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Note

Influence of pharmaceutical gel vehicles containing oleic acid/sodium oleate combinations on hairless mouse skin, a histological evaluation

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Abstract

Aqueous gel preparations containing oleic acid/sodium oleate combinations were applied three times daily to hairless mice (CD1 strain). Six groups of animals ($n = 9$ or $n = 10$) were treated topically with six experimental vehicles containing 2, 3 and 4.5% oleic acid (OA) at two different pH values, 7.3 and 7.7. Sodium lauryl sulfate (5%) in a similar gel preparation was used as the positive control ($n = 5$), while untreated animals were used as the negative control ($n = 6$). After three treatment days, the skin samples were collected and processed for histological evaluation. It was seen that the severity and frequency of histological changes in the skin treated with OA-containing vehicles were directly correlated with increased pH/ ionization (i.e. decreased OA/sodium oleate ratio) and with overall OA concentration.

Keywords: Oleic acid; Topical preparation; Gel vehicle; Hairless mice; Skin irritation; Dermal toxicity

1. Introduction

The study of penetration enhancers and vehicle formulations has become an important research area, in which intensive attempts are being made to overcome the barrier properties of the skin [1]. One of the methods currently being studied is to modify the architecture of stratum corneum by interaction of accelerants, such as free fatty acids, with the lipid and keratin constituents [2].

Oleic acid (OA), a *cis*-unsaturated free fatty acid, is known to selectively perturb the inherent lipid structure of the stratum corneum as a separate fluid phase [3], resulting in a decrease in lipid transition temperatures [4] and an increase in the absorbance frequency of infrared stretching vibrations [5]. It has been postulated that this proposed mechanism of enhancing penetration takes place for several drugs. For instance, it was found that hydration and aqueous-based accelerants were relatively ineffective, while pretreatment with 5% oleic acid in propylene glycol yielded a 35-fold increase in the penetration of 5-fluorouracil [6]. In another study, oleic acid increased the rate and extent of 4-cyanophenol penetration across human skin in vivo as evaluated by attenuated total-reflectance infrared spectroscopy [7].

Most penetrants, including oleic acid, are chemicals that interact with skin constituents and might, therefore, cause reversible or irreversible damage to the viable cells. An extensive histopathological study on 18 potential penetration enhancers revealed that 10% oleic acid as an aqueous dispersion induced a significant change in nude mice skin tissue, indicating a possible skin irritation effect [8].

In the present study, we formulated several pH-controlled combinations of water-soluble oleic acid/sodium oleate in topical aqueous gels, constituting smooth, consistent and 'easy to apply' dermal preparations. Unlike dispersed oleic acid preparations, these formulations were 'one-phase' stable systems (at least for 6 months at 40°C) with a cosmetic and pharmaceutical importance. Since a large portion of the oleic acid in such systems was in a water-soluble ionized form, it was reasonably presumed that they would have been relatively less permeable through the stratum corneum, resulting in a reduction in dermal toxicity. We, therefore, evaluated the histopathological changes of the skin of hairless mice exposed to these gels as well as the relative skin irritancy of the oleic acid/oleate combination systems after thrice daily treatment for three consecutive days.

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Table 1
Summary of the vehicle preparations tested on each group of hairless mice

Gel preparation[a]	n	% oleic acid	pH[b]	Other
Vehicle 1	9	2.0	7.3	–
Vehicle 2	9	2.0	7.7	–
Vehicle 3	9	3.0	7.3	–
Vehicle 4	10	3.0	7.7	–
Vehicle 5	9	4.5	7.3	–
Vehicle 6	10	4.5	7.7	–
Vehicle 7	5	–	7.5	SLS[c]
Control	6	–	–	–

[a] All vehicles were gelled with Methocel K-15.
[b] The pH was adjusted to 7.3 or 7.7 with the appropriate citric acid/sodium hydroxide ratio.
[c] SLS, sodium lauryl sulfate.

2. Materials and methods

2.1. Materials

All materials were of pharmaceutical grade, specified according to the British Pharmacopoeia, unless otherwise stated. Oleic acid was purchased from Unichema (The Netherlands), propylene glycol was obtained from Dow Chemical Co. (Switzerland), and sodium lauryl (dodecyl) sulfate from Merck (Darmstadt, Germany). The gelling agent, Methocel® K-15M, was purchased as a USP grade product from Colorcon (Italy). Citric acid and sodium hydroxide were both analytical grades.

