

Preparation of tripterine nanostructured lipid carriers and their absorption in rat intestine

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The purpose of this study was to develop an optimized nanostructured lipid carrier formulation (NLC) for tripterine, and to estimate the potential of NLCs as oral delivery system. Tripterine-loaded NLCs were prepared by the solvent evaporation method. The average drug entrapment efficiency, particle size and zeta potential of the optimized tripterine-loaded NLCs were $78.64 \pm 0.37\%$, 109.6 ± 5.8 nm and -29.8 ± 1.3 mV, respectively. The tripterine-loaded NLCs showed spherical morphology with smooth surface under the transmission electron microscope (TEM). The crystallization of drug in NLC was investigated by differential scanning calorimetry (DSC). The drug was in an amorphous state in the NLC matrix. According to the *in vitro* release study, the tripterine-loaded NLCs showed a delayed release profile of tripterine. The rat intestinal perfusion model was used to study the absorption of tripterine solution and tripterine-loaded NLCs. The P_{eff}^* (effective permeability) of tripterine-loaded NLCs in the duodenum, jejunum, ileum and colon was approximately 2.1, 2.7, 1.1, 1.2-fold higher than that of tripterine solution, respectively. The 10%ABS (percent absorption of 10 cm of intestine) of tripterine-loaded NLCs in the duodenum, jejunum, ileum and colon was approximately 2.2, 2.3, 1.2, 1.3-fold higher than that of tripterine solution, respectively. The intestinal toxicity of tripterine formulated in the NLCs was investigated and compared with the tripterine solution by the MTT assay with Caco-2 cell models. According to the result, the tripterine-loaded NLCs could greatly decrease the cytotoxicity of the drug. In conclusion, the NLC formulation remarkably improved the absorption of tripterine and showed a better biocompatibility.

1. Introduction

Tripterygium wilfordii Hoog f. (*Celastraceae*) is used in traditional Chinese medicine for treatment of swelling, fever, chills, sores, joint pain, inflammation and rheumatoid arthritis. Tripterine (Fig. 1) is one of the main bioactive components of *Tripterygium wilfordii* Hoog f., and now is widely used in chronic nephritis, thrombocytolytic purpura and dermatologic diseases in Chinese medicine (Radhamani et al. 2011). Recently, studies have shown that tripterine has a significant effect on the treatment of immune diseases (Li et al. 2008, 2005), skin diseases (Brinker et al. 2008) and cancer (Chang et al. 2003; Radhamani et al. 2011). However, tripterine has a low bioavailability due to its negligible solubility in water. Tripterine also has many severe adverse effects, e.g. diarrhea, headache, nausea and infertility, especially if used at high concentrations (Lehtonen et al. 2010). Therefore, in order to overcome these deficiencies, it is necessary to design a formulation possible to improve the oral absorption and bioavailability as well as reducing the side effects of tripterine.

Lipid-based drug delivery systems are expected as promising oral carriers because of their potential to increase the solubility and improve oral bioavailability of poorly water-soluble and/or lipophilic drugs (O'Driscoll et al. 2008). In recent years, as a new generation of lipid nanoparticles which consists of a mixture

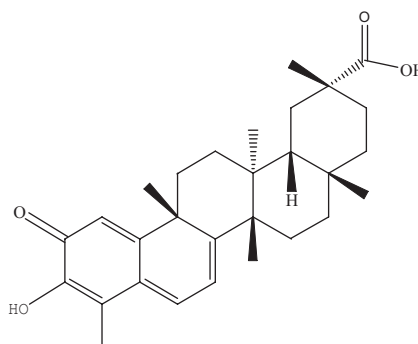


Fig. 1: Chemical structure of tripterine

of different lipid molecules, nanostructured lipid carriers has been developed to overcome the disadvantages of traditional lipid nanoparticles such as limited drug loading, risk of gelation and drug leakage during storage caused by lipid polymorphism. NLCs system also possesses many advantages of traditional lipid nanoparticles, such as good biocompatibility, controlled drug release, and the possibility of production on large industrial scale (Müller et al. 2002a, b). Lipid formulations loaded with poorly water-soluble drugs for the oral route have been investigated and

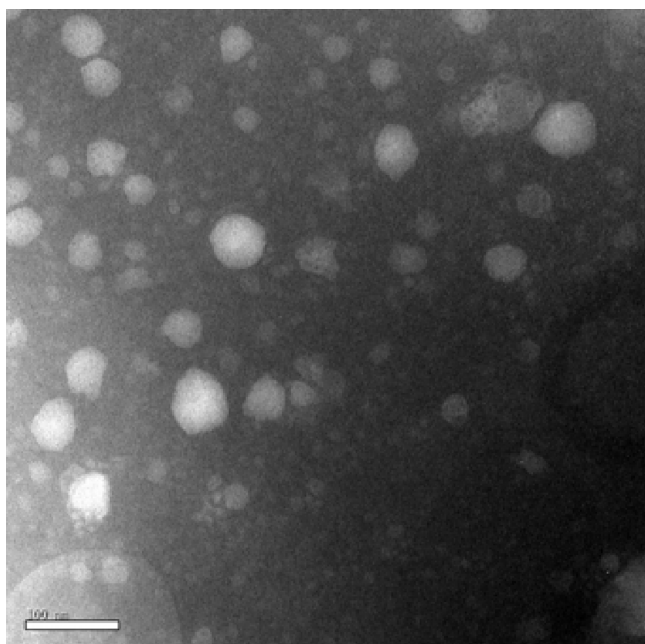


Fig. 2: TEM photograph of tripterine-loaded NLCs (NLC-5)

reported to improve the oral bioavailability of drugs, but there are only few reports on NLC systems for oral administration yet. In view of this, we made efforts to develop tripterine-loaded NLCs and to see if the nanostructured lipid carriers could increase the absorption of tripterine.

