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Self-Assembled Cubic Liquid Crystalline Nanoparticles for Transdermal Delivery of Paeonol

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: The aim of this study was to optimize the preparation method for self-assembled glyceryl monoolein-based cubosomes containing paeonol and to characterize the properties of this transdermal delivery system to improve the drug penetration ability in the skin.

Material/Methods: In this study, the cubic liquid crystalline nanoparticles loaded with paeonol were prepared by fragmentation of glyceryl monoolein (GMO)/poloxamer 407 bulk cubic gel by high-pressure homogenization. We evaluated the Zeta potential of these promising skin-targeting drug-delivery systems using the Malvern Zeta sizer examination, and various microscopies and differential scanning calorimetry were also used for property investigation. Stimulating studies were evaluated based on the skin irritation reaction score standard and the skin stimulus intensity evaluation standard for paeonol cubosomes when compared with commercial paeonol ointment. *In vitro* tests were performed on excised rat skins in an improved Franz diffusion apparatus. The amount of paeonol over time in the *in vitro* penetration and retention experiments both was determined quantitatively by HPLC.

Results: Stimulating studies were compared with the commercial ointment which indicated that the paeonol cubic liquid crystalline nanoparticles could reduce the irritation in the skin stimulating test. Thus, based on the attractive characteristics of the cubic crystal system of paeonol, we will further exploit the cosmetic features in the future studies.

Conclusions: The transdermal delivery system of paeonol with low-irritation based on the self-assembled cubic liquid crystalline nanoparticles prepared in this study might be a promising system of good tropical preparation for skin application.

MeSH Keywords: Cubic Liquid Crystalline Nanoparticles • HPLC • Irritation • Paeonol • Transdermal Delivery

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Background

Transdermal drug delivery is one of the major methods in external applications of traditional Chinese medicines and also is one of the characteristics and advantages of traditional Chinese medicine use.

Paeonol (2-hydroxy-4-methoxyacetophenone) (Figure 1), also known as Danpi in Chinese, is one of the main active ingredients from the barks of *Paeonia suffruticosa Andr.*, and the dried root or entire plant of *Pycnostelma Panicu latum (Bunge) K Schum*. It was reported that paeonol has significant anti-atherosclerosis, anti-inflammation, anti-allergy, and antitumor effects [1]. A study showed that paeonol had a significant inhibitory effect on skin vascular inflammatory reaction in guinea pigs and passive skin allergy in rats [1,2] by inhibition of the release of inflammatory mediators, such as histamine, serotonin, bradykinin, and arachidonic acid. Wang et al. [3] investigated the mechanism of the inhibitory effects of paeonol on skin inflammation by RT-PCR. Unfortunately, due to the poor water solubility, stability, and high volatility, paeonol was limited in its curative effects and applications. It is necessary to use a new modern medicine dosage formula and new technologies to improve the paeonol dosage form preparation.

Paeonol transdermal drug delivery can avoid the adverse effects of traditional hormonal drugs and is easy to use with long-lasting effect. The dosage form of available skin application of paeonol in current clinical use is mainly ointment. Recently, to extend its applications and improve its clinical therapeutic effects, the study of paeonol dosage form drew great attention. The transdermal delivery of paeonol has been studied using several drug carriers, such as microsponge [4], proniosomes [5], and the liposome [6]. However, cubic crystal nanoparticles loaded with paeonol have not been reported previously.

Transdermal preparation has been a popular research focus in China and abroad, and develops rapidly. Conventional external preparation is good for its large dosage load. However, it cannot deliver the drug with sustained release in the skin, and thus its clinical use is limited. Recently, the use and mechanisms of liposome, phospholipid complex, and some monomers that can include traditional Chinese medicine were studied.

For example, Mei et al. [7] showed the use of microemulsion of *Tripterygium wilfordii*, a material which can increase the skin permeability and anti-inflammatory activity, for topical delivery of triptolide. Ling-yun Xu et al. [8] evaluated *Tripterygium wilfordii* lactone microemulsion gel for its pharmacodynamics and showed that the preparation has good anti-inflammatory, analgesic, and immune inhibition effects, with mild skin irritation. Today, transdermal preparation with Chinese medicine has made great progress, with many choices available.

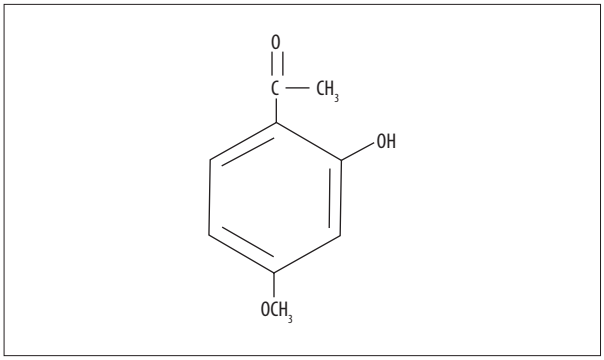

Figure 1. Chemical structure of paeonol.

However, there is no widely recognized transdermal preparation for drug delivery which has a clarified mechanism, stable quality, and controllable process. Moreover, microemulsion and liposome also have their limits, such as demulsification in microemulsion and susceptibility to oxidation of liposome, which result in low coating rate and thus hinder the development of external preparations of Chinese medicine.

As a new type of transdermal delivery system, cubic crystal is drawing great attention. Maia et al. [9] and Jenning et al. [10] proposed the concepts of skin-targeted and epidermis-targeted systems to promote drug infiltration and absorption in the skin or skin layer, and reduce the drug entering into circulation. With improvement for the skin-targeting of drugs, the carrier technology has become a novel transdermal drug delivery system and is under intense research.

The phase area of a cubic crystal is very large. Its internal structure is a 3-dimensional extension of continuous network structure with double water phases [11,12]. The viscosity and film strength of cubic crystal are so big that it helps to stabilize the lipid cubic crystal-coated drugs. Today, the most advantageous cubic phase system is the glycerol monoolein (GMO) and water system.

According to some scholars [13,14], the cubic crystal system membrane surface area of drug-loading GMO/water formation is so large that it can coat with different polarities and doses of the drugs. Water-soluble drugs can be coated in cubic crystals of polarity and fat-soluble drugs can be coated in cubic crystals with lipid double membrane. The use of cubic crystal system loaded with drugs in the body has the advantage of sustained and controlled drug release, and external use has drawn the attention of researchers [15–17]. Nielsen et al. showed that cubic crystals have good adhesion performance for up to 6 h in the rabbit vaginal mucosa. In 2005, for the first time, Esposito et al. [18] reported that the cubic crystal model is applicable to anti-inflammatory drugs for transdermal drug delivery, such as indomethacin.

