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In vitro and in vivo evaluations of the efficacy and safety of skin permeation enhancers using flurbiprofen as a model drug

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Abstract

The efficacy and safety of commonly used enhancers were systemically evaluated by in vitro and in vivo methods in this study. Flurbiprofen was used as the model drug to examine the enhancing capacity of these enhancers. Both in vitro permeation by Franz cells and in vivo kinetics of skin disposition were performed to determine the flurbiprofen permeation by enhancers. Unsaturated fatty acids showed the greatest enhancement of flurbiprofen permeation. The enhancing effect of D-limonene was slightly lower than that of the fatty acids. Azone and L-α-lecithin even reduced the skin deposition by flurbiprofen application. In vitro prostaglandin E_2 (PGE_2) release by cell culture, in vivo transepidermal water loss (TEWL) and colorimetry, and skin morphological changes were determined to examine the irritation of the skin by enhancers. The results showed that skin disruption and inflammation did not necessary correspond to the enhancing efficiency of the enhancers. Moreover, some discrepancies were observed in these irritant profiles when using various methods. The fatty acids generally showed the most irritating properties, followed by Azone, D-limonene, and L-α-lecithin. A complete portrait of the efficacy and safety of commonly used enhancers was therefore established in this study.

Keywords: Percutaneous absorption; Flurbiprofen; Enhancer; Irritation; Bioengineering method; Cell culture

1. Introduction

Poor permeability in the stratum corneum (SC) limits the usefulness of the transdermal drug administration route (Finnin and Morgan, 1999). Drug permeation through the SC can be increased with skin permeation enhancers. Practical use of enhancers requires the careful balancing of skin toxicity and the permeation enhancement benefit (Boelsma et al., 1996). Although there is a large amount of information available on the efficiency and toxicity of enhancers, there is little information on the overall evaluations of these two aspects of enhancers. The aim of this study was to systemically assess the efficacy and safety of commonly used enhancers by various evaluations.

Flurbiprofen was selected as a model drug to examine the effect of enhancers on skin permeation by this drug. Flurbiprofen, one of the most potent inhibitors of platelet aggregation currently available, is used to treat gout, osteoarthritis, rheumatoid arthritis, and sunburn (Poul et al., 1993). Suitable long-term percutaneous absorption of flurbiprofen at a controlled rate is needed because of its short half-life (3.9 h) and because it can cause gastrointestinal discomfort (Chen and Fang, 2000; Heyneman et al., 2000). This

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study focuses on skin permeation by flurbiprofen and the drug reservoir within the skin.

The present study utilizes in vitro Franz cells to explore the influence of enhancers on skin permeation by flurbiprofen. The amount of drug retained within the skin reservoir was also determined in vitro and in vivo. Assessment of the skin irritant potential of enhancers was performed using in vivo methods, including transepidermal water loss (TEWL) and colorimetry. The advantages of bioengineering evaluations are represented by the possibility of objectively collecting data and by monitoring readings on a linear scale with recorders (Fang et al., 1997).

Enhanced skin permeability possibly results from two effects: (1) the skin's biologic or physiopathological responses of its defensive function, and (2) physicochemical interactions which have altered the chemical structure or composition of lipids and/or proteins in the SC (Xu and Chien, 1991). Both effects can induce irritation or toxicity in skin which should be of concern. The inflammation of human skin fibroblasts by enhancers was investigated in order to explore the mechanism of biologic responses in skin to enhancer treatments. Histological examinations by light microscopy were used to assess the physicochemical effects of these enhancers on skin.

2. Materials and methods

2.1. Materials

Flurbiprofen, carboxymethyl cellulose sodium salt (CMC), oleic acid, linoleic acid, and linolenic acid were purchased from Wako Chemical Co. (Japan). D-Limonene and L-α-lecithin were supplied by Sigma Chemical Co. (MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Biowest Co. (France). Prostaglandin E_2 (PGE_2) kit (Correlate-EIA®) was from Assay Designs, Inc. (MI, USA). All other chemicals and solvents were of analytical grade.

2.2. Preparation of hydrogels

To prepare CMC hydrogels, a 5% (w/v) concentration of polymer was added into half of the total amount (47%) of pH 7 citrate-phosphate buffer, after which the mixture was stirred continuously for 1 h. After 24 h, the residual pH 7 buffer (47%) and 1% (w/v) flurbiprofen were added to the mixture with continuous stirring for 1 h. Vehicles containing an enhancer at a concentration of 5% (v/v or w/v) were also prepared.

2.3. In vitro percutaneous absorption

In vitro skin permeation experiments were carried out using a Franz diffusion assembly. The shaved back skin of female Wistar rats (180–200 g) was mounted on the receptor compartment with the SC side facing upwards into the donor compartment. The donor medium was 1 g of vehicle containing flurbiprofen with or without enhancers. The receptor medium was 10 ml of pH 7.4 citrate-phosphate buffer. The available diffusion area between compartments was 0.785 cm^2. The stirring rate and temperature were kept at 600 rpm and 37 °C, respectively. At appropriate intervals, 300-μl aliquots of the receptor medium were withdrawn and immediately replaced with an equal volume of fresh buffer. The amount of flurbiprofen retained in the skin was determined at the end of the in vitro permeation experiment (12 h). The applied site of skin was washed 10 times using a cotton cloth immersed in methanol. A sample of skin was weighed, cut with scissors, positioned in a glass homogenizer containing 1 ml of methanol, and ground for 5 min with an electric stirrer. The resulting solution was centrifuged for 10 min at 10,000 rpm. The supernatant was analyzed by HPLC.

2.4. In vivo topical application

An accurately weighed 0.4 g amount of hydrogels containing flurbiprofen was spread uniformly over a sheet of non-woven polyethylene cloth (2.5 × 2.5 cm^2, Johnson & Johnson Co., USA), which was then applied to the shaved back area of the rat. The polyethylene cloth was fixed with Tegaderm® adhesive dressing (St. Paul, MN, USA) and Fixomull® stretch adhesive tape (Beiersdorf AG, Hamburg, Germany). Four pieces of cloth with application durations of 2, 4, 6, and 8 h, respectively, of hydrogels were applied to each rat. The procedure of extraction of drug from the skin was the same as that of the in vitro experiments.

2.5. HPLC analysis of flurbiprofen

The flurbiprofen content was analyzed using an HPLC system consisting of a Hitachi L-7110 pump, a Hitachi L-7200 sample processor, and a Hitachi L-7400 UV detector. A 25-cm-long, 4-mm inner diameter C18 column (LichroCart 250-4, Merck) was used. The mobile phase consisted of a 40% aqueous phase adjusted to pH 2.6 with acetic acid; and 60% acetonitrile at a flow rate of 1 ml/min. The wavelength of the UV detector was set at 245 nm.

2.6. In vivo irritation evaluations

The applied method of hydrogels was the same as that for the in vivo topical application. After 24-h administration, the hydrogel was removed, and the application site was gently cleaned by cotton wool swab. After withdrawal of the hydrogel for 30 min, TEWL and colorimetry were determined. TEWL was measured quantitatively using a Tewameter® (TM210, Courage & Khazaka Co., Germany). The TEWL was calculated automatically and expressed in $g/m^2/h$. An adjacent untreated site was used as the baseline standard for each determination.

A spectrocolorimeter (CD100, Yokogawa Electrical Co., Japan) was used to measure skin erythema/irritation caused by the enhancers. The measuring head was held perpendicularly to the skin to avoid any pressure. As a reference, an untreated site was examined in each determination. The color is expressed in a three-dimensional coordinate system. According to the CIE (Commission Internationale de l'Eclairage), erythema caused by skin is expected to result in an increase in values on the a^*-axis (Snater et al., 1995). After measurements, the applied site was again covered with polyethylene cloth, Tegaderm® and Fixomull® without hydrogel. Recovery from the skin erythema/irritation was examined once a day for 5 days.

2.7. Cell culture

Human skin fibroblasts (Hs68) were obtained from the Food Industry Research and Development Institute (Hsin-Chu, Taiwan). Cells were maintained in DMEM supplemented with 10% heat-inactivated FCS and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B) in a humidified incubator at 37 °C and 5% CO_2. For further experiments, cells were seeded at a density of 10^5 cells/ml in 24-well Costar plates and cultured in DMEM containing 10% heat-inactivated FCS.

Stock solutions of enhancers dissolved in ethanol (except for the fatty acids which were dissolved in propylene glycol (PG) for the solubility consideration) were prepared. The final concentration of the stock solution in the culture medium was 0.015 and 0.025% (v/v). Skin fibroblast cultures were exposed to test enhancers for 24 h for determination of PGE_2 levels. PGE_2 was measured in cell culture supernatant by use of commercially available ELISA according to the manufacturer's instructions.

2.8. Histological examination by light microscopy

Histological changes in Wistar rat skin were examined after pretreatment with 5% enhancers in hydrogels for 6 h to evaluate the effect of the acute toxicity of these enhancers on skin morphology. Immediately after pretreatment, the specimen of the exposed area was examined histologically. The adjacent untreated skin area was also assessed as the control group. Each specimen was fixed in a 10% pH 7.4-buffered formaldehyde solution for at least 48 h. The specimen was cut vertically against the skin surface. Each section was dehydrated using ethanol, embedded in paraffin wax, and stained with hematoxylin and eosin. In each skin sample, three different sites were examined and evaluated under light microscopy (Nikon Eclipse 4000, Japan).

2.9. Statistical analysis

The statistical analysis of differences between different treatments was performed with the Mann–Whitney U test by SPSS® software. A 0.05 level of probability ($P < 0.05$) was taken as the level of significance. The Kruskal–Wallis test and Pearson correlation were also utilized in the present study.

3. Results and discussion

3.1. In vitro percutaneous absorption

The percutaneous absorption of flurbiprofen from pH 7 buffer with or without polymers was investigated.

Table 1
Effect of polymers and enhancers on flurbiprofen flux and amount in the skin after topical application in vitro

Formulation	Flux ($\mu g/cm^2/h$)	Amount in skin at 12 h ($\mu g/mg$)$\times 10^2$
pH 7 solution	8.27 ± 1.07	69.72 ± 8.19
CMC hydrogel	6.21 ± 0.65	61.62 ± 19.03
+ Oleic acid	47.11 ± 15.19*	85.18 ± 16.49
+ Linoleic acid	49.88 ± 8.54*	94.32 ± 10.76*
+ Linolenic acid	56.26 ± 11.82*	62.56 ± 9.69
+ Azone	5.54 ± 0.45	15.72 ± 2.52**
+ Limonene	33.31 ± 6.74*	78.99 ± 5.05
+ L-α-Lecithin	6.98 ± 0.32	24.57 ± 6.57**

The polymer and enhancer concentrations in the hydrogel formulation were 5%. Each value represents the mean ± S.D. ($n = 4$).
* $P < 0.05$ higher than the control group (CMC hydrogel).
** $P < 0.05$ lower than the control group (CMC hydrogel).

The cumulative amount–time profiles were plotted. The slopes of the resulting linear plots were calculated, and the flux ($\mu g/cm^2/h$) was determined from the slope. Hydrogels prepared by CMC showed lower ability to release drug than did the pH 7 solution as shown in Table 1, indicating that the cross-linkage formed by polymers in hydrogel may interact with flurbiprofen.

A series of commonly used enhancers, including unsaturated fatty acids, Azone, cyclic monoterpene, and phospholipids, were used to promote the percutaneous absorption of flurbiprofen. Oleic acid, linoleic acid, and linolenic acid which possess the same alkyl chain but one, two, and three double bonds, respectively, were the fatty acids tested here. As shown in Table 1, these three fatty acids all enhanced the flux of flurbiprofen, and the enhancement levels were similar for the three formulations. Fatty acids slightly enhanced the drug reservoir within the skin (Table 1). Moreover, only linoleic acid showed a significant difference in the skin deposition as compared to the control group ($P < 0.05$). The use of Azone led to a great reduction in the skin uptake of flurbiprofen and no effect on the flux as compared to hydrogels without enhancers (Table 1). This fact can be explained if one takes into account that Azone exerts an effect on the hydration of the SC (Sugibayashi et al., 1992). This condition facilitates the permeation of hydrophilic compounds; nevertheless, for the more-lipophilic compounds, such as flurbiprofen, partitioning into the "hydrated" SC is made more difficult (Díez-Sales et al., 1996).

The cyclic monoterpene, D-limonene, enhanced the flux to a greater extent without affecting tissue uptake or partitioning into the skin (Table 1). Phospholipids are reported to enhance the skin permeation of many drugs. However, no or a negative influence on the skin permeation of flurbiprofen by L-α-lecithin was observed in this study (Table 1). It is plausible that many phospholipids are unable to permeate into the skin from aqueous hydrogels, and thus an extra barrier is found on the skin surface (Valjakka-Koskela et al., 1998).

3.2. In vivo topical application

As shown in Fig. 1, only a slight increase in the in vivo skin uptake of drug after treatment with enhancers was seen which is similar to the result of in vitro permeation experiments. Almost all curves leveled off after a 2 h application of hydrogels; that is, a longer duration of flurbiprofen administration caused no further increase in the skin depot. The limited space of the skin reservoir for the drug being saturated may explain this phenomenon. Drug amounts in skin at 2, 4, and 6 h after application of hydrogels with linoleic acid were significantly higher ($P < 0.05$) as compared with that of the control group (Fig. 1). Moreover, the drug amounts after treatment with oleic acid only showed significant increase ($P < 0.05$) at 2 and 6 h. The in vivo skin depot was generally correlated with the in vitro skin depot, which showed a trend of linoleic acid > oleic acid > linolenic acid.

Both in vitro and in vivo results showed that Azone could lead to a reduction of drug reservoir in the skin as shown in Fig. 1 and Table 1. On the other hand, D-limonene only promoted the skin depot of flurbiprofen at 2 h of application ($P < 0.05$). L-α-Lecithin produced lower skin uptake of flurbiprofen than did the control group although no significant difference ($P > 0.05$) was observed between them.

3.3. In vivo irritation evaluations

The proposed mechanisms involving promotion of drug permeation by enhancers which may cause skin responses to irritation are: (1) induction of inflammatory mediator release in epidermal cells in the presence or absence of cytotoxicity, and (2) direct physicochemical interaction with the SC eliciting corrosive

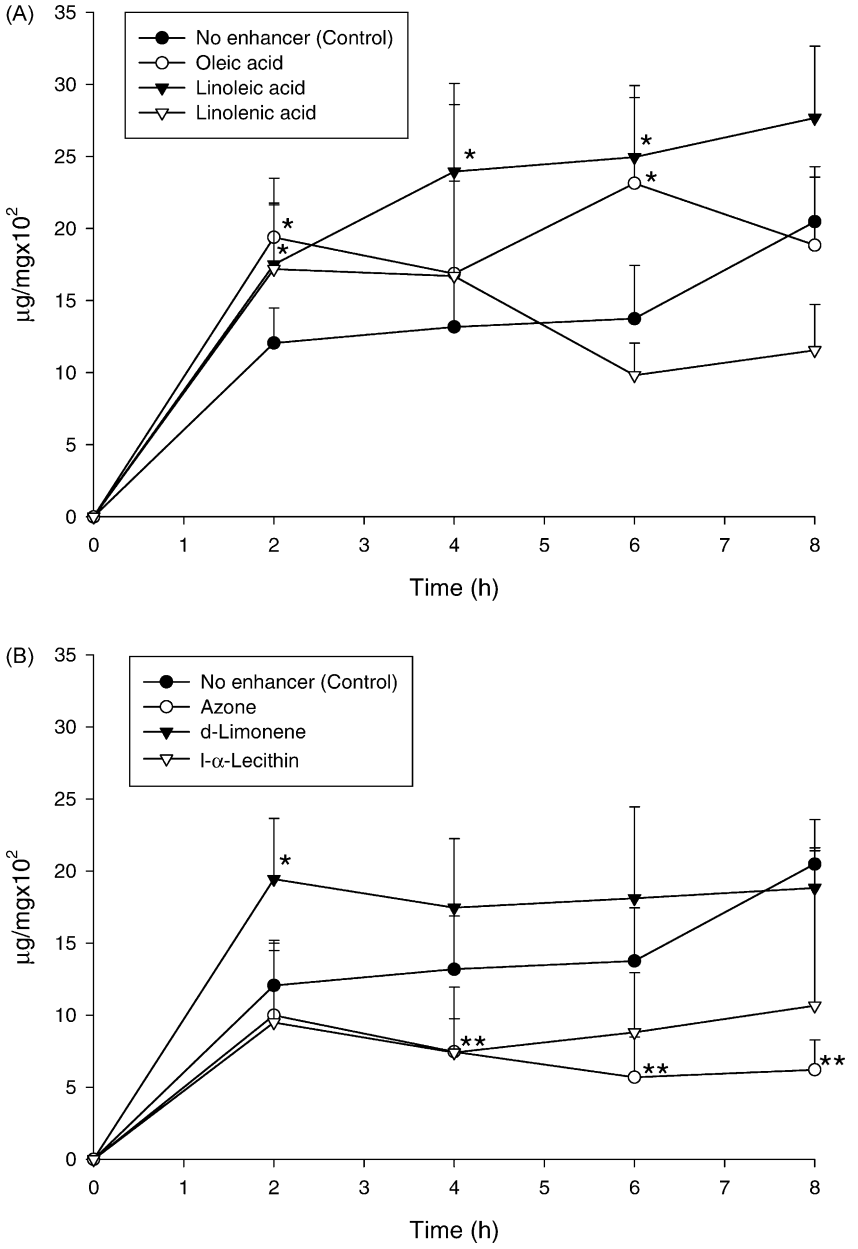

Fig. 1. Kinetics of flurbiprofen uptake within skin after topical application of hydrogels with enhancers of fatty acids (A); and Azone, D-limonene and L-α-lecithin (B). Each value represents the mean ± S.D. ($n = 5$). $^{*}P < 0.05$ higher than the control group; $^{**}P < 0.05$ lower than the control group.

damage. Hence a series of experiments for evaluating the safety of these enhancers was conducted in vitro and in vivo in the following sections. TEWL was performed to assess SC damage, and a good correlation between chemical damage to the skin barrier and an increase in TEWL was demonstrated. On the other hand, the a^{*}-coordinates of colorimetry have been demonstrated to correlate well with inflammatory

reactions of the skin (Serup and Agner, 1990; Fang et al., 2002).

As shown in Fig. 2, the ΔTEWL (TEWL value of treated site minus the TEWL value of an adjacent untreated site) determined over 5 days was evaluated after 24-h application of hydrogels. The hydrogels without enhancers were divided into two formulations: vehicles with or without 1% flurbiprofen. Curves of both ΔTEWL and Δa^* of the two control groups were similar during the 5 days (Figs. 2A and 3A). This result indicates that flurbiprofen itself caused no skin irritation as determined by these methods. The ΔTEWL of the control groups at day 1 (the hydrogel just removed) was approximately 10 $g/m^2/h$, then the △TEWL values descended near the baseline (0 $g/m^2/h$) from days 2 to 5. This may have been due to the high amount of the aqueous phase in the hydrogels hydrating the SC, causing the evaporation of excess water when measuring TEWL.

The treatment of enhancers generally induced ascendant values of ΔTEWL and Δa^* at the beginning, then the values gradually dropped to the baseline as the skin recovered to its normal status (Figs. 2 and 3). In order to compare TEWL values and color differences induced by these enhancers, a unique datum rather than multiple kinetics is needed. Calculation of the area under the curve is statistically debatable because of the insufficient plots for calculation. Hence the algebraic sums of the mean adjusted values were selected as end results ($\Sigma\Delta$TEWL and $\Sigma\Delta a^*$) as shown in the legends of Figs. 2 and 3 (Queille-Roussel et al., 1991). The three fatty acids demonstrated a significant increase ($P < 0.05$) in the extent of water loss from the SC relative to the control groups during 4 days post-enhancer removal (Fig. 2A). The SC layers may have completely recovered to a normal condition by day 5. The fatty acids either increased the degree of disorder of the intercellular lipid hydrocarbon chains or formed phase-separated fluid domains within the liquid-crystalline lipid structures reversibly causing a loss of diffusion resistance within the SC barrier (Schneider et al., 1996). Linolenic acid increased $\Sigma\Delta$TEWL by a factor of 7.2, whereas the other fatty acids produced a lower increase. This trend differed from that of the enhancement of flurbiprofen permeation in vitro and in vivo, indicating that the degree of SC barrier disruption (TEWL) is not necessarily correlated to the efficiency of the enhancement.

When fatty acids were applied to the skin, detectable erythema was noted by the colorimeter as shown in Fig. 3A. This suggests that some inflammatory responses may have occurred in the epidermis. Linolenic acid was found to be most irritating at the beginning (day 1). However, no significant differences were seen among $\Sigma\Delta a^*$ values of the three fatty acids during 5 days. According to the data of TEWL and colorimetry, the fatty acids tested here influenced both the physiopathological and physicochemical states of the skin. The detailed mechanisms are cited in the next sections.

Some controversies exist about the safety of Azone. It has been reported that Azone can be applied without toxicity or discomfort to the skin (Stoughton, 1982; Phillips and Michniak, 1995). However, other reports have shown the opposite results (Díez-Sales et al., 1996; Finnin and Morgan, 1999). In this present study, Azone still exhibited a moderate irritation to the skin as shown in Figs. 2B and 3B. Azone significantly increased $\Sigma\Delta$TEWL by a factor of 2.0. In addition to its ability to disrupt the structured lipids of the SC, Azone was reported to increase retention of water in the lipid matrix of the SC (Takeuchi et al., 1995). Azone showed a significantly higher erythema score on days 1–5 in comparison with the control group (Fig. 3A). An 8.4-fold increase in $\Sigma\Delta a^*$ was observed which was greatly higher than that of $\Sigma\Delta$TEWL. This indicates that Azone may have induced a greater level of inflammatory responses to the epidermis than the disruption of the SC barrier.

Both D-limonene and L-α-lecithin demonstrated no significant increase ($P > 0.05$) in ΔTEWL and Δa^* relative to the control group (Figs. 2B and 3B), which implies that the skin barrier was not affected by these two compounds. Although D-limonene and L-α-lecithin showed a similar profile on in vivo evaluations, the enhancement of flurbiprofen permeation by these two enhancers greatly differed. D-Limonene modulated flurbiprofen permeation through a 5.3-fold increase (Table 1). This suggests that the skin permeation of drugs can possibly be enhanced with limited changes in skin physiology and physicochemistry. It was found that the rank order of enhancing efficiency by these enhancers did not agree well with the rank order of skin disruption and irritation. Hence a correlation between efficiency and skin barrier function

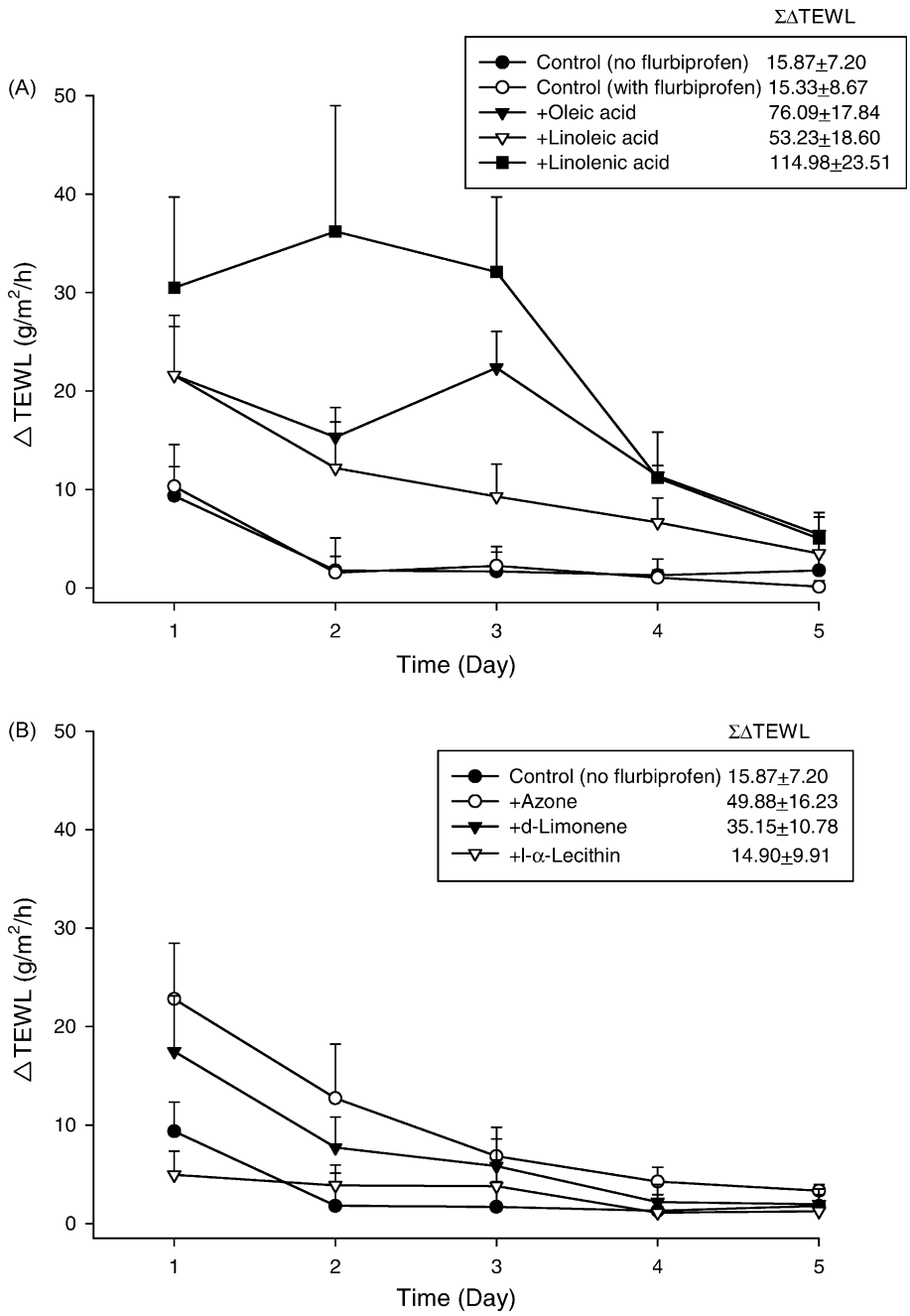

Fig. 2. Kinetics of ΣΔTEWL during 5 days after topical application of hydrogels with enhancers of fatty acids (A); and Azone, D-limonene and L-α-lecithin (B) for 24 h. Each value represents the mean ± S.D. ($n = 6$).

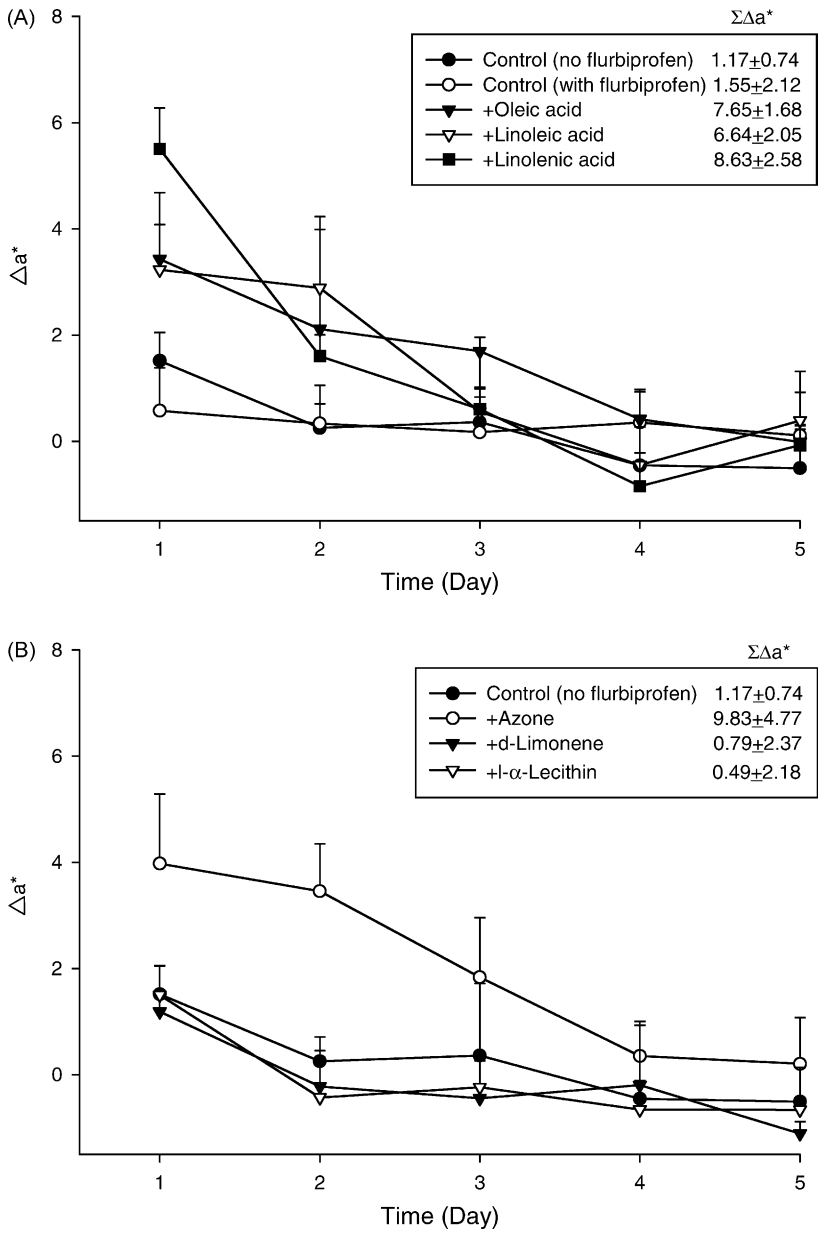

Fig. 3. Kinetics of Δa^* by colorimetry during 5 days after topical application of hydrogels with enhancers of fatty acids (A); and Azone, D-limonene and L-α-lecithin (B) for 24 h. Each value represents the mean ± S.D. ($n = 6$).

should not be assumed beforehand. A moderate correlation ($r = 0.7824$) between $\Sigma\Delta$TEWL and $\Sigma\Delta a^*$ was also observed for these enhancers. Differentiation between physiopathological and physicochemical responses is complicated because the skin reactions appear very heterogeneous particularly with respect to epidermal damage (Agner and Serup, 1989). In order to elucidate this differentiation, skin inflammation and disruption on the level of cell biology and skin morphology were examined in the following.

3.4. In vitro cell culture of skin fibroblasts

Local inflammatory cells first attack foreign toxins in the early stage. Then local inflammatory mediators, such as bradykinin, serotonin, and prostaglandins are released (Xu and Chien, 1991). The presence of human skin fibroblasts made in response to inflammation is a potential means of quantitating irritation (Bason et al., 1991). In this study, the PGE_2 release of cultured human fibroblasts by enhancers was investigated. Because of solubility considerations, the fatty acids should be dissolved in PG to treat cells, while the others were dissolved in ethanol. As shown in Fig. 4A, PG itself moderately increased ($P < 0.05$) the PGE_2 level. It was found that PG solvate the α-keratin and irritated the epidermal and dermal structures, and so it is classified as a moderate irritant (Wahlberg and Nilsson, 1984; Takeuchi et al., 1995). Since PG is a vehicle commonly used in pharmaceutical and cosmetic formulations, the use of PG should be cautious due to its capacity to cause inflammatory responses. On the other hand, ethanol caused no irritation of skin fibroblasts as shown in Fig. 4B.

Fig. 4 exemplifies the PGE_2 release profiles generated by a series of enhancers. When fibroblasts were incubated in the presence of fatty acids for 24 h with various concentrations in culture medium (0.0015 and 0.0025%), the release of PGE_2 from fibroblasts was significantly increased ($P < 0.05$) as compared to the basal group (Fig. 4A). Linoleic acid showed the greatest irritant response to fibroblast cells, followed by oleic acid and linolenic acid, which both showed similar profiles. Various doses of fatty acids did not affect the expression of fibroblasts at the PGE_2 level.

Azone at 0.0015% induced a significant increase ($P < 0.05$) in the release of PGE_2 from fibroblasts (Fig. 4B). However, 0.0025% Azone did not increase the PGE_2 level as compared to the control group. It is proposed that a high concentration of Azone may cause cytotoxicity followed by cell death of skin fibroblasts. Neither D-limonene nor L-α-lecithin affected the expression of PGE_2 levels. This result is related to the low skin irritation by these two compounds as evaluated by in vivo bioengineering methods. In a comparison between the levels of parameters $\Sigma\Delta$TEWL and PGE_2 induced by all enhancers tested, a low correlation was found ($r = 0.3606$ for 0.0015% and $r = 0.3733$ for 0.0025%) between in vivo skin barrier disruption and in vitro cell inflammation. Hence PGE_2 release in fibroblast cultures was not indicative of irritancy potential. A low correlation was also observed between $\Sigma\Delta a^*$ and 0.0025% enhancers on PGE_2 release ($r = 0.4076$). However, a moderate relationship was shown between $\Sigma\Delta a^*$ and 0.0015% enhancers on PGE_2 release ($r = 0.7345$). This may have been due to cell death caused by 0.0025% Azone which could be excluded ($r = 0.7020$ between $\Sigma\Delta a^*$ and 0.0025% enhancers after exclusion of Azone). The results of colorimetry were predominantly determined by the erythema level in the subepidermal vascular plexus and papillary loops (Agache and Dupond, 1994; Fang et al., 1997). We concluded that the inflammatory responses generated by skin fibroblasts, mainly consisting of the viable epidermis/dermis, may reflect that skin irritation assessed by colorimetry is more meaningful than the index of disruption of the SC by TEWL.

Enhancers can induce inflammations and cells and mediators can cause some chemical, structural, and/or compositional alterations of cutaneous lipids and proteins, thus changing the skin permeability (Zhao and Singh, 2000). Based on this consideration, the relationship between the enhancing efficiency and PGE_2 level released by these enhancers was calculated. Neither flurbiprofen flux nor skin uptake showed a relationship with the inflammatory responses by 0.0015% enhancers ($r = 0.3739$ for flux and $r = 0.3081$ for skin uptake). There may be some relation between flurbiprofen permeation profiles and PGE_2 treated by a higher concentration of enhancers ($r = 0.6972$ for flux and $r = 0.6899$ for skin uptake). However, at all events, the enhancing efficiency still did not correlate well with the inflammatory signals.

Prostaglandins are a diverse group of autocrine and paracrine hormones that mediate many cellular and physiological processes (Adams et al., 1996). Two prostaglandin synthases that catalyze the formation of prostaglandin H2 (an intermediate in formation of prostaglandins) from arachidonic acid are cyclooxygenase-1 (Cox-1) and cyclooxygenase-2 (Cox-2) (Tsujii and DuBois, 1995; Adams et al., 1996). Cox-2 is efficiently induced in migratory cells responding to pro-inflammatory stimuli and is considered to be an important mediator of inflammation (Langenbach et al., 1995). Increases in PGE_2 formation by enhancers were blunted by NS398, a selective

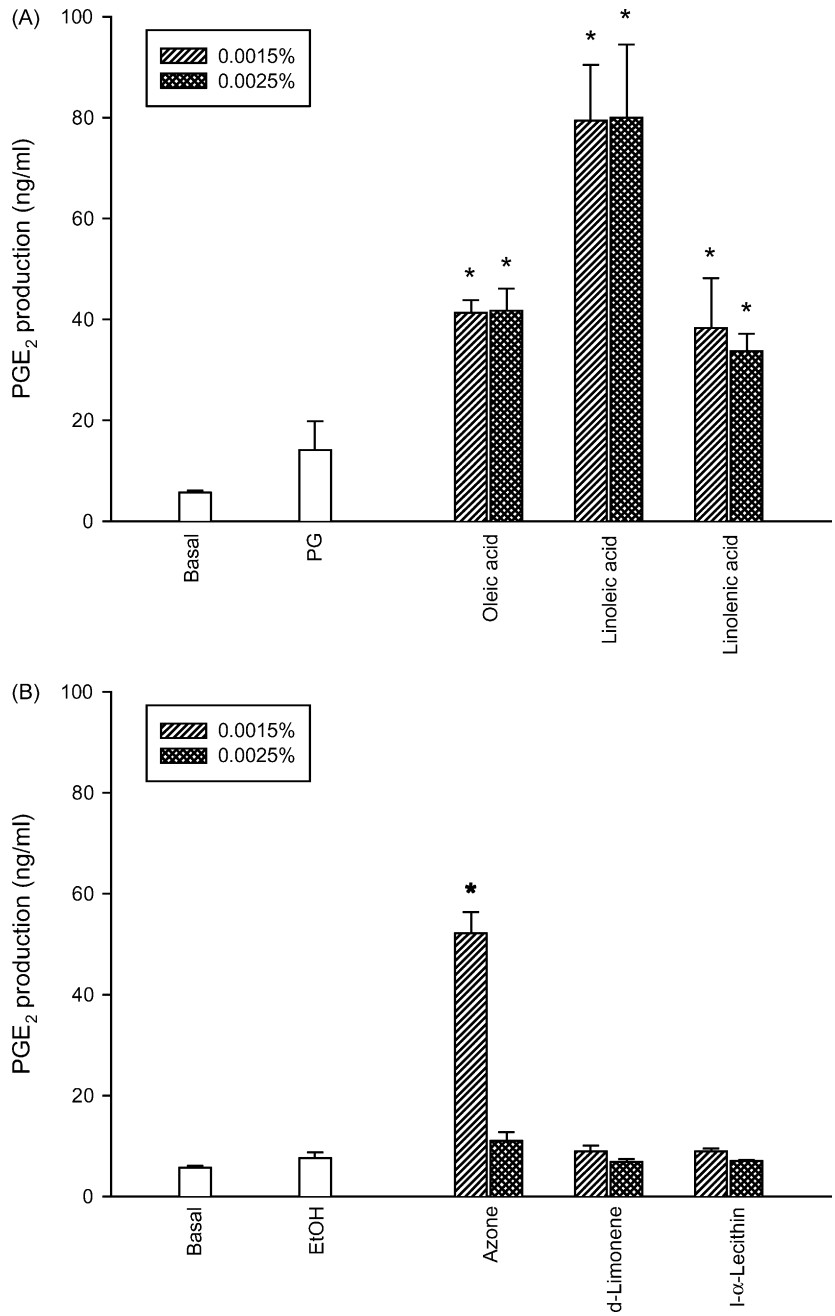

Fig. 4. PGE_2 production by skin fibroblasts in cultured medium after treatment with enhancers at various concentrations: (A) enhancers dissolved in PG, and (B) enhancers dissolved in ethanol. Each value represents the mean ± S.D. ($n = 4$). $^{*}P < 0.05$ higher than the control group.

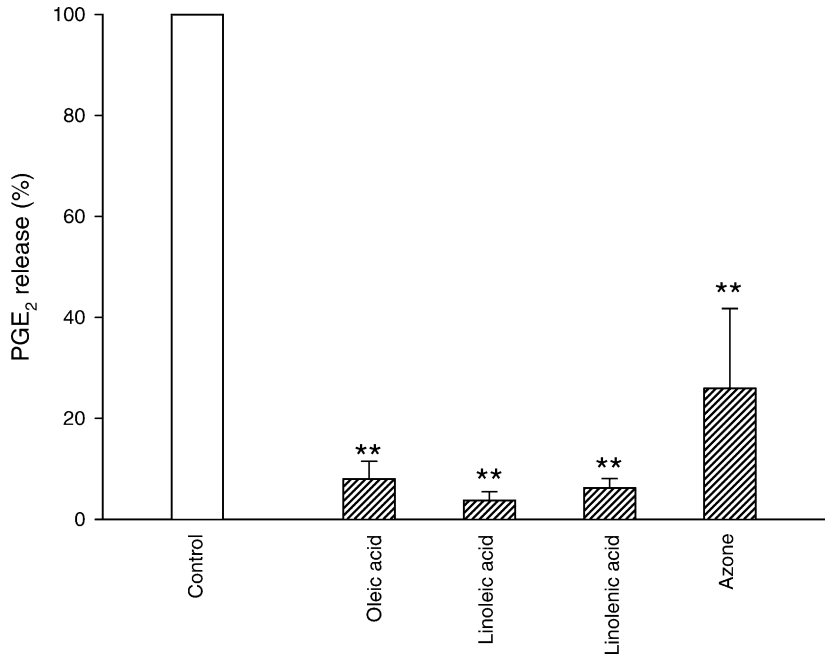

Fig. 5. PGE_2 production by skin fibroblasts in cultured medium after treatment with enhancers with NS398. Each value represents the mean ± S.D. ($n = 4$). $^{**}P < 0.05$ lower than the control group.

Cox-2 inhibitor (Futaki et al., 1994), indicating that Cox-2 is involved in enhancer-mediated inflammatory responses (Fig. 5).

3.5. Histological examination by light microscopy

The skin irritation by enhancers after a 6-h exposure was histopathologically investigated to explore the possible role of physicochemical responses of the skin to enhancers. Light microscopy indicated no observable damage to whole skin in the untreated group (data not shown). As shown in Fig. 6A, oleic acid increased the neutrophiles and lymphocytes in the epidermis/dermis (inflammatory cell infiltration). This is a phenomenon of an inflammatory response caused by oleic acid which was confirmed by cell culture results in this study. Hyperplasia of the stratum granulosm and epidermis was observed after treatment with oleic acid. Enhanced epidermal proliferation has been observed by several investigators (Rijzewijk et al., 1988; Xu and Chien, 1991). It was reported that epidermal injury results in recruitment of epidermal cells from the quiescent stage into the mitotic cycle by activation of the phosphoinositol cycle. Inflammatory mediators such as PGE_2 can increase the epidermal proliferation which may be consistent with results of this study. Disruption and fragmentation of SC layers were also observed, which may have contributed to the enhancing effect on flurbiprofen permeation.

As shown in Fig. 6B, thickened epidermis was observed after treatment with linoleic acid which was similar to that seen with oleic acid. Proliferation of collagen in the dermis was also observed, which is an indicator of post-inflammatory responses. The size of collagen was also augmented after linoleic acid treatment. Dehydration of the skin may produce the enlargement of collagen. As shown in Fig. 2A, linoleic acid exhibited the lowest $\Sigma\Delta$TEWL level among three fatty acids tested, which may be indicative of morphologic observations of dehydration of the skin. As compared to oleic acid, disruption of the SC layers and hyperemia in the epidermis were found to be slighter with linoleic acid treatment (Fig. 6B).

Fig. 6C demonstrates microscopic results after treatment with linolenic acid. The SC was largely lost and appears as a thin compact layer. Inflammated cells

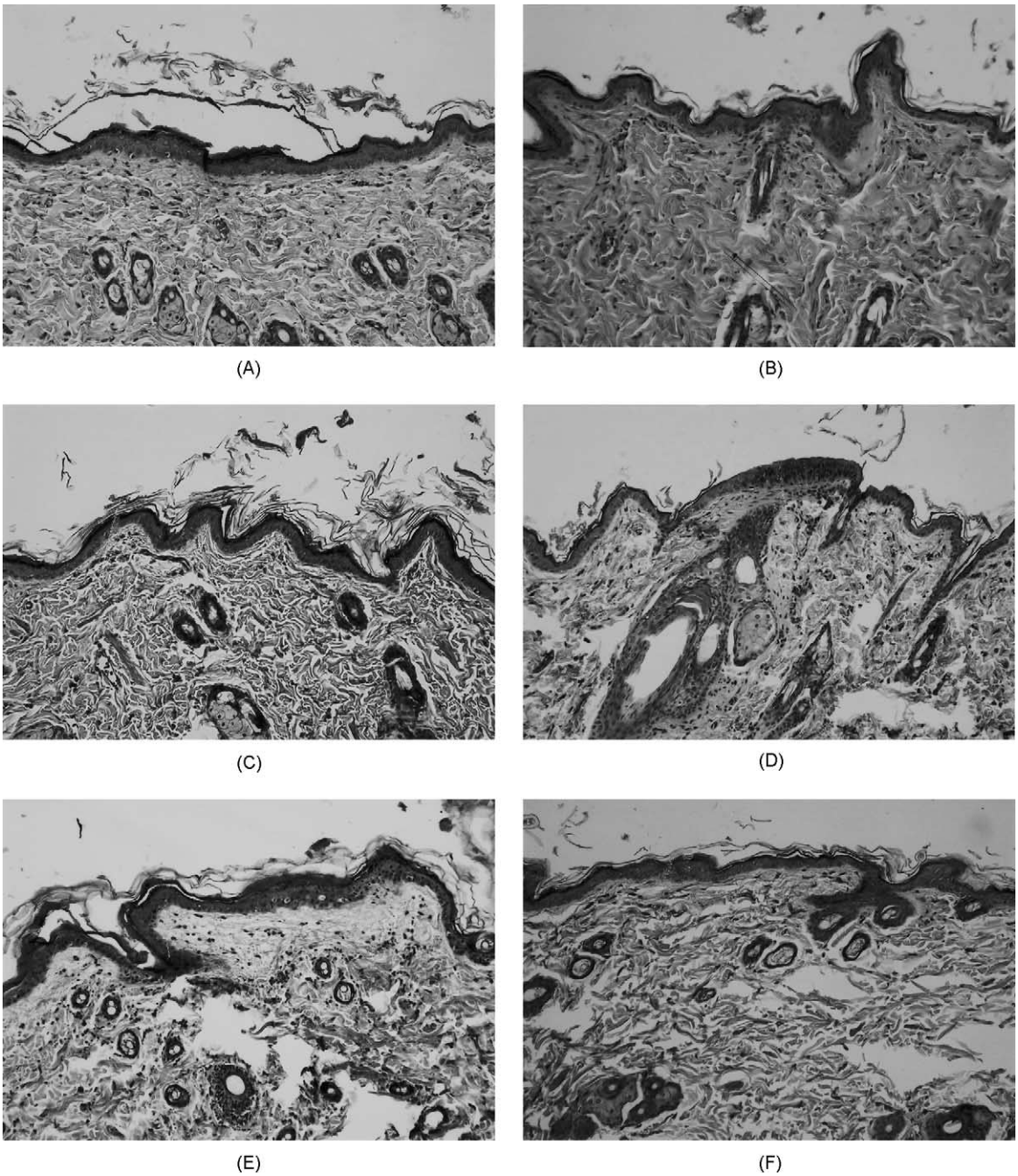

Fig. 6. Light microscopic photographs (100×) of rat skin after treatment with 5% enhancer in hydrogels: (A) oleic acid, (B) linoleic acid, (C) linolenic acid, (D) Azone, (E) D-limonene, and (F) L-α-lecithin.

were found in the superficial layers including the stratum granulosm and epidermis. Some necrosis was also observed in the skin. It is generally considered that the three fatty acids examined here all showed a certain extent of hyperemia and SC disruption. This result was verified by the in vitro and in vivo irritant evaluations in the present study. These effects on the skin contribute to the capacity to enhance drug permeation.

Morphologic changes in the skin with Azone treatment are focused in the epidermis (Fig. 6D). Proliferation was observed in the superficial layer of skin. Neutrophils and lymphocytes increased in the epidermis, indicating the inflammation of skin cells. Acute inflammation of skin cells was observed in D-limonene-treated skin (Fig. 6E). This result was not reflected by PGE_2 induced by D-limonene. The SC layers were also slightly scattered by D-limonene resulting in the promotion of flurbiprofen permeation and an increase in $\Sigma\Delta$TEWL. There was almost no change in skin morphology by L-α-lecithin as compared with untreated skin (Fig. 6F). No inflammatory responses were observed in L-α-lecithin-treated skin, suggesting non-irritating properties of L-α-lecithin as determined by a series of in vitro and in vivo evaluations.