The aim of this study was to develop a NLC delivery system as a potential oral formulation of tripterine and assess its intestinal absorption and toxicity. In this study, stearic acid (SA) and isopropyl myristate (IPM) were chosen as the solid and liquid cores. A variety of different emulsifiers were used to prepare NLC, including Pluronic F68 (PF68), Tween-80, D- α -tocopherol polyethylene glycol succinate (TPGS), and soybean lecithin (SLT). It has been reported that TPGS is a good emulsifier in preparation of polymeric nanospheres (Mu et al. 2002), but there is no report on TPGS for NLCs. Thus in this study, we tried to use TPGS as the emulsifier in NLCs system for better preparation. The physicochemical characteristics of the obtained NLCs, such as the drug loading capacity, encapsulation efficiency, size, zeta potential, and differential scanning calorimetry (DSC) results were investigated. According to the result, we chose superordinary NLCs for further investigation. Here, we used a rat intestinal perfusion model to study the absorption mechanisms of tripterine-loaded NLCs, because this model is routinely used to investigate drug absorption and is recognized by FDA as viable models of human intestinal absorption (Yan et al. 2008). Finally, the formulation of NLC was evaluated on the drug release properties *in vitro* and the intestinal toxicity was determined by cell viability experiment.

2. Investigations, results and discussion

2.1. Particle size and zeta potential measurements

Tripterine-loaded NLCs contain 4% (wt%) tripterine. Lipid nanoparticles were produced by the solvent evaporation method. Stearic acid (SA) was used as a solid matrix for the nanoparticles. Isopropyl myristate as the liquid matrix was mixed with stearic acid to form NLCs. The proportion of SA and IPM was set at 3:1 wt%. SLT, Tween-80 and TPGS were used as emulsifiers of nanoparticles in different systems. The results showed that different types of emulsifiers had a great influence on the quality of NLCs. When SLT, Tween-80 and TPGS were used

as emulsifiers separately (NLC-1, NLC-2, NLC-3), the particle size of NLCs was increased and the zeta potential values of NLCs were above -20 mV, which indicated that a bad stability of NLCs. Thus the emulsifier matrix was selected for the nanoparticles. As shown in Table 1, NLC-4 and NLC-5 had smaller particle size than the other formulations, which was 93.7 ± 6.5 nm and 109.6 ± 5.8 nm, respectively, both of them were less than 120 nm. Surface charge has an important influence on the stability of a nanoparticle system. Generally, high surface charge reflected stronger repulsion interactions among nanoparticles. The zeta potential values of different NLCs was between -11.9 ± 2.1 mV and -30.2 ± 0.9 mV, as a result, the NLC-4 and NLC-5 could be more stable in the aqueous dispersion environment than NLC-6. With respect to particle size and zeta potential, the formula of NLC-4 and NLC-5 was better than the others. The mean particle size, zeta potential and entrapment efficiency (EE) of the resulting products are reported in Table 1.

2.2. Visualization by transmission electron microscopy (TEM)

The result of TEM imaging of tripterine-loaded NLCs (NLC-5) is shown in Fig. 2. It was obvious that the particles had a spherical shape with smooth surface and without any aggregation or adhesion.

2.3. Drug encapsulation efficiency

An important issue with respect to the use of lipid nanoparticles as drug carriers is their capacity for drug loading. As summarized in Table 1, the entrapment efficiency of NLC-4, NLC-5 and NLC-6 was between $32.95 \pm 0.42\%$ and $78.64 \pm 0.37\%$. When SLT and TPGS were used as the emulsifier matrix (NLC-5), the drug loading was approximately 2.4, 1.9-fold higher than NLC-4 (SLT and Tween-80 group) and NLC-6 (TPGS and Tween-80 group), respectively. The entrapment efficiency of NLC-1, NLC-2 and NLC-3 was not determined, because they were easy to emerge floccule when SLT, Tween-80 and TPGS were used as emulsifiers separately.

Emulsifiers which were composed of TPGS and SLT resulted in higher entrapment efficiency ($p < 0.05$) when compared to formulations with tween-80 and SLT. It was observed that the TPGS system showed greater entrapment efficiency than the Tween-80 system ($p < 0.05$). Although the physicochemical characteristic of the drug molecule plays an important role, the surfactant character also has significant effect on the localisation of the drug molecule. The emulsifier could reduce the leakage of the drug molecule from the oily droplets which could make improvement of the encapsulation efficiency of the drug in the NLCs. In the present case, the bulky and large surface area of TPGS resulting from its big lipophilic alkyl tail (polyethylene glycol) and hydrophilic polar head portion (tocopherol succinate) could effectively protect the diffusion or partition of the hydrophobic tripterine from polymer to external phase. The encapsulation efficiency of tripterine in the NLCs can thus be significantly improved. According to the result of particle size, zeta potential and EE, NLC-5 was chosen for further investigation because of its small particle size, highest EE and good stability.