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Cubic crystal phase can improve osmotic rate and sustained drug release in skin, and its good *in situ* biological adhesion characteristic comes from the similarity between the vulnerable temporary surface protection of an ulcer and the vulnerable layer of the liquid cubic crystal, which make it superior to other percutaneous drug delivery systems [19]. Estracanholli et al. [20] and Peng et al. [12] proved that the cubic crystal had good percutaneous penetration performance by dermal testing. Cubic crystal gel and nanoparticles both could be used in a transdermal delivery system, but the high adhesion and poor spreadability of the gel has limited its application [21]. The cubic crystal nanoparticles delivery system of GMO/water complex with comprehensive advantages over liposomes and microemulsion/nanoemulsion [22], especially in external use, is being studied by many researchers.

The cubic crystalline system of GMO/water complex may be a promising approach for the delivery of paeonol to the skin due to sustained drug release, improved drug penetration across skin layers, and minimum adverse effects in this system. Not yet commercialized, cubic crystal in nanoparticle form has great value for academic research and industrial application. In this study, a drug-loaded cubic crystal nanoparticle system was prepared, and its transdermal absorption properties and stimulation on skin were investigated in order to provide a more efficient and convenient dosage form for clinical treatment.

Material and Methods

Materials and reagents

Paeonol (>99% pure) was purchased from Dahua Weiye Co. Ltd. (Wuhan, China). GMO was ordered from BaoMan Co. Ltd. (Shanghai, China), and Poloxamer 407 was provided by YuanYe Co. Ltd. (Shanghai, China). The Milli-Q water was prepared by Millipore purifying system (Molsheim, France) throughout this study. HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). All other reagents used in this study were of analytical grade.

Animals

Male Sprague-Dawley (SD) rats weighing 200 g to 230 g were supplied by the Lab Animal Services Center of Zhejiang Province. White New Zealand rabbits weighing 2.5 to 3.0 kg were purchased from the Lab Animal Services Center of Bengbu Medical College (Bengbu, China). They were all kept in animal facility rooms under standard controlled environment conditioned with suitable temperature, humidity, and light. All animals were housed in fixed cages, and food and water were provided *ad libitum*. The animals were kept in the facility for more than 1 week before start of the experiments.

Analytical system

The analysis used reverse-phase high-performance liquid chromatography (HPLC) (Shimazu, Kyoto, Japan). The mobile phase was a mixture of methanol and double-distilled water with ratio in volume of 75:25 for methanol: water. The mobile phase was subjected to ultrasound before filtering through a 0.45-μm Millipore filter and degassed prior to use. The determination of the samples was achieved by HPLC using packed column 250×4.6 mm with particle size of 5 μm (Kromasil C18; Biomics™ Co. Ltd., Nantong, China). The wavelength was 274 nm and injection volumes were 20 μl for all samples. The flow rate was 1.0 ml/min and all the analyses were performed at room temperature.

Preparation of paeonol cubosomes

Ordinary cubic gel and drug-loaded cubic crystal nanoparticle system were modified from established methods according to the previously reported prescription and preparation technology in the literature [12,21,23,24]. In brief, GMO and Poloxamer 407 (P407) were melted at a ratio of 9:1 in a 60°C water bath. When the sample was almost completely melted, 60°C deionized water was added gradually to the mixture and mixed for 1 min to achieve a homogenous state. Then the sample was allowed to sit at room temperature for more than 1 week. Subsequently, a pale yellow transparent gel was formed. The ordinary cubic gel was prepared by disruption of mechanical stirring. Fragmentation of the gel was performed in water by intermittent probe sonication for 10 min, using pulse mode with 400 W energy input. The resultant milky coarse fragmented gel was homogenized thoroughly using a high-pressure homogenizer (JN-02HC; JUNENG Co. Ltd., Guangzhou, China) at 60°C with high pressure to obtain an opalescent dispersion of cubosomes. The final products were stored at room temperature until use. In the case of preparing samples containing drug, prescribed paeonol was first dissolved in deionized water. Then GMO and P407 at a ratio of 9: 1 were melted at 60°C in a water bath and mixed evenly. The prepared deionized water containing paeonol was added gradually. The remaining process was the same as the preparation of the blank cubosomes.

Characterization of cubosomes

Particle size, zeta potential and encapsulation efficiency

Particle size distribution (Z-average), polydispersity (PDI), and zeta potential were determined by photon correlation spectroscopy using a Zeta sizer of NanoZS90 (Malvern Instruments, Malvern, UK), which is based on the principles of Brownian motion. The model pattern of cubosomes, including honeycomb structure, bicontinuous watershed, and closed lipid bilayer, can better present its mechanism (Supplementary Figure 1).

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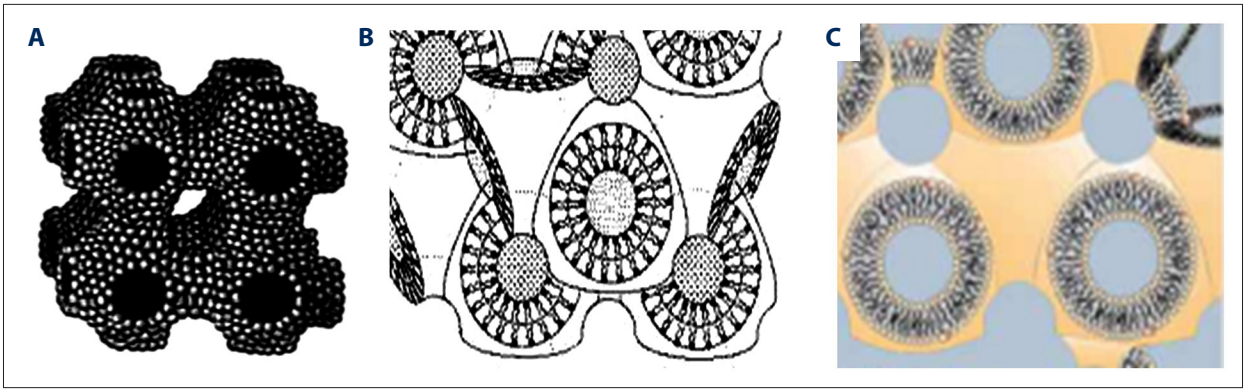

Supplementary Figure 1. The model pattern of cubosomes. (**A**) honeycomb structure; (**B**) bicontinuous watershed; (**C**) closed lipid bilayer.

Samples were diluted with deionized water prior to measurement, and the dispersant viscosity was set at 0.8872 cP at 25°C. The results presented here were the average of 3 successive circulation measurements.

The drug encapsulation efficiency was determined using ultrafiltration. Firstly, we accurately measured 0.4 ml of cubosomes containing drug, then we transferred it to the inner tube of the Millipore filter with 3.6 ml deionized water added in the outer tube later. The amount of drug loaded in the cubosomes was calculated as the difference between the total amount used in preparation of the cubosomes and the amount in the filter, as determined by HPLC after being centrifuged at 4000 rpm for 30 min and filtered through a 0.45-μm microporous membrane.

