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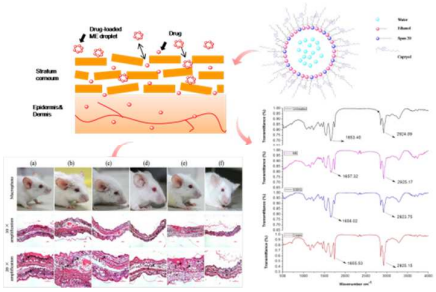

Topical administration of the optimal microemulsion could effectively enhance skin location amount of azelaic acid without causing skin irritation.

Improved percutaneous delivery of azelaic acid employing microemulsion as nanocarrier: Formulation optimization, *in vitro* and *in vivo* evaluation

Huixian Ma[a], Meng Yu[a], Fengping Tan[a**], Nan Li[a*]

[a]Tianjin Key Laboratory of Drug Delivery and High-Efficiency, School of Pharmaceutical Science and Technology, Tianjin University, 300072 Tianjin, PR China

[*]Corresponding author at: School of Pharmaceutical Science and Technology, Tianjin University, 300072, Tianjin, PR China.

E-mail address: linan19850115@163.com

Tel.: +86-022-27404986

** Co-corresponding author at: School of Pharmaceutical Science and Technology, Tianjin University, 300072, Tianjin, PR China.

E-mail address: tanfengping@163.com

Tel.: +86-022-27405160

Abstract: The present study aimed to develop and optimize a microemulsion (ME) nanocarrier system as topical vehicle of azelaic acid (AZA) to improve its skin location and therapeutic efficacy. D-optimal mixture experimental design was utilized to optimize ME for realizing maximum skin retention and appropriate droplet size. Three formulation variables: Smix X1 (a mixture of Span 20/Ethanol, 1:9, w/w), water X2 and Oil X3 (Capryol 90) were included in the design; while the three responses contained skin retention (Y1), AZA amount in collection medium after 24 h (Y2) and mean particle size (Y3). The values of formulation components (X1, X2 and X3) were 50.3%, 13.5% and 36.2%, respectively. *In vitro* studies, the optimal ME revealed much higher release rate, enhanced skin targeting and penetration effect of AZA relatively to control formulations (ethanol solution based gel and commercial cream). Attenuated total reflectance fourier-transform infrared spectroscopy study further confirmed us that vehicles could transport the active agents across stratum corneum (SC) layer by changing the amount and arrangement of lipid within SC. In addition, skin irritation test and pharmacodynamics studies were conducted, and the results suggested that the optimal ME exhibited prominent therapeutic effect over control formulations without any irritant response.

Keywords:

Azelaic acid; microemulsion; formulation optimization; skin targeting effect; therapeutic efficacy.

1. Introduction

Rosacea is a common chronic inflammatory dermatosis characterized by transient or persistent central facial erythema, visible blood vessels, and often papules and pustules.[1] The cause of rosacea is still unknown, however, available evidence supports that genetic and environmental factors (such as sun exposure, drinking alcohol, and cosmetics) should be responsible for the etiology of this skin disorder.[2] Rosacea affects mostly facial skin which leads to the much trouble on the social contact of suffers in a prominent manner.[3] Moreover, the current treatment of rosacea has been claimed to be empiric and imperfect.

Azelaic acid (1,7-heptanedicarboxylic acid, AZA) is a saturated, straight-chained C9-dicarboxylic acid that has been reported to be the active pharmaceutical ingredient in a number of prescription drugs for the treatment of rosacea.[4] AZA, however, with its commercial formulations of 15% gel (FINACEA®) and 20% cream (Skinoren®), restrains its penetration across the stratum corneum (SC) due to poor bioavailability mainly caused by low drug dissolved fraction and poor skin permeability.[2,5] Theoretically, suitable percutaneous permeation is an essential factor for pharmaceutical agents to achieve satisfactory therapeutic effect. Topical delivery systems aiming to promote AZA cutaneous penetration are necessary to maximize its biological efficacy. Meanwhile, considering the local nature of skin disorders, it is advisable to reside drug at the site of application for localized delivery.

Effective penetration of the active agents through the SC is a major challenge in topical drug delivery.[6] On the matter a number of research works have been done to

increase skin penetration through the SC, such as chemical modification,[7] penetration enhancer or retardant,[8] micro-needles,[9] and microwaves.[10] Recently, nanoscale vehicles have attracted significant attention as delivery strategies for active molecules, e.g. liposomes,[11] solid lipid nanoparticles,[12] and microemulsion.[13,14,15,16]

Microemulsion (ME) has been proved to have a significant potential to increase the penetration of lipophilic, hydrophilic, and amphiphilic substances into and through the skin compared to conventional vehicles.[17,18] MEs are optically isotropic and thermodynamically stable nanosized structure mixtures of aqueous phase, oil phase and amphiphile(s).[19,20] Several mechanisms have been proposed to explain the advantages of ME superior to conventional vehicles. First, the ingredients of ME could interfere the diffusional barrier of the SC and improve cutaneous permeation of drug by acting as permeation enhancers.[13] Second, the increased thermodynamic activity of drugs incorporated in ME formulations is a significant driving force for drug release and skin penetration.[21] Third, small droplet size could settle down to close contact with the skin which leads to a considerable increase of surface area and hence improves absorption.[22] Also, continuously and spontaneously fluctuating interface of ME enables high drug mobility and subsequently enhances drug diffusion process. [23]

The aim of this work was to optimize a ME nanocarrier system for AZA, which provided skin targeting effect and maximum dermal therapeutic effect. ME formulations were developed by constructing pseudo-ternary phase diagrams and optimized by D-optimal design based on maximum drug amount in skin layers,

appropriate skin penetration and small particle size. The optimized formulation was characterized by droplet size, size distribution and pH value. Attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) study was carried out to elucidate the interaction mechanism between ME and skin. Besides, the optimized ME formulations, ethanolic solution based gel and commercial cream were evaluated for *in vitro* skin permeation studies, skin sensitivity test, and pharmacodynamics study for comparison purpose.

2. Materials and Methods

2.1. Materials

Azelaic acid (MW 188, 99% purity) was purchased from Huabei reagent Co., Ltd (Tianjin, China). Capryol® 90 with a purity of 98% was a kind gift from Gattefossé (Saint-Priest, France). Ethanol (99% purity) and Span 20 (with a purity of 98%) were obtained from Jiangtian pharmaceutical reagent Co., Ltd (Tianjin, China). Klucel® MF was purchased from Hercules, Inc. (Wilmington, DE, USA). 20% AZA commercial cream (Skinoren®) was obtained from Bayer Co., Ltd (Taiwan). All other reagents were of analytical grade.

2.2. Skin membranes and animals

The abdominal porcine skin was obtained from pig less than one month old. After removing the hair and the subcutaneous tissue, the skin was washed with normal saline, divided into small pieces and stored at - 20 ^{o}C until use. Wistar rats (about 200 ± 20 g) and Male Kun-Ming mice (weighing 20 ± 2 g) were purchased from Chinese Academy of Medical Sciences (Tianjin, China) and used for skin irritation

test and *in vivo* therapeutic effects of anti-rosacea, respectively. All work performed with animals was in accordance with and approved by the Institutional Animal Care and Use of Tianjin University.

2.3. Construction of pseudo-ternary phase diagram and preparation of formulations

The pseudo-ternary phase diagram was constructed based on the oil phase (Capryol 90), surfactant (Span 20), cosurfactant (ethanol) and water. The mass ratio of surfactant to cosurfactant (Smix) was fixed at 1:9. Then, the oil phase was mixed with Smix at w/w ratios ranging from 1:9 to 9:1. Finally, 1 g of oil/Smix mixture in appropriate ratio was titrated with water drop by drop under magnetic stirring at ambient temperature. The resultant mixtures were examined according to their visual appearance. Usually, the system which was a transparent and low viscous solution was defined as ME region. In contrast, the turbid sample was identified as conventional emulsion. The boundary point between ME region and emulsion region was determined and corresponding component ratio was recorded to plot the pseudo-ternary phase diagram.

When preparing drug-loaded ME formulations, 10% (w/w) AZA was dissolved in the oil/Smix mixture. Then, appropriate amount of water was added to prepare ME formulations under magnetic stirring.

An ethanolic solution based gel (ESBG) containing the same AZA concentration (10%, w/w) was prepared and utilized as control formulation. Klucel® MF was added to bidistilled water under stirring until complete incubation. The obtained gel was

diluted with equal amount of ethanol solution followed by the addition of AZA, resulting in a final AZA concentration of 10% (w/w).

2.4. Formulation optimization of AZA-loaded MEs

D-optimal mixture experimental study was designed based on a three component system: Smix X1 (a mixture of Span 20/ethanol, 1:9, w/w)，aqueous phase X2 (water) and the oil phase X3 (Capryol 90). The total concentration of the three components summed to 100%. Based on the obtained ME region in the phase diagram, the range of each component was selected as follows: X1 (40-80%), X2 (0-30%), and X3 (20-60%) (This district was shown in Fig. 1). The skin retention amount of AZA at 24 h (Y1), AZA amount in collection medium after 24 h (Y2) and mean particle size (Y3) were used as the responses (dependent variables). The responses of all model formulations were treated with Design-Expert software (version 7; Stat-Ease, Inc, Minneapolis, MN). Suitable models for D-optimal design containing linear, quadratic, special cubic and cubic models. The best fitting mathematical model was selected by comparing statistical parameters including the standard deviation (SD), the multiple correlation coefficient (R^2), adjusted multiple correlation coefficient (adjusted R^2) and the predicated residual sum of square (PRESS), proved by Design-Expert software. Since the PRESS value indicated how well the model fits the data, the value of the selected model should be smallest among these models.[24] The base design consisted of 16 runs (Table 1).

2.5. Evaluation of prepared formulations

Malvern Mastersizer (Nano ZS90, Malvern Instruments, Malvern, UK) was

158 used for determining of droplet size for D-optimal design. The optimized ME
159 formulation was characterized for droplet size, size distribution profile and zeta
160 potential. The pH values of the optimized ME formulation, ESBG and marketed
161 cream were detected using a digital pH-meter (PHS-3C, Shenbang Instrument
162 Corporation, Shanghai, China) at 25 ± 2 ℃.

163 **2.6. Stability assay**

164 The optimal AZA loaded ME was preserved in glass vial with a sealing
165 cap and was kept under long-term condition at 25 ± 2 ℃/60 ± 5% RH. The
166 physical stability of the ME formulation was assessed for appearance,
167 droplet size and polydispersity index (PDI) at predetermined time
168 interval of 0, 1, 2, and 3 months. For chemical stability, concentration
169 of AZA in the ME was determined by HPLC analysis at each predetermined
170 time interval.

171 **2.7. *In vitro* skin permeation studies**

172 Porcine skin samples were mounted on Franz Diffusion Cells with the SC side
173 facing the donor chamber (diffusion area = 1.77 cm^2). The receptor medium was 17.6
174 mL of normal saline under constant magnetic stirring at 500 rpm. After equilibration
175 of skin samples with normal saline for 1 h at 37 ± 0.5 ^oC, finite doses (25 mg cream
176 and 50 mg of the optimal ME formulation and ESBG, which correspond to 5 mg AZA,
177 respectively) were applied to skin surface (n = 6). At predetermined time intervals (4,
178 6, 8, 10, 12 and 24 h), approximately 0.5 mL of the receptor medium was withdrawn
179 for HPLC analysis and equal volume of fresh normal saline was compensated. The

remaining formulation on the skin surface was wiped with cotton ball soaked with methanol/water (40/60, v/v) after incubation for 24 h. The tape-stripping method was employed to remove SC layer.[22] The skin was stripped with 15 pieces of adhesive tape and all the tapes except for the first one were digested with methanol/water (40/60, v/v), then filtered for analysis. After removal of SC, the remaining skin samples were minced, vortexed with 5 mL of methanol and centrifuged to extract residual AZA in the epidermis and dermis. The supernatants were collected and filtered for analysis. The permeation rate of AZA (flux, μg/cm^2 h) through porcine skin was calculated from the slope of linear portion of the cumulative amount permeated through the skins per unit area versus time plot.

2.8. HPLC method

A Water e2695 series HPLC with UV 2489 detector (Waters, USA) was used for AZA method validation. The optimized chromatographic conditions were present as follows: 250 mm × 4.6 mm stainless steel C18 column (I.D., 5 μm, Thermo, USA); column temperature at 35 ^{o}C; 20 μl injection volume; detection wavelength set at 215 nm; mobile phase consisted of acetonitrile and phosphate buffer (pH 3.0, 50 mM) at 25:75 (v/v); flow rate of 1.0 ml/min.

For in vitro studies, the peak area (y) correlated linearly with AZA concentration (x, μg/ml) in the range of 5.0 - 100.0 μg/ml with a mean correlation coefficient of 0.9999. The regression equation of the calibration curve was y = 586.54x - 317.69 with recovery of 99.36%.

2.9. Attenuated total reflectance fourier transform infrared

spectroscopy (ATR-FTIR) study

To prepare SC sample for ATR-FTIR study, the SC was firstly separated by placing the skin sample in 0.5% trypsin (type I, Sigma Aldrich) in phosphate-buffered saline pH 7.4 for 4 h.[25] The obtained SC sheet was cleaned with deionized water and dried in desicator for 12 h. Then, the SC samples were incubated with different formulations by means of diffusion cells for 24 h as section 2.6 described. All experiments were performed in triplicate. The samples were mixed with KBr to make pellets and were measured on an FTIR spectrometer (Bruker EQUINOX, Germany) with a spectral resolution of 4 cm^{-1}. The absorbance was measured in the region from 400 cm^{-1} to 4000 cm^{-1} at 37 ^{o}C.

2.10. Skin irritation test

To determine the skin compliance of the developed formulations, skin irritation test was carried out based on histopathological examination. The hair on the dorsal side (2 cm × 3 cm) of Wistar rats was carefully removed without damaging the skin.[26] The control group was treated with normal saline while other groups were treated with the optimized ME formulation, ESBG and commercial cream (containing 5 mg of AZA), respectively, three times a day for three days consecutively (n = 3). These formulations were uniformly spread within the area of 1.77 cm^2. After 3 days, the animals were observed for any signs of itching or change in skin such as erythema, papule, and dryness. Then, the rats were sacrificed using carbon dioxide gas. The test skin was removed, fixed and stored in

formaldehyde (10%, v/v). Tissue specimens were processed routinely and embedded in paraffin wax. Parafin blocks were cut serially at 10 μm. Sections were stained with hematoxylin and eosin (H&E) and examined by light microscope (Olympus BX-51, Japan).

2.11. Pharmacodynamics studies

Croton oil inflammation model was performed to induce rosacea model.[27,28] Briefly, 10 μL of croton oil in acetone (5% v/v) was painted on the inner surface of the right ears in group a-e, while the left ears were used as control. Fifteen minutes later, 60 μL of blank ME, AZA-loaded ESBG (10%, w/w) and AZA-loaded ME (10%, w/w) were topically applied to group b, c, d, respectively. Commercial cream (20%, 25 mg) was administrated to group e. At 4, 8 and 24 h, ear thickness was measured near the top of the ear distal to the cartilaginous ridges. Change in ear thickness from control was taken as an edema index. The ear tissue samples were collected after 24 h and submitted to histopathological analysis.

2.12. Data analysis

At least three to six replicates of each experiment were used. All results were reported as mean ± SD. Paired two-tailed Student's t-test was employed to calculate the statistical significance. The level of significance was set as $p < 0.05$.

3. Results and discussion

3.1. Construction of pseudo-ternary phase diagram

Pseudo-ternary phase diagrams were constructed to determine the components and concentration range for 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. The ME region was observed near the surfactant vertex characterized by high surfactant content and low water content. In other words, water-in-oil (W/O) MEs were easily formed at high Smix content. The maximal water solubilization capacity of this W/O system was nearly 40%, which might be ascribed to the excellent intersolubility of water and ethanol. Different ME formulations in the area surrounded by blue lines were prepared and optimized based on D-optimal design.

3.2. Formulation optimization of MEs using D-optimal design

D-optimal design is an efficient method for the optimization of pharmaceutical formulations, which could clarify the relationship between independent variables and dependent variables in a formulation. In our study, D-optimal mixture experimental design was conducted to rapidly obtain the optimal ME formulation. Smix (a mixture of Span 20/ethanol, 1:9, w/w) (X1), water (X2) and Capryol 90 (X3) were chosen as formulation variables, at the mean time the skin retention (Y1), AZA amount in collection medium after 24 h (Y2) and mean particle size (Y3) were selected as responses (dependent variables). The responses of these formulations were summarized in Table 1.

The independent and response variables were related using polynomial equation with statistical analysis through Design-Expert software (version 7; Stat-Ease, Inc, Minneapolis, MN). The equation that fitted to the data was as follows:

$$Y = b_1X1 + b_2X2 + b_3X3 + b_4X1X2 + b_5X1X3 + b_6X2X3 + b_7X1X2X3 + b_8X1X2(X1-X2) + b_9X1X3(X1-X3) + b_{10}X2X3(X2-X3) \quad (1)$$

where b_1 to b_{10} are the coefficients computed from the observed experimental values of Y. Coefficients with one factor represents the effect of that particular factor while the coefficients with more than one factor represents the interaction between those factors. Positive sign in front of the factors indicates synergistic effects while negative sign indicates antagonistic effect of the factors.[29]

In our study, drug accumulation in skin layers was considered to be the most significant factor to evaluate the efficiency of formulations. As shown in Table 1, skin retention amount of AZA released from ME formulations varied from 111.49 to 593.26 μg, inferring that the three independent factors possessed a profound effect on AZA skin retention amount. The approximation of response values of Y1 based on Sp. cubic model was the most suitable due to its smallest PRESS value (Table 2). The related regression equation was:

$$Y1 = 204.20X1 + 546.05X2 + 111.33X3 + 510.31X1X2 + 170.11X1X3 + 314.46X2X3 + 5007.79X1X2X3 \quad (2)$$

The positive values of all coefficients confirmed the synergistic effect of the three independent variables on Y1. Besides, it was obvious that the term X1X2X3 had the highest effect on this response with the largest coefficient of 5007.79, which could be confirmed by the 2D contour diagram that illustrated the effect of varying ratios of X1, X2, and X3 on the skin retention of MEs (Fig. 2a). As was indicated by the central

solid portion of the plot, ME formulations at moderate level of oil, Smix and water could perform higher skin retention, which represented higher therapeutic efficacy.

The AZA amount in collection medium after 24 h (Y2) of the different ME formulations ranged from 161.67 to 792.74 μg (Table 1). As presented in Table 2, quadratic model was the most appropriate mathematical model for Y2 with obtained regression equation:

$$Y = 217.90X1 + 736.56X2 + 230.15X3 + 1048.47X1X2 + 290.91X1X3 + 337.94X2X3 \quad (3)$$

The coefficient of X1X2 for this response was the largest one, indicating the positive effect of combination of Smix and water content on the drug penetrated into the receptor medium. From the 2D contour plots (Fig. 2b), we could observe that moderate levels of three factors indicated relatively lower cumulative AZA amount in receptor medium, which represented less systemic side effects. For mean particle size, cubic model was the most suitable model based on the largest R^2 value ($R^2 = 0.9980$, Table 2). The regression equation was presented as follows:

$$Y = -0.034X1 + 6.26X2 + 0.020X3 + 31.91X1X2 - 0.69X1X3 + 18.79X2X3 - 97.55X1X2X3 - 15.26X1X2(X1-X2) - 5.20X1X3(X1-X3) + 38.89X2X3(X2-X3) \quad (4)$$

According to 2D contour plots (Fig. 2c), the water content provided the largest contribution to the mean droplet size. In other words, increasing amount of water resulted in nonlinear escalations in particle size. In fact, mean droplet size of formulations containing less than 4% water content as well as water-free systems

312 (mixtures of the surfactant, cosurfactant and oil, S/COS/O-mix) was not measurable.
313 As water content increased, the droplet size of ME formulations also increased,
314 indicating a swelling process taken place within the droplets at high aqueous
315 contents.[30]

316 In order to obtain optimal ME formulations with maximum skin targeting effect
317 and minimum skin permeation, the response Y1 should be maximized (> 600 μg)
318 while Y2 should be minimized (< 600 μg). The S/COS/O-mixtures resulted in
319 significantly lower AZA permeation relative to ME droplets (Fig. 2a b), which
320 demonstrated that the presence of droplets in nanosize had a prominent contribution to
321 the percutaneous penetration of drugs.[31] Thus, the response Y3 should have optimal
322 intermediate range (5-10 nm) to ensure the formation of ME droplets, resulting in
323 maximum skin retention with less systemic side effects. Based on these conditions,
324 the three responses were then combined to determine an all over optimum region (Fig.
325 3). According to the selection criteria, an ME which satisfied with optimal drug skin
326 retention, appropriate permeated amount of drug and droplet size was considered to be
327 the optimal formulation. An optimal response was found with Y1, Y2 and Y3 of
328 571.64 μg, 573.97 μg and 3.78 nm at X1, X2 and X3 value of 50.3%, 13.5% and
329 36.2%, respectively (Table 3). In order to assess the reliability of the
330 developed mathematical model, microemulsion formulation was formed
331 corresponding to above mentioned factor levels. Experimental values of
332 Y1, Y2 and Y3 were 593.57 μg, 584.69 μg, and 3.83 nm, respectively. The
333 predicted and experimental values demonstrated small percentage error of 3.69%,

1.83% and 1.32%, respectively. In addition, a good agreement was obtained between the model prediction and experimental observation. The optimal ME formulation was used for next steps, while ethanolic solution based gel (ESBG) and 20% AZA commercial cream (Skinoren®, Bayer Co., Ltd, Taiwan) were used as control formulations.

3.3. Evaluation of prepared formulations

The appearance of the optimal ME was clear and transparent by visual observation (Fig. 4). The particle size, PDI value and zeta potential of the optimal ME were 3.83 nm, 0.216 and -4.99, respectively, ratifying its excellent homogeneity and stability. The pH values were determined as 3.44, 3.13 and 4.15 for cream, ESBG and ME, respectively (Table 4). Among the three formulations, the pH value of ME was consistent with that of human skin surface (typically slightly above pH = 5), resulting in less skin irritant potential to a certain degree.[9]

3.4. Stability assay

The optimized ME formulation was stable when stored at 25 ± 2 ℃/60 ± 5% RH for three months where there was no obvious change in visual appearance (Table 5). Besides, the main changes of droplet size and PDI were also not observed during 3 months. The concentration of AZA in the optimal ME was above 98.69% ± 3.96 during 3 months, which demonstrated that there was no degradation.

3.5. *In vitro* skin permeation studies

The penetration behaviors of AZA from the optimized ME, ESBG and

356 commercial cream were evaluated for comparison purpose. The cumulative permeated
357 amount of AZA through porcine skin after 24 h was calculated and plotted against
358 time. As shown in Fig. 5, the cumulative amount of AZA in the receptor chambers
359 was steadily increased over time. The optimal ME and ESBG presented a comparable
360 penetration behavior through the skin, which was significantly higher than that of
361 AZA marketed cream ($P < 0.05$ for ESBG; $P < 0.01$ for ME). Moreover, the ME
362 formulation and ESBG provided higher permeation rate than cream, which
363 represented a possible rapid therapeutic effect. The results demonstrated that the
364 tested ME formulation and ESBG had potent enhancement effect for the topical
365 administration of AZA.

366 Drug accumulation in different skin layers (SC and viable skin layers) after 24 h
367 application of the three formulations was determined (Fig. 6). The total skin retention
368 was defined as the sum of the amounts in the SC and viable skin layers (epidermis and
369 dermis, ED). The three formulations could be arranged in a descending order in
370 relation to the percentage of total skin retention after 24 h as follows: ME (11.87%) >
371 ESBG (4.74%) > cream (3.41%) (Table 4). As depicted in Fig. 6, there was no
372 significant difference between ESBG and AZA cream after 24 h application ($P > 0.5$
373 for both SC data and ED data). However, the drug content in the skin layers
374 (both in SC and viable skin layers) treated with the optimized ME was
375 significantly higher compared to cream suspension ($P < 0.01$) and ESBG (P
376 < 0.05), which was inconsistent with the permeation tendency through the
377 skin.

As AZA was water insoluble, it could not completely dissolve in the cream and mainly suspended in this dosage form.[5] However, in the optimal ME and ESBG, AZA mainly existed in dissolved form due to their co-solvent and physicochemical properties. In generally, only the dissolved fraction of an active agent in a vehicle could enter the skin.[32] Therefore, both the optimal ME and ESBG resulted in significantly higher skin permeability than commercial cream. For AZA retention in skin layers, however, ESBG as well as cream resulted in significantly lower amount than the optimal ME, which be ascribed to the microstructure of MEs.

3.6. Attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) study

ATR-FTIR study was conducted to study the skin-vehicle interaction and reveal the mechanism of enhanced cutaneous penetration based on various vehicles. In IR spectra of skin treated different formulation and untreated (control), the changes in peak position and intensity (peak height) of bands were compared, including CH_2 stretching (around 2924 cm^{-1}, represent the asymmetric stretching CH_2 vibrations) and amide 1 stretching (around 1653 cm^{-1}, sensitive to H-bond change in the SC).[25,33]

The change in peak intensity of band was considered to be important because it provided information about the lipid amount presenting in the SC. As shown in Fig. 7, after the treatment with ME, the peak height of CH_2 stretching (around 2924 cm^{-1}, 0.82%) was significantly increased compared to control (0.77%), implying lipid extraction in SC and enhancement in drug cutaneous

permeation;[25] while the SC treated with ESBG and cream displayed decreased peak intensity (0.73% for ESBG and 0.42% for cream), suggesting lipid strengthening in SC and subsequently significant retardation effect on percutaneous transport. Analysis of amide 1 model pointed to a shift to higher wavenumber when SC treated with ME (from 1653.40 to 1657.32 cm^{-1}) and ESBG (from 1653.40 to 1656.32 cm^{-1}) relative to untreated SC. The shift indicated the weakening of the H-bonds between the amide linkages within the SC, which favored substance penetration into skin.[25] The region corresponding to CH_2 asymmetric vibration (around 2924 cm^{-1}) provided information about conformational order of the SC lipid chains.[34] After treated with ME formulation, the band shifted to higher values in comparison with control, suggesting the permeation enhancement due to disorder in the lipid arrangement. However, the band of SC treated with ESBG showed a shift to lower values, supporting the enhancement of stable organization of lipids.

3.7. Skin irritation test

Although most of the ingredients used in ME preparation were pharmaceutically approved, they might also irritate the skin at higher concentrations.[35] As a result, histopathological examination was performed to valuate any irritant potential of the optimized ME compared to control formulations.[36] After 3 days, rats in all the groups showed no apparent edema, erythema and other irritant response. Microscopic images of rat skin treated with various formulations were shown in Fig.8. Compared to normal skin (Fig.8a), the SC layer of rat skin treated with ME and ESBG

422 became thinner but without any apparent change in epidermis and dermis
423 (Fig. 8c, d). Besides, the SC, epidermis and dermis layers were normal
424 following cream application (Fig. 8b). In addition, the skin treated with
425 cream, ESBG and ME showed no sign of inflammation cells. The result
426 suggested that the optimized ME might be safe to be used for topical AZA
427 delivery.

428 **3.8. Pharmacodynamics studies**

429 Cutaneous polymorphonuclear leukocyte inflammation was induced by croton
430 oil to evaluate the therapeutic effect of ME formulation on rosacea based on reduced
431 ear redness, edema, et al.[27,28] Mice untreated (group f) and treated with croton
432 oil only (group a) were used as negative and positive control,
433 respectively. Croton oil could produce intense redness, accompanied by large
434 number of infiltrated inflammatory cells in viable skin layers, edema, and even severe
435 skin ulcer (Fig. 9a). The application of blank ME4 could not improve inflammation
436 compared to positive control ear (Fig. 9a, b). On the contrary, AZA-loaded ME
437 exhibited significant inhibitory effect on inflammation response based on the
438 significantly reduced number of inflammatory cells in the whole skin layers (Fig. 9d),
439 which was superior to ESBG and commercial cream treated ears (Fig. 9c,e). It seemed
440 that there was no significant difference between AZA-loaded ME treated ear and
441 negative control ear (Fig. 9f) in both macro photos and micro photos. In addition,
442 the application of AZA-loaded ME (group d) also significantly inhibited the increase
443 of the ear thickness (ear edema) compared to the other formulations ($P < 0.01$ for

group a,b; $P < 0.05$ for group c, e) (Fig. 10). The results indicated that the optimized ME formulation significantly improved the therapeutic effect compared to market cream.

AZA, a bioactive molecule used in many skin disorders, restrains its penetration across the stratum corneum due to poor bioavailability mainly caused by low drug solubility and poor skin permeability. In order to enhance AZA solubility in the vehicles, ionization and monosodium salt of AZA were investigated, respectively.[5,37] In our study, however, AZA was completely solubilized in the optimal ME without any physical or chemical treatment. Besides, gel[3], liquid crystal[38] and nanoscale vehicles (including microemulsion[37], ethosomes and liposomes[39]) were developed as alternative topical formulations of AZA. In these studies, the effect of developed vehicles on the cutaneous permeation of AZA was investigated only using excised skin model in *in vitro* study. However, the therapeutic efficacy of AZA based on topical vehicles has to be proven, since there are many other variables that could affect the efficacy when used *in vivo*. Thus, in our study, *in vivo* pharmacodynamics studies were further conducted. The results indicated that the optimized ME formulation containing AZA significantly improved the therapeutic effect on rosacea.

Both metronidazole (MTZ) and AZA are considered to be the first-line treatment of rosacea. In our previous work, we have developed and optimized a ME to enhance targeting localization of MTZ in skin layers

and improve therapeutic efficacy of MTZ.[27] However, some comparative researches demonstrated that AZA was superior to MTZ in improving inflammatory lesions and erythema of rosacea.[2] Generally speaking, there were three obvious differences between these two research articles. Firstly, MTZ, with logP value of -0.18, shows highly hydrophilic property, resulting in limited permeation into and through the skin caused by lipophilic barrier of SC; while AZA is a lipophilic drug (logP value is 1.45) and restrains skin penetration mainly due to its poor solubility. Secondly, considering significantly different properties between MTZ and AZA, we developed oil-in-water (O/W) ME for MTZ and water-in-oil (W/O) ME for AZA, respectively. The components of W/O ME in this study were also different from that of O/W ME in our previous study. Last but not least, in addition to the similar optimization and pharmacodynamics studies in our both studies, ATR-FTIR was further carried out to investigate the molecular vibrations of the SC components and reveal the mechanism of enhanced cutaneous penetration based on ME vehicle in current study.

In addition, porcine skin was chosen as in vitro penetration model in our study not only due to its physiological, biochemical and histological similarities to human skin, but also because of less variability than other skin models.[40,41] In contrast, the skin model from mice exhibited an extremely high density of hair follicles which might affect precutaneous absorption of molecules. Thus, hairy rodent skin is usually used in *in vivo* studies rather other in vitro studies.[40] Nevertheless, *in vivo* studies are

still performed on this specie. We realized the potential limitations caused by different animal models used for *in vitro* (porcine skin) and *in vivo* (mice). However, the optimal therapeutic effect of the optimal ME on rosacea might indicate the improved AZA retention in mice skin, demonstrating that these two models may have good correlations for the permeation of AZA to a certain degree.

4. Conclusion

In current study, the application of ME systems for topical delivery of AZA was investigated. D-optimal mixture experimental design was applied to rapidly obtain the optimal AZA-loaded ME formulation realizing maximum skin accumulation, appropriate penetration into receptor medium and globule size. The optimal ME composed of 50.3% Smix (a mixture of Span 20/Ethanol, 1:9, w/w), 13.5% water and 36.2% Capryol 90. Contrary to ESBG and commercial cream, the optimized ME significantly enhanced AZA retention in the skin and penetration through the skin in *in vitro* permeation studies. ATR-FTIR study indicated that the improved AZA release from the optimal ME was mainly due to the disturbed SC barrier function via lipid extraction, weakening H-bond between the amide linkages and disordering lipid arrangement of SC. Additionally, the results of skin irritation test and pharmacodynamics study inferred that the AZA-loaded optimized ME formulation was safe and more effective in the treatment of croton oil-induced rosacea than commercial cream and ESBG. Taken together, the optimal W/O ME might be a promising topical vehicle of AZA for improved therapeutic effect of anti-rosacea.

Acknowledgment

We acknowledge the National Basic Research Project (2014CB932200) of the MOST for financial support.

Declaration of Interest section

The authors report no declarations of interest.

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598 **Table 1**

599 The formulations of mixture design and their characterization results.

No.	Smix (X_1)	Water (X_2)	Oil (X_3)	Skin retention (μg) (Y_1)	AZA amount in collection medium at 24 h (μg) (Y_2)	Particle size (nm) (Y_3)
1	68.27	3.49	28.24	317.86	353.42	0
2	80	0	20	202.91	261.67	0
3	65.37	14.63	20	403.56	663.65	9
4	40	9.81	50.19	289.17	472.65	1.48
5	80	0	20	202.91	161.67	0
6	53.98	13.59	32.43	593.26	573.95	4.36
7	50.49	0	49.51	120.67	193.38	0
8	53.98	13.59	32.43	591.47	583.93	4.36
9	65.37	14.63	20	503.56	663.65	8.55
10	50	30	20	543.89	792.74	12
11	40	0	60	111.49	222.33	0
12	40	20.28	39.72	385.43	520.91	7.75
13	40	0	60	123.67	246.30	0
14	40	30	30	514.58	699.58	12
15	60.32	0	39.68	214.40	327.17	0
16	60.32	0	39.68	234.45	359.23	0

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609 **Table 2**

610 Model summary statistics of the measured response.

Response	Model	SD	R^2	Adjusted R^2	PRESS
Y1	Linear	95.18	0.7362	0.6957	161000
	Quadratic	69.83	0.8908	0.8362	104600
	Special cubic	34.37	0.9762	0.9603	31320.77
	Cubic	40.45	0.9780	0.9450	575200
Y2	Linear	83.69	0.8305	0.7655	145400
	Quadratic	51.00	0.9580	0.9371	65780.87
	Special cubic	52.97	0.9593	0.9321	71972.51
	Cubic	57.44	0.9681	0.9202	1726000
Y3	Linear	1.34	0.9274	0.9162	32.66
	Quadratic	1.19	0.9556	0.9335	48.09
	Special cubic	1.01	0.9714	0.9523	39.18
	Cubic	0.32	0.9980	0.9951	64.77

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Table 3

Predicted and experimental values for the optimized microemulsion.

Response	Predicted value	Experimental values	Error % [a]
Y_1	571.64	593.57	3.69
Y_2	573.97	584.69	1.83
Y_3	3.78	3.83	1.32

[a] Error% was calculated using the formula [(Experimental value - Predicted value)/Experimental value] × 100

Table 4

pH value and the permeation parameters of commercial cream, ESBG and the optimized ME formulation.

Formulation	pH value	Flux (μg/cm^2 h)	Amount in collection medium at 24 h （μg）	ER	Total skin retention (%)
Cream	3.44 ± 0.052	4.29 ± 0.23	129.48 ± 14.56	--	3.41 ± 0.31
ESBG	3.13 ± 0.058	10.73 ± 2.68*	401.875 ± 26.99*	3.10	4.74 ± 0.35
ME	4.15 ± 0.071	15.64 ± 2.49**	584.69 ± 40.87**	4.52	11.87 ± 0.76**

ER: enhancement ratio for drug permeation=Flux in ESBG or ME/Flux in cream.

* $P < 0.05$, when compared to control

** $P < 0.01$, when compared to control

Table 5

Storage stability of AZA ME under long-term condition. Data represent mean ± SD for three batches.

Parameters	M[a] 0	M[a] 1	M[a] 2	M[a] 3
Appearance	Transparent	Transparent	Transparent	Transparent
Droplet size (nm)	3.83 ± 0.071	3.92 ± 0.095	3.98 ± 0.083	4.15 ± 0.075
PDI	0.216 ± 0.002	0.198 ± 0.003	0.295 ± 0.004	0.167 ± 0.003

[a] M stands for month

Figure captions

Fig. 1 Pseudo-ternary phase diagram showing a w/o microemulsion region (area surrounded by pink line) made of Capryol 90 (oil phase), water, and the mixture of Span 20 (surfactant) and ethanol (cosurfactant) at a fixed mass ratio of 1:9. Area surrounded by blue line was used for D-optimal design.

Fig. 2 2D contour plots for the effects of variables on the skin retention after 24 h (μg) (a), AZA amount in collection medium after 24 h (μg) (b) and particle size (nm) (c) of W/O ME.

Fig. 3 Overlay plot for the effect of different variables on the three responses: skin retention after 24 h (μg) (Y_1), AZA amount in collection medium after 24 h (μg) (Y_2) and particle size (nm) (Y_3).

Fig. 4 Typical appearance and particle size distribution of the optimal ME formulation.

Fig.5 Permeated amount of AZA in the receptor medium at various time points: comparison of commercial cream, ESBG and the optimal ME. Results are expressed as mean ± SD, n = 6. * $p < 0.05$, ** $p < 0.01$.

Fig.6 Percentage amount of azelaic acid distributed in stratum corneum (SC), epidermis and dermis (ED) after 24 h exposure of commercial cream, ESBG and the optimal ME. Results are expressed as mean ± SD, n = 6. * $p < 0.05$, ** $p < 0.01$.

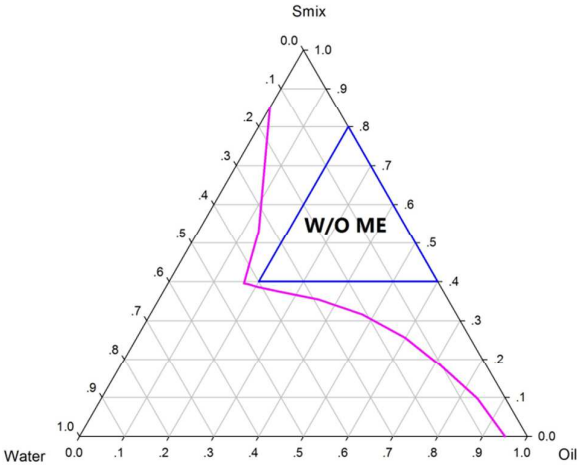
Fig.7 Representative ATR-FTIR spectra of untreated porcine skin SC (control) and SC treated with the optimal ME, ESBG and commercial cream.

Fig.8 Microscopic images of rat skin treated with (a) normal saline, (b) commercial cream, (c) ESBG and (d) the optimal ME.

Fig. 9 Photomicrograph of mice ears **(**upper panel), H&E-stained mice ear tissue at a magnification of 10 × (middle panel) and magnification of 20 × （bottom panel）sensitized with

various formulations. Mice ear treated with (a) croton oil, (b) croton oil and blank ME, (c) croton oil and drug loaded ESBG, (d) croton oil and drug loaded ME, (e) commercial cream. And mice ear untreated used as control (f).The number 1 indicated inflammatory cells. The number 2 and number 3 indicated skin ulcer and edema, respectively.

Fig.10 Ear thickness differences between left (untreated) and right (treated) ears of mice treated with different formulations respectively.



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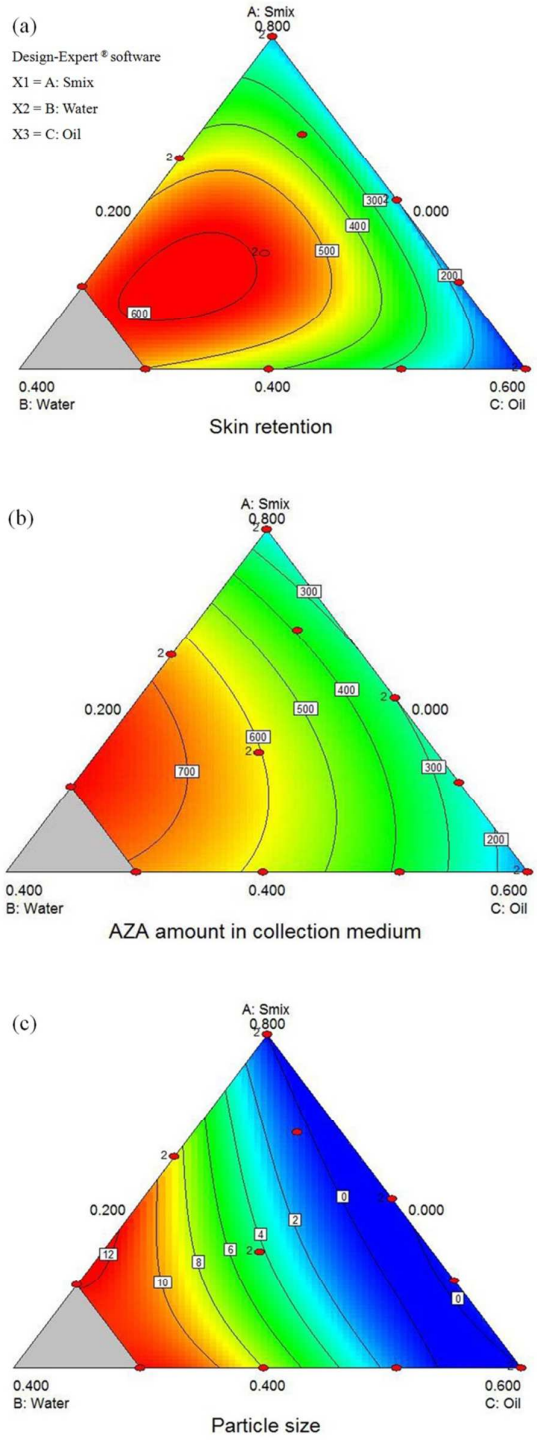

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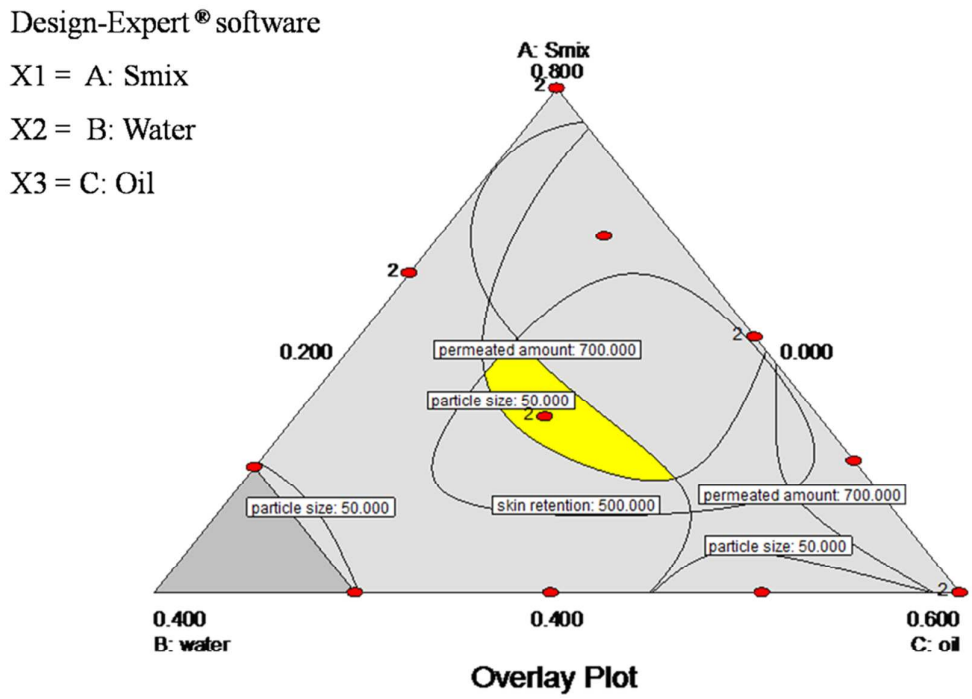

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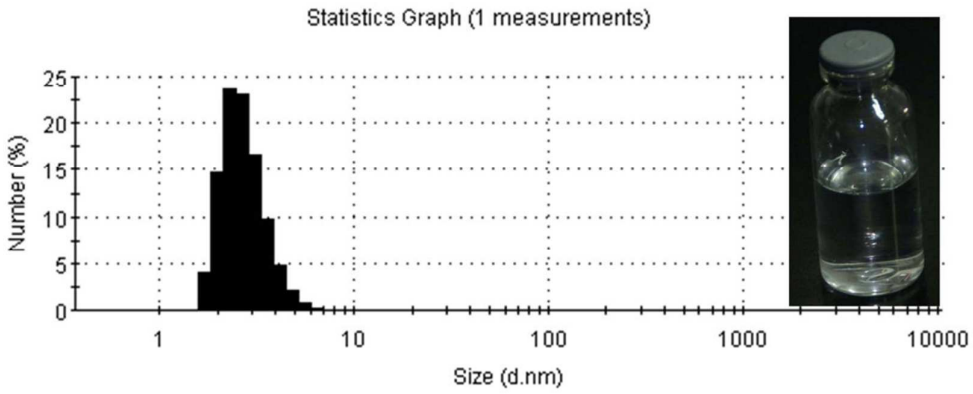

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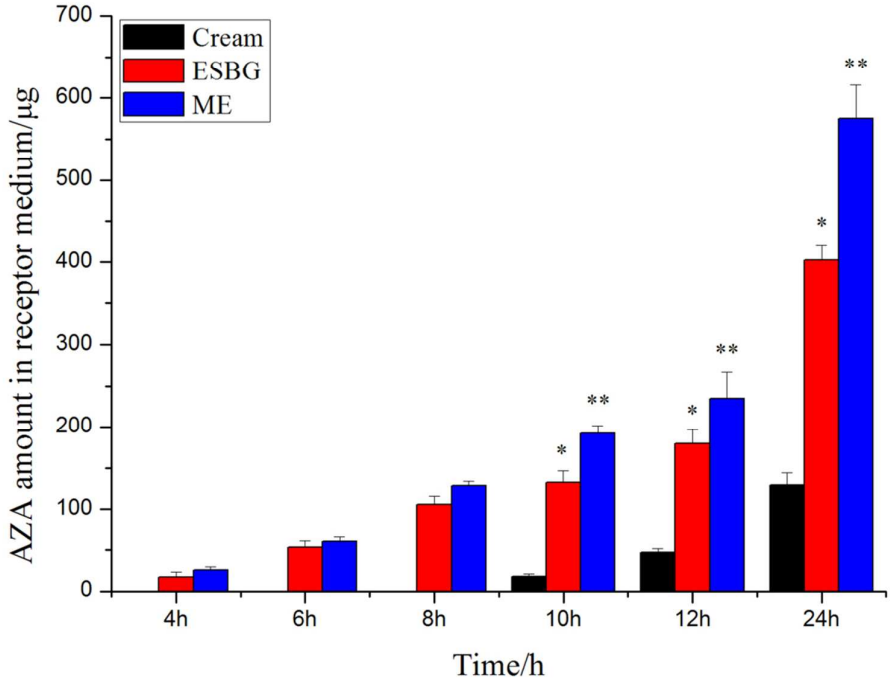

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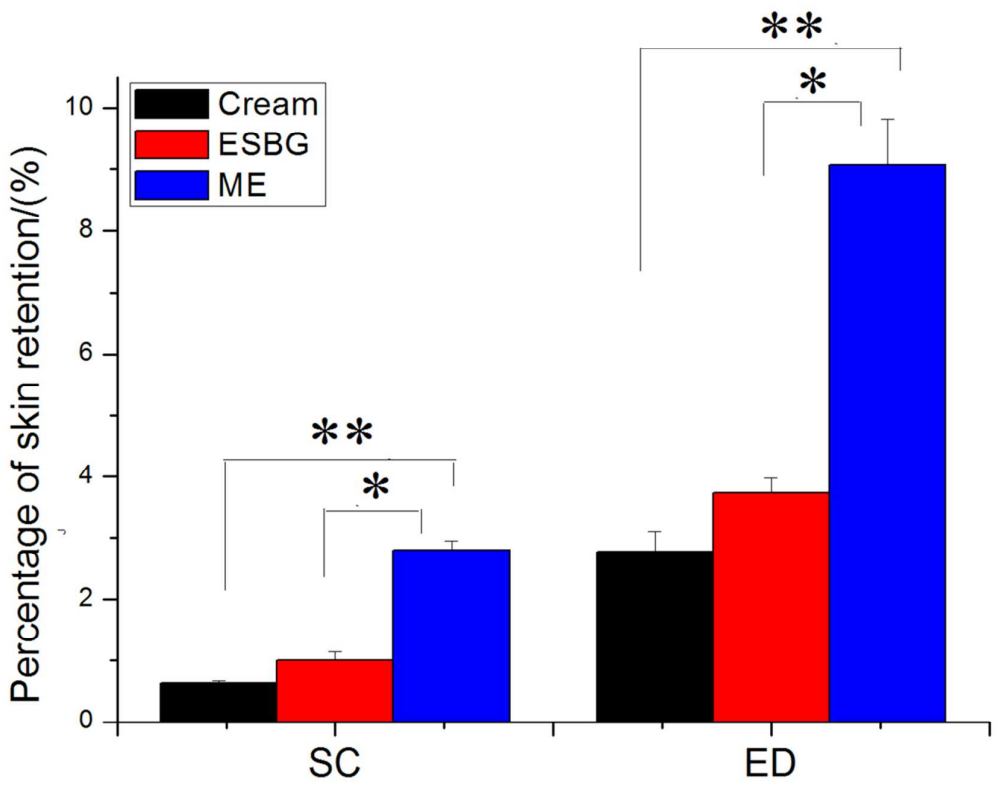

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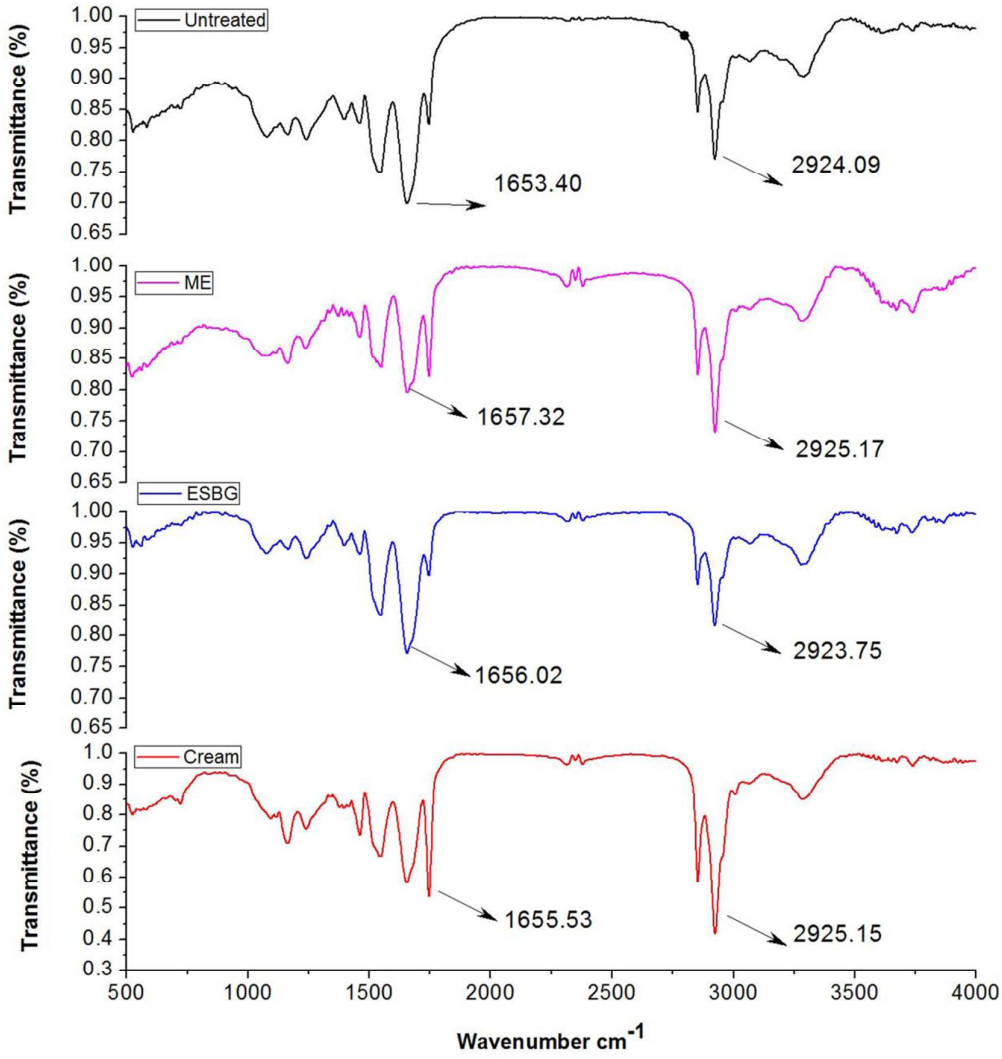

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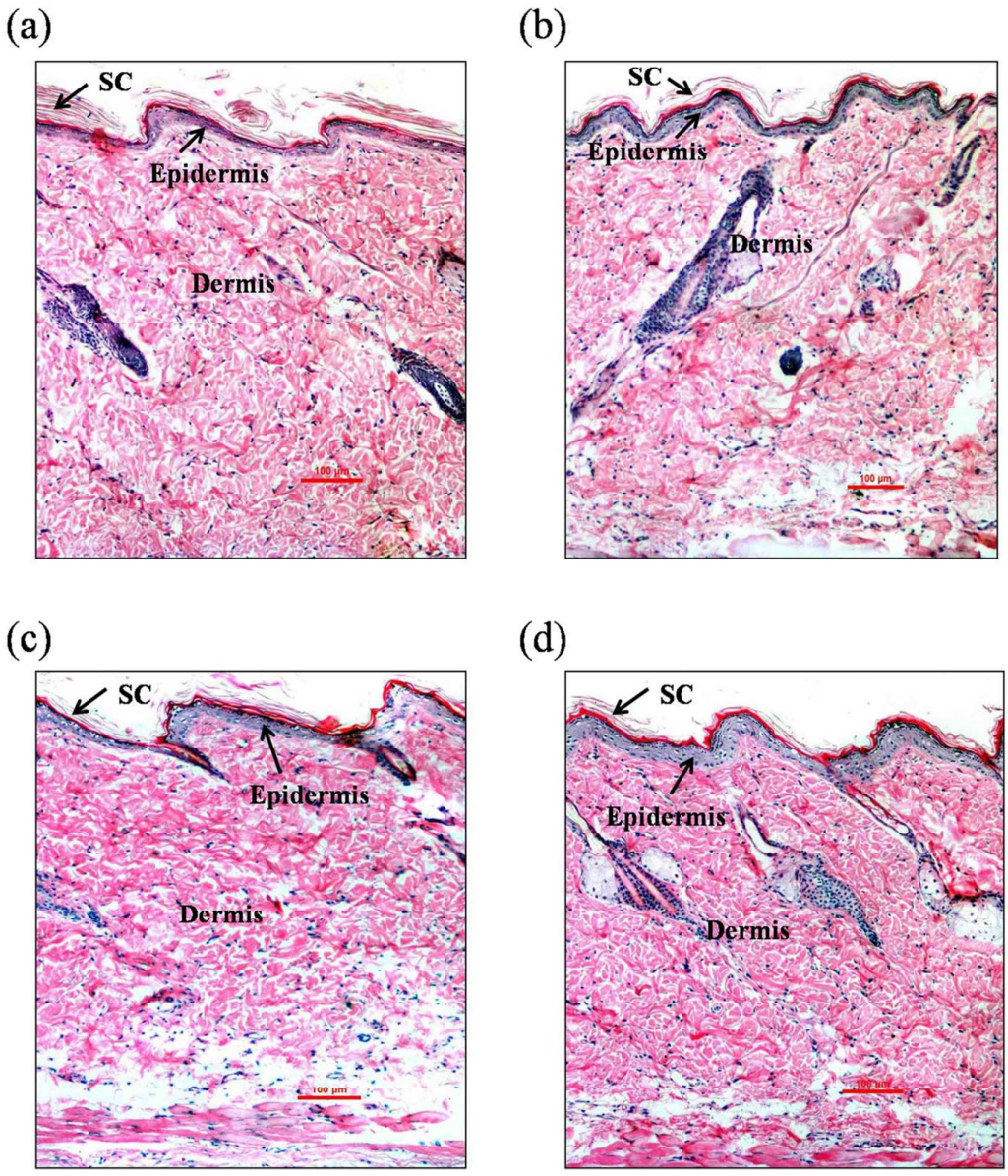

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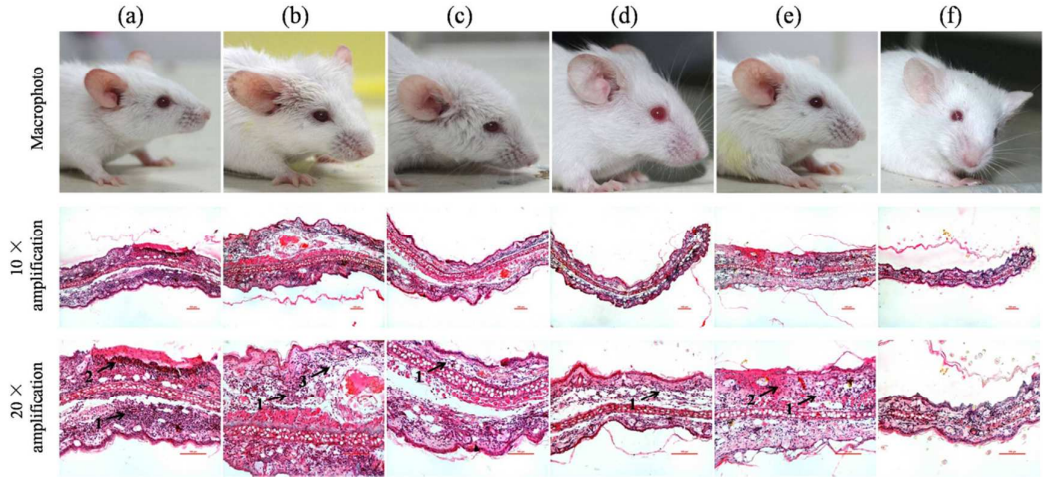

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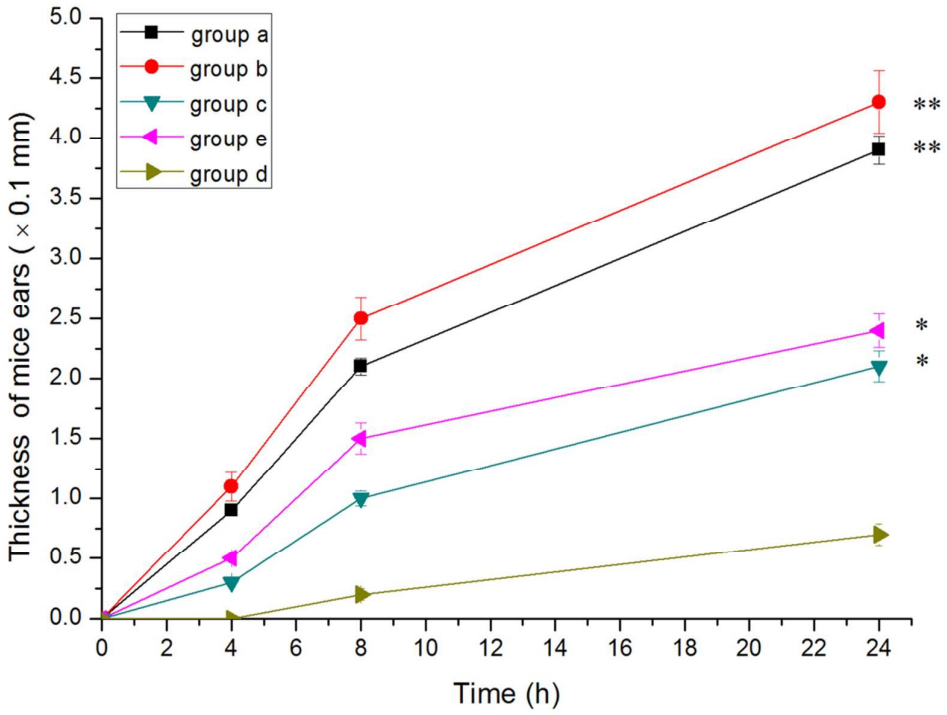

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