2.4. Characterization by differential scanning calorimetry (DSC)

DSC has been used to characterize the melting and crystallization behavior of crystalline material like lipid nanoparticles (Hu et al. 2006; Han et al. 2008). In the case of NLC, DSC character-

Table 1: Characterization of the lipid nanoparticles by particle size, zeta potential, entrapment efficiency and drug loading

	SLT (%)	Tween-80 (%)	TPGS (%)	Mean size (nm)	Zeta potential (mv)	EE (%)	DL (%)
NLC-1	20%	0	0	209.4 ± 9.4	-17.8 ± 1.2	/	/
NLC-2	0	20%	0	321.4 ± 14.3	-8.9 ± 1.3	/	/
NLC-3	0	0	20%	195.6 ± 13.7	-3.5 ± 0.8	/	/
NLC-4	10%	10%	0	93.7 ± 6.5	-30.2 ± 0.9	32.95 ± 0.42	1.65 ± 0.02
NLC-5	10%	0	10%	109.6 ± 5.8	-29.8 ± 1.3	78.64 ± 0.37	3.93 ± 0.02
NLC-6	0	10%	10%	138.3 ± 8.2	-11.9 ± 2.1	45.13 ± 0.54	2.08 ± 0.03

Each value represents the mean ± SD (n = 3).

ization can illuminate its structure through the mixing behavior of solid lipids with liquid lipids.

Figure 3 shows the thermograms obtained from bulk material (mannitol, tripterine, SA, IPM, SLT, TPGS, and lyophilized samples (tripterine-loaded NLCs).

The melting peak of the tripterine was around 175.7 °C and 211.4 °C which was shown in the thermograms (Fig. 3A). The presence of tripterine crystalline state was confirmed by the melting peak of tripterine in the physical mixtures II (Fig. 3C). However, the thermograms of the tripterine-loaded NLCs (Fig. 3D) did not show the melting peak of the tripterine. Compared with A, B, C, D, the melting peak of samples in nanometer system was different from physical mixtures. The results indicated that tripterine in the tripterine-loaded NLC was not in crystalline state but in amorphous state and the similar results were reported by Venkateswarlu and Manjunath (2004) and Jennings et al. (2000) stating that lipid nanoparticles could not allow the drug to crystallize.

2.5. *In vitro* release of the drug from lipid nanoparticles

In order to develop a NLC system with general applicability, it is of great importance to understand release mechanism and kinetics. The levels of *in vitro* tripterine release from nanoparticles are shown in Fig. 4. The amount of tripterine released from nanoparticles was plotted as a function of time. Because of the

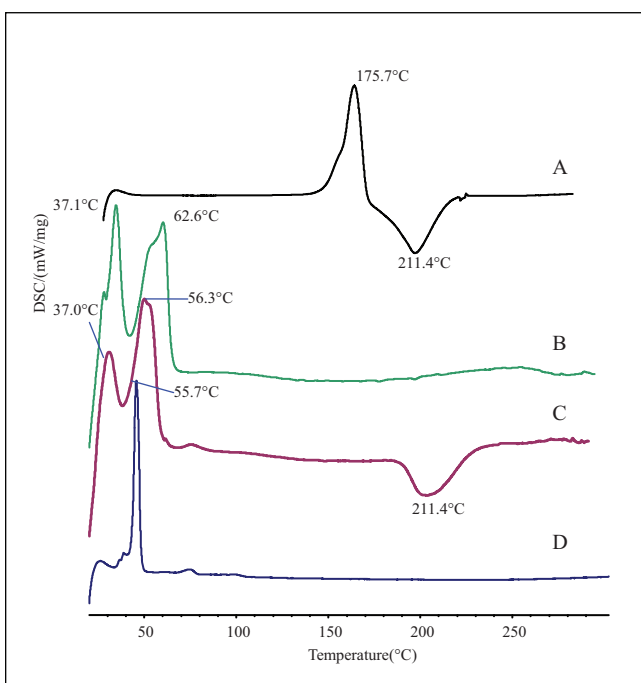


Fig. 3: Overlaid DSC thermograms of tripterine (A), physical mixtures I (B), physical mixtures II (C), tripterine-loaded NLC (D)

low solubility of tripterine in water, tripterine solution mixture of Tween-80 (0.5%) was selected as the control group. The control group showed quick release of tripterine, and the amount of the released drug reached 96.4% after 24 h, which was significantly higher than that of NLCs (77.6%) (Fig. 4). The release profiles of drug from tripterine solution and tripterine-loaded NLC were fitted to zero order kinetics model, first order kinetics model Higuchi kinetics model and Ritger and Peppas kinetics model, respectively (Table 2). Based on the fitting result, the Ritger and Peppas kinetics model ($\ln R = k \ln t + C$) (Ritger and Peppas 1987) was chosen to describe the release profiles of drug from tripterine-loaded NLC because of good correlation ($r = 0.9838$). The value of k was 0.6848 ($0.45 < k < 0.85$), which suggested that tripterine release from NLC was a delayed release process by the effect of the mixing of drug diffusion and lipid matrix erosion.