2.2. Pharmaceutical Preparation

Oleic acid (2, 3, or 4.5%) was mixed in 4/6 propylene glycol/water. The oleic acid was first solubilized in the mixture by titration with 10N sodium hydroxide solution to pH 8.5. Then the pH was re-adjusted to 7.3 or 7.7 with 20% citric acid solution. Methocel K-15M (1.5%) was gradually dissolved to obtain a clear and smooth gel. Table 1 presents the various vehicle preparations.

2.3. Animals

All animal procedures were conducted in accordance with approved institutional protocols. Sixty-seven male hairless mice (30 g, CD-1 strain) were obtained from The Weizmann Institute, Rehovot, Israel. Each animal was housed separately. Cages were labeled to identify the occupants and the experimental group by animal number and color code. The animals were fed ad libitum throughout the study. Drinking water was supplied to the cages via glass bottles.

2.4. Treatment groups

The 67 animals were randomly assigned to eight treatment groups. Six groups of mice ($n = 9$ or $n = 10$) were treated topically with various oleic acid-containing gel vehicles (see Table 1). An untreated animal group ($n = 6$) was used as the negative control, and a group ($n = 5$) treated with 5% sodium lauryl sulfate (a strong irritant) in a gel vehicle, was used as the positive control.

2.5. Experimental procedure

About 50 mg of each vehicle preparation were applied under no occlusion over the dorsum of each animal three times daily for 3 consecutive days (nine treatments). This regimen was chosen to simulate a short-term treatment by a chronic administration of these topical vehicles. The amount of the gel applied was fixed so as to thoroughly cover 1 cm^2 of skin area. On the fourth day, the animals were killed. Samples (1 cm^2) of the applied skin areas were taken, flattened on cardboard and preserved in 4% buffered formaldehyde. Following dehydration and being embedded in paraffin wax, the sections were cut to a thickness of 4–5 μm, stained with haematoxylin and eosin (H&E) and examined microscopically. Sections were cut parallel to the craneo-caudal axis of the animal and included subcutis and muscular layer. The findings were scored as described previously [8].

The mean histological score (mean score) was calculated according to the following formula:

$$\text{mean score} = \frac{\sum A_i n_i + \sum B_i n_i + \sum C_i n_i + \ldots}{n}$$

where, A, B, C, etc. are the scored histological parameters (Table 2); n = group size. The histological results (as frequencies of positive or negative effects) were evaluated statistically for each parameter using the Fisher exact test for 2×2 contingency tables ($P = 0.05$).

3. Results and discussion

The overall severity of the microscopic findings for all preparations tested was mild to moderate (mean score $<$ 10). Relatively, the most severe findings were observed after skin exposure to 5% SLS, which was used as an irritant reference. In this study, only the following skin parameters were found to have changed: epidermal-thickening (A); increase in all the layers of the stratum granulosum (B); hyperkeratosis (C) and infiltration of the dermis (J). The results are presented in Table 2. Photomicrographs illustrating typical histological changes observed in skin tissues are shown in Fig. 2.

It can be seen that for each oleic acid concentration, the score for parameter B increased more significantly when the pH increased ($P < 0.05$) than the scores measured for other parameters. Fig. 1 shows the mean histological score obtained for the various vehicles, demonstrating an increase as the oleic acid concentrations and the pH (only at 3 and 4.5%) increase. This was not observed at 2% oleic acid application, where no significant changes were noted. It is demonstrated, therefore, that gel vehicles containing 2%

Table 2
Frequency of histological changes caused by each vehicle application

	Control $n = 6$	2% OA[a] pH 7.3 $n = 9$	2% OA pH 7.7 $n = 9$	3% OA pH 7.3 $n = 9$	3% OA pH 7.7 $n = 10$	4.5% OA pH 7.3 $n = 9$	4.5% OA pH 7.7 $n = 10$	5% SLS[b] $n = 5$
Epidermal changes								
A. Epidermal thickening								
0	6	7	9	2	2	2	–	1
1	–	–	–	1	–	–	–	–
2	–	2	–	6	8	7	10	3
3	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	1
5	–	–	–	–	–	–	–	–
B. Increase in cell layers of the stratum granulosum								
0	6	8	9	8	2	5	–	1
1	–	1	–	1	8	4	9	3
2	–	–	–	–	–		1	1
3	–	–	–	–	–		–	–
C. Hyperkeratosis								
0	6	7	9	3	2	–	–	–
1	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–
3	–	2	–	6	8	9	10	4
4	–	–	–	–	-	–	–	1
5	–	–	–	–		–	–	–
Dermal change								
J. Infiltration of the dermis								
0	6	7	9	9	10	8	10	2
1	–	2	–	–	–	1	–	2
2	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	1
4	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–
Mean histological score	0	1	0	4	5	5	6	7

[a] OA, oleic acid.
[b] SLS, sodium lauryl sulfate.

oleic acid, at both pH 7.3 and 7.7, were not irritant to mouse skin, while vehicles having higher concentrations (3 and 4.5%) caused mild to moderate pathological changes with a pH dependency. The histological changes were solely related to epidermal thickening and hyperkeratosis but not to infiltration of the dermis, implying that this latter parameter (and actually all dermal properties) were not affected by oleic acid as they had been by 5% sodium lauryl sulfate. The most frequent cases with the greatest microscopic changes of the skin occurred in animals treated with vehicles containing 4.5% oleic acid at pH 7.7. A mild skin irritancy can be claimed for the mice treated with vehicles containing 3% oleic acid at pH 7.3; the incidence of this class of irritation was relatively lower than that in exposed to a higher pH value or a higher oleic acid concentration. The data also showed that vehicles containing 3 and 4.5% oleic acid at pH 7.7 and 7.3, respectively, caused similar histological pictures, and the differences between them lie only in their frequency of incidence. This indicates that the pH or the ratio of sodium oleate to oleic acid in the gel determines the severity of the skin irritation. The apparent pK_a of oleic acid in 40% propylene glycol aqueous solution was about 7.5 (as determined by the Henderson-Hasselbach relationship and data obtained from back titrations of

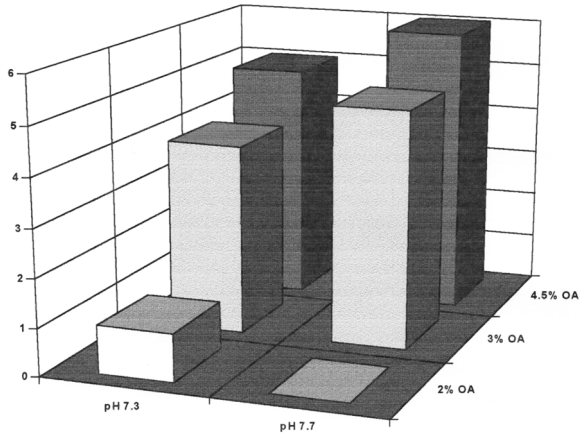

Fig. 1. Influence of pH and oleic acid (OA) concentrations on the mean histological score.

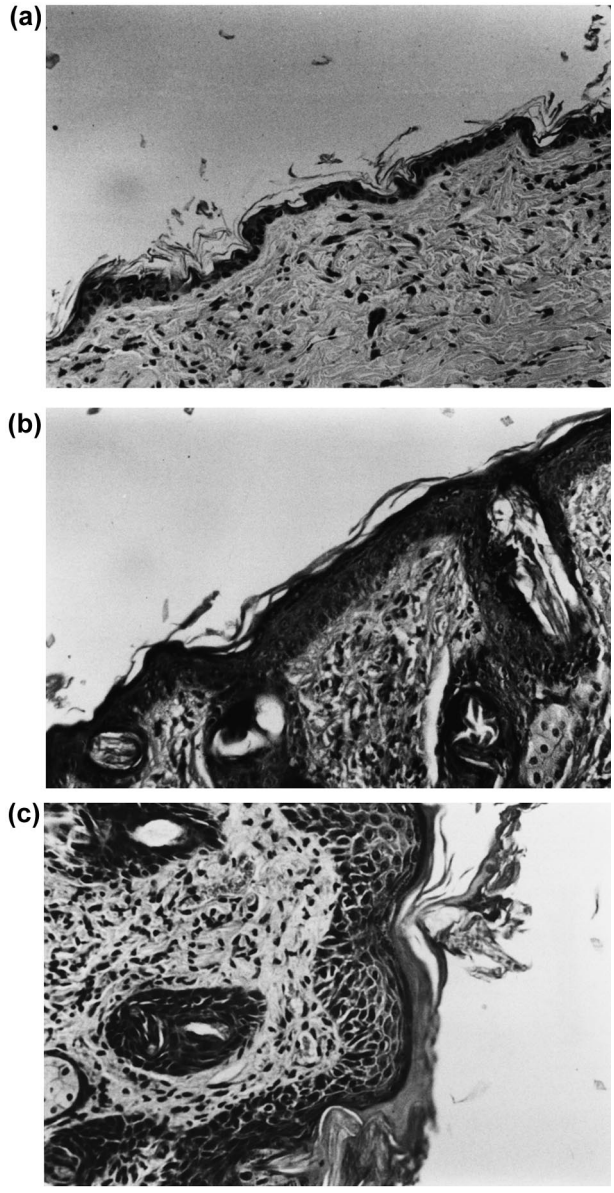

Fig. 2. Photomicrograph illustrating the thickness of the epidermal and keratin layers of an untreated control hairless mouse (a); photomicrograph illustrating the epidermal and keratin layers changes in a hairless mouse to which 4.5% oleic acid, pH 7.7, had been topically applied (b). The epidermis is thickened approximately twofold as compared to the untreated control skin, while the keratin layer is thickened (hyperkeratotic), half loose, half compact; and photomicrograph illustrating the epidermal and keratin layers changes in a hairless mouse to which 5% sodium lauryl sulfate had been topically applied (c). The epidermis is thickened approximately threefold as compared to the untreated control skin, while the keratin layer is compactly severely thickened (H&E, × 250)

completely ionized oleate). Around this value, the higher the value of the pH, the higher is the oleate/oleic acid ratio. This means that oleate ions have a higher irritant and proliferative effect on the skin than oleic acid. In other words, not less but even more severe skin damage occurs while the ionization of oleic acid increases. Since it would have been postulated intuitively that an ionized form of oleic acid could not easily partition into the skin through its lipid pathway, thus leading to relatively less damage, an explanation for our different results is needed. A possible explanation may be that a coacervation of propylene glycol with the polar fatty acid ions enhances the permeation of both substances. The coacervation phenomenon followed by a phase separation can be visually followed up in a non-viscous solution containing 50% propylene glycol and 4.5% oleic acid at pH 7.3–7.7. In addition, It was obvious that the toxicity was abruptly eliminated at 2% oleic acid concentration even at pH 7.7, perhaps because at this concentration there were less interactions with propylene glycol to form a coacervate, and the polar carboxylic groups of the free fatty acid are more expressed in reducing skin penetration.

Penetration enhancing effects are caused by structural alteration of the stratum corneum, the main barrier of the skin. However, the stronger the effect, the greater the changes in the deeper cutaneous layers, followed by histological damage. Although it is difficult to quantify the effect of various accelerating agents on drug penetration according to the histological observations, histopathology is an effective tool for the evaluation of the optimal concentration of a particular enhancer in producing desired permeable defects in the stratum corneum with minimal damage to the epidermal and dermal layers. It should be noted that measurement of transepidermal water loss (TEWL) has been widely used as a relatively faster and non-invasive way to identify structurally damaged skin, however, in some studies it failed to correlate with epidermal proliferation [9] and skin permeability [10]. Free fatty acids, like oleic, whose effect on the skin permeability of co-applied drug is related to lipid perturbation [4,5], have been recognized as useful topical penetration enhancers for pharmaceuticals. In our work, we have provided further supporting evidence, that although effective, oleic acid induces some inflammatory effect on the skin in a concentration-dependent manner. Interestingly, we have shown that oleate salt causes more damage to the skin than its acid form, a finding that has some implications as to its potential as a more effective penetration enhancer.

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