The drug encapsulation efficiency was calculated as follows:

$$E.E.\ (\%)=[(W_{total}-W_{free})/W_{total}]\times 100\%$$
$$\approx[(C_{total}V_{total}-C_{free}V_{free})/C_{total}V_{total}]\times 100\%$$

E.E. is the drug encapsulation efficiency; W_{total} and W_{free} are the total amount of the drug in the cubosome and in the filter, respectively; C_{total} and C_{free} are the concentrations of the drug in the cubosome and in the filter, respectively; V_{total} and V_{free} are the volumes of the drug in the cubosome and in the filter, respectively.

Transmission electron microscopy (TEM)

In order to characterize the morphology and distribution of cubosomes, the prepared samples were analyzed using TEM (JOEL JEM-2100, Tokyo, Japan). The TEM test uses negative staining technique, which can tell us the inner cubic structure of the self-assembled nanoparticles. Before observation, samples were diluted with water, dropped on the bronze, and dyed by 2% PTA solution (pH 7.0). After sitting for about 10 min for drying, samples were observed in minimal amount and the images were recorded digitally by a CCD camera.

Polarized light microscope (PLM)

PLM was used to detect whether the self-assembled cubic system had the polarization phenomenon. Samples were dropped on the microscopic slide and covered with a coverslip slowly. We adjusted the polarization angle to 90 degrees, and then performed the observation and the charting.

Differential scanning calorimetry (DSC)

DSC (VP-DSC, Microcal, USA) was used to accurately measure the heat. With the agitation part (pressure perturbation accessory for VP-DSC), heat capacity of solution samples at a constant temperature can also be accurately measured. Samples were sealed in aluminum crimp cells and heated at a rate of 10°C/min from 0°C to 150°C in nitrogen. The results and dates were recorded and then plotted using OriginPro 8 software.

Storage stability studies

The stability of the samples was tested under conditions different in temperature – cold, low, and room temperatures. The samples were placed at cold temperature of –20°C, low temperature of 4°C in refrigerator, and room temperature of 20°C, respectively. All of the samples for storage testing had their stability determined by evaluation of the particle size, zeta potential, and encapsulation efficiency after 24 h or 1 month. And all of the samples were determined for their content at the same time.

Stimulating studies

White New Zealand rabbits used in the stimulating study were divided into a complete skin group and a damaged skin group, with 4 animals in each. Twenty-four hours before the experiment, the rabbits were shaved on the back. Two areas, each of 3×3 cm in size, were cleared on the 2 sides of the back of animals. Animals in the damaged skin group were shaved with

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a razor to cause mild bleeding. The bleeding extent was consistent on both sides. Commercial ointment was applied to the left sides and self-assembled nanoparticles were applied to the right sides.

The skin irritation test for single-dosing

Six hours after being applied with paeonol commercial ointment or self-assembled nanoparticles, the rabbits were cleansed on the application sites with warm aseptic water to remove the drug at 1, 24, 48, and 72 h later. Erythema or edema at application sites were evaluated by macroscopic observation according to the New Drug Toxicology Studies Guiding Principles for Skin Irritation Reaction Score [25]. Skin stimulus intensity grading point=(total erythema score+total edema score)/the number of animals. Erythema score: 0 point, no erythema; 1 point, mild erythema (barely visible); 2 points, moderate erythema (highly visible); 3 points, severe erythema; 4 points, Purple red spot to mild eschar formation. Edema scores: 0 point, no edema; 1 point, mild edema (barely visible); 2 points, moderate edema (obviously skin uplift); 3 points, severe edema (skin uplift 1 mm, clear contours); 4 points, serious edema (skin uplift more than 1 mm and expanded skin bulge). Skin stimulus intensity grading point: 0–0.49 point, no irritation; 0.5–2.99 points, mild irritation; 3.0–5.99 points, moderate irritation; 6.0–8.0 points, severe irritation. Thus, based on the score standards, the status of the erythema or edema on the experimental area on the back of the animals was recorded and the skin stimulus intensity grading point was calculated for the evaluation of the stimulating intensity of the drugs on the animal models.

The skin irritation test for multiple dosing

Drug was administrated to the animals for 7 days in the 2 different dosage forms. Then the animals were sacrificed and the tested skin was taken and then fixed in 10% formaldehyde. After that, the samples were embedded in paraffin, sectioned, and HE stained, followed by histologic examination. Different changes in complete skin group or damaged skin group in the 2 dosage forms were examined under a microscope.

Transdermal permeation and retention studies

The SD rats were sacrificed by cervical dislocation. Then we immediately removed the hair on the abdomen and abdominal skin was cut out. After subcutaneous adipose tissues were removed, the cleared skin were rinsed by pure water and normal saline repeatedly, and then cut into pieces of appropriate size, and stored in cold.

In vitro transdermal experiments

Modified Franz diffusion apparatus was used to study the *in vitro* transdermal penetration of different preparations, with a receptor compartment volume of 5 ml and an effective area of 0.85 cm^2. The SD rat's abdominal skin was fixed between donor compartment and receptor compartment. Cuticle layer contacting with drugs was located in the donor compartment. In the experiments, the donor compartment was added of the same amount of cubic cubosome, ordinary gel and commercial ointment, respectively. The recirculated water was set at 37±0.5°C. The receptor compartment was filled with phosphate buffer solution (PBS, pH 7.4) at similar temperature and the solution in the receptor compartment was stirred at 300 rpm. The different formulations (paeonol cubic cubosome 0.5 ml, paeonol ordinary gel 0.5 g, paeonol commercial ointment 0.5 g) were placed in the donor compartment. Samples (1 ml) were withdrawn at 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 h from the receptor compartment and replaced immediately with an equal volume of fresh pH 7.4 PBS at 37°C. During the experiment, attention was paid to the bubble in the receptor compartment. The collected samples were analyzed by HPLC after being filtered through a 0.45-μm microporous membrane. By comparing the 3 preparations for transdermal permeation behavior within 24 h, the per unit accumulation osmotic quantity area of the skin was calculated. The cumulative osmotic quantity of paeonol (Qn) that permeated across the skin was plotted versus time (t). The slope of the linear portion of the graph was calculated. The steady-state infiltration rate J_s ($ug \cdot cm^{-2}h^{-1}$) was determined according to:

$$J_s = dQn/A \times dt$$

Where dQn/dt is the liner portion of the slope (μg/h), A is the skin surface area.

In vitro retention experiments

Cuticle and epidermis/dermis were examined for drug retention in this experiment. After the *in vitro* transdermal penetration study, the diffusion apparatus was dismantled, and the skins were reused and washed with normal saline quickly to remove the remaining drug. The skins were then dried with filter paper, and 3M tapes were used to collect the cuticle samples by taping and stripping away the cuticle layer 15 times. The remaining skin was the epidermis/dermis layer. The 3M tapes with cuticle samples and epidermis/dermis samples were cut into pieces and tissue-homogenized. After that, the samples were mixed with 5 mL methanol and ultrasonicated for 30 min. Then the extracts were centrifuged at 4000 rpm for 30 min and 20 μl of the supernatants were injected for HPLC analysis after filtration by a 0.45-μm microporous membrane.

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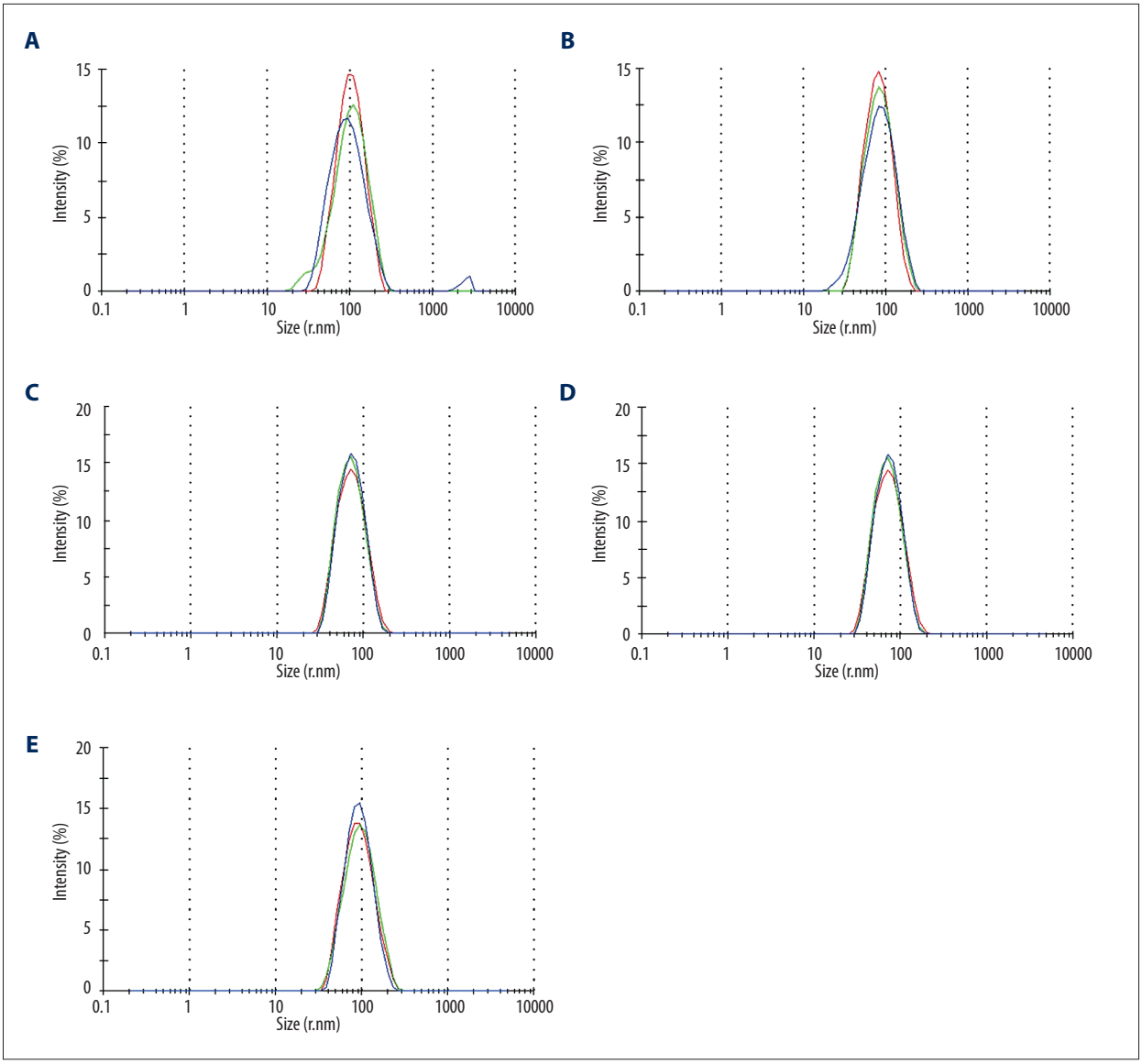

Figure 2. Size distribution of paeonol cubosomes and blank cubosomes. Homogenization conditions: (**A**) 800 bar for 6 cycles; (**B**) 800 bar for 9 cycles; (**C**) 800 bar for 12 cycles; (**D**) 1200 bar for 9 cycles; (**E**) 800 bar for 9 cycles of blank cubosomes.

Results

Optimization of the self-assembled cubic liquid crystalline nanoparticles containing paeonol

Effects of conditional constructions on particle size and zeta potential of cubic liquid crystalline nanoparticles

It was reported in the literature that the cubic liquid crystalline system can coat drugs with different polarity and doses. In order to obtain the best cubosome particles of the system, we investigated the influence of experimental parameters, such as homogenization pressure and number of homogenization cycles, on the morphological and dimensional characteristics of the cubosomes. As shown in Figure 2, there were a few particles larger than 1 μm found in cubosomes, which were homogenized at 800 bar for 6 cycles. The homogenization cycles were increased to 9 at 800 bar and the cubosome particle size and size distribution were obtained. Further increase of the homogenization cycles to 12 at 800 bar did not show a significant effect on the change of the particle size. Subsequently, we increased the homogenization pressure to 1200 bar from 800 bar for 9 cycles, and there was not much change compared with the homogenization cycles of 9 at 800 bars. The same method was applied to the cubosomes without drugs. Before applying the samples to Zeta sizer for detection, we investigated the dilution ratio of 20, 50, 100, and 200 multiples in deionized water. After examination of each dilution,

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Table 1. Results of particle size under different homogenization conditions.

Cubosome dispersion	Mean ±SD	Polydispersity index (PDI)
800 bar for 6 cycles	85.18±4.91	0.19±0.04
800 bar for 9 cycles	83.74±1.56	0.16±0.004
800 bar for 12 cycles	82.45±1.12	0.15±0.03
1200 bar for 9 cycles	84.33±2.44	0.14±0.02
Blank cubosome	96.02±2.05	0.15±0.01

Table 2. Particle size and zeta potential of paeonol cubosomes and blank cubosomes.