2.6. Absorption of tripterine-loaded NLCs and tripterine solution in intestine

We used rat intestinal perfusion model to study the absorption of tripterine solution and tripterine-loaded NLCs. The P_{eff}^* and 10%ABS values of tripterine solution and tripterine-loaded NLCs are shown in Table 3. The P_{eff}^* of tripterine solution was 0.319 ± 0.033 , 0.332 ± 0.015 , 0.160 ± 0.021 and 0.126 ± 0.047 in the duodenum, jejunum, ileum and colon, respectively, the P_{eff}^* values were pretty low (< 1), which suggested that tripterine is poorly absorbed from the intestine. For four different intestinal segments, the absorption of tripterine in the duodenum and jejunum were better than that in ileum and colon. Comparatively speaking, the P_{eff}^* of tripterine-loaded NLCs in the duode-

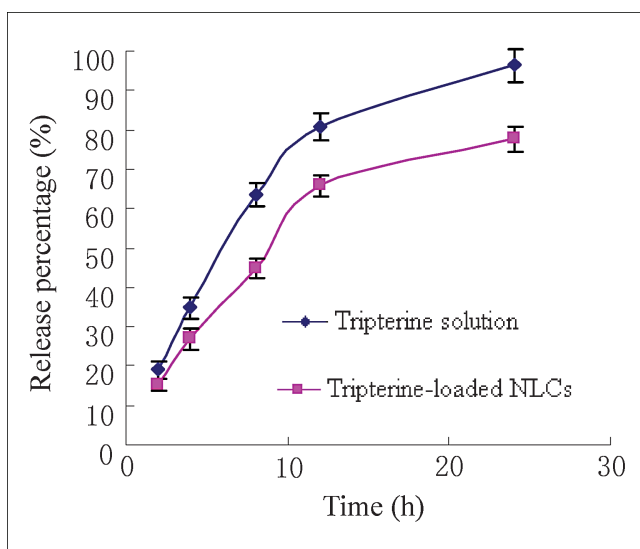


Fig. 4: *In vitro* release profile of tripterine from tripterine solution and tripterine-loaded NLCs. Each value represents the mean ± S.D. (n = 3)

Table 2: Fitting of accumulated release percentage of tripterine solution and NLC

Equation	Tripterine solution	Tripterine-loaded NLC
Zero order equation	$R = 0.039t + 0.2438$, $r = 0.8934$	$R = 0.0278t + 0.1823$, $r = 0.9323$
First order equation	$\ln(1-R) = -0.1432t + 0.1102$, $r = 0.9996$	$\ln(1-R) = -0.0615t - 0.1127$, $r = 0.9529$
Higuchi equation	$R = 0.2287t^{1/2} - 0.0792$, $r = 0.9213$	$R = 0.1873t^{1/2} - 0.0867$, $r = 0.9352$
Ritger- Peppas equation	$\ln R = 0.6761 \ln t - 2.0118$, $r = 0.9473$	$\ln R = 0.6848 \ln t - 2.2801$, $r = 0.9838$

num and jejunum was approximately 1.1, 1.7-fold higher than that of tripterine solution, respectively, and also the 10%ABS of tripterine-loaded NLCs in the duodenum and jejunum was approximately 1.2, 1.3-fold higher than that of tripterine solution. This indicated that intestinal absorption of tripterine was significantly enhanced by NLC, and NLCs showed a promising potential for promoting intestinal absorption of poorly water-soluble drugs.

The possible reasons for low intestinal absorption of tripterine were its poor water-solubility, and the barrier of the physicochemical environment of gastrointestinal tract and intestinal epithelium (Roger et al. 2009). Therefore, we summarized the possible mechanism of the improved intestinal absorption of tripterine by employing NLC formulation. Firstly, NLC can improve the solubility of tripterine and the particle size range of NLC formulation was almost less than 120 nm, the smaller size nanoparticles have efficient gastrointestinal tissue uptake by other routes, like uptake by intracellular pathways. They could gain entry in the tissue through intracellular spaces and especially in larger defects of the mucosa. The reduction in particle

size also resulted in a great increase in surface area of the particles which could increase the contact area of NLC and intestinal epithelium. Meanwhile, the small particle size of NLC was positive to a sufficient and steady absorption in the intestinal tract. Thus it seems that the mechanism of gastrointestinal uptake of these particles is a particle size dependent exclusion phenomena with smaller size particles more likely to be internalized inside the cells and tissue (Desai et al. 1996).

Secondly, NLC was composed of solid and liquid lipids which were structurally similar to fat rich in food. The lipids could induce bile secretion in the small intestine and the tripterine-loaded NLCs were associated with bile salt to form mixed micelles which helped the intact NLCs get into the intestinal epithelium (Plain and Wilson 1984; Müller et al. 2006; Zhuang et al. 2010). The uptake of intact NLCs played a dominant role in the promoted absorption. Another reason of the improved absorption might be attributed to the use of surfactant TPGS in the NLC formulations. TPGS might increase the intestinal epithelial permeability by disturbing the cell membrane and reversibly open the tight junction of intestinal epithelial cell (Bittner et al. 2002).

