ARTICLE

An Optimized Inexpensive Emollient Mixture Improves Barrier Repair in Murine Skin

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Abstract

Maintenance of epidermal permeability barrier homeostasis is the most crucial cutaneous function, as it allows life in a terrestrial environment. Defective epidermal permeability barrier results not only in excessive water loss, but also in the induction of cutaneous inflammation and an increased risk of infections. Together these could help explain the increased risk of death in premature and low birth weight infants whose skin is functionally compromised. Improvement of permeability barrier function by topical barrier repair therapies could become a valuable approach to reduce neonatal mortality, and to prevent/treat dermatoses, accompanied by barrier abnormalities, and to prevent microbial pathogen colonization/invasion. Yet, the current barrier enhancing products are not optimal. In the present study, we optimized the ratio of several inexpensive ingredients, previously shown to be effective individually for barrier homeostasis, and then assessed the efficacy of this mixture, as well as its mechanisms of action in normal murine skin. Although basal barrier function, skin surface pH and stratum corneum hydration remained unchanged, our results show that pretreatment of normal murine skin with this optimized mixture accelerates barrier recovery. The barrier-enhancing effects and potent antimicrobial activities of this optimized mixture could be attributed at least in part to a parallel

stimulation of epidermal differentiation and antimicrobial peptide expression. Since the individual ingredients in this mixture are inexpensive, this optimized mixture show promise as a means of reducing neonatal mortality in low-income settings, but it also could be more widely used to prevent skin disorders associated with epidermal permeability barrier abnormalities.

Key Words: Epidermal Permeability Barrier, Emollient, Antimicrobial peptides, Skin pH, premature

Running Title: Topical Emollient Improves Barrier Function

All authors declare no conflicts of interest.

Introduction

Mortality rates for pre-term (<37 weeks gestational age) and low birth weight infants (<2500g), are higher than for their term and normal birth weight counterparts[1]. This is particularly true in low income countries. For example, world-wide, more than 60% of preterm births occur in Africa and South Asia[2], and neonatal deaths accounts for 50% of the infant mortality rate in Africa and other low income countries[3,4]. Although neonatal mortality rates have been declining recently, declines are slower than for older children and efforts to decrease neonatal mortality rates are a global priority[5]. Improving the care of small neonates has been identified as one of the interventions likely to have the greatest impact on neonatal mortality[6].

Studies have found that the application of a range of emollients reduce neonatal mortality among preterm infants, decrease infection rates and increase weight[7]. The mechanisms of action are unclear. The benefits of emollients for reducing neonatal mortality could be attributed to the prevention of infection[8-10], it could also be due to the improvement of epidermal permeability barrier. This hypothesis is supported by a negative correlation that has been found between transepidermal water loss (TEWL), an indicator of epidermal permeability barrier, with both birth weight[11] and gestational age[12-14], and the higher neonatal mortality rates found in male compared to female neonates[15], the latter exhibiting superior barrier function[16]. Additionally, it is known that compromised permeability barrier increases the risk of infections[17], and increases the loss of body fluid and body weight[18,19]. It also increases energy consumption in preterm infants[18]. Given the findings that topical emollients reduce systemic infections and neonatal mortality in preterm infants[5,9,20], and the likelihood that this is due to enhanced epidermal permeability barrier, products to improve epidermal permeability represent an effective strategy to reduce neonatal mortality rates, particularly in preterm and low birth weight infants, living in low income countries.

Some products are already available to improve epidermal permeability, including sunflower oil and Aquaphor[8,13]. Topical applications of these barrier enhancing products has been shown to reduce mortality rates by 26-32% in preterm infants[21]. Although sunflower seed oil is effective, its lipid composition, a key determinant of its efficacy, varies with the source of the oil. Moreover, the high proportion of fatty acids in this and other oils can disturb the normal ratio of lipids in stratum corneum, resulting in disruption of permeability barrier and skin irritation[22]. Aquaphor and petrolatum can instantly lower TEWL due to their occlusive properties, but occlusion can

inhibit endogenous restoration of natural permeability barrier[23]. Other physiologic lipid-based products, such as Epiceram®, are also effective in improving barrier function, but their costly ingredients render them unaffordable in many developing countries. In the present study, we optimized ratios of several inexpensive ingredients, each of which was shown previously to improve barrier function individually[24,25], and evaluated the effects of this optimized mixture on epidermal permeability barrier homeostasis in normal murine skin, as well as, the mechanistic basis of its benefits.

Materials and Methods

Materials: 6-8 weeks old female albino hairless (SKH1) mice were purchased from Charles River Laboratories (Wilmington, MA). Ethanol and propylene glycol were purchased from Fisher Scientific (Fairlane, NJ). The optimized mixture, containing sunflower oil, glycerol, borage oil, petrolatum and lanolin, was prepared in-house and vertexed prior to topical application. Epiceram, a product known to improve barrier function in atopic dermatitis[26-28], from PuraCap was used as positive control. Affinity-purified, rabbit anti-mouse antibodies to filaggrin, involucrin, and loricrin was purchased from BabCo (Richmond, CA). Anti-mouse cathelin-related antimicrobial peptide (CAMP) antibody was from Santa Cruz Biotechnology (Texas, USA). Secondary biotinylated goat anti-rabbit IgG and ABC-peroxidase kit were purchased from Vector laboratories (Burlingame, CA, USA). The anti-proliferating cell nuclear antigen (PCNA, Ki-67) was purchased from CalTag Laboratories (Burlingame, CA, USA).

Experimental protocols and functional studies: All animal procedures were approved by the Animal Studies Subcommittee (IACUC) of the San Francisco Veterans Administration Medical Center and performed in accordance with their guidelines. Mice were maintained in a temperature- and humidity-controlled room, and given standard laboratory food and tap water *ad libitum*. To determine whether repeatedly topical applications of the optimized mixture improve epidermal function in normal mouse skin, mice were topically treated with the optimized mixture twice daily for three days. Untreated mice served as normal controls, and mice treated with Epiceram served as positive controls. Basal epidermal biophysical properties were assessed by measuring transepidermal water loss (TEWL) and stratum corneum hydration using respective probes (TM300 for TEWL and CM825 for hydration) connected to MPA5 (C&K, Cologne, Germany). pH900 (C&K, Cologne, Germany) was used to measure skin surface pH. For barrier recovery, TEWL was measured at 0 and 2 hours after tape stripping, which result in a 10-fold increase in TEWL, and percent barrier recovery rates were calculated as described previously[29]. To determine whether single application of the optimized mixture improve epidermal function in barrier-damaged mouse skin, mice were tape-stripped and immediately followed by topical application of either the optimized mixture or Epidceram. Tape-stripped mice left air exposure served as controls. TEWL was measured at 0, 1 and 5 hours after tape stripping. The results were normalized by untreated controls, setting the untreated controls as 100%.

Immunohistochemistry: The methods for assessment of both the differentiation markers and proliferation were carried out as published previously[30,31]. Briefly, after deparaffinization and blocking with 4% bovine serum albumin, 5μm paraffin sections were incubated with primary rabbit anti-mouse antibodies (Covance/BabCo. Berkeley, CA) at the dilutions of 1:2000 for

filaggrin, 1:1000 for involucrin, and 1:500 for loricrin, overnight at 4°C. After washing with 10mM citrate buffer, sections were incubated with goat anti-rabbit antibody (1:400) for 30 min at room temperature, followed by ABC-peroxidase (Vector, Burlingame, CA) reaction. For PCNA staining, sections were incubated with biotinylated monoclonal anti-PCNA antibody (CalTag Laboratories, Burlingame, CA) for 2 hours at room temperature. The sections were visualized with a Zeiss (Axioplan 2) microscope (Jena, Germany). Digital images were captured with AxioVision software 2.05 (Carl Zeiss Vision, Munich, Germany).

Electron Microscopy: Skin biopsies from vehicle- and antagonist-treated mice (as above) were fixed in Karnovsky's fixative overnight, and post-fixed with either 0.25% ruthenium tetroxide or 1% aqueous osmium tetroxide, containing 1.5% potassium ferrocyanide, as described previously[31,32]. Ultrathin sections were examined using an electron microscope (Zeiss 10A, Carl Zeiss, Thornwood, NY) operated at 60 kV.

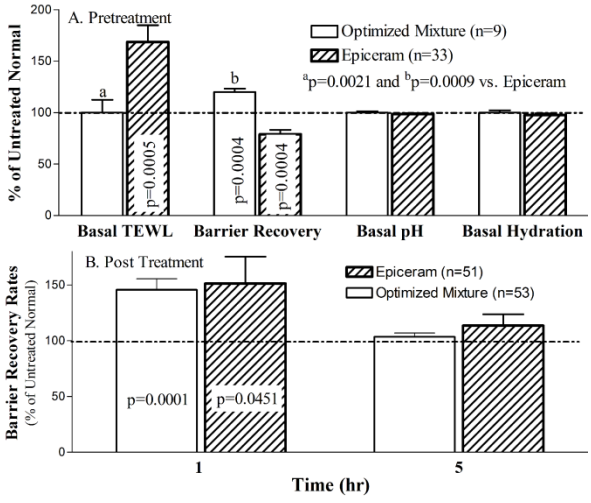
Statistical Analyses*:* Data are expressed as the means ± SEM. Unpaired two-tailed student t test with Welch's correction was used to determine significant differences when two groups were compared.

Results

Optimized mixture accelerates permeability barrier recovery

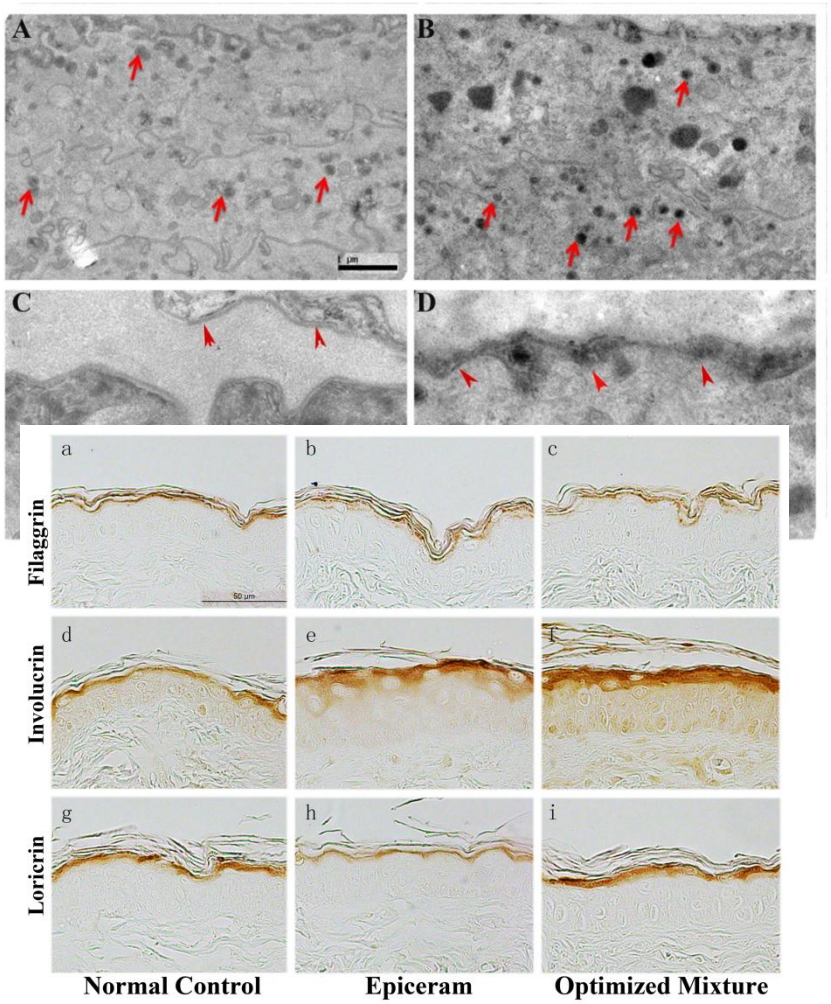
After three days of the treatment with the optimized mixture, the overall gross appearance of skin was similar between treated and untreated mice. Inflammation was not evident histologically, nor was epidermal proliferation abnormal, as assessed by H&E and PCNA staining, respectively, (data not shown). We then assessed the impact of this optimized mixture on epidermal biophysical properties in normal skin. As shown in Figure 1A, pretreatment with the mixture did not alter basal biophysical properties in normal murine skin. In contrast, permeability barrier recovery significantly accelerated 2 hours after barrier disruption, as compared with untreated controls. Surprisingly, repeated topical applications of Epiceram, a known barrier enhancer, elevated baseline TEWL and delayed barrier repair, in comparison to either untreated control or the optimized mixture treatment (Fig 1A).

Since epidermal permeability barrier is compromised in both preterm and low birth weight infants, we next determined whether this optimized mixture improves epidermal permeability barrier in skin with perturbed barrier. Our results showed that a single topical application of either the optimized mixture or Epiceram significantly reduced TEWL one hour after barrier disruption (Figure 1B). But no differences were seen by 5 hours after application. These results demonstrate that topical application of the optimized mixture improves epidermal permeability homeostasis in both normal and perturbed skin.



Topical optimized mixture stimulates epidermal differentiation

Because epidermal differentiation-related proteins are key determinants for epidermal permeability barrier[33], we next determined whether the topical optimized mixture upregulates the expression of epidermal differentiation-related proteins, which provide a mechanism whereby this mixture improves permeability barrier homeostasis. Indeed, the intensity of immunostaining for involucrin was markedly enhanced following 3days of treatment with either the topical optimized mixture or Epiceram (Fig 2e, f vs. 2d), while the intensity of epidermal filaggrin (Fig 2a vs. 2b,c) staining remained unchanged. The optimized mixture did not alter the expression of loricrin while a slight reduction in loricrin expression was evident following Epiceram applications. These results suggest that topical optimized mixture could improve barrier function in part by upregulation of epidermal involucrin expression.



Topical optimized mixture stimulates lamellar body formation

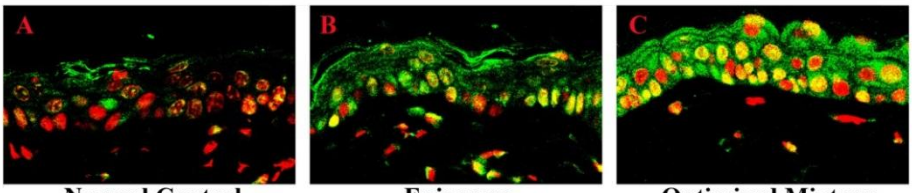
Since formation of both the permeability and antimicrobial barrier requires secretion of lamellar body content[34], we next assessed the changes in lamellar body formation following 3days of topical treatments with optimized mixture. As seen in Figure 3B, more lamellar bodies are present in the cytosol of the stratum granulosum following topical treatment with the optimized mixture in comparison to untreated skin (Figure 3A). Moreover, acceleration of membrane bilayer maturation became evident in optimized mixture-treated skin (arrows in Figure 3D) in comparison to untreated skin (arrows in Figure 3C). These results suggest that the optimized mixture-induced enhancement of epidermal permeability barrier homeostasis can be attributed, at least in part, to stimulation of lamellar body formation and accelerated membrane bilayer maturation.

Topical optimized mixture upregulates the expression of epidermal antimicrobial peptide

Previous studies have demonstrated that epidermal antimicrobial peptides such as CAMP not only provide a barrier against infection, but also are crucial for permeability barrier. Moreover, the expression levels of CAMP are lower in preterm infants than in full term babies[35]. Thus, we next assessed whether enhanced expression of epidermal CAMP occurs after the treatment with the optimized mixture, providing an additional, potential mechanism for the improvement of barrier and further providing the rational for clinical usage in treating preterm infants. As shown in Figure 4, a dramatic increase in the intensity of CAMP immunostaining became evident following treatment with both optimized mixture and Epiceram for 3 days in comparison with untreated controls (Fig 4A vs. 4B & C). These results demonstrate that the topical optimized mixture enhances the expression of a key epidermal antimicrobial peptide, i.e., CAMP.

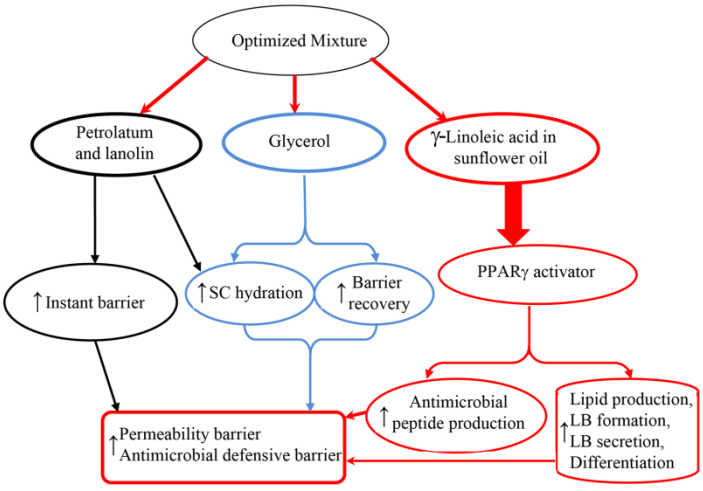
Discussion

Previous studies have shown that improving epidermal permeability barrier through topical applications of emollients reduces neonatal mortality rates in preterm infants[21,36], increases body weight[7], prevents systemic infection[37], and delays the development of atopic dermatitis[38]. Although emollients such as sunflower oil and Aquaphor show promise as a means of improving barrier function[8] , they still have certain limitations. For example, fatty acid (a key determinant for permeability barrier) composition in sunflower oil varies with the sources, consequently affecting its clinical efficacy. Aquaphor can improve permeability barrier, but with no benefits for the reduction of infection risk[10] whereas both defective permeability barrier and infection are the risk factors for neonatal deaths. Therefore, development of the products benefiting both permeability and antimicrobial barrier becomes imperative. In the present study, we developed an optimized mixture that improves epidermal permeability barrier in both intact and barrier-damaged skin. The underlying mechanisms by which this optimized mixture improves epidermal permeability barrier include stimulation of epidermal differentiation, upregulation of epidermal antimicrobial peptide expression, and increased lamellar body formation. Epidermal differentiation-related proteins are critical components of “bricks” of the straum corneum, providing a scaffold necessary for the post-secretory organization of extracellular lipids[33,39]. Pertinently, deficiency of either filaggrin or involucrin compromises epidermal permeability barrier [40,41]. Here, we show that topical applications of the optimized mixture dramatically increase the expression of involucrin. In addition, epidermal antimicrobial peptides provide not only an antimicrobial barrier, but also contribute to permeability barrier[42]. We showed previously that CAMP deficiency delays permeability barrier recovery in mice[42]. Thus, upregulation of epidermal CAMP expression could be another potential mechanism for the improved barrier function in skin treated with the optimized mixture.



Lamellar bodies in stratum granulosum are the only known organelles that deliver lipids and CAMP to the stratum corneum interstices[42,43]. Maturation of membrane bilayers is required for the formation of a competent permeability barrier. In the present study, increased number of lamellar body and acceleration of membrane bilayer maturation become evident in skin treated with the optimized mixture, providing an additional mechanism by which the optimized mixture could improve permeability barrier function. All these changes in epidermal function, induced by the optimized mixture, are likely mediated by peroxisome proliferator-activated receptor (PPARs), because both the borage oil and sunflower seed oil in the mixture, contain linoleic acid and □-linoleic acid, PPARγ activators. Activation of PPARγ stimulates epidermal differentiation, and lamellar body formation and secretion. In particularly, PPARγ is required for 1□, 25-dihydroxyvitamin D3-induced involucrin expression[44]. Likewise, PPARγ also mediates 1a, 25-dihydroxyvitamin D3-induced hBD-3 and cathelicidin expression in keratinocytes[45,46]. The putative mechanisms whereby this optimized mixture improves epidermal permeability barrier are illustrated in Figure 5.

It is worth noting that the novelty of this mixture is that all the ingredients are needed for optimal efficacy, thereby eliminating additional ingredients, that would increase cost and potentially cause adverse effects. For example, all ingredients in this mixture can increase stratum corneum hydration, which are required to improve in certain skin disorders, such as atopic dermatitis, psoriasis, ichthyosis and aged skin. Lower concentrations of petrolatum and lanolin instantly reduce TEWL without producing excessively occlusive effect, which could potentially harm skin defense responses to external insults. These two ingredients are particularly useful for correcting barrier function in barrier-damaged skin. Moreover, lower concentrations of sunflower oil and borage oil reduce greasiness and potential irritant effects without sacrificing benefits. Although there is no clinical data to ascertain the efficacy of this optimized mixture in humans, the following beneficial effects of the optimized mixture on epidermal function still provide a strong rationale for its clinical applications.



1. <u>Accelerating barrier repair without altering basal barrier function:</u> Optimal permeability barrier is required for epidermal homeostasis. A supper protection such as occlusive products can reduce TEWL, but also inhibit cellular metabolism. Of course, compromised barrier causes skin diseases. This optimized mixture maintains barrier function at its normal level while accelerating barrier repair upon requirement (barrier disruption). Therefore, this mixture could serve as a skin emergency kit and could be routinely applied to normal skin. The barrier could be quickly repaired on once barrier gets damaged such as in aged skin where barrier recovery is delayed.
2. <u>Improving barrier in barrier damaged skin:</u> Barrier disruption not only stimulates epidermal proliferation and induces inflammation, but also facilitates bacteria invasion and allergen

penetration. Thus, this mixture could be very useful for the prevention of inflammation and infection in barrier damaged skin such as infants, particularly premature infants who predispose to skin infection.

3. Upregulating epidermal antimicrobial peptide expression: In addition to impact on permeability barrier function, epidermal antimicrobial peptides play a key role against microbial infection. The infants, especially premature infants, are more vulnerable to bacterial infection, largely due to premature innate immunity. And infection is among the major causes of infant mortality. Moreover, both atopic dermatitis and ichthyosis are at higher risk for certain types of infection. Since this optimized mixture can upregulate epidermal antimicrobial peptide expression and improve barrier function, it could be useful for infants and other dermatoses.

Conclusions

Because this inexpensive formulation improves epidermal permeability barrier function and antimicrobial defense, it could be useful for the treatment of neonatal and aged skin, as well as inflammatory dermatoses characterized by barrier abnormalities.

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Figure Legends

Figure 1. Topical optimized mixture improves epidermal permeability barrier homeostasis. Mice were treated as described in materials and method section. Fig 1A depicts the influences of topical optimized mixture and Epiceram on statum corneum biophysical properties after 3 days of treatment. . Fig 1B displays the effects of topical optimized mixture and Epiceram on permeability barrier homeostasis after a single application to barrier disrupted murine skin. Results were normalized by untreated controls as shown by dotted line. N and significances are indicated in the figures.

Figure 2. Topical optimized mixture stimulates epidermal differentiation. After 3 days of treatments with either topical optimized mixture or Epiceram, skin samples were taken for immunohistochemical staining of differentiation marker proteins. Figures 2a,d, g are untreated normal controls; Figures 2b,e,h are Epiceram-treated skin, and 2c,f,i are optimized mixture-treated skin. Magnification bar=50 □m.

Figure 3. Topical optimized mixture stimulates lamellar body formation. After 3 days of treatments，skin biopsies were taken for ultrastructural analysis. Figures 3A & B represent lamellar bodies, as indicated by arrows, in untreated and optimized mixture-treated, respectively. Figures 3C & D display lipid processing (see arrow) in untreated and optimized mixture-treated, respectively. Magnifications for Figures 3A & B are the same and magnification bar =1 □m; Magnifications for Figures 3C & D are the same and magnification bar =0.5 □m.

Figure 4. Topical optimized mixture upregulates the expression of epidermal antimicrobial peptide. After 3 days of treatments with either topical optimized mixture or Epiceram, skin samples were taken for immunohistochemical staining of CAMP. Figures 4a,b,

and c are untreated normal control-, Epiceram- and optimized mixture-treated, respectively. All pictures were taken at the same magnification. Magnification bar=40 □m.

Figure 5. The putative mechanisms by which topical optimized mixture improves permeability barrier homeostasis.

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