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Efficacy and irritancy of enhancers on the in-vitro and in-vivo percutaneous absorption of curcumin

Jia-You Fang, Chi-Feng Hung, Hsien-Chih Chiu, Jhi-Joung Wang and Te-Fu Chan

Abstract

Curcumin is a predominant compound derived from the rhizomes of *Curcuma longa* L., and shows antibacterial, anti-inflammatory and antineoplastic activity. The in-vitro and in-vivo skin absorption of curcumin was investigated after application of enhancers using Wistar rat as an animal model. The enhancers selected in this study included terpenes, flavonoids and cholestanol. The irritant profiles of these enhancers were also established by transepidermal water loss (TEWL) and histological observations. Cyclic monoterpenes generally showed stronger enhancement of curcumin permeation than the other enhancers. Modulation of concentration and pretreatment duration of enhancers possibly indicated that the enhancers have varied ability and mechanisms to enhance curcumin permeation. Terpineol produced the highest TEWL values among the enhancers tested, whereas ketocholestanol produced no, or only a negligible, increase in TEWL as compared with control. The results showed that skin disruption and inflammation did not necessarily correspond to the enhancing efficiency of the enhancers.

Introduction

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is the main colorant found in the rhizomes of *Curcuma longa* L. (Zingiberaceae). It is widely used in traditional medications, cosmetics and textiles (Suhaimi et al 1995). Curcumin has proven to be promising as it exhibits antibacterial, anti-inflammatory and antineoplastic properties (Ammon & Wahl 1991; Huang et al 1995). It appears that when given orally, curcumin is far less active than after intraperitoneal administration. This may be due to poor absorption of curcumin by the gastrointestinal tract (Ammon & Wahl 1991). Recent studies have also demonstrated that curcumin exerts therapeutic effects on wound healing and skin tumours after topical application (Huang et al 1997; Santibáñez et al 2000). Hence the percutaneous route may be suitable for administration of curcumin for both local and systemic therapeutic uses.

Although percutaneous absorption is a promising route for curcumin, there have been few studies investigating the design of curcumin topical application. The aim of this study was to assess the permeation characteristics of curcumin across skin. The transdermal route is not more widely used because of the inherent barrier properties of the skin. This study also focuses on the use of enhancers to modulate the skin permeation of curcumin and the drug reservoir within the skin. A series of enhancers, including terpenes, flavonoids and cholestanol, were used to promote the percutaneous absorption of curcumin. Practical use of enhancers requires the careful balancing of skin toxicity and the permeation enhancement benefit (Boelsma et al 1996). This study systemically assessed the efficacy and safety of these enhancers by various evaluations.

In-vitro Franz cells were utilized to explore the permeation characteristics of curcumin with or without enhancers. 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 the in-vivo bioengineering method of transepidermal water loss (TEWL). The advantages of this method is that it is possible to objectively collect data and monitor readings on a linear scale with recorders

Pharmaceutics Laboratory, Graduate Institute of Natural Products, Chang Gung University, Tao-Yuan, Taiwan

Jia-You Fang, Hsien-Chih Chiu

School of Medicine, Fu Jen Catholic University, Taipei Hsien, Taiwan

Chi-Feng Hung

Department of Medical Research, Chi-Mei Medical Center, Tainan Hsien, Taiwan

Jhi-Joung Wang

Department of Obstetrics and Gynecology, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan

Te-Fu Chan

Correspondence: J.-Y. Fang, Graduate Institute of Natural Products, Chang Gung University, 259 Wen-Hwa 1st Road, Kwei-Shan, Tao-Yuan, Taiwan. E-mail: fajy@mail.cgu.edu.tw

(Fang et al 2002). Histological examination by light microscopy was used to assess the physicochemical effects of these enhancers on the skin and their mechanism of action.

Materials and Methods

Materials

Curcumin, β-myrcene, eugenol, l-menthol, 1,8-cineole, hesperetin, phloretin and ketocholestanol were purchased from Sigma Chemical Co. (St Louis, MO). Terpineol, carveol, farnesol and nerolidol were obtained from Aldrich Chemical Co. (St Louis, MO). Carboxymethylcellulose sodium salt (CMC-Na) and carboxymethylcellulose ammonium salt (CMC-NH_4) were from Wako Chemical Co. (Osaka, Japan). All other chemicals and solvents were of analytical grade.

In-vitro skin permeation

In-vitro skin permeation experiments were carried out using a Franz diffusion cell. Skin taken from Wistar rats (180~200 g) was mounted on the receptor compartment with the stratum corneum side facing upwards into the donor compartment. The donor medium was 1 mL of vehicle containing 0.015 M curcumin in an ethanol–citrate-phosphate buffer, pH 7.4, mixture (25:75). The receptor medium was 10 mL of ethanol–citrate-phosphate buffer, pH 7.4 (50:50). The ethanol–citrate-phosphate buffer, pH 7.4 (25:75) vehicle with enhancers was used to pretreat skin for various lengths of time before applying curcumin. The available diffusion area between cells was 0.785 cm^2. The stirring rate and temperature were maintained at 600 rev min^{-1} and 37 °C, respectively. At appropriate intervals, 300-μL samples of the receptor medium were withdrawn and immediately replaced with an equal volume of fresh buffer. The amount of curcumin was determined by HPLC.