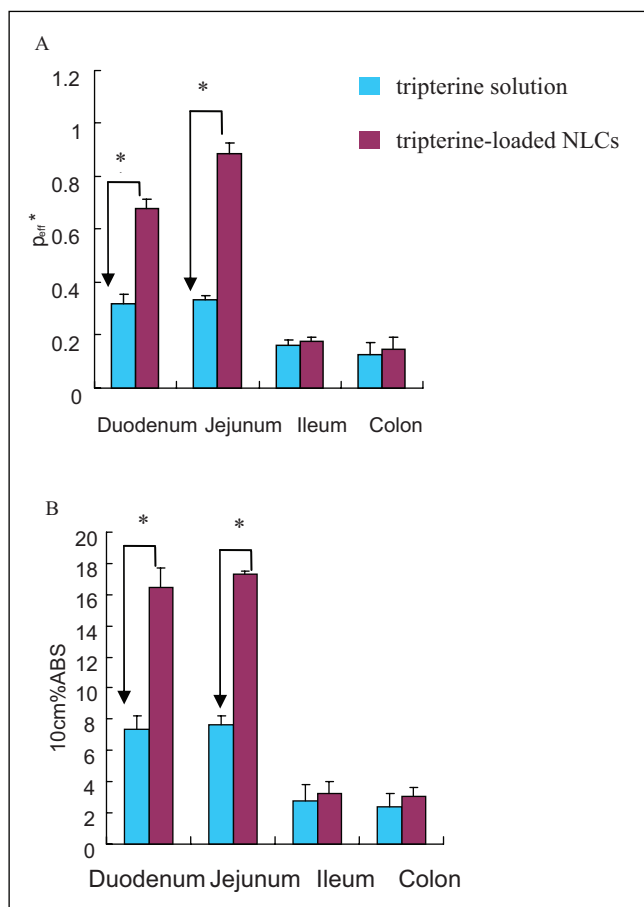


Fig. 5: Comparison of P_{eff}^* and 10cm%ABS between tripterine solution and tripterine-loaded NLCs. Each value represents the mean \pm S.D. ($n = 4$), $*P < 0.05$ (A: P_{eff}^* ; B: 10%ABS)

2.7. *In vitro* cell viability assay

The *in vitro* cytotoxicity of tripterine formulated in NLCs was investigated and compared with the tripterine solution by cell viability experiments. Figure 6 shows the percentage of cell viability of Caco-2 cells incubated with the tripterine formulated in the NLCs versus tripterine solution at different drug concentrations. From the results, we can see that at the same concentration, the tripterine solution produced stronger inhibitory effect on the cells growing than tripterine-loaded NLCs, and the cell viability rates were $9.64 \pm 0.98\%$, $12.23 \pm 1.53\%$ and $13.37 \pm 1.74\%$ at the concentration of 40 $\mu\text{g/mL}$, 20 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$, respectively. Blank NLCs and 0.5% (w/v) Tween-80 solutions

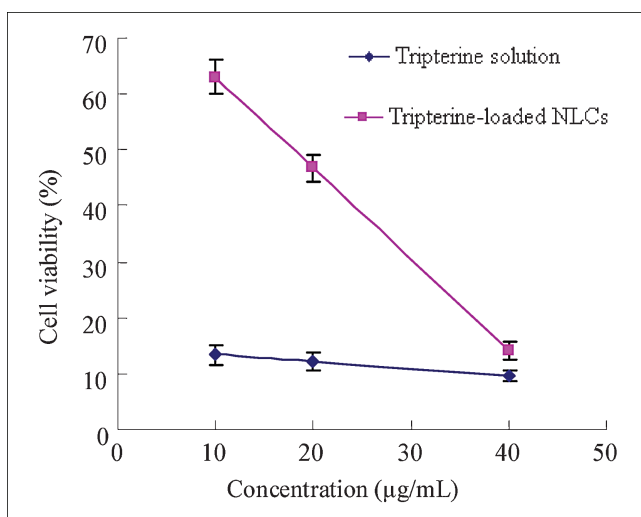


Fig. 6: Cell viability of tripterine solution and tripterine-loaded NLCs on caco-2 cells using the MTT assay. Each value represents the mean \pm S.D. ($n = 4$)

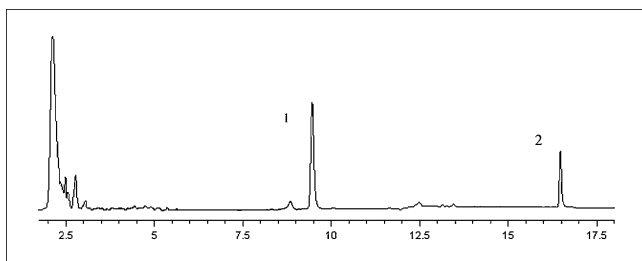


Fig. 7: The HPLC chromatograms of perfusate samples (1. tripterine; 2. prednisolone)

had no significant toxicity on cells, because the cell viability rates of the blank NLCs and 0.5% (w/v) Tween-80 solutions were large than 90%. Tripterine-loaded NLCs greatly decreased the cytotoxicity of the drug, and the percentage of cell viability of tripterine-loaded NLCs at 40 $\mu\text{g/mL}$, 20 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$ was approximately 0.5, 2.8, 3.7-fold higher than that of tripterine solution, respectively. The IC_{50} (50% cell growth inhibition) of tripterine solution was 0.34 $\mu\text{g/mL}$, whereas the tripterine-loaded NLCs was 2.11 $\mu\text{g/mL}$. As depicted in Fig. 6, the cell viability of both tripterine NLCs and solution decreased when the concentration of the drug was increased, and an excellent linear relationship was obtained. The cell viability of tripterine-loaded NLCs had a dramatically increase from 40 $\mu\text{g/mL}$ to 10 $\mu\text{g/mL}$ whereas the cell viability of tripterine solution kepted in a low level, which demonstrated that tripterine-loaded NLCs had lower cytotoxicity than tripterine solution. These suggested that the NLCs were biocompatible with Caco-2 cells and, therefore, well tolerated by the gastrointestinal tract, and the similar results have been reported by Silva et al. (2011), Nassimi et al. (2010), and Rouxhet et al. (2009).

