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Research Article

A Novel Topical Targeting System of Caffeine Microemulsion for Inhibiting UVB-Induced Skin Tumor: Characterization, Optimization, and Evaluation

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Abstract. The purpose of the present study was to develop an optimal microemulsion (ME) formulation as topical nanocarrier of caffeine (CAF) to enhance CAF skin retention and subsequently improve its therapeutic effect on UVB-induced skin carcinogenesis. The pseudo-ternary phase diagram was developed composing of Labrafil M 1944 CS as oil phase, Cremophor EL as surfactant, tetraglycol as cosurfactant, and water. Four ME formulations at water content of 50, 60, 70, and 80% were prepared along the water dilution line of oil to surfactant ratio of 1:3 and characterized in terms of morphology, droplet size, and electric conductivity. A gel at the same drug loads (1%, *w*/*w*) was used as control. *Ex vivo* skin permeation studies were conducted for ME optimization. The optimized formulation (ME4) was composed of 5% (*w*/*w*) Labrafil M 1944 CS, 15% (*w*/*w*) Smix (2/1, Cremophor EL and tetraglycol), and 80% (*w*/*w*) aqueous phase. The skin location amount of CAF from ME4 was nearly 3-fold higher than control ($P<0.05$) with improved permeated amount through the skin. The skin targeting localization of hydrophilic substance from ME4 was further visualized through fluorescent-labeled ME by a confocal laser scanning microscope. In pharmacodynamics studies, CAF-loaded ME4 was superior in terms of increasing apoptotic sunburn cells ($P<0.05$) as compared with control. Overall results suggested that the ME4 might be a promising vehicle for the topical delivery of CAF.

KEY WORDS: apoptosis; caffeine; CLSM study; hydrophilic drug; microemulsion; percutaneous delivery.

INTRODUCTION

The incidence of the skin cancer has surged over the last several years mostly due to increased sun exposure (1). The ultraviolet B radiation (UVB), a minor component of sunlight, should bear the main responsibility for this phenomenon (2). Available evidences supported that UVB radiation exposure could mutagenize DNA directly, alter intracellular signal transduction, and induce the activation of transcription factors, leading to the development of cancer cells (1). The inhibition of UVB-induced skin tumor is still uncertain due to its complex pathological process. However, regulation of apoptosis has been proved to be one relevant factor in UV carcinogenesis. In fact, apoptosis inhibitor expressed in transgenic mice could accelerate the development of UV-induced squamous cell carcinomas (3).

Caffeine (1,3,7-trimethylxanthine; CAF) is an alkaloid, which is most commonly present in tea leaves, coffee, and cocoa beans (4). CAF has demonstrated an inhibitory effect on UV-induced skin carcinogenesis by functioning as a sunscreen and a stimulatory effect on apoptosis in the epidermis of UVB-treated mice (2,3,5–8). Lu *et al.* have proved that topical application of CAF selectively induced apoptosis in tumor without affecting neighboring nontumor site (9). However, CAF represents highly hydrophilic property, resulting in restricted cutaneous permeation and poor skin retention caused by lipophilic barrier of the stratum corneum (SC) (10). For the successful treatment of UV-induced skin cancer, it is recommended to deliver more CAF into the skin layers by overcoming SC barrier. Thus, the development of topical delivery systems that could efficiently deliver CAF into the skin is necessary.

New delivery systems such as microemulsion (ME), nanoparticle, and nanovesicular systems have been addressed to facilitate drug delivery into and through the skin (11). ME is defined as a system of water, oil, and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (12). In recent years, MEs have attracted much interest as topical drug vehicle because of their significant advantages, including thermodynamic stability, high solubilization capacities for both hydrophilic and hydrophobic molecules, easy formation, and small droplet size. In fact, ME had been widely used in pharmaceutical applications and could increase drug permeation into and through the skin (13). It is generally accepted that ME is a practical delivery platform for improving target specificity, therapeutic activity, and reducing toxicity of both hydrophilic and lipophilic substances (14–19).

To inhibit UVB-induced skin tumor, Shakeel and Ramadan developed nanoemulsion formulations for transdermal delivery of CAF. In this previous study, only percutaneous

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permeation in *in vitro* skin permeation studies was investigated (20). In our study, however, ME formulations were prepared along the water dilution line of oil to surfactant ratio of 1:3 and optimized based on *ex vivo* skin permeation studies. Then, the optimized ME formulation was evaluated via confocal laser scanning microscopy (CLSM) study and pharmacodynamic performance. So, the current study was carried out with two goals: (1) to develop a suitable ME for improved skin targeting effect of CAF and (2) to evaluate the therapeutic effect of the optimized ME depending on *in vivo* performance.

MATERIALS AND METHODS

Materials

CAF (MW 194.19, 99% purity) was obtained from Zhongan Pharmaceutical Co., Ltd (Tianjin, China). Labrafil M 1944 CS was obtained from Gattefossé (Saint-Priest, France). Cremophor EL with a purity of 98% was purchased from BASF SE (Ludwigshafen, Germany). Tetraglycol with a purity of 99% was provided by HEOWNS (Tianjin, China). Klucel® MF was purchased from Hercules, Inc. (Wilmington, DE, USA). All other reagents were of analytical grade.

Skin Preparation and Animals

Skin Preparation

Fresh full-thickness porcine skin was obtained from a pig born less than 2 months. After the pig was sacrificed, the abdominal skin was harvested and washed with normal saline. Then, the hair was trimmed off with electrical clippers carefully, and the skin samples were cut into smaller pieces. The thickness of the skin was measured as 0.95±0.12 mm. Before *ex vivo* skin permeation studies, subcutaneous fat and muscles needed to be removed from tested skin samples.

Animals

The animal protocols were in accordance with the National Institute of Health's guidelines regarding the principles of animal care. Female Kun-Ming (KM) mice weighing 20±2 g were used for pharmacodynamics studies, which were purchased from the Chinese Academy of Medical Sciences (Tianjin, China).

Solubility Studies

The solubility of CAF in various components (Labrafil M 1944 CS, Cremophor EL, tetraglycol, and phosphate buffered saline pH 7.4) was determined. An excess amount of CAF was dispersed into 2 ml solvents in different vials. The resulting mixture was shaken for 72 h in a water bath at 25±2.0°C. The equilibrated suspension was then centrifuged for 10 min at 12000 rpm. The supernatant was obtained followed by filtration through a 0.45-μm membrane filter. The drug concentration was determined using HPLC analysis after appropriate dilution. Each experiment was performed in triplicate.

Construction of Pseudo-Ternary Phase Diagram and Preparation of MEs

The pseudo-ternary phase diagram was constructed employing Labrafil M 1944 CS as the oil phase, water, Cremophor EL, and tetraglycol as the surfactant and cosurfactant (Fig. 1). The mixture of surfactant and cosurfactant (Smix) was prepared at a mass ratio of 2:1. First, the mixture of surfactant and cosurfactant (Smix) at a mass ratio of 2:1 was prepared. Next, Smix was mixed with oil at the weight ratios of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9 (*w*/*w*). Then, these mixtures were titrated with water under magnetic stirring at room temperature. The resulting mixtures were evaluated visually, and oil-in-water (O/W) ME phase was defined as the easily flowable and transparent region.

To prepare ME formulations, water dilution line of oil to surfactant ratio of 1:3 (DL1/3) was drawn from the water apex to the opposite oil side of the phase diagram (Fig. 1). Four ME formulations at water content of 50, 60, 70, and 80% were prepared. When preparing drug-loaded ME formulations, appropriate amount of CAF was first dissolved in water. Then, the obtained mixture was added into oil/Smix mixture by water titration method. The final ME formulations contained 1% (*w*/*w*) CAF. A gel at the same drug loads (1%, *w*/*w*) was prepared and used as control. To prepare the gel, CAF was dissolved in an aqueous phase. Then, Klucel® MF (0.75%, *w*/*w*) was added to the solution while stirring until the solution was gelled.

Characterization of CAF-Loaded ME Formulations

The MEs were characterized for various physicochemical attributes. The morphology of CAF-loaded ME4 (the optimized formulation) was studied using transmission electron microscopy (FEI-Tecnai G2 F20, NED). The average droplet

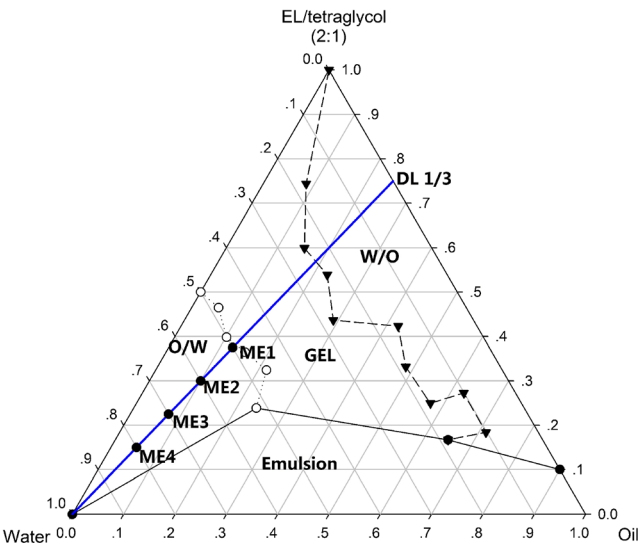

Fig. 1. Pseudo-ternary phase diagram composed of Labrafil M 1944 CS (oil phase), water, mixture of Cremophor EL (surfactant), and tetraglycol (cosurfactant) at a mass ratio of 2:1. The *diagram* indicated microemulsion regions (O/W and W/O ME), gel region (*GEL*), emulsion region, and the positions of selected O/W ME formulations. Line *DL 1/3* represented a water dilution line at oil to Smix mass ratio of 1:3 (*w*/*w*)

Table I. Solubility of Caffeine in Oil, Surfactant, Cosurfactant, and Buffer (Mean±SD, $n=3$)

Reagents	Solubility (mg/ml)
Labrafil M 1944 CS	3.82±0.047
Cremophor EL	6.60±0.089
Tetraglycol	17.57±0.11
Phosphate buffered saline (PBS; pH 7.4)	24.40±0.010

size and polydispersity index of drug-loaded MEs were measured by Malvern Mastersizer (Nano ZS, Malvern Instruments, UK). Electrical conductivity (EC) of MEs was determined at 25±2°C using a conductometer (AP-2, HM Instruments, China).

Ex Vivo Skin Permeation Studies

Franz diffusion cells providing a diffusion area of 1.77 cm^2 were used for *ex vivo* skin permeation studies. Pretreated porcine skin samples were mounted between donor and receptor compartments of the diffusion cells. Phosphate buffered saline (PBS; pH 7.4) was selected as the reception medium to imitate human body physiology environment and maintain sink condition due to its excellent solubilization capacities for CAF (The solubility of CAF in PBS was shown in Table I). The receptor medium was maintained at 37±0.5°C and was magnetically stirred at 500 rpm. ME formulations and control (0.1 g, containing 1 mg of caffeine) were applied onto the skin surface, respectively. At predetermined time intervals (2, 4, 6, 8, 10, 12, 24 h), 300 μl aliquot of receptor medium was withdrawn and replaced immediately with equal volume of fresh medium to maintain total volume unchanging. All the permeation samples were filtered through 0.45 μm membrane filters and then injected into an HPLC system for detection. For each formulation, six replicates were performed.

At the end of *ex vivo* permeation experiment (i.e., 24 h), the remaining formulation was removed from the skin surface by wiping the surface with a cotton ball soaked with PBS (pH 7.4). Then, the SC was removed employing the tape-stripping method (21). Repeated tape-striping (average 14 strips) was continued until SC layer disappeared. All the strips were digested in 10 ml PBS. Then, the residual skin sample was minced manually with scissors and sonicated with ultrasound for 20 min in 5 ml of methanol:PBS 1:1 mixture (*v*/*v*). The obtained CAF extract was filtered through 0.45 μm membrane filters and injected into an HPLC system for analysis.

Permeation Data Analysis

Average cumulative amount of drug permeated per unit skin surface area (μg/cm^2) was plotted as a function of time (t) for each formulation, and the cumulative permeation amount at 24 h, Q_{24}, was calculated. The extrapolation of the linear part to the time axis gave the lag time, and the slope yielded the pseudo steady-state permeation flux J_{ss} (μg/cm^2/h).

Confocal Laser Scanning Microscopy (CLSM) Studies

To validate the drug delivery behavior in skin layers, fluorescent dye rhodamine B was used as a substitute of hydrophilic drug to predict drug distribution using CLSM (22,23). ME4 and control formulations (0.1 g, respectively) labeled with 0.02% rhodamine B were applied to porcine skin which was mounted on Franz cell. At predetermined time intervals (2, 12, and 24 h), the dye remaining on the skin surface was removed carefully to avoid contamination by the fluorescent marker. Then, the skin samples were frozen and cut into vertical slices of 10 μm thickness. Rhodamine B distribution in different skin layers was detected using Confocal Laser Scanning Microscopy (20× magnifications, LEICA TCS SP5, Leica, Germany). The fluorescence was recovered in the red band exciting the specimens at 560 nm.

In Vivo Pharmacodynamics Studies

The study of stimulatory effect on UVB-induced apoptotic sunburn cells after topical administration of caffeine was carried out by focusing UVB radiation (UVB-313, BM Corporation, China) on the shaved dorsal skin surface of female KM mice (24). The total energy dose of UVB was 90 mJ/cm^2 which was detected by LS-123 research radiometer/photometer (Linshang corporation, Shenzhen, China). KM mice were divided into four experimental groups with each group consisting of three mice. The four experimental groups were treated as follows: group 1=normal skin group; group 2=UVB group; group 3=UVB+blank ME4 (UVB+vehicle); group 4=UVB+CAF-loaded ME4 (UVB+CAF). Immediately after UVB irritation, 120 μl of either blank ME4 and CAF-loaded ME4 (1%, *w*/*w*) was applied to the mice dorsal site (about 25 mm length and 5 mm width) in group 3 and group 4, respectively (24,25). After 6 h, the skin specimens were excised, embedded, and stained by hematoxylin and eosin for the observation of apoptotic cells in the epidermis. The percentage of apoptotic sunburn cells was calculated from the number of these cells per 100 cells counted from the entire 25 mm length of the epidermis for each skin section (25).

HPLC Analysis

HPLC analysis was performed using Waters 2695 HPLC system equipped with a Waters 2489 UV/visible detector (Waters, USA) as well as a SunFire C18 5 μm 4.6×250 mm column (Ireland). The elution mixture was 85:15 (*v*/*v*) water:acetonitrile with a flow rate of 1 ml/min at 35±2°C. The injection volume was 20 μl, and the retention time was 6.5 min at a wavelength of 273 nm. Prior to injection into HPLC system, the elution mixture was filtrated through 0.45 μm membrane filter (Millipore, Jinteng, China) and sonicated for 3 min for degassing.

For *ex vivo* permeation studies, the peak area (y) correlated linearly with CAF concentration (x, μg/ml) in the range of 0.5–50 μg/ml with a mean correlation coefficient of 0.9999. The regression equation of the calibration curve was $y=29157x-1761.6$ with lowest detection and quantitation limit at 5 and 15 ng/ml, respectively.

Statistical Data Analysis

The *ex vivo* skin permeation results were reported as mean±SD ($n=6$) while the *in vivo* studies data are presented

as mean±SD (n =3). Data was statistically analyzed employing Student's t test. Differences between formulations were considered significant when $P<0.05$.

RESULTS

Solubility Studies

Table I showed the solubility of CAF in the oil, surfactant, cosurfactant, and buffer. The solubility of CAF was the highest in PBS 7.4 (24.40±0.010 mg/ml) followed by tetraglycol (17.57±0.11 mg/ml). For Cremophor EL and Labrafil, the solubility of CAF was only 6.60±0.089 mg/ml and 3.82±0.047 mg/ml, respectively.

Preparation of MEs

The construction of a pseudo-ternary phase diagram was essential to determine the range of concentrations and ratios of components in the existence region of ME. Based on the optimization study of pseudo-ternary phase diagrams in our lab (unpublished data), the optimized pseudo-ternary phase diagram is shown in Fig. 1. As shown, the diagram was partitioned into three regions, representing water-in-oil (W/O) ME, gel, and O/W ME, respectively. The O/W ME region was near the water vertex and characterized by high water content (>50 wt.%) with low oil content (<20 wt.%). Most O/W ME could be diluted by water infinitely. In addition, we could observe that Smix content was relatively low in this region. In other words, O/W ME was easily formed at low Smix content.

When choosing ME vehicle, some basic guidelines, such as water content, Smix content, and droplet size, etc., need to follow. In our study, four O/W ME formulations (ME1–ME4) at 10% increment in water concentration were prepared along the water dilution line of oil to Smix ratio of 1:3 (DL1/3). CAF solution-based gel was used as control formulation. The compositions of ME were listed in Table II.

Microemulsion Characterization

The morphology of the CAF-loaded ME4 (our optimal formulation) was imaged by TEM. As shown in Fig. 2, the droplets were spherical and discrete with uniform distribution in the outer continuous aqueous phase. The average droplet sizes of drug-loaded MEs ranged from 27.45 to 103.80 nm (Table II). Besides, the relatively low polydispersity index of ME formulations (<0.3) demonstrated a high level of droplet homogeneity and stability. The EC values of the MEs increased with the water content, reaching 223.66 μS/cm in ME4 containing 80 wt.% of solubilized water while 75.33 μS/cm in ME containing 50 wt.% of solubilized water.

Ex Vivo Skin Permeation Studies

The permeation profiles of CAF through pig skin from various ME vehicles and control were presented in Fig. 3 while the percutaneous permeation parameters were listed in Table III. The permeated amount of CAF in the receptor medium steadily increased over time for all the formulations and the permeation profiles followed zero order release kinetics (Fig. 3a). Besides, the permeation rates of CAF at a steady state via ME formulations were much higher than those of CAF aqueous solution gel. The formulations could be arranged in a descending order in relation to the cumulative amount of CAF permeated through 24 h (Q_{24}) as follows: ME4 (374.18 μg/cm^2)>ME3 (331.17 μg/cm^2)>ME2 (273.22 μg/cm^2)>ME1 (245.69 μg/cm^2)>control (240.44 μg/cm^2) (Table III). No significant differences were observed between formulations.

The CAF distribution in the skin layers was measured after 24 h exposure of ME and control formulations. The skin retention is the sum of the amounts found in the SC, epidermis, and dermis. As Table III showed, ME4 resulted in the highest skin location amount (177.28±14.24 μg/g skin tissue) among all the ME formulations, which was nearly 3-fold more than the control (68.94±9.65 μg/g skin tissue) with statistically significant difference ($P<0.05$). Compared to control, the significant enhancement of CAF level from ME4 was not only in the SC but also in the epidermis and dermis ($P<0.05$) (Fig. 3b). Above all, it could be concluded that ME4 demonstrated improved skin permeability with enhanced skin retention compared with other ME and control formulations. Thus, ME4 was selected as the optimized formulation and subjected to further studies.

Confocal Laser Scanning Microscopy (CLSM) Studies

Visualizing distribution of rhodamine B within the skin layers was studied to investigate skin targeting effect of hydrophilic substances from ME4 formulation. Fluorescence images in Fig. 4 demonstrated fluorescence intensities in the tissues after 2, 12, and 24 h when rhodamine B was applied based on the control (Fig. 4a–c) and ME4 formulations (Fig. 4d–f). Cutaneous uptake and distribution of fluorescence gradually increased in skin layers with time in the case of both formulations. Nevertheless, ME4 clearly represented much higher and deeper penetration of the florescent markers

Table II. The Compositions and Characteristic Parameters of Different O/W Microemulsion Formulations

	(%) *w/w* of ingredients					
Number	Oil	Smix	Aqueous phase	Average size (nm)	PDI	EC (μs/cm)
ME1	12.5	37.5	50	103.80±13.15	0.27±0.056	75.33±1.15
ME2	10	30	60	79.38±9.56	0.23±0.025	100.00±2.65
ME3	7.5	22.5	70	38.02±8.15	0.21±0.013	134.33±0.58
ME4	5	15	80	27.45±4.35	0.15±0.011	223.66±1.15

Smix is the mixture of surfactant and cosurfactant at a mass ratio of 2:1. Aqueous phase: double-distilled water
PDI polydispersity index, *EC* electrical conductivity

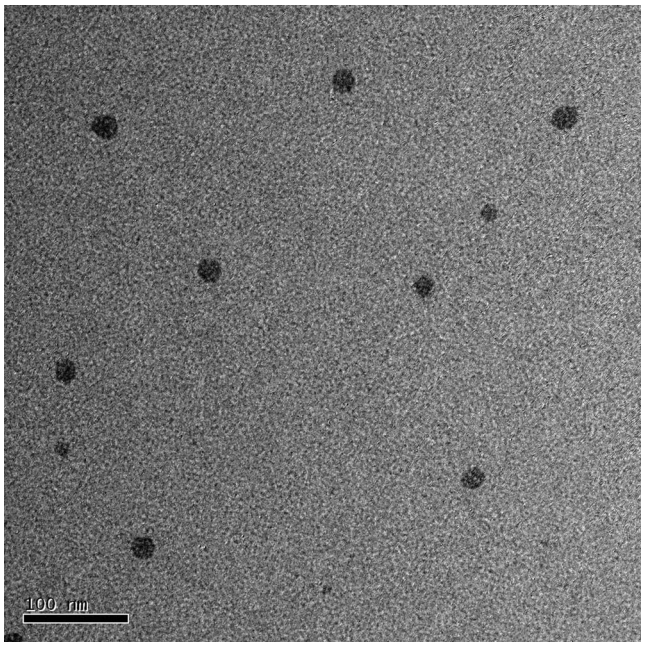

Fig. 2. Transmission electro micrograph (TEM) of ME droplets from CAF-loaded ME4 formulation. The *scale bar in the bottom of the picture* represents 100 nm in length

relatively to control. The fluorescence area of ME4-treated skin even overlaid the whole epidermis and dermis domain after 24 h. This phenomenon suggested that ME4 could promote the permeation of hydrophilic substances in a more pronounced manner compared to control. The obtained results were consistent with *ex vivo* skin retention studies, indicating an improved capacity of ME4 to deliver hydrophilic substances to the target site.

In Vivo Pharmacodynamics Studies

Pharmacodynamics studies were performed to investigate the effect of CAF-loaded ME4 on increasing UVB-induced apoptosis to prevent skin tumor. Compared to group 1 (normal skin), the groups treated with 90 mJ/cm^2 UVB (groups 2–4) resulted in the formation of apoptotic sunburn cells (Fig. 5). As for group 2 (UVB) and group 3 (UVB+vehicle), the mean percentage of apoptotic sunburn cells per 100 cells in the epidermis was 0.53 and 0.67%, respectively (Fig. 6). There was no statistically significant difference between these two experimental groups ($P>0.05$). However, the addition of caffeine after UVB irritation (group 4, UVB+CAF) significantly increased the number of such cells compared to group 2 and group 3. The mean percentage of apoptotic cells in group 4 was 1.53% which was almost 3 times and 2.5 times higher relative to group 2 and group 3, respectively ($P<0.05$). Taking causal relationship between apoptotic sunburn cell and skin cancer inhibition into account, the results of this study confirmed us that the optimal ME was suitable for topical administration of CAF and may partially diminish skin cancer.

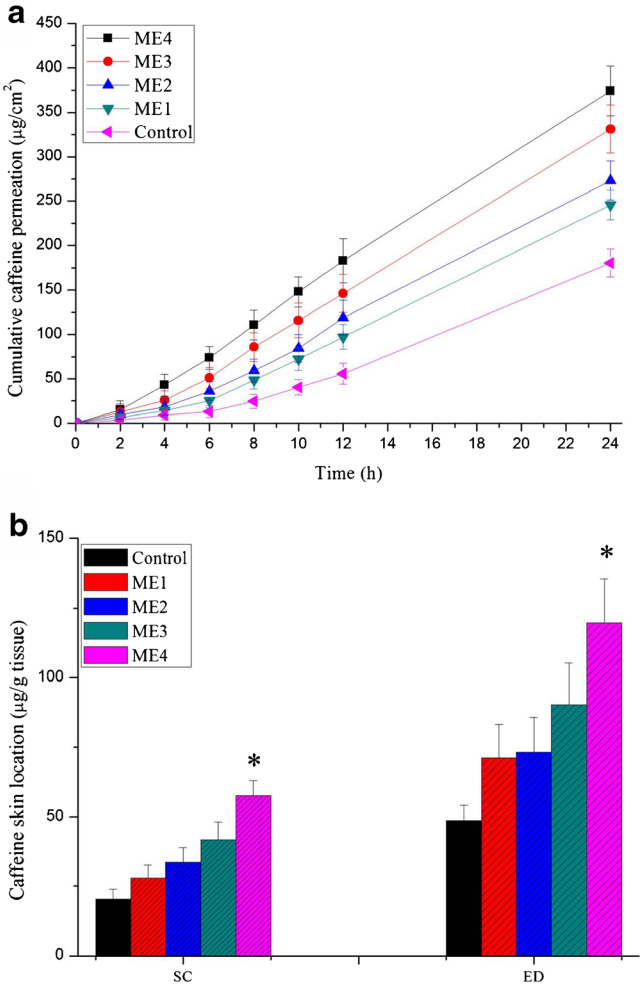

Fig. 3. **a** *Ex vivo* cumulative amount of CAF permeated through porcine skin *vs.* time curves of microemulsion and control formulations. **b** Cutaneous retention of CAF from MEs and control after the *ex vivo* permeation study (*SC* stratum corneum, *ED* viable epidermis and dermis). *Each symbol* and *bar* represented the mean±SD ($n=6$). *$P<0.05$, when compared to control

DISCUSSION

The present study describes the potential application of O/W ME as the topical carrier system for CAF delivery. Four ME formulations at water content of 50, 60, 70, and 80% were prepared along the water dilution line of oil to surfactant ratio of 1:3 and characterized for morphology, droplet size, and electrical conductivity (EC) values. The dimension of nanoparticle was considered to be the most important parameter because chemical penetration into skin could occur through pilosebaceous pores (10–70 μm), sweat gland pores (60–80 μm), and most commonly through the lipidic matrix that fills a gap (about 75 nm) between the SC dead corneocytes (26). Besides, particle of small size could settle down to close contact with the skin, leading to a considerable increase in surface area and improving its percutaneous absorption (27).

For the EC measurement, the values increased with water content in ME formulations. In O/W ME type, the conductive entity is the continuous aqueous phase. As the aqueous content increases, the increased aqueous phase resulted in the

Table III. *Ex Vivo* Skin Permeation Parameters of Different O/W Microemulsion Formulations and Control Formulation (Mean±SD, $n=6$)

Number	J_{ss} (μg/cm^2/h)	Q_{24} (μg/cm^2)	Skin retention (μg/g skin tissue)
Control	10.14±1.87	240.44±15.38	68.94±9.65
ME1	12.39±1.19	245.69±16.05	99.06±10.86
ME2	13.28±1.43	273.22±22.04	106.75±12.10
ME3	15.40±3.27	331.17±27.11	132.09±12.67
ME4	16.08±3.52	374.18±27.70	177.28±14.24*

*$P<0.05$

enhancement of EC. In fact, this phenomenon was in agreement with a previous report (28). This previous study showed that the EC values of O/W MEs increased linearly as aqueous content incorporated in formulations further increased. In addition, this previous study also demonstrated the low EC value in many cases of ME system of nonionic surfactant, which may explain the low EC values in our study.

In *ex vivo* skin permeation studies, it should be noted that the frozen/thawed pig skin model may be more permeable to both ME and control formulations (8). However, ME formulations generated faster permeation of CAF than control. This phenomenon may be related to physiochemical properties of MEs. Apart from the contribution of ME ingredients which acted as permeation enhancers in drug percutaneous permeation (29), the low interfacial tension of amphiphilic layer not only improved the contact to the skin but also facilitated the transport of drug from vehicle to the SC with the aid of the fluctuating oil-water interface (30). Since water content is not directly related with CAF percutaneous delivery, the difference between ME formulations in drug penetration may be ascribed to the influence of the apparent size and thermodynamic activity of MEs. Both of small droplet size and increased thermodynamic activity are significant driving forces for the drug passive penetration through the skin (29, 31). Therefore, ME4 which had relatively smaller mean droplet size and lower amount of surfactant mixture (representing increased thermodynamic activity) resulted in much higher skin permeability compared with other ME formulations.

In addition to O/W MEs, water-in-oil (W/O) nanoemulsions, containing Lauroglycol-90, Transcutol-HP, IPA, and water, were investigated for topical application of CAF (20). In this previous study, the optimal nanoemulsion

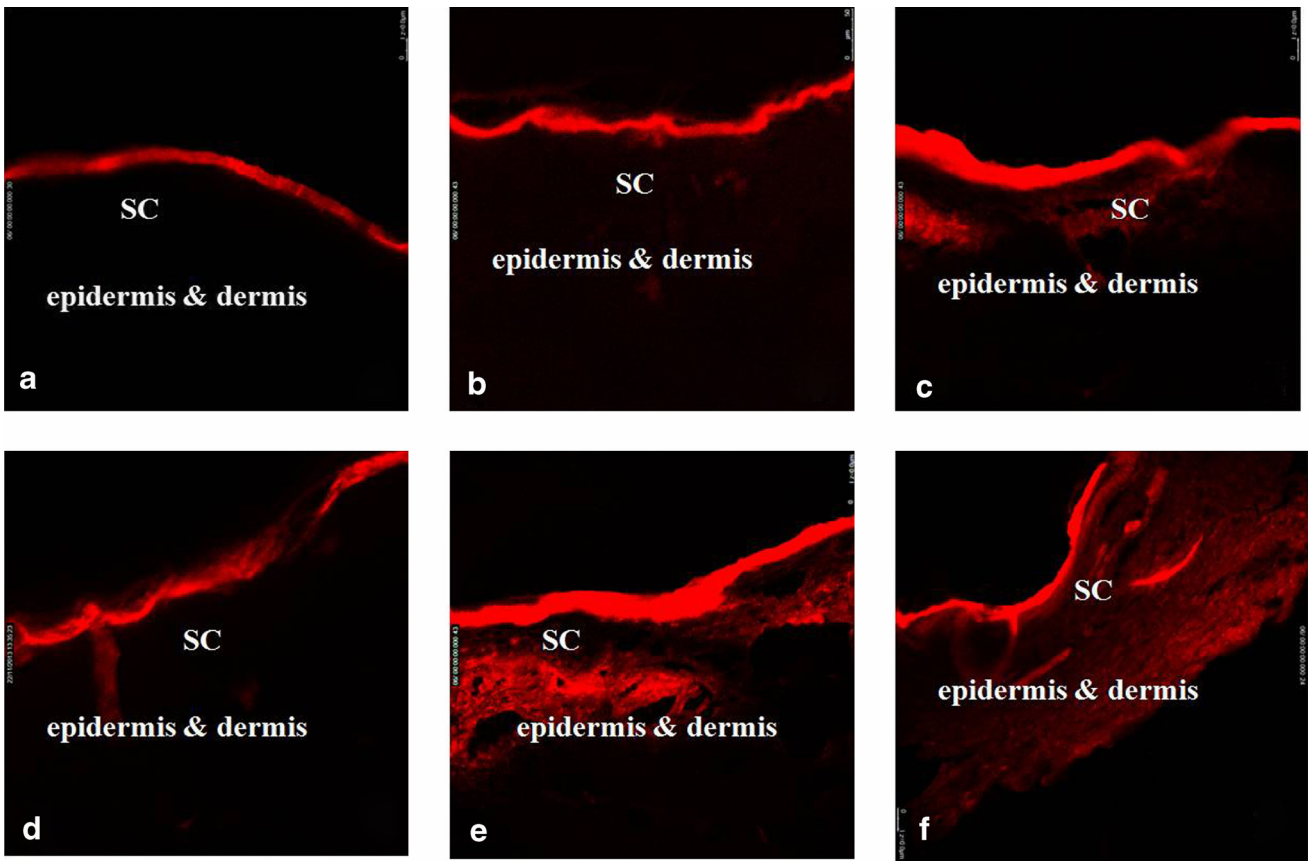

Fig. 4. CLSM images represented fluorescence intensities of hydrophilic fluorescent dye rhodamine B in the stratum corneum (*SC*), epidermis, and dermis determined at each predetermined time intervals after application of control: 2 h (**a**), 12 h (**b**), and 24 h (**c**) and optimal ME4 formulation: 2 h (**d**), 12 h (**e**), and 24 h (**f**)

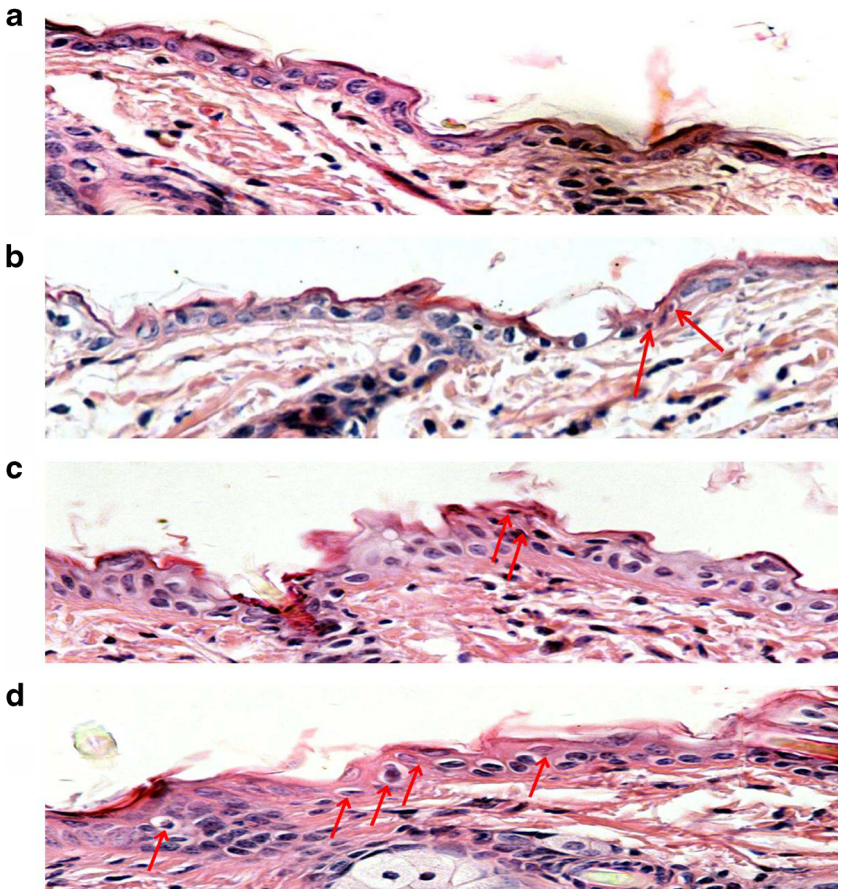

Fig. 5. Microscopic images of **a** the normal skin epidermis cells, apoptotic sunburn cells in epidermis of skin treated with **b** UVB, **c** UVB+Vehicle, and **d** UVB+CAF. Apoptotic cells which were indicated by *red arrows* were observed morphologically in H&E stained skin section

(C12 formulation) presented relatively smaller droplet size (around 20 nm) similar to ME4 formulation (around 27 nm). Both C12 and ME4 formulations resulted in significantly higher skin permeability compared to CAF aqueous solution. The transdermal flux of C12 formulation was 147.55±8.21 μg/cm^2/h when 10 mg CAF was applied on rat skin, while the flux of ME4 formulation was 16.08±3.52 μg/cm^2/h when 1 mg CAF was applied on pig skin. However, no information was provided about skin location amount as well as *in vivo* animal model for W/O nanoemulsions.

In vitro skin permeation studies could provide valuable information for drug transport into and through the skin. Therefore, it is essential to choose predictive *in vitro* penetration models. Excised human skin is properly regarded as the "gold standard" for *in vitro* penetration experiments related to human dermal risk assessment (32). However, suitable human skin samples are not readily accessible to most researchers. As a result, excised animal skin models from a pig, mice, and rat are frequently used as surrogates. Among them, porcine skin is the most relevant animal model for human skin not only due to its physiological, biochemical, and histological similarities to human skin but also because of its less variability than other skin models (21, 33). The skin models from mice and rat exhibit an extremely high density of hair follicles which may affect percutaneous absorption of molecules. In spite of disadvantages of rats and mice in *ex vivo* skin permeation studies, *in vivo* studies are still performed on these species. We realized the potential limitations caused by different animal models used for *ex vivo* (porcine skin) and *in vivo* (mice).

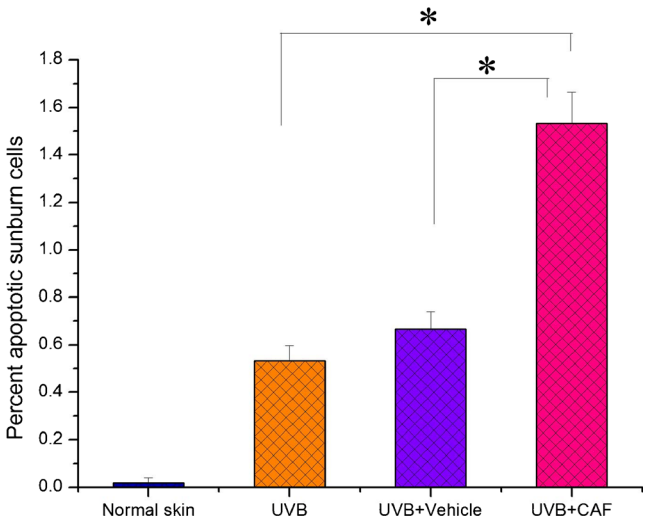

Fig. 6. Results of apoptotic sunburn cells quantitation in epidermis of normal skin, skin treated with UVB, skin treated with UVB+Vehicle and skin treated with UVB+CAF. Values are mean±SD (n=3). *P<0.05

However, the higher skin retention of ME4 (unpublished data) and its enhanced stimulatory effect on increasing apoptotic sunburn cells in *in vivo* studies were consistent with the improved skin targeting effect of ME4 in *ex vivo* studies, demonstrating that these two skin models might have good correlations for the penetration of CAF to a certain degree.

For most topical pharmaceuticals and cosmetics, penetration through the skin barrier is essential for developing their effects. However, hydrophilic molecules, accounting a large portion of pharmaceuticals and cosmetics, could not penetrate through the skin easily (22). For hydrophilic drugs, the intracellular pathway contributed most of the total skin permeability (19). MEs have been proved as one of the topical delivery systems which could promote drug permeation by possibly opening both intra- and intercellular pathways (22). In this study, an optimal O/W ME was developed for percutaneous study of CAF with highly hydrophilic property. CAF from the optimal ME formulation was proved to be concentrated in skin layers, leading to the improved therapeutic effect on skin tumor compared to control. The results indicated the potential of O/W ME as a vehicle of hydrophilic drug for cutaneous therapeutics.

CONCLUSION

Owing to high hydrophilic property, CAF was hard to permeate across the SC and retain in the skin. In this study, we developed an ME system comprising of Labrafil M 1944 CS as the oil phase and Cremophor EL and tetraglycol as the surfactant and cosurfactant, respectively, at the Smix ratio of 2:1. Several ME formulations at water content of 50, 60, 70, and 80% were prepared along the water dilution line of oil to surfactant ratio of 1:3 and subjected to *ex vivo* skin permeation studies for optimization. *Ex vivo* studies demonstrated that ME4 was most efficient in promoting CAF penetration into and through the skin compared to control. Thus, ME4 was selected as the optimized formulation. Then, skin targeting effect of hydrophilic substances from ME4 was further proved by CLSM studies. Moreover, the pharmacodynamics data suggested that the topical application of CAF-loaded ME4 resulted in a significantly increase in UVB-induced apoptotic sunburn cells. In conclusion, the optimized ME4 formulation with skin targeting effect could be favorably utilized for inhibiting UVB-induced skin tumor and be a promising topical vehicle of hydrophilic drugs for skin diseases.

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