Sample	Average size (nm)	Zeta potential (mv)	PDI
Paeonol cubosome	84.65±2.08	–33.6±0.96	0.13±0.03
Blank cubosome	93.57±1.21	–40.8±1.29	0.18±0.04

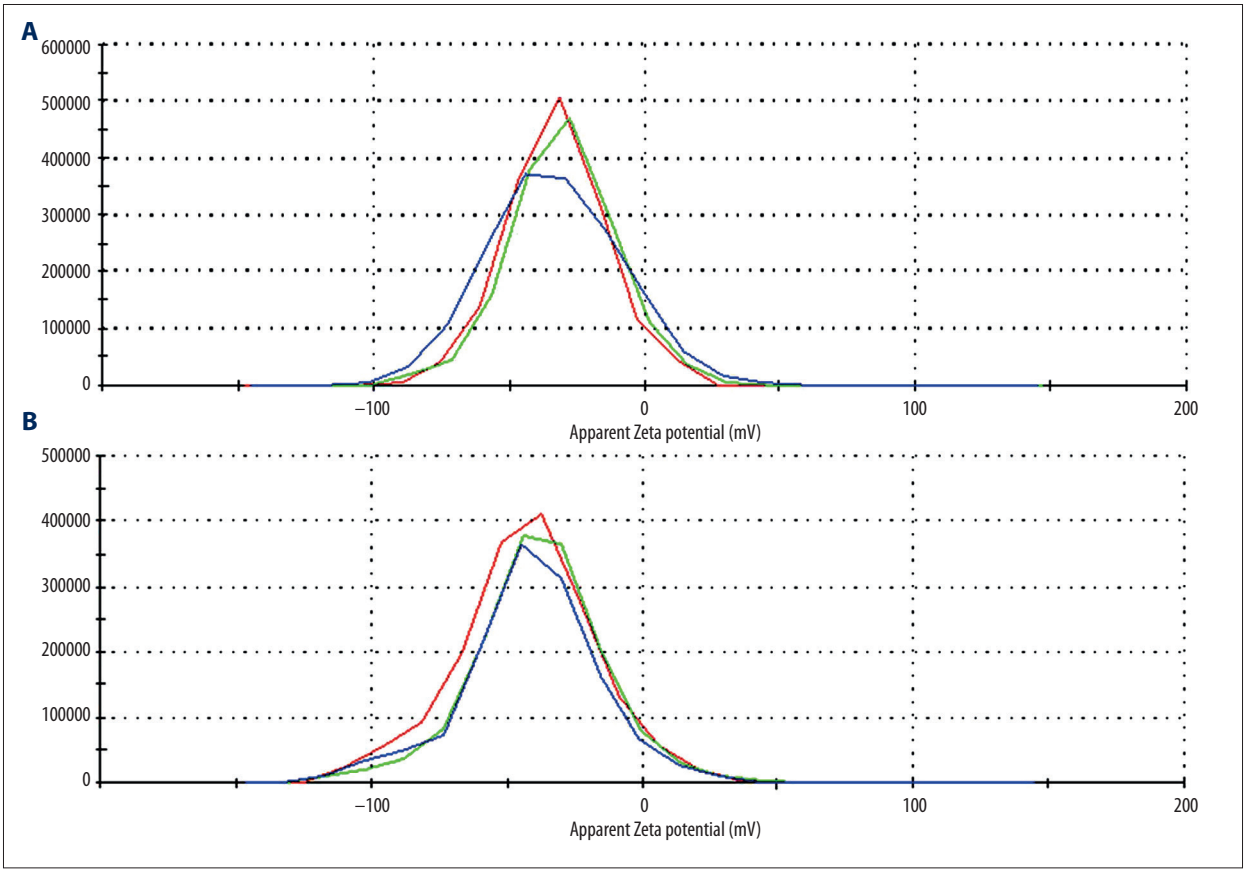

Figure 3. Zeta potential distribution of paeonol cubosomes (**A**) and blank cubosomes (**B**).

we found that 100 and 200 multiple dilutions were of better performance and finally choose 100 multiple dilution as the working dilution.

Based on the best optimized formulation, the results of the particle size of blank cubosome and cubosome containing paeonol at 800 bar for 9 cycles are shown in Table 1. The particle size of paeonol cubosome is a little smaller than that of the blank one. Table 2 shows that the absolute zeta potential value of the blank cubosome is a little bigger than that of the paeonol cubosome. The mean and standard deviation (SD) of particle diameter (Table 1), zeta potential data (Table 2), and

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Table 3. Effects of paeonol content on particle size and zeta potential of the cuosomes (n=3).

Paeonol (g)	Mean particle size (nm)	PDI	Zeta potential (mv)
0.24	85.51±2.19	0.12±0.02	–26.5±1.04
0.32	84.29±1.50	0.09±0.01	–31.4±1.57
0.40	84.33±2.97	0.16±0.02	–38.4±2.36

Table 4. Effects of peaonol content on entrapment efficiency (n=3).

Paeonol (g)	Entrapment efficiency (%)	Actual drug-loading (%)
0.24	90.89±2.09	6%
0.32	93.28±3.66	8%
0.40	91.37±2.84	10%

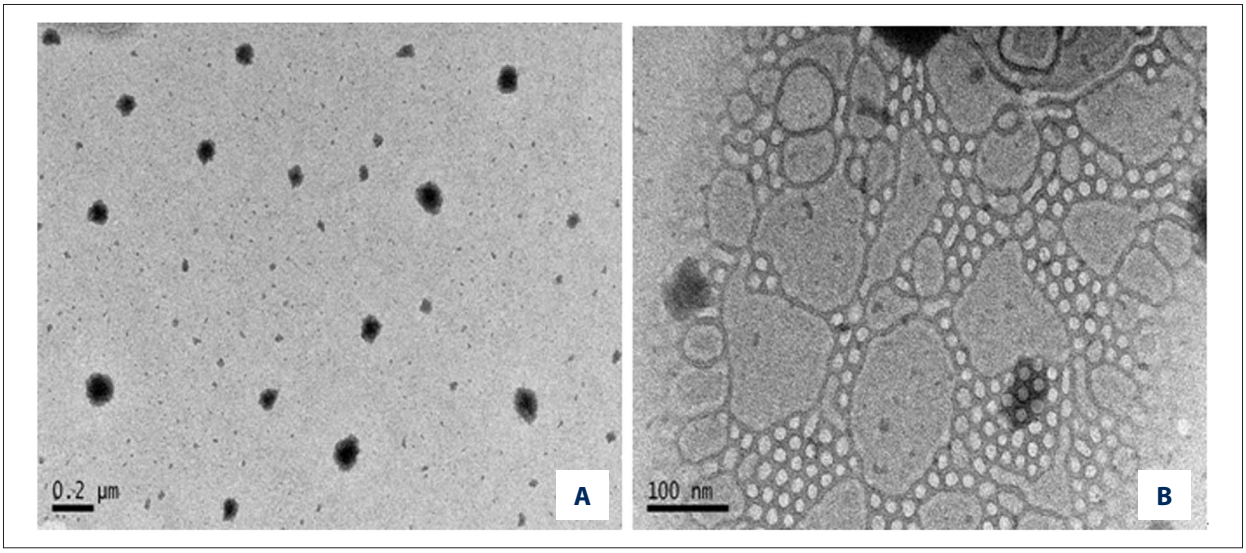

Figure 4. Distribution and the shape of paeonol GMO-based cubosomes (**A**) and the inner structure of paeonol GMO-based cubosomes (**B**).

zeta potential distribution (Figure 3) for paeonol cubosomes and blank cubosomes were as follow:

Effects of paeonol loading on particle size and zeta potential

The influence of loading different doses (0.24 g, 0.32 g, and 0.4 g) of paeonol to the cubic crystal system on the particle size and zeta potential during sample preparation was investigated under condition of homogenization at 800 bar for 9 cycles. The results in Table 3 indicated that there was not much difference in particle sizes and zeta potentials of samples with increased doses of paeonol from 0.24 g to 0.4 g. The tendency of the change in the data was consistent with those shown in Tables 1 and Table 2. With increases in dose, there was not much change in the particle size,whereas absolute zeta potential value increased.

Effects of paeonol loading on encapsulation efficiency

Paeonol was encapsulated or entrapped in GMO-based cubosomes. Table 4 shows that the entrapment efficiency of samples with different doses was above or close to 90%, which indicated that the system had a high coating rate with high dose loading [13,14,26]. The influence of loading different paeonol doses (0.24 g, 0.32 g, and 0.4 g) to the cubic crystal system on entrapment efficiency during sample preparation was investigated under condition of homogenization at 800 bar for 9 cycles. When the dosage increased to 10%, the drug entrapment efficiency decreased because extra high dosage can affect opposite party liquid crystal layers of lipid molecules, and can also likely change the nanoparticle morphology and structure.

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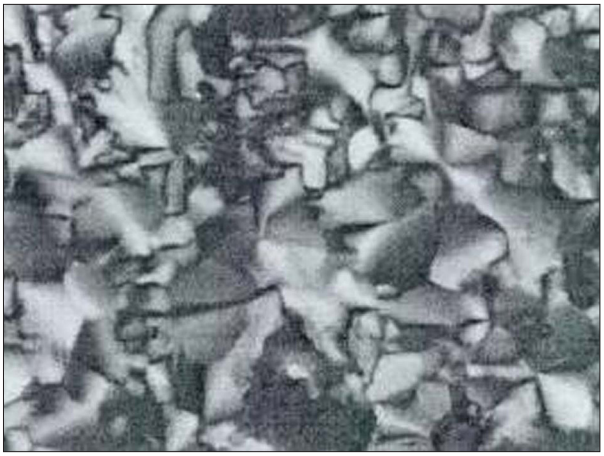

Figure 5. PLM of paeonol GMO-based cubosomes.

Characterization of GMO-based cubosomes

Transmission electron microscopy (TEM)

The samples prepared under condition of homogenization at 800 bar for 9 cycles were examined by TEM to investigate the inner structure of these self-assembled nanoparticles. Figure 4A shows that the paeonol cubosomes are nearly perfectly uniform in size and are evenly distributed, and the shape of the cubosomes is similar to balls, about 80 nm in diameter. Figure 4B show the cubic crystal special inner structure. The internal structure of the nanoparticles is a 3-dimensional extension continuous network with double water phase and unique double channel [11].

Polarized light microscope (PLM)

The PLM provides information on characterization of molecular polarization based on liquid crystal optical birefringence phenomenon. Cubic liquid crystal molecules have an isotropic arrangement and thus have no birefringence, so the sight field is dark. As shown in PLM photo of Figure 5, the optical characteristic of the sample in the photo is isotropic and there is no polarization, which indicates that the crystal is cubic in morphology.

Differential scanning calorimetry (DSC)

Differential scanning calorimetry was a rapid and reliable method to screen paeonol, P407, GMO with P407, the mixture of paeonol and GMO with P407, and the preparations of paeonol cubosome and blank cubosome.

Furthermore, DSC can show details of the maximum absorption with possible interactions. The DSC thermograms of Paeonol (A), GMO and P407 (B), their mixture (C), and the preparation of paeonol cubosome (D) are shown in Figure 6. As shown in

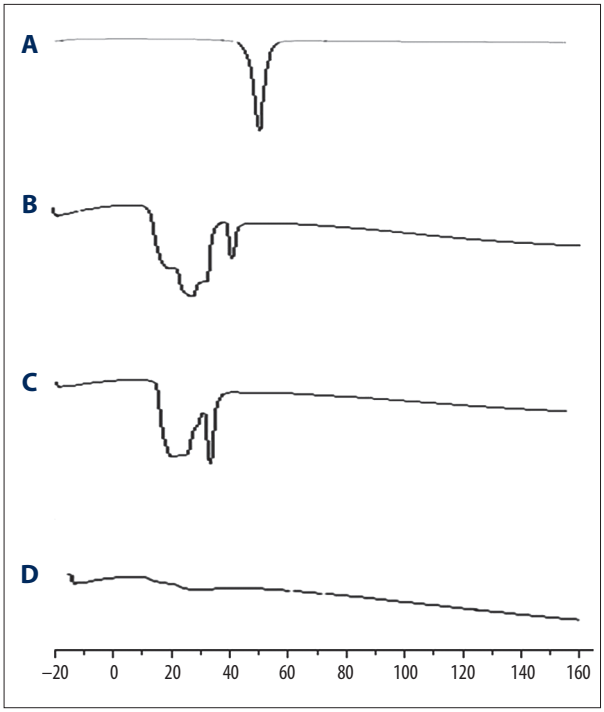

Figure 6. The DSC thermograms of Paeonol (**A**), GMO and P407 (**B**), physical mixture (**C**), paeonol cubosome (**D**).

Figure 6D, the characteristic peaks of the drug disappeared, which indicated the formation of the cubosome.

Storage stability studies of paeonol cubosomes

Storage stability of paeonol cubosomes was tested using samples loaded with 4 different doses of drug. The amount of drug loaded in each sample was determined before the test. After that, the samples were placed in the storage sites. After 24 h, there was no major change observed for all of the samples. Subsequently, after storage for more than 1 month, there was a slight change in the samples. The change of the cold-temperature (–20°C) sample was greater than in the low-temperature (2–8°C) and room-temperature samples. The main change was in the drug amount, decrease by about 5%. There were few major changes in particle size, zeta potential, and encapsulation efficiency (Supplementary Table 1), indicating that the system was relatively stable at low temperature and room temperature during 1 month.

Stimulating studies

The scores of the stimulating studies shown in Tables 5 and 6 were based on the skin irritation reaction score standard and the skin stimulus intensity evaluation standard. The results show that the cubosomes had no irritation in the complete skin group but had mild irritation in the damaged skin group, and the irritation disappeared after 24 h. The commercial

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Supplementary Table 1. Storage stability of paeonol cubosome (n=3).

Group		Drug amount (μg/ml)	Particle size (nm)	Zeta potential (mv)	Encapsulation efficiency (%)
Before experiment		21.60±0.52	82.09±2.03	–31.2±2.61	92.35
After experiment	24 h	21.54±0.36	82.61±1.37	–30.5±0.96	91.76
	A month at –20°C	16.13±0.55	83.56±3.95	–35.7±3.64	90.02
	A month at 2–8°C	20.85±0.38	80.12±2.28	–33.5±1.56	92.07
	A month at room temperature	21.27±0.19	82.19±1.33	–32.1±1.87	92.15

Table 5. The evaluation result of the complete skin group (n=4).

Market ointment group				Cubosome group			
Time	Score	Number	Result	Time	Score	Number	Result
1 h	1.25	4	Mild irritation	1 h	0.25	1	No irritation
24 h	0.25	1	No irritation	24 h	0.25	1	No irritation
48 h	0.25	1	No irritation	48 h	0.00	0	No irritation
72 h	0.00	0	No irritation	72 h	0.00	0	No irritation

Table 6. The evaluation result of the damaged skin group (n=4).

Market ointment group				Cubosome group			
Time	Score	Number	Result	Time	Score	Number	Result
1 h	2.00	4	Mild irritation	1 h	1.25	3	Mild irritation
24 h	1.00	3	Mild irritation	24 h	0.25	1	No irritation
48 h	0.25	1	No irritation	48 h	0.00	0	No irritation
72 h	0.0	0	No irritation	72 h	0.00	0	No irritation

ointment had mild irritation up to nearly 48 h in the damaged skin group. Compared with the commercial ointment group, the stimulus of the cubosome group was of no obvious local irritation, and was less than in the commercial paeonol ointment group.

In the skin irritation test for multiple dosing as shown in Figure 7, the microscopic examination of complete skin group for both dosage forms indicated that there was no degeneration, necrosis, or congestion, and no inflammatory cell or blood vessel infiltration. However, the damaged skin groups both had moderate inflammatory cell infiltration and cell thickening, but the tissue structure of the contact surface in skin was normal. For the damaged skin group, the stimulating degree of paeonol cubosome treatment was less than that of the commercial paeonol ointment treatment. After stopping the application of the drug, the skin irritation and stimulation were restored. The results were in conformity with the requirements for the degree of stimulation for tropical preparations to the skin. The skin stimulating test result shows that the tropical treatment is safe, and justifies further development and application of drugs for external use.

Transdermal permeation and retention studies

Figure 8 showed that transdermal permeation of paeonol cubosome is much greater than that of paeonol ordinary gel but not commercial ointment (P=0.039 and 0.082, respectively). The failure to observe a significant difference between paeonol cubosome and commercial ointment may be caused by variation of the results. More tests may be performed in the future to observe the transdermal permeation results. Twenty-four hours after *in vitro* the transdermal permeation test, we continued to conduct the retention study. Table 7 shows the results.

Paeonol cubosome and paeonol commercial ointment were examined in the retention study. The results show that the retention of paeonol cubosome was extremely significantly higher than that of paeonol commercial ointment (P<0.01) when

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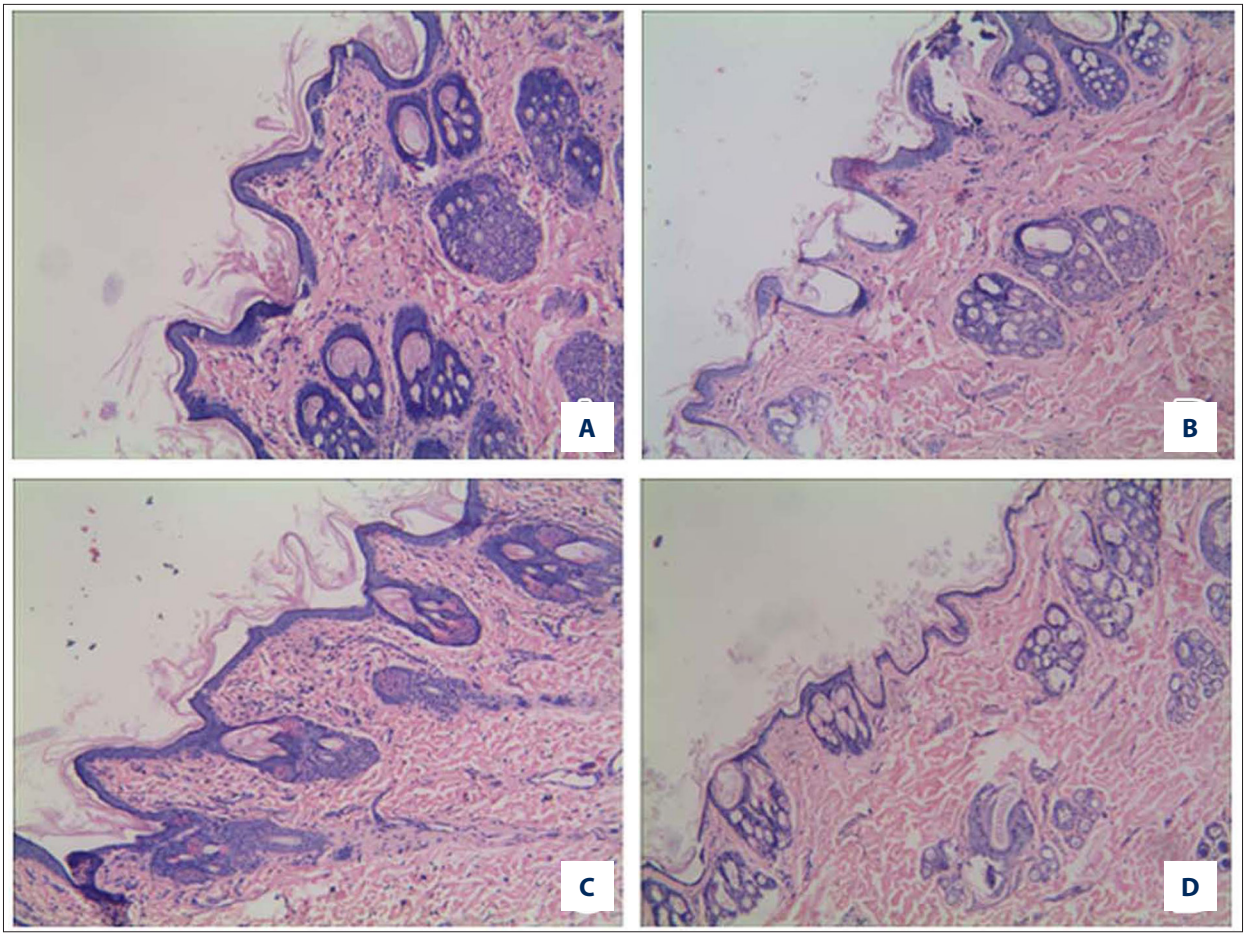

Figure 7. The figures of pathological skin irritation test: (**A**) Commercial ointment group of damaged skin; (**B**) Commercial ointment group of complete skin; (**C**) Cubosomes of damaged skin; (**D**) Cubosomes of completed skin.

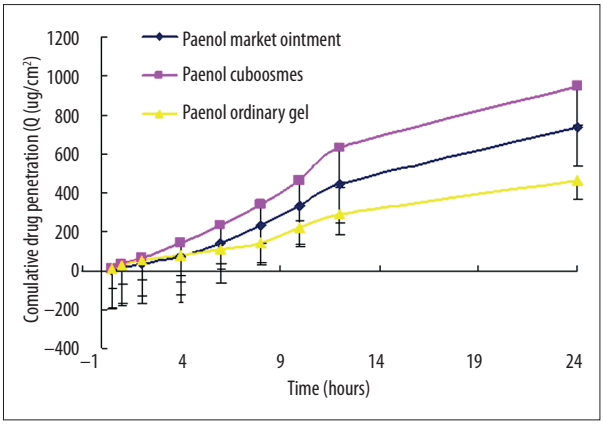

Figure 8. The accumulated permeation studies of paeonol cubosome, commercial ointment and paeonol ordinary gel in SD rats.

analyzed in SPSS 16.0 software, which indicates that the performance of cubic crystal targeting was good; thus, the concept of skin targeting [9,10] was proposed. The paeonol cubosomes not only promoted drug absorption in the skin, but also reduced the drug entering the circulation. In future experiments, we will use micro-dialysis to characterize the drug distribution in subcutaneous tissues and investigate the therapeutic effect of the drug on the local lesion cells or tissues in order to improve the therapeutic index. In the literatures it was reported that the smaller the particle size was, the greater the drug could be retained in the skin [27,28]. The cubic liquid crystal carrier system technology can improve drug targeting for skin application, which is a promising novel transdermal drug delivery system and an important topic under intensive research nowadays.

The experiment confirmed that the paeonol cubic crystal nanoparticles can enhance the transdermal osmotic quantity significantly, and the drug-loaded cubic crystal system has a good transdermal absorption performance, consistent with previous literature reports [29]. It was indicated in the experiment that, after 24 h, the amount of paeonol cubosome in the cuticle layer and epidermis/dermis is significantly higher than that of paeonol commercial ointment. Slow-controlled release proved to be a characteristic of cubic crystal nanoparticles. We

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Table 7. The retention results of paeonol cubosomes and paeonol market ointment in skin and cuticle after 24 h (n=3).

Samples	Epidermal/dermal (ug)	Cuticle (ug)
Paeonol cubosomes	236.72±11.46	92.79±7.45*
Paeonol market ointment	159.33±9.38	54.18±5.90*

* P<0.01.

have already completed the experiment with skin *in vitro*, and then we will conduct the *in vivo* experiments in the future study.

Discussion

Paeonol, one of the main active ingredients from the barks of *Paeonia suffruticosa Andr.*, was demonstrated to have many biological activities, including anti-atherosclerosis, anti-inflammation, anti-allergy, and antitumor effects [1]. Due to avoidance of adverse effects, the paeonol transdermal drug delivery system is widely used, with long-lasting effect. In recent years, several drug carriers, such as microsponge [4], proniosomes [5], and the liposome [6], have been studied in the transdermal delivery of paeonol. However, the cubic crystal nanoparticles loaded with paeonol has not been reported previously. In this study, we characterized the properties of the self-assembled cubic liquid crystalline nanoparticles containing paeonol and showed that the nanoparticles could reduce the irritation in the skin stimulating test, suggesting they might be a promising system of good tropical preparation for skin application.

The cubic liquid crystalline system is recently recognized as a new type of transdermal delivery system. Previous studies proposed the concepts of skin-targeted and epidermis-targeted systems to promote drug infiltration and absorption in the skin or skin layer, and reduce the amount of drug entering into circulation [9,10]. In this study, we first performed optimization of the self-assembled cubic liquid crystalline nanoparticles containing paeonol through analysis of the effect of conditional constructions, paeonol loading on particle size and zeta potential, as well as the effect of paeonol loading on encapsulation efficiency. To obtain the best cubosome particles of the system, we investigated the influence of experimental parameters, such as homogenization pressure and number of homogenization cycles, on the morphological and dimensional characteristics of the cubosomes and showed there were a few particles larger than 1 μm found in cubosomes homogenized at 800 bar for 6 cycles. Further increase of the homogenization cycles to 12 at 800 bar did not show significant effect on the change of the particle size. No significant differences of particle sizes and zeta potentials were observed when treated with increased doses of paeonol. Furthermore, the drug entrapment efficiency was decreased when the loading dosage of paeonol was increased to 10%.

In recent years, a glycerol monoolein (GMO) and water system have been identified as the most advantageous cubic-phase system. The cubic crystal nanoparticles delivery system of GMO/water complex has various comprehensive advantages over microemulsion/nanoemulsion and liposomes [22], especially in external use. In this study, we also characterized GMO-based cubosomes. Transmission electron microscopy analysis showed that the paeonol cubosomes are nearly perfectly uniform in size and are evenly distributed, and the shape of the cubosomes is similar to balls, about 80 nm in diameter. In addition, the internal structure of the nanoparticles is of 3-dimensional extension, with a continuous network with double water phase and unique double channel. Cubic liquid crystal molecules are an isotropic arrangement with no birefringence, and cubic in morphology, as demonstrated by polarized light microscope analysis. In the skin irritation test for multiple dosing, there was no degeneration, necrosis, or congestion, and no inflammatory cell and blood vessel infiltration between different doses. Once the drug administration is ceased, the skin irritation and stimulation can be restored. Furthermore, transdermal permeation and retention studies were also performed in this study and showed that transdermal permeation of paeonol cubosome is much greater than that of paeonol ordinary gel but not commercial ointment. Additionally, the retention study demonstrated that retention of paeonol cubosome was extremely significantly higher than that of paeonol commercial ointment, indicating the performance of cubic crystal targeting was good; thus, the concept of skin targeting [9,10] was proposed.

Conclusions

This study aimed to prepare and improve the transdermal drug delivery system for paeonol by using the self-assembled paeonol cubosomes, which should increase drug bioavailability in the skin. Compared with the commercial ointment, the self-assembled cubosomes allowed sustained drug release for up to 24 h, and the irritation was smaller than that of commercial ointment. Thus, cubic phase of GMO and water is a promising carrier for the delivery of paeonol. The paeonol also has the pharmacologic effect of inhibition of pigmentation. These properties indicate that it is a new drug-loaded system with excellent pharmaceutical and cosmetic properties, which may need continued research. However, the mechanism of the drug penetration is still unknown and further studies on these aspects are still necessary.

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