3. Experimental

3.1. Materials and animals

3.1.1. Materials

Tripterine (purity >99%) was provided by ZeLang medicine technology co.ltd (China, Nanjing). Prednisolone (purity >99%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (China). Stearic acid (SA), isopropyl myristate (IPM), Pluronic F68, Tween-80, acetone and ethanol were obtained from Shanghai Chemical Reagent Corporation (Shanghai, China), soybean lecithin (SLT) was supplied by Shanghai Advanced Vehicle Technology Company (Shanghai, China); D- α -tocopherol polyethylene glycol succinate (TPGS) and Hanks' balanced salt solution (HBSS; powder form) were purchased from Sigma. All other materials (typically analytical grade or better) were used as received.

3.1.2. Animals

Male Sprague-Dawley rats weighing between 250 and 300 g were obtained from the SLEK Lab Animal Center of Shanghai (Shanghai, China). The rats were fed with water and a standard diet. The rats were fasted overnight before the day of the experiment.

3.2. Methods

3.2.1. Preparation of lipid nanoparticles

Blank NLCs were produced by the solvent evaporation method. The proportion of SA and IPM was set at 3:1 wt%. SLT, Tween-80, TPGS was used as emulsifiers. Each formulation was dissolved in a mixed solvent of acetone and ethanol, and was added to an aqueous surfactant solution containing of PF68 (1.0 wt%) at 65 $^{\circ}\text{C}$ under gentle magnetic stirring at 1200 rpm for 4 h. An external water bath (0–2 $^{\circ}\text{C}$) was used to maintain the sample temperature for 2 h in order to stabilize the NLC. The total volume of the final product was 100 mL. Tripterine-loaded NLCs which contains 4% tripterine were produced in the same manner. The obtained blank NLCs and tripterine-loaded NLCs dispersions were used for subsequent studies.

3.2.2. Particle size and zeta potential measurements

Particle size and zeta potential of various NLC formulations were measured by photon correlation spectroscopy (Nano-ZS ZEN3600 zetasizer, Malvern Instruments Corp., UK) at 25 $^{\circ}\text{C}$ under a fixed angle of 90 $^{\circ}$ in polystyrene cuvettes. The measurements were obtained using a He-Ne laser of 633 nm. Zeta potential was measured in folded capillary cells using the Nano-ZS ZEN3600 zetasizer. The NLC sample was prepared by diluting the sample with double-distilled water, and the conductivity of the same solution was adjusted to 50 S/cm by 0.1 mmol/L sodium chloride solution for zeta potential measurement. The zeta potential values were calculated using the Smoluchowski equation.

3.2.3. Visualization by transmission electron microscopy (TEM)

The microstructures of NLC, tripterine-loaded NLCs were examined by TEM (Tecnai 12, Philips company, Holland) with negative stain method. Samples were diluted appropriately with aqueous phase before preparation for TEM. Firstly, a drop of the each sample was applied to a copper grid coated with carbon film and air-dried, then 2% (w/v) phosphotungstic acid (PTA) solution was dropped onto the grids. After being negative stained and air-dried under room temperature, the samples were accomplished for the TEM investigation.

3.2.4. Drug encapsulation efficiency

Entrapment efficiency (EE) was determined to assess the extent of tripterine incorporation in the nanoparticles. EE of the tripterine-loaded NLCs were determined by measuring the concentration of the free unloaded tripterine in the aqueous phase of the nanoparticle suspension. Briefly, approximately 0.5 mL of the nanoparticle dispersion was placed in the outer chamber of the filter assembly with molecular weight cut-off of 12K (Pall, USA). The filter assembly was then centrifuged at 5000 rpm (approximately 3000 \times g) for 15 min. The NLCs along with the encapsulated tripterine remained in the outer chamber whereas the aqueous dispersion medium containing the free unloaded tripterine moved to the sample recovery chamber through the filter membrane. After separation, the amount of the free tripterine in methanol was determined by HPLC. The amount of free drug was designated as W_{free} . Equal volume of tripterine-loaded NLCs suspension was accurately taken, dissolved and diluted with methanol. Then, drug content in the resultant solution was determined by HPLC, and the calculated drug amount was designated as W_{total} . The HPLC analytical method for tripterine was described as follows: In brief, the HPLC system included a binary pump, an auto sampler, and an photodiode array detector all from Agilent (USA). A Agilent HC-C18 column (150 \times 4.6 mm i.d.; particle size 5 μm) was used as the stationary phase. The mobile phase was an acetonitrile: 0.4% phosphoric acid (80:20; v/v) mixture at a flow rate of 1 mL/min. The UV detection wavelength was 425 nm (tripterine). Consequently, the drug encapsulation efficiency (EE) and drug loading (DL) could be calculated by the following equations (Luo et al. 2006):

$$\text{EE}\% = \frac{W_{\text{total}} - W_{\text{free}}}{W_{\text{total}}} \times 100\% \quad (1)$$

$$\text{DL}\% = \frac{W_{\text{total}} - W_{\text{free}}}{W_{\text{lipid}}} \times 100\% \quad (2)$$

where W_{free} is the amount of free tripterine, W_{total} is the amount of tripterine in the system, W_{lipid} is the weight of the lipid.

3.2.5. Characterization by differential scanning calorimetry (DSC)

DSC analysis was performed using DSC 204 (NETZSCH, German). A scan rate of 10 $^{\circ}\text{C}/\text{min}$ was employed in the temperature range of 25–300 $^{\circ}\text{C}$. Standard aluminum sample pans were used. About 10 mg sample was taken for analysis. An empty pan was used as reference. Preparation of samples for thermal analysis:

- (A) Tripterine
- (B) Physical mixtures I. SA, IPM, TPGS, SLT, PF68 and mannitol.
- (C) Physical mixtures II. Tripterine, SA, IPM, TPGS, SLT, PF68 and mannitol. The ratio of tripterine to matrix was similar to that of weight ratio in NLC formulation.
- (D) Lyophilized samples. Tripterine-loaded NLCs were frozen at –70 $^{\circ}\text{C}$ for 8 h. Then the samples were freeze-dried at –52 $^{\circ}\text{C}$ and a pressure of 7.5 mmHg during 40 h. The tripterine-loaded NLC powder was collected and stored at 4 $^{\circ}\text{C}$.

3.2.6. In vitro release kinetics of the drug from lipid nanoparticles

The drug released from the carriers was measured using a Franz diffusion cell. A dialysis Tubing (Union Carbide Corporation, with a molecular

weight cutoff of 8000–14000) was mounted between the donor and receptor compartments. The donor medium consisted of 4 mL of vehicle containing tripterine-loaded NLCs. The receptor medium consisted of 10 mL of 0.5% Tween-80 in pH 7.4 buffer to maintain sink conditions. The dialysis tubing was put into Franz diffusion cell which included receptor medium. The stirring rate and temperature were 600 rpm and 37 °C, respectively. At appropriate intervals (2, 4, 8, 12, and 24 h), 1 mL aliquots of the receptor medium were withdrawn and immediately replaced with equal volumes of fresh buffer. The amount of drug released was determined by an HPLC method as described in Section 3.2.4.

3.2.7. Animal surgery

The surgical procedures were approved by the Animal Ethics Committee of Jiangsu Provincial Academy of Chinese Medicine. Anesthesia was induced by an i.m. injection of urethane (0.5 g/mL). After the rat was anesthetized, four segments of intestine, duodenum, jejunum, ileum, and colon (8–12 cm for each of them) were simultaneously cannulated, each with two cannulae. After cannulation, the small intestinal segments were placed carefully into the abdominal cavity, avoiding crimping or kinking of the segments. The incision was then covered by a normal saline-wetted paper towel. A piece of plastic wrap was put on the towel to keep the intestinal segments moist. To keep the temperature of the perfusate constant, the inlet cannulate was kept warm by a 37 °C circulating water bath (Hu et al. 1998; Liu et al. 2006; Jia et al. 2004).

3.2.8. Intestinal absorption of tripterine-loaded NLCs and tripterine solution

This is a single-pass perfusion method. Four segments of the intestine (duodenum, jejunum, ileum, and colon) were perfused simultaneously with a perfusate containing the compound of interest using an infusion pump (Harvard Apparatus, Cambridge, MA, USA) at a flow rate of 0.15 mL/min. After a 30-min washout period, which is usually sufficient to achieve the steady-state absorption, four samples were collected from the outlet cannulae every 30 min afterward. After perfusion, the length of the intestine was measured. The outlet concentrations of the test compounds in the luminal perfusate were determined by HPLC.

The conditions for the HPLC analysis of tripterine in the perfusion samples were as follows: system, Agilent 1100 HPLC with photodiode array detector; Agilent HC-C18 column (150 × 4.6 mm i.d.; particle size 5 μm) was used as the stationary phase (Agilent, Palo Alto, CA, USA). Mobile phase A, acetonitrile; mobile phase B, 0.4% phosphoric acid; gradient, 0–5 min: 30% A, 5–20 min: 30–80% A; flow rate, 1 mL/min; The UV detection wavelength was 425 nm (tripterine), 246 nm (prednisolone as internal standard); injection volume, 20 μL. The HPLC chromatograms of perfusate samples are shown in Fig. 7. In general, these methods were selective and reproducible with day to day variability of less than 3%. The accuracy and precision were greater than 98%. The tested linear response ranges for tripterine was 1 to 40 μg/mL.

3.2.9. Data analysis

In the perfused rat intestinal model, P_{eff}^* and 10%ABS are two representation of the intestinal membrane permeability. P_{eff}^* and 10%ABS of the compounds are calculated using the following equations:

$$P_{\text{eff}}^* = \frac{1 - C_m/C_o}{4Gz} \quad (3)$$

$$10 \text{ cm } \% \text{ ABS} = \frac{(1 - Q_m/Q_o) \times 10}{VL} \quad (4)$$

where C_o and C_m are inlet and outlet concentrations, respectively; Q_m and Q_o are inlet and outlet drug content; V is related with flow rate (Q) and water flux, Gz , or Graetz number ($Gz = \frac{\pi DL}{2Q}$), is a scaling factor that incorporates flow rate (Q), intestinal length (L), and diffusion coefficients (D) to make the permeability dimensionless. C_m was adjusted for water flux, data points were discarded if the water flux exceeded 0.5%/cm of intestine.

3.2.10. In vitro cell viability assay

In the present study, we used the Caco-2 cell line to study the effect of different concentrations of tripterine solution and tripterine-loaded NLCs (40 μg/mL, 20 μg/mL, 10 μg/mL) on cell toxicity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Mosmann 1983; Dolbear and Vanderlaan 1994). Briefly, the cells within the exponentially growing period were harvested and plated in 96-well plates at a concentration of 1×10^4 cells/well for Caco-2 cells (Meca et al. 2010). They were then incubated for 24 h at 37 °C. After overnight incubation, the cells were divided into four treatment groups, including: (a) tripterine solutions;

(b) tripterine-loaded NLCs; (c) 0.5% (w/v) Tween-80 solutions; (d) blank NLCs. Cells in the wells were treated with various concentrations of tripterine and tripterine-loaded NLCs for 36 h. Then, 10 μL of MTT (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. After the supernatant was discarded, 100 μL DMSO was added to each well. The absorbance value at 490 nm was determined using a microplate reader (Naruse et al. 2002) (Bio-Rad Instruments, Hercules, CA, USA). Cell viability rate of the various concentrations were calculated using the following equation and IC_{50} values was obtained using statistical software SPSS v15.0.

$$\text{Cell viability rate} = (T/C) \times 100\% \quad (5)$$

where T means average OD value of the sample well, C means negative control average OD value of the well.

3.2.11. Statistical analysis

One-way ANOVA (SPSS 15.0 software) was used to analyze data for multiple comparisons. Unpaired student's t test (Microsoft Excel) was used to analyze the data when there were only two groups in the experiments. The prior level of significance was set at $p < 0.05$.

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References

- Amselem S (1999) Solid coprecipitates for enhanced bioavailability of lipophilic substances [P]. US: 5891469, 1999-04-06.
- Bittner B, Guenzi A, Fullhardt P, Zuercher G, González RC and Mountfield RJ (2002) Improvement of the bioavailability of colchicine in rats by co-administration of D- α -tocopherol polyethylene glycol 1000 succinate and a polyethoxylated derivative of 12-hydroxy-stearic acid. *Arzneimittelforschung* 52: 684–688.
- Brinker AM, Ma J, Lipsky PE, Raskin I (2007) Medicinal chemistry and pharmacology of genus Tripterium (Celastraceae). *Phytochemistry* 68: 732–766.
- Chang FR, Hayashi KI, Chen IH, Liaw CC, Bastow KF, Nakanishi Y, Nozaki H, Cragg GM, Wu YC, Lee KH (2003) Antitumor agents. 228. fine new agarofurans, Reissantins A-E, and cytotoxic principles from *Reissantia buchananii*. *J Nat Prod* 66: 1416–1420.
- Chen J, Lin H, Hu M (2003) Metabolism of flavonoid via enteric recycling: Role of intestinal disposition. *Pharmacol Exp Ther* 304: 1228–1235.
- Chen Y, Zhao YH, Jia XB, Hu M (2008) Intestinal absorption mechanisms of prenylated flavonoids present in the heat-processed *Epimedium koreanum* Nakai (Yin Yanghuo). *Pharm Res* 25: 2190–2199.
- Desai MP, Labhasetwar V, Amidon GL, Rief RJ (1996) Gastrointestinal uptake of biodegradable microparticles: effect of particle size. *Pharm Res* 13: 1838–1845.
- Dolbear F, Vanderlaan M (1994) Techniques for measuring cell proliferation. In: Tyson CA, Frazier JM (Eds.) *In Vitro Toxicity Indicators, Methods in Enzymology* 1B: 178–200.
- Han F, Li S, Yin R, Liu H, Xu L (2008) Effect of surfactants on the formation and characterization of a new type of colloidal drug delivery system: nanostructured lipid carriers. *Colloids Surf. A: Physicochem. Eng. Aspects* 315: 210–216.
- Hu FQ, Jiang SP, Du YZ, Yuan H, Ye YQ, Zeng S (2006) Preparation and characteristics of monostearin nanostructured lipid carriers. *Int J Pharm* 314: 83–89.
- Hu M, Roland K, Ge L, Chen J, Li Y, Tyle P, Roy S (1998) Determination of absorption characteristics of AG337, a novel thymidylate synthase inhibitor, using a perfused rat intestinal model. *J Pharm Sci* 87: 886–890.
- Jenning V, Thünemann AF, Gohla SH (2000) Characterisation of a novel solid lipid nanoparticle carrier system based on binary mixtures of liquid and solid lipids. *Int J Pharm* 199: 167–177.
- Jia X, Chen J, Lin H and Hu M (2004) Disposition of flavonoids via enteric recycling: enzyme-transporter coupling affects metabolism of biochanin A and formononetin and excretion of their phase II conjugates. *J Pharmacol Exp Ther* 310: 1103–1113.
- Lehtonen M, Paimelad T, Kaarniranta K (2010) Celastrol: Molecular targets of Thunder God Vine. *Biochem Biophys Res Commun* 399: 430–442.
- Li H, Zhang YY, Tan HW, Jia YF, Li D (2008) Therapeutic effect of tripterine on adjuvant arthritis in rats. *J Ethnopharmacol* 118: 479–484.
- Li H, Zhang YY, Huang XY, Sun YN, Jia YF, Li D (2005) Beneficial effect of tripterine on systemic lupus erythematosus induced by active chromatin in BALB/c mice. *Eur J Pharmacol* 512: 231–237.

- Lindmark T, Nikkila T, Artursson P (1995) Mechanisms of absorption enhancement by medium chain fatty acids in intestinal epithelial Caco-2 cell monolayers. *J Pharmacol Exp* 275: 958–964.
- Liu ZQ, Jiang ZH, Liu L, Hu M (2006) Mechanisms responsible for poor oral bioavailability of paeoniflorin: role of intestinal disposition and interactