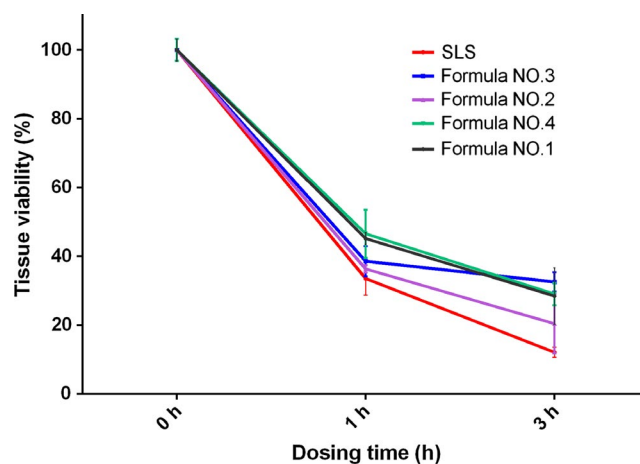


FIGURE 5 The skin barrier function recovery profile of different formulas application with the diaper dermatitis-like skin equivalents

IL-1 α level was detected in the diapered skin compared to nondiapered skin.²¹ Therefore, our skin equivalents induced by SLS could mimic the skin irritation caused by over-hydration, urine, feces, friction, and fecal enzymes in diaper areas. It could also be used as an interesting tool for screening actives which would be helpful to decrease the incidence of diaper dermatitis, especially the irritant diaper dermatitis (IDD).

In the aid of the reconstructed skin equivalents, we studied the inhibition of inflammatory cytokines, improvement on skin barrier function, and tissue viability of designed formulas. From the results of inhibition of the inflammatory cytokine secretion and anti-irritation function, the formula NO.4 displayed significant efficacy. The results implied that anti-inflammation was of great importance for repair of the diaper dermatitis. The formula NO.4 included higher anti-inflammation actives and the same concentration of skin barrier promotion actives as compared with other formulas.

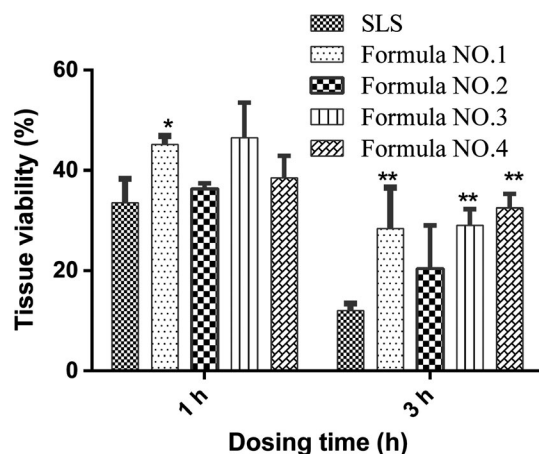


FIGURE 6 The skin barrier function recovery of different formulas with the diaper dermatitis-like skin equivalents at different exposure time. (* $P < 0.05$, ** $P < 0.01$, compared with SLS group)

This could be attributed to the effect of anti-inflammatory components, α -(-)-bisabolol and derivatives of glycyrrhetic acid. The former is one kind of natural monocyclic sesquiterpene and currently in use in various formulations, especially in cosmetics. It can reduce inflammatory cytokine production such as TNF- α and ameliorate skin inflammation.²⁶ The derivative of glycyrrhetic acid in the designed formulas was mainly stearyl glycyrrhetinate, the anti-inflammation function of which has been reported in several patents.²⁷ the formula NO.1, No.3, and NO.4 can lighten the skin barrier injury caused by SLS irritation. Among them, the formula NO.1 was composed of higher ratio of skin lipids analogs and lower concentration of anti-inflammation actives than the formula NO.4. The formula NO.3 was composed of high ratio of ZnO and lower concentration of anti-inflammation actives. According to the tissue viability and ET50 profile results after 1 h and 3 h of exposure by irritant Trion X-100, it has been seen that the formula NO.4, contained higher ratio of ZnO and anti-inflammatory actives, displayed the best efficacy of skin barrier recovery. We may deduce that enough anti-inflammation treatment and keep the skin of diaper area away from the reduplicated irritation was a prerequisite to the skin dermatitis treatment. Anti-inflammation of different formulas keeps in line with the skin barrier recovery. Because an acute inflammatory eruption caused by urine, enzymes in feces, friction, and chemical irritants are a critical driving force in the exacerbation of diaper dermatitis, actives of anti-irritation can also decline IL-1 α secretion by segregating further irritation.

In conclusion, it can be concluded that skin equivalents induced by SLS could be used as an effective tool for screening actives or cosmetic formulas which would be helpful to decrease the inflammation and repair the diaper dermatitis, especially in infants. The skin care formulas prepared containing anti-inflammation and skin barrier recovery actives showed significantly better efficacy for treatment about diaper dermatitis. These results were also validated by our study of consumer feedback.

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