

# The Ultrastructural Changes of Stratum Corneum Lipids after Application of Oleic Acid in Propylene Glycol

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**Background:** The stratum corneum presents a significant barrier to transdermal drug delivery. Approaches to improve percutaneous absorption of drugs have included iontophoresis and skin penetration enhancers. Oleic acid has been studied as a skin penetration enhancer for drugs, primarily via its action mainly on the stratum corneum lipid structure.

**Objective:** The purpose of this study was to assess the interaction between oleic acid and stratum corneum lipids in vivo.

**Methods:** Male hairless mice were treated topically with oleic acid. Barrier function was assessed by transepidermal water loss measurement and ultrastructural observation with ruthenium tetroxide (RuO<sub>4</sub>) staining.

**Results:** Oleic acid in propylene glycol had a profound effect on epidermal barrier function and was found to be concentration dependent. Moreover, ultrastructural examination with RuO<sub>4</sub> post-fixation demonstrated that there were marked alterations in the stratum corneum lipid structure.

**Conclusion:** This study provides direct evidence that oleic acid increases the epidermal permeability through a mechanism involving the stratum corneum lipid membrane perturbation via the lacunae formation within the stratum corneum.

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**Key Words:** Barrier function, Oleic acid, Propylene glycol, Ruthenium tetroxide (RuO<sub>4</sub>), Stratum corneum lipids

The stratum corneum (SC), the outermost layer of the skin, provides a number of functions. The main one is the prevention of entry of toxic chemicals from the environment by the formation of an essential permeability barrier to transepidermal water loss (TEWL). This barrier is formed by the subcellular organization of epidermal structural proteins and lipids into a two-compartment system, i.e. by a continuous production of intercellular lipid matrices surrounding lipid-depleted, protein-enriched cor-

neocytes<sup>1,2</sup>. Current evidence suggests that these lipids are mainly composed of ceramides, cholesterol and free fatty acids in approximately equal quantities, organized into a series of broad lamellar membranes that play a vital role in the function of the barrier<sup>3</sup>.

The formidable SC barrier allows only a limited number of drugs to be delivered in therapeutic amounts through the skin<sup>4</sup>. Therefore, reduction of the SC barrier function is predicted to increase the therapeutic efficacy of dermatological formulations. As a consequence, a number of chemical enhancers have been developed by obtaining significant improvements in the kinetics and/or extent of percutaneous absorption. Oleic acid (OA), a widely used chemical enhancer, is believed that it enhances penetration for drugs primarily via its

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action mainly on the SC lipid structure. Currently, the biophysical technique of attenuated total-reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy has been used to examine certain features of the SC after application of OA<sup>5,6</sup>. However, the interaction between OA and SC lipids induces ultrastructural changes to SC lipids and these have not been fully understood. The use of ruthenium tetroxide (RuO<sub>4</sub>) fixation allows for detailed studies of the spatial organization of the intercellular lipids of the SC.<sup>7,8</sup> In this study, we examined the intimate structural changes of SC lipids after treatment of OA with a RuO<sub>4</sub> staining technique.

## MATERIALS AND METHODS

### Materials

Hairless male mice, 8-12 weeks old, were used for this study. They were fed a regular mouse diet and water *ad libitum*. All animal experiments complied with the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). Reagent-grade oleic acid and propylene glycol were purchased from Sigma Chemical Co. (St. Louis, Mo). Ruthenium tetroxide, embedding resins were purchased from Polysciences, Inc. (Warrington, PA).

### Experimental Protocols and Electron Microscopy

Under anesthesia, the skin of the experimental mice (6 mice in each group) were treated with the following concentrations of 0.1M, 0.15M, 0.2M and 0.3M OA in PG, each for 30, 40, 50, 60 and 120 min. Other groups were treated with pure OA and PG as well as 0.3M OA in PG for 2 hours, separately. The formulations were applied as follows: 50 $\mu$ l solution was dropped on 15mm diameter filter paper, and covered with a Tegaderm® wrap. After the treatment, the patches were removed, wiped with a paper towel, and TEWL measurements were performed 10 min after the removal of patches with a Tewameter TM210 (Courage + Khazaka, Germany).

After treatment with the protocols described above, skin samples were taken from treated and untreated sites for electron microscopy. Samples were minced to <1mm<sup>3</sup> and fixed in modified Karnovsky's fixative overnight, washed in 0.1M

cacodylate buffer, and post-fixed in 0.25% RuO<sub>4</sub> in 0.1M cacodylate buffer for 45 min. in a dark at room temperature. After fixation, all samples were dehydrated in graded ethanol solutions, and embedded in an Epon-epoxy mixture. Ultrathin 60- to 80-nm sections were examined in an electron microscope (HITACHI-500) after further contrasting with uranyl acetate-lead citrate.

Statistical significances were determined using the Student's t-Test.

## RESULTS

### The Effects of OA on Skin Barrier Function

In this study, we first assessed the ability of OA to influence the epidermal barrier function in different concentrations. As shown in Fig. 1, OA did not influence the epidermal barrier function at the lower concentration of 0.1M in PG based on the TEWL measurement, at least for the 120 min of treatment. At the higher concentrations of 0.15M, 0.2M and 0.3M OA, the increased TEWL was clearly noted. When pure PG alone was applied to the intact skin for 2 hours, no change in TEWL was observed. After 2 hours of treatment with pure OA, the TEWL level was increased two-fold in comparison with the normal level ( $p < 0.0001$ , normal TEWL =  $9.3 \pm 1.3 \text{ gm/m}^2 \text{ per hr}$ ). Whereas, application of 0.3M OA in PG showed a significant increase in TEWL ( $74.3 \pm 5.2 \text{ gm/m}^2 \text{ per hr}$ ,  $p < 0.00001$ , Fig. 2).

We next measured barrier recovery in 0.3M OA-treated animals. As shown in Fig. 3, after 0.3M OA treatment for 2 hours, there was no delay in the rates of barrier recovery, indicating that there was no toxic effect to barrier recovery after OA treatment.

### Ultrastructural Observation

To gain insights into the mechanisms by which OA disrupts the epidermal barrier, we examined the ultrastructure of OA in PG-treated epidermis. After 2 hours of PG treatment, minimal changes in the SC intercellular lipid lamellar membrane structure were found. As shown in Fig. 4, the basic unit lamellae were separated by amorphous materials in comparison with the normal lipid lamellae (Fig. 4A and Fig. 4B). Moreover, following pure OA treatment, normal-appearing lipid lamellar membranes (Fig. 5A) and lacunae were also ob-

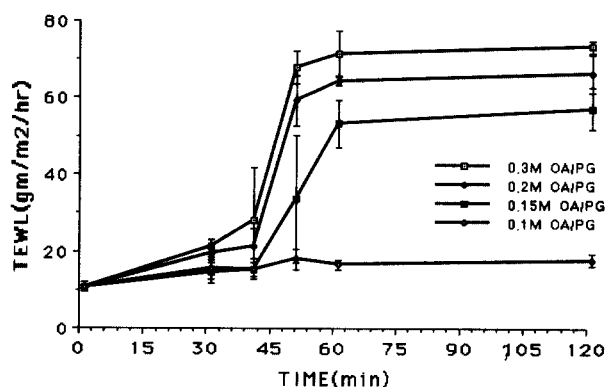


Fig. 1. Changes in TEWL after application of different concentrations of OA according to application times. Groups were treated with 0.1M, 0.15M, 0.2M and 0.3M OA in PG, each for 30, 40, 50, 60 and 120 min. There were no significant changes of TEWL after 120 min treatment with 0.1M OA. At the higher concentrations, TEWL increased in relation to the concentration of OA and application time. Each point represented the mean ( $\pm$  SEM).

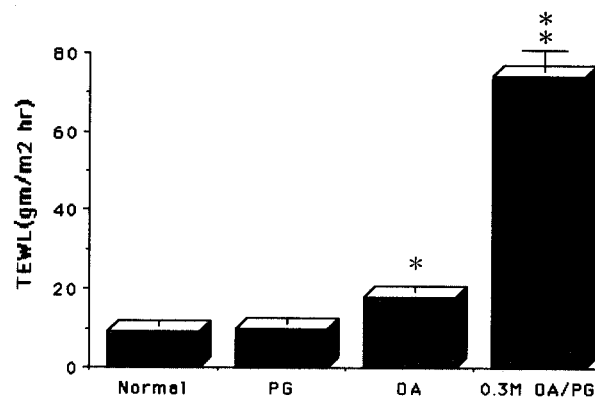


Fig. 2. Effect of administration OA on barrier function. Animals were treated with pure PG and OA, 0.3M OA in PG for 2 hours, separately. PG did not show any significant changes in TEWL, after pure OA treatment. The TEWL level increased two-fold in comparison with the normal (\* $p < 0.0001$ ), whereas administration of 0.3M OA/PG drastically elevated the TEWL (\*\* $p < 0.00001$ ). Results were represented as mean ( $\pm$  SEM).

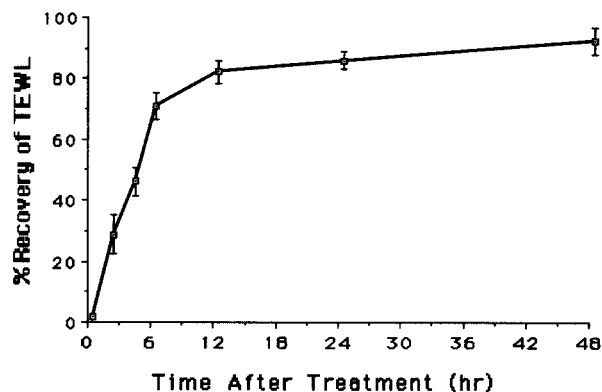


Fig. 3. Recovery rates of TEWL after topical application of 0.3M OA for 120 min. OA treatment caused an immediate marked increase in TEWL, which returned to normal within 48 hours. Each point represented the mean ( $\pm$  SEM).

served in some place within the SC (Fig. 5B). In contrast, after treatment of 0.3M OA in PG, the SC intercellular lipid membrane structure altered drastically and the intercellular domains of the SC revealed an absence of normal appearance; instead, elongated lacunae appeared within the widened SC interstices (Fig. 4A). Furthermore, fragments of lamellae and amorphous electron-dense material were limited to the intercellular

Fig. 4. Electron micrograph of murine epidermis. A shows the normal stratum corneum intercellular lipid membrane, while B shows the pure PG-treated epidermis. Note the basic unit lipid lamellae (arrow heads) separated by amorphous materials (asterisks). Magnification: A,  $\times 153,470$ ; B,  $\times 89,410$ ; inset,  $\times 105,550$ .

**Fig. 5.** Survey micrograph of pure OA-treated epidermis. Note the normal-appearing lipid lamellar membranes (A) and lacunae (B, stars) coexisting within the stratum corneum. Magnification: A,  $\times 108,940$ , B,  $\times 88,420$ .

spaces of the SC (Fig. 4B). These results demonstrated that application of OA in PG led to disruption of the intercellular lipid membrane structure in the SC.

## DISCUSSION

The permeability barrier function of mammalian keratinizing epithelia is regulated by a system of lipid-enriched, membrane bilayers in the intercellular spaces of the SC<sup>1</sup>. Studies have shown that the quantities of these lipids, rather than the number of cell layers or thickness of the cornified layer, determine the permeability characteristics of a particular skin or mucosal site<sup>9,10</sup>, and are generally recognized as the primary barrier to facile transdermal drug delivery<sup>11</sup>. Therefore, a number of physical and chemical enhancers have been developed. Approaches to improve skin absorption have included mainly iontophoresis, and barrier perturbation with chemical penetration enhancers.

The monounsaturated fatty acid OA is a commonly used chemical enhancer, and the mechanism of its action has been widely studied *in vitro*<sup>11-13</sup>. These studies have shown that OA primarily mod-

**Fig. 6.** Ultrastructure of the epidermis after 2 hours 0.3M OA in PG treatment. Note the elongated lacunae appearing within the widened stratum corneum interstices (A, asterisks). Moreover, fragments of lipid lamellae (B, arrow heads) and amorphous electron-dense material (B, asterisks) were limited to the intercellular spaces of the stratum corneum. Magnification: A and B,  $\times 105,550$ .

ulates the extracellular lipid domain of the SC. It appears that the predominant effect of the fatty acid is the formation of a phase-separated domain, resulting in the creation of permeable interfacial defects through which more facile transport can occur<sup>13,14</sup>. Furthermore, other infrared spectroscopy and differential scanning calorimetry experiments also indicate that a more general lipid disordering is caused by OA<sup>11</sup>.

Previous studies have demonstrated that there is a well-established synergy between the enhancer activities of OA and PG vehicles<sup>15</sup>. It has been suggested that PG is able to perturb more hydrophilic regions (e.g. protein-rich domains) of the SC. In addition, it is often used as a cosolvent for more lipophilic enhancers such as OA, providing the opportunity for synergy and amplification of the effect<sup>15,16</sup>. Takeuchi and co-workers<sup>17</sup> analysed the behavior of PG in the dermis by ATR-FTIR spectroscopy. They showed that PG penetrated through the skin barrier and did not give any sig-

nificant peak in the spectrum after 2 hours or even after 10 hours of treatment. Thus the results suggested that PG did not affect the epidermal barrier function, and our results further confirmed this issue by TEWL measurements. Moreover, barrier recovery after acetone treatment or tape-stripping appeared to be a two-phase process, with 50-60% recovery occurring over the first 6 hours, and a relatively slow rate of recovery requiring 24-48 hours thereafter<sup>18,19</sup>. Our results show that after application of OA, the barrier recovery is similar to the acute barrier disruption model.

The ultrastructural studies provided new information of potential insights into the mechanism of barrier perturbation with the OA/PG system. Application of RuO<sub>4</sub> post-fixation permitted elucidation of a sequence of changes in the SC intercellular lipid membrane structure<sup>7,8</sup>. After application of PG alone, the separation in the basic unit membrane appeared. We hypothesized that PG did influence the stratum corneum lipid structure, whereas such an influence might be too small to manifest itself in TEWL. In other words the RuO<sub>4</sub> staining technique might be more superior to TEWL measurement, which could reveal this ultrastructural change of the SC lipids. Moreover, after pure OA treatment, the normal-appearing lipid lamellar membrane and lacunae coexisted within the SC, suggesting that the pure OA predominantly affected the SC lipids, but the amount of OA taken up by the SC was limited. This finding was also consistent with the epidermal barrier functional evaluation, which showed detectable amounts of TEWL after pure OA treatment (cf. Fig. 2). Finally when OA/PG coapplied, a number of lacunae appeared throughout the SC, and accompanied the significantly elevated TEWL levels. These results demonstrated that the lacunae within the SC might play an important role in the defect of barrier function.

In summary, the results presented here demonstrate that OA, applied to murine skin in vivo, under conditions which increase the epidermal permeability, does not generally alter the SC lipid membrane structure. OA/PG system can perturb the SC lipid membrane structure, and it can increase the epidermal permeability through a mechanism involving lipid membrane perturbation via the formation of lacunae within the SC.

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