4. Conclusions

The efficacy and safety of commonly used permeation enhancers were systemically evaluated using a series of in vitro and in vivo methods. It was found that enhancers with particular chemical characteristics produced particular enhancing and irritant responses. In general, the relationship between enhancement efficiency and irritation of these enhancers was very low. The efficacy of enhancement showed a trend of fatty acids > D-limonene > L-α-lecithin ≥ Azone for flurbiprofen permeation. On the other hand, irritation and inflammation of the enhancers showed a trend of fatty acids > Azone > D-limonene > L-α-lecithin. We concluded that D-limonene may be the best choice as the enhancer for flurbiprofen permeation due to its moderate enhancement and low irritation to skin. Azone is the most widely known enhancer, and it is often used by transdermal research groups because of its good overall enhancing capacity. However, an opposite result was observed in the present study as Azone inhibited the skin permeation and uptake of flurbiprofen and showed decisive irritation and/or toxicity to skin in vitro and in vivo.

There were some discrepancies in the irritant evaluations of enhancers by different methods, including in vitro cell cultures, in vivo bioengineering methods, and histological examinations. Irritant responses of the skin are complicated because reactions appear very heterogeneous particularly with respect to epidermal damage. Hence, careful and extensive consideration should be exercised when selecting enhancers for formulations since the different evaluation methods may indicate different irritation behaviors. However, the determinations of TEWL and in vitro PGE_2 level are suggested for practical use because of the better ability of both methods to distinguish the irritation among various enhancers.

References

Adams, J., Collaco-Moraes, Y., de Belleroche, J., 1996. Cyclooxygenase-2 induction in cerebral cortex: an intracellular response to synaptic excitation. J. Neurochem. 66, 6–13.

Agache, D.G., Dupond, A., 1994. Recent advances in non-invasive assessment of human skin blood flow. Acta. Derm. Venereol. Suppl. 185, 47–51.

Agner, T., Serup, J., 1989. Skin reactions to irritants assessed by non-invasive bioengineering methods. Contact Dermat. 20, 352–359.

Bason, M.M., Gordon, V., Maibach, H.I., 1991. Skin irritation: in vitro assays. Int. J. Dermatol. 30, 623–626.

Boelsma, E., Tanojo, H., Bodde, H.E., Ponec, M., 1996. Assessment of the potential irritancy of oleic acid on human skin: evaluation in vitro and in vivo. Toxicol. In Vitro 10, 729–742.

Chen, H.Y., Fang, J.Y., 2000. Therapeutic patents for topical and transdermal drug delivery systems. Exp. Opin. Ther. Patents 10, 1035–1043.

Díez-Sales, O., Watkinson, A.C., Herráez-Domínguez, M., Javaloyes, C., Hadgraft, J., 1996. A mechanistic investigation of the in vitro human skin permeation enhancing effect of Azone. Int. J. Pharm. 129, 33–40.

Fang, J.Y., Leu, Y.L., Wang, Y.Y., Tsai, Y.H., 2002. In vitro topical application and in vivo bioengineering evaluation of nonivamide hydrogels using Wistar rat as an animal model. Eur. J. Pharm. Sci. 15, 417–423.

Fang, J.Y., Tasi, M.J., Huang, Y.B., Wu, P.C., Tsai, Y.H., 1997. Percutaneous absorption and skin erythema: quantification of capsaicin and its synthetic derivatives from gels incorporated with benzalkonium chloride by using non-invasive bioengineering methods. Drug Dev. Res. 40, 56–67.

Finnin, B.C., Morgan, T.M., 1999. Transdermal penetration enhancers: application, limitation, and potential. J. Pharm. Sci. 88, 955–958.

Futaki, N., Takahashi, S., Yokoyama, M., Arai, I., Higuchi, S., Otomo, S., 1994. NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. Prostaglandins 47, 55–59.

Heyneman, C.A., Lawless-Liday, C., Wall, G.C., 2000. Oral versus topical NSAID in rheumatic diseases. Drugs 60, 555–574.

Langenbach, R., Morham, S.G., Tiano, H.F., Loftin, C.D., Ghanayem, B.I., Chulada, P.C., Mahler, J.F., Lee, C.A., Goulding, E.H., Kluckman, K.D., 1995. Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. Cell 83, 483–492.

Phillips, C.A., Michniak, B.B., 1995. Topical application of Azone analogs to hairless mouse skin: a histopathological study. Int. J. Pharm. 125, 63–71.

Poul, J., West, J., Buchanan, N., Grahame, R., 1993. Local action transcutaneous flurbiprofen in the treatment of soft tissue rheumatism. Br. J. Pharmacol. 32, 1000–1003.

Queille-Roussel, C., Poncet, M., Schaefer, H., 1991. Quantification of skin-colour changes induced by topical corticosteroid preparations using the Minolta chromameter. Br. J. Dermatol. 124, 264–270.

Rijzewijk, J.J., Bauer, F.W., Boezeman, J.B.M., Happle, R., Mier, P.D., 1988. Recruitment of quiescent cells following epidermal injury is initiated by activation of phosphoinositol cycle. J. Invest. Dermatol. 90, 44–47.

Schneider, I., Dobner, B., Neubere, R., Wohlrab, W., 1996. Evaluation of drug penetration into human skin ex vivo using branched fatty acids and propylene glycol. Int. J. Pharm. 145, 187–196.

Serup, J., Agner, T., 1990. Colorimetric quantification of erythema—a comparison of two colorimeters with a clinical scoring scheme and laser Doppler flowmetry. Clin. Exp. Dermatol. 15, 267–272.

Snater, E., Janssen, E.A.A.J., Van der Valk, P.G.M., Van de Kerkhof, P.C.M., 1995. Transepidermal water vapour loss is not increased during and following dithranol irritation. Br. J. Dermatol. 132, 908–912.

Stoughton, R.B., 1982. Enhanced percutaneous penetration with 1-dodecylazocycloheptan-2-one. Arch. Dermatol. 118, 474–477.

Sugibayashi, K., Nakayama, S., Seki, T., Hosoya, K., Morimoto, Y., 1992. Mechanism of skin penetration-enhancing effect by laurocapram. J. Pharm. Sci. 81, 58–64.

Takeuchi, Y., Yasukawa, H., Yamaoka, Y., Taguchi, K., Fukushima, S., Shimouaka, Y., Nishinaga, H., Morimoto, Y., 1995. Behavior of propylene glycol in dermis after treatment of rat intact skin surface with fatty acids, fatty amines or Azone dissolved in propylene glycol. Biol. Pharm. Bull. 18, 304–309.

Tsujii, M., DuBois, R.N., 1995. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell 83, 493–501.

Valjakka-Koskela, R., Kirjavainen, M., Mönkkönen, J., Urtti, A., Kiesvaara, J., 1998. Enhancement of percutaneous absorption of naproxen by phospholipids. Int. J. Pharm. 175, 225–230.

Wahlberg, J.E., Nilsson, G., 1984. Skin irritancy from propylene glycol. Acta. Derm. Venereol. 64, 286–290.

Xu, P., Chien, Y.W., 1991. Enhanced skin permeability for transdermal drug delivery: physiopathological and physico-chemical considerations. Crit. Rev. Ther. Drug Carrier Sys. 8, 211–236.

Zhao, K., Singh, J., 2000. Mechanisms of in vitro percutaneous absorption enhancement of tamoxifen by enhancers. J. Pharm. Sci. 89, 771–780.