The amount of curcumin retained in the skin was determined at the end of the in-vitro permeation experiment (24 h). The skin was washed 10 times using a cotton cloth immersed in methanol. A sample of skin was weighed, cut with scissors, placed 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 rev min^{-1}. The supernatant was analysed by HPLC.

Preparation of hydrogels

Hydrogels were used as curcumin vehicles in the in-vivo study and a portion of in-vitro study. A 3 or 5% (w/v) concentration of CMC-Na or CMC-NH_4 was added to half the total amount of ethanol–citrate-phosphate buffer, pH 7.4 (25:75), after which the mixture was stirred continuously for 1 h. After 24 h, the residual ethanol–citrate-phosphate buffer, pH 7.4 buffer and 0.015 M curcumin were added into the mixture with continuous stirring for 1 h.

In-vivo topical application

A 1.5-mL ethanol–citrate-phosphate buffer, pH 7.4 (25:75) solution of enhancers was pipetted onto a sheet of non-woven polyethylene cloth ($3 \times 3\,cm^2$; Johnson & Johnson Co., USA) and applied on the back area of the rat for 1 h. An accurately weighed amount (0.4 g) of hydrogels containing curcumin was spread uniformly over a polyethylene cloth ($2.5 \times 2.5\,cm^2$), which was then applied to the treated sites after removing the enhancer solution. The polyethylene cloth was fixed with Tegaderm adhesive dressing (3M, St Paul, MN) and Fixomull stretch adhesive tape (Beiersdorf AG, Hamburg, Germany). Two pieces of cloth containing hydrogels with application durations of 2 and 8 h, respectively, were applied to each rat. The procedure of extraction of drug from the skin was the same as for the in-vitro experiments.

HPLC analysis of curcumin

The HPLC method was modified from Hiserodt et al 1996. The curcumin content was analysed using an HPLC system consisting of a Hitachi L-7110 pump, a Hitachi L-7200 sample processor and a Hitachi L-7480 fluorescence detector. A 25-cm long, 4-mm inner diameter C18 column (LichroCart 250-4, Merck) was used. The mobile phase consisted of a 45% aqueous phase adjusted to pH 2.5 with acetic acid and 55% acetonitrile at a flow rate of 0.7 mL min^{-1}. The wavelength of the fluorescence detector was set at an excitation of 420 nm and an emission of 544 nm. The detection limit for curcumin was 40 ng mL^{-1}.

In-vivo TEWL determination

The method of applying the enhancer solutions in the TEWL determination was the same as for the pretreatment procedures in the in-vivo topical application. The durations of application were 2 and 8 h. Quantitative measurements of TEWL were carried out with an evaporimeter (Tewameter 300; Courage & Khazaka, Köln, Germany) after removal of enhancer solutions. The TEWL was automatically calculated and expressed in $g\,m^{-2}\,h^{-1}$. An adjacent untreated site was used as the baseline standard for each determination.

Histological examination by light microscopy

Histological changes in Wistar rat skin were examined immediately after pretreatment with 5% enhancers for 2 and 8 h. The adjacent area of untreated skin 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).

Statistical analysis

The statistical analysis of differences between different treatments was performed using 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 was also used. In the in-vivo topical experiments and in-vivo TEWL study (sample size = 6), the one-way analysis of variance was utilized to determine the statistical comparison.

Results and Discussion

Effect of enhancers on in-vitro percutaneous absorption of curcumin

The passive diffusion of curcumin across the skin was investigated. Cumulative amount–time profiles were plotted. The slopes of the resulting linear plots were calculated, and the flux ($\mu g\ cm^{-2}\ h^{-1}$) was determined from the slope. As shown in Table 1, curcumin itself exhibited a very low flux and skin deposition after in-vitro topical application for 24 h. This suggests that enhancing methods were needed for topical curcumin to achieve the desired therapeutic effects. Natural products are of considerable interest to the pharmaceutical industry. Hence a series of natural enhancers, including terpenes, flavonoids and cholestanol, were used to promote the percutaneous absorption of curcumin. An ethanol–citrate-phosphate buffer, pH 7.4 (25:75) mixture with 5% enhancers was used as a pretreatment vehicle for the in-vitro permeation study. Solvents such as ethanol, in combination with an enhancer, accumulate in the tissue and increase the partitioning of the drug due to the greater affinity of drug for the solvents (Barry 1991).

The effects of these enhancers after 1 h of pretreatment on the percutaneous absorption parameters of curcumin (flux, total permeated amount at 24 h, drug in skin reservoir at 24 h) are shown in Table 1. First, the influence of pretreatment with ethanol on the transport behaviour of curcumin was determined. After pretreatment with 25% ethanol, the skin partitioning of curcumin increased approximately 2.9 times, whereas no significant difference ($P > 0.05$) was observed in drug flux. Lipid extraction and osmotic expansion may be the most plausible explanations for the increased amount of drug within the skin (Williams & Barry 1992; Magnusson et al 1997).

Terpenes are constituents of essential oils that provide a series of clinically acceptable enhancers for lipophilic and hydrophilic drugs (Zhao & Singh 1998). Terpene enhancers mainly increase drug diffusivity in the skin by disrupting the highly ordered intercellular lipid structure of the stratum corneum (El-Kattan et al 2001). All the evaluated terpenes had significant effects on the flux or skin deposition of curcumin (or both) relative to the control (Table 1). Terpineol provided the best enhancing activity on curcumin flux, followed by carveol and nerolidol. Terpineol increased the flux 4.15-fold relative to the control. However, this was not significantly different from the other two enhancers ($P > 0.05$). Eugenol, L-menthol, and farnesol were the second group, only mildly enhancing the curcumin flux. Unlike other terpenes, β-myrcene did not increase curcumin flux relative to the control. β-myrcene is classified as an acyclic monoterpene, which

Table 1 Effect of pretreatment with enhancers and hydrogels on curcumin flux and amount of drug in the skin after topical application to rat skin in-vitro.

Formulation	Category	Flux ($\mu g\ cm^{-2}\ h^{-1}$) $\times 10^2$	Total permeated amount at 24 h ($\mu g\ cm^{-2}$)	Amount in skin at 24 h ($\mu g\ mg^{-1}$) $\times 10^2$
No pretreatment	—	6.46 ± 3.10	1.58 ± 0.44	3.02 ± 1.25
Ethanol–pH 7.4 buffer (25:75)	—	9.91 ± 1.75	2.53 ± 0.41	8.73 ± 2.33
β-Myrcene	Monoterpene	10.06 ± 2.69	2.51 ± 0.65	12.51 ± 2.65
Eugenol	Cyclic monoterpene	22.86 ± 4.71*	5.63 ± 1.12*	22.68 ± 10.37*
L-Menthol	Cyclic monoterpene	18.19 ± 5.10*	4.56 ± 2.84	26.88 ± 6.79*
1,8-Cineole	Cyclic monoterpene	21.30 ± 1.63*	5.20 ± 0.43*	22.58 ± 4.24*
Terpineol	Cyclic monoterpene	41.10 ± 7.80*	10.00 ± 2.05*	10.52 ± 2.47
Carveol	Cyclic monoterpene	38.14 ± 5.72*	9.28 ± 1.35*	9.01 ± 2.17
Farnesol	Sesquiterpene	21.82 ± 4.02*	5.38 ± 0.97*	11.71 ± 4.86
Nerolidol	Sesquiterpene	32.58 ± 7.08*	8.21 ± 0.47*	7.65 ± 2.01
Hesperetin	Flavonoid	11.21 ± 7.44	2.92 ± 1.78	8.49 ± 0.70
Phloretin	Flavonoid	4.91 ± 3.51	0.92 ± 4.90	7.15 ± 1.35
Ketocholestanol	Cholestanol	28.15 ± 4.92*	6.03 ± 0.84*	10.41 ± 1.22
3% CMC-Na	Hydrogel	6.83 ± 2.22	1.76 ± 0.56	2.59 ± 1.15
5% CMC-Na	Hydrogel	0.68 ± 0.12†	0.16 ± 0.03	1.36 ± 5.00
3% CMCN-H_4	Hydrogel	0†	0†	1.30 ± 0.62

The enhancer concentration in the hydrogel formulation was 5%. *$P < 0.05$ compared with the ethanol–pH 7.4 buffer (25:75) pretreatment group; †$P < 0.05$ compared with non-pretreated group. Each value represents the mean ± s.d. ($n = 4$).

differs from cyclic monoterpenes and sesquiterpenes tested in this study. It is of interest to note that Arellano et al (1996) found that acyclic terpenes are the best enhancers for diclofenac permeation across skin. Their result was contrary to that of our investigation, indicating that a universal effect of a series of terpenes to enhance transdermal drug delivery is not possible. This may be due to the various model drugs, experimental designs and skins utilized in different studies and laboratories.

For topical application, the drug skin content is considered an important parameter and in this study, curcumin skin deposition was determined at 24 h (Table 1). L-Menthol provided the highest curcumin skin content, which was not significantly different from that produced by eugenol and 1,8-cineole ($P > 0.05$). However, these three cyclic monoterpenes provided a skin deposition that was significantly higher than that provided by the other enhancers ($P < 0.05$). The reason for the highest enhancement being produced by L-menthol may be the improvement in the partitioning of the drug to the stratum corneum when combined with ethanol (Gao & Singh 1998; Sinha & Kaur 2000). Contrary to the result of curcumin flux at the highest level, the curcumin skin content after pretreatment with terpineol was comparable with that provided by the control ($P > 0.05$). This may indicate that the uptake of curcumin molecules by skin could be quickly released to the receptor of the Franz cell by treatment with terpineol. Another observation is that the enhancers that were most effective in promoting curcumin skin content (eugenol, 1,8-cineole and L-menthol) were completely different to those promoting curcumin flux (terpineol, carveol and nerolidol). The mechanisms of action of terpineol may also explain this phenomenon.

To date, most investigations of essential oils have focused on the monoterpene constituents. It has been shown that both mono- and sesquiterpene enhancers increase the percutaneous absorption of drugs (Arellano et al 1996; Moser et al 2001). The sesquiterpenes (farnesol and nerolidol) also showed different enhancing behaviour to that of cyclic monoterpenes. The sesquiterpenes could not increase skin uptake of curcumin but did enhance curcumin permeation across the skin (Table 1). This result was similar to that of an earlier study, which demonstrated that farnesol and nerolidol significantly increased skin permeation of 5-fluorouracil, but that the effect on the partition coefficient to the skin was not important (Cornwell & Barry 1994).

Phloretin and ketocholestanol, categorised as flavonoid and cholestanol, respectively, are two novel enhancers that can promote skin permeation of progesterone and lidocaine (lignocaine) (Valenta et al 2001a, b). It was suggested that incorporation of phloretin and ketocholestanol into the stratum corneum results in decreased lipid order. In our study, hesperetin, phloretin and ketocholestanol were selected to examine the enhancing effect on curcumin permeation. None of the flavonoids increased the skin deposition and flux of curcumin as shown in Table 1 ($P > 0.05$). On the other hand, ketocholestanol significantly enhanced curcumin flux by 2.84-fold ($P < 0.05$) without affecting the skin uptake of curcumin.

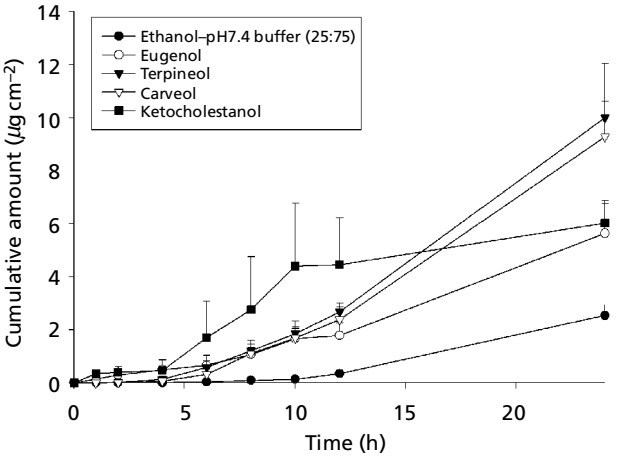

Figure 1 Cumulative amount of curcumin detected in the receptor compartment vs time after pretreatment of rat skin with enhancers. All data represent the means of four experiments ± s.d.

The lack of an enhancing effect by phloretin on curcumin permeation may have been due to insufficiency of the 1-h pretreatment time since in a previous study (Valenta et al 2001a) the skin samples had been treated for 12 h by phloretin before application of drug to induce successful enhancement of permeation. Another possible explanation is the pretreated vehicle used in this study (ethanol–citrate-phosphate buffer, pH 7.4 (25:75)). There are several reports that the activity of phloretin is higher at pH 5 than at pH 7 or pH 10 because the active form of phloretin is the un-ionized form (Verkman & Solomon 1980; Bechinger & Selig 1991; Valenta et al 2001a).

As shown in Table 1, various enhancers exhibit different behaviour in enhancing the flux or drug skin content (or both) of curcumin. Cyclic monoterpenes generally showed higher activity in promoting curcumin permeation than did the other enhancers. Hence, three cyclic monoterpenes—eugenol, terpineol and carveol—were selected to perform further studies because of their enhancing activity on curcumin permeation (Figure 1). The selection of ketocholestanol for further study was because curcumin permeation across skin pretreated with ketocholestanol was largely increased during the initial 12-h application, although the increase of permeated amount over 12–24 h was limited (Figure 1).

Effect of pretreatment duration on in-vitro percutaneous absorption of curcumin

Figure 2 summarizes the effects of the duration of pretreatment with four selected enhancers on the percutaneous absorption of curcumin. Different pretreatment durations produced comparable curcumin fluxes for the three cyclic monoterpenes ($P > 0.05$). This indicates that a 15-min pretreatment is sufficient to achieve effective percutaneous absorption of curcumin since a longer duration may restrict its permeation to the level of 15 min. A different response was observed for ketocholestanol since

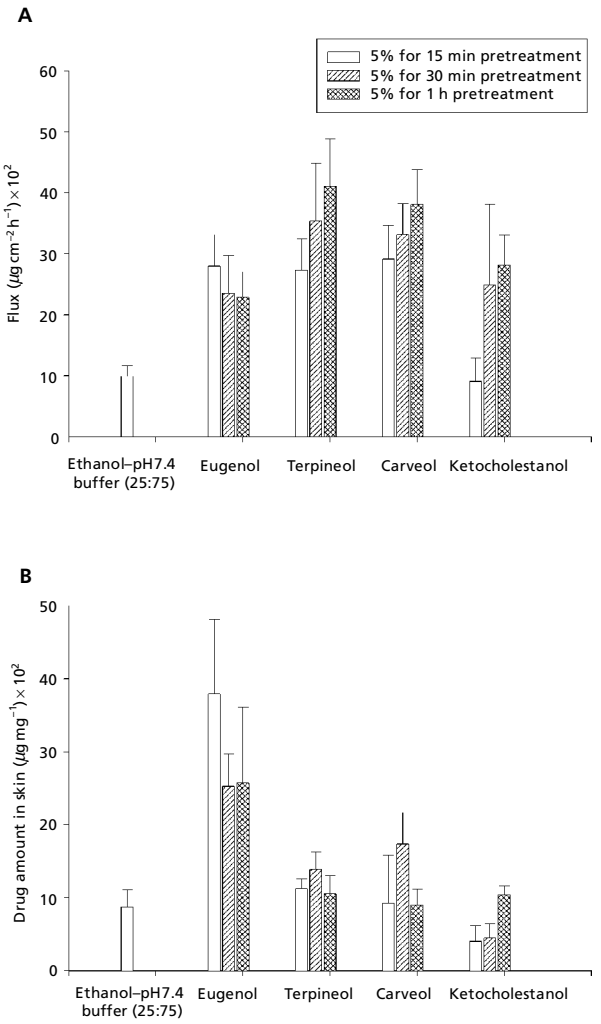

Figure 2 Flux (A) and drug amount within rat skin (B) of curcumin after pretreatment of skin with 5% enhancers for various periods of time. All data represent the means of four experiments ± s.d.

the 15-min duration did not increase the permeated amount of curcumin (Figure 2A). The minimum pretreatment duration for ketocholestanol to develop its enhancing effect was 30 min, with the curcumin fluxes for both 30 min and 1 h pretreatment being similar ($P > 0.05$) and also significantly higher than that of 15 min ($P < 0.05$).

The enhancing effect of various pretreatment durations on curcumin skin deposition was the same as that on curcumin flux for eugenol (Figure 2B). Varying the duration of pretreatment with terpineol had no effect on the skin deposition of curcumin ($P > 0.05$). The same was observed for ketocholestanol. Only carveol exhibited the enhancing activity on curcumin deposition in skin after 30 min pretreatment. The result demonstrates that the enhancing effect of carveol was not proportional to the pretreatment duration. A longer pretreatment duration may cause more significant morphological alterations in the skin structure. With carveol, the severe skin disruption produced by longer duration of pretreatment did not always accompany a higher enhancing effect on drug permeation. The same result was observed in studies with azone, which showed that greater skin damage results in a lower drug partitioning in skin as well as lower skin permeability (Hou & Flynn 1989; Xiong et al 1996).

Effect of pretreatment concentration on in-vitro percutaneous absorption of curcumin

The effect of enhancer concentration on curcumin permeation was investigated at the determined pretreatment duration of 15 min for cyclic monoterpenes since 15 min pretreatment was sufficient for them to enhance percutaneous absorption of curcumin; the pretreatment duration for ketocholestanol was set at 1 h. The flux of curcumin generally increased following the increase of enhancer concentration (Figure 3A). Eugenol, terpineol and ketocholestanol at 5% provided curcumin fluxes that were significantly higher than those at 1% and 3% ($P < 0.05$). Moreover, the 1% and 3% concentrations produced comparable curcumin flux. Carveol at 5% provided higher curcumin flux, which did not significantly differ from that at the 3% concentration ($P > 0.05$).

There was a linear relationship between the concentration of eugenol and its corresponding enhancement of the curcumin amount within skin (Figure 3B). Concentrations from 1% to 5% of the other enhancers had no significant enhancing activity on drug skin content. By comparing Figures 2 and 3, it was seen that modulation of the enhancer concentration was more sensitive in governing the percutaneous absorption of curcumin than was pretreatment duration.

In-vitro percutaneous absorption of curcumin from hydrogels

One particular problem common to many drugs designed for use on skin is poor retention at the site of application in the in-vivo or clinical situation (or both). This problem may be resolved by the incorporation of bioadhesive polymers within the system. Anionic carboxymethylcellulose (CMC) is a synthetic water-soluble cellulose largely used as a matrix for drug-delivery systems (Doelker 1987; Wang et al 2001). The permeation of curcumin from CMC with different counter ions of Na^+ and NH_4^+ was compared. As shown in Table 1, no significant difference ($P > 0.05$) was observed between the flux and skin reservoir of curcumin from pH 7.4 buffered solution and 3% CMC-Na hydrogel. This suggests that the cross-linkage structure formed by 3% CMC-Na after hydration does not interact with curcumin molecules. However, the hydrogel composed of 3% CMC-NH_4 completely retarded the curcumin flux without affecting the partitioning of curcumin into the skin. This may be due to the higher viscosity of hydrogels formed by CMC-NH_4 than CMC-Na (Wang et al 2001), resulting in a more rigid hydrogel structure and a decrease in drug release rate.

A high proportion of CMC-Na (5%) was also tested as a vehicle for curcumin and this retarded curcumin

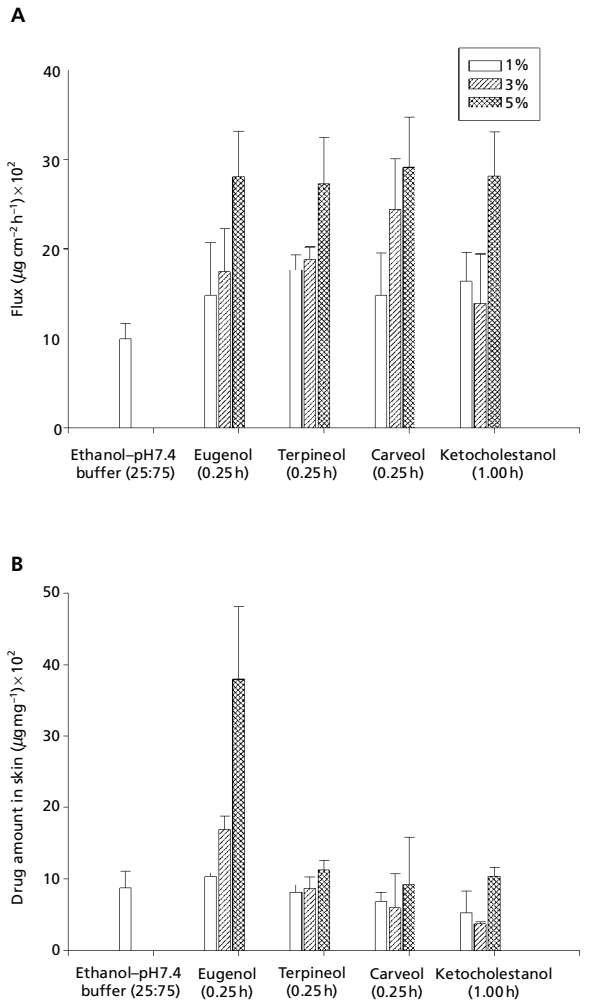

Figure 3 Flux (A) and drug amount within rat skin (B) of curcumin after pretreatment with enhancers at various concentrations. All data represent the means of four experiments ± s.d.

permeation (Table 1), possibly due to the increased difficulty of permeation from a more sticky cellulose matrix. The 3% CMC-Na hydrogel was selected for further in-vivo study because of its comparable flux and skin deposition to those of solution vehicle. CMC-Na also exhibits good bioadhesion to the skin, which may prolong the time of location at the site of application (Doelker 1987; Jones et al 1997).

In-vivo topical application of curcumin

The effect of enhancers (e.g., eugenol, terpineol, carveol and ketocholestanol) on the in-vivo topical application of curcumin is shown in Table 2. The skin reservoir of curcumin after topical application of curcumin hydrogels was significantly higher ($P < 0.05$) for all the enhancer-treated groups than for the control. However, the skin deposition at 2 h was not significantly different ($P > 0.05$) among the enhancer-treated groups. This result was not consistent

Table 2 Curcumin uptake within rat skin after topical application of hydrogels, with or without enhancer pretreatment, in-vivo

Formulation	Amount in skin at 2 h ($\mu g\ mg^{-1}$) $\times 10^2$	Amount in skin at 8 h ($\mu g\ mg^{-1}$) $\times 10^2$
No pretreatment	5.16 ± 1.16	3.27 ± 0.44
Eugenol	12.14 ± 2.29*	7.11 ± 3.05*
Terpineol	11.85 ± 0.90*	8.00 ± 1.96*
Carveol	10.10 ± 1.99*	4.92 ± 1.58
Ketocholestanol	11.99 ± 3.39*	6.30 ± 2.91

Each value represents the mean ± s.d. ($n = 6$). *$P < 0.05$ compared with the non-pretreated group.

with the data from in-vitro studies, which showed that eugenol had the highest enhancement of curcumin partitioning to the skin (Table 1). This may have been because the skin reservoir of curcumin was fully saturated, contributing to the retardation of the entrance of drug into the already saturated skin. Skin deposition after 8 h of application of curcumin hydrogels was lower than that after 2 h of application for all enhancers tested (Table 2). This may indicate that the rate of desorption was faster than the rate of skin partitioning after 2 h application of curcumin. The enhancement of the skin's drug capacity remained significant after 8 h of application for eugenol and terpineol as compared with the control ($P < 0.05$). On the other hand, the enhancing activity of carveol and ketocholestanol declined to the level of the non-pretreatment group after 8 h application (Table 2). As compared with the profiles of in-vitro permeation (Table 1), eugenol still showed good enhancement of the in-vivo topical application of curcumin. The enhancing activity of terpineol was comparable with that of eugenol in the in-vivo experiment, which differed from the result in the in-vitro permeation.

In-vivo TEWL evaluations

The efficacy of enhancers on curcumin permeation was fully evaluated as described above. Safety of enhancers is a prerequisite for fulfilling their promise of further use. In-vivo TEWL and skin histology evaluation was used to evaluate the safety of these enhancers. Measurement of TEWL can be an effective marker for evaluating the health and efficiency of the stratum corneum barrier in-vivo (Kalia et al 2001; Fang et al 2002). The determination of TEWL was made 2 and 8 h after applying 5% enhancer solutions. Baseline values of untreated sites were subtracted from the achieved TEWL measurement to give the actual changes in TEWL (ΔTEWL, Table 3). The ΔTEWL values significantly increased after a 2-h application of all enhancer solutions (except for ketocholestanol) to rat skin. Terpineol increased the ΔTEWL at 2 h by the greatest level, whereas the other cyclic monoterpenes produced a lower increase. This trend differed from that of the enhancement of curcumin permeation in-vitro and in-vivo, indicating that the degree of stratum

Table 3 ΔTEWL at 2 h and 8 h after topical application of enhancers to rat skin.

Formulation	ΔTEWL ($g\,m^{-2}h^{-1}$) at 2 h	ΔTEWL ($g\,m^{-2}h^{-1}$) at 8 h
No pretreatment	0.22 ± 0.86	1.13 ± 0.83
Eugenol	6.12 ± 2.04*	3.64 ± 2.18
Terpineol	16.93 ± 9.07*	8.67 ± 5.29*
Carveol	4.52 ± 1.70*	3.57 ± 2.04*
Ketocholestanol	0.57 ± 1.75	0.29 ± 1.38

Each value represents the mean ± s.d. ($n = 6$). *$P < 0.05$ compared with the non-pretreated group.

corneum barrier disruption (TEWL) was not necessarily correlated to the efficiency of the enhancement. The level of drug partitioning to enhancer-pretreated skin may be the predominant mechanism for the increased drug permeation in the pretreated experimental design. The ΔTEWL values of eugenol and carveol at 2 h were comparable and moderately higher ($P < 0.05$) than that of the control. Ketocholestanol demonstrated no significant increase ($P > 0.05$) in ΔTEWL at 2 h relative to the control (Table 3), indicating that the effect of 5% ketocholestanol may be sufficiently low to avoid any skin irritation.

Treatment with enhancers generally induced ascendant values of ΔTEWL initially, then the values gradually dropped to a lower level, as indicated by ΔTEWL at 8 h (Table 3). Terpineol still showed the highest ΔTEWL at 8 h of all enhancers tested. The ΔTEWL dropped towards the baseline value at 8 h as the skin recovered to its normal status following eugenol and carveol treatments. Although eugenol and carveol showed a similar profile for skin disruption evaluations, the enhancement of curcumin permeation by these two enhancers differed greatly. Eugenol showed higher in-vitro curcumin partitioning into the skin than did the other enhancers, whereas terpineol showed a limited increase. This suggests that the skin permeation of drugs can possibly be enhanced with limited changes in skin physiology and physicochemistry. A similar result was observed for ketocholestanol, which caused no skin irritation as determined by ΔTEWL (Table 3), but which significantly enhanced curcumin skin absorption in-vitro and in-vivo (Tables 1 and 2).

Histological examination by light microscopy

The skin irritation caused by enhancers after 2 h and 8 h exposure was histopathologically investigated. As compared with non-treated skin (Figure 4A), the stratum corneum layers were partially lost after 2 h of treatment with 5% eugenol (data not shown) and stratum corneum fragmentation was significantly extended after 8 h of treatment (Figure 4B, arrows). No other changes were observed in the histology of eugenol-treated skin. Ablation of stratum corneum may have contributed to the enhancing effect of eugenol on curcumin permeation. The higher ΔTEWL values as compared with the control also confirmed disruption of the stratum corneum after eugenol treatment. Moderate superficial inflammatory cell infiltration (hyperaemia) was found in skin treated with terpineol for 2 h (data not shown). Degeneration of epidermal cells was also observed. Some spongiotic vesicles in the epidermis were observed after 8 h treatment with terpineol, similar to the skin histology of contact dermatitis (Figure 4C, arrows). This disruption of the skin morphology by terpineol may account for the highest ΔTEWL among all enhancers tested.

Carveol also showed an effect on skin histology (Figure 4D). Approximately one-fourth of the carveol-treated area of skin had a scattered, loose stratum corneum after 2 h treatment (data not shown). Some microcrusts also formed. Acute inflammatory cell infiltration in the upper dermis, as well as partial, confluent necrosis of epidermal keratinocytes, was observed after 8 h of carveol treatment (Figure 4D, arrows). The formation of subepidermal cleft was also noted. There was no (or only negligible) physical damage to skin after 2 and 8 h treatment with ketocholestanol (Figure 4E), resulting in a ΔTEWL comparable with that of the control group. In general, morphological alterations of the skin structure increased in the order of non-treated group ≤ ketocholestanol < eugenol < terpiterpineol ≤ carveol.

Although terpineol and carveol showed similar levels in the changes of skin morphology, the ΔTEWL of terpineol was significantly higher (*t*-test, $P < 0.05$) than that of carveol (Table 3). This indicates that there were some discrepancies in the irritant evaluations of enhancers using different methods. Irritant responses of the skin are complicated because reactions appear very heterogeneous, particularly with respect to epidermal damage.

Conclusions

The percutaneous absorption of curcumin across rat skin treated with enhancers from solutions and hydrogels was examined in this study. The efficacy and safety of these enhancers were systemically evaluated using a series of in-vitro and in-vivo methods. The amount of curcumin that permeated across the skin and that deposited within the skin were determined. The enhancers significantly increased the absorption of curcumin by the skin. Cyclic monoterpenes generally increased curcumin permeation or skin deposition (or both) to a greater extent than did the other types of enhancer. The results also revealed that the enhancers have different enhancing behaviour and mechanisms of action on the skin. An enhancer concentration above 3% or 5% may be sufficient to enhance curcumin permeation. Modulation of enhancer concentration was more effective in controlling the skin absorption of curcumin than was modulating pretreatment duration. The 3% CMC-Na hydrogels produced a comparable curcumin flux and skin partitioning to the aqueous solution, indicating the lack of a barrier function of the cross-linkage formed by 3% CMC-Na polymers. In the irritancy evaluations, terpineol exhibited the highest toxicity according to its ΔTEWL and skin histology. Ketocholestanol

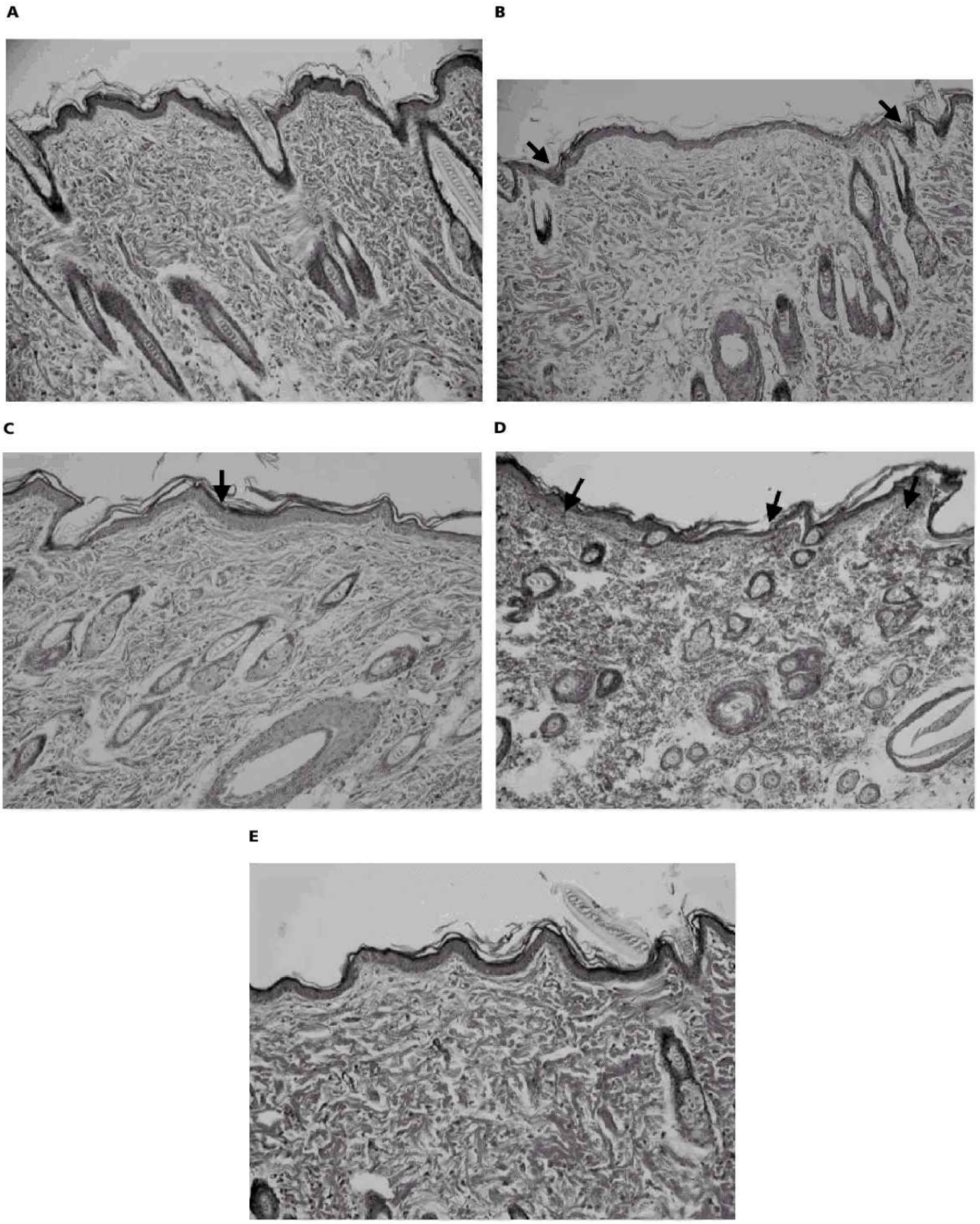

Figure 4 Microscopic photographs (×100) of Wistar rat skin after no pretreatment (A) and after 8 h treatment with 5% eugenol (B), 5% terpineol (C), 5% carveol (D) or 5% ketocholestanol (E).

may be an ideal enhancer because of its enhancing activity and non-irritant properties. Eugenol can also serve as a practical enhancer because it had higher enhancing activity than ketocholestanol with limited irritation to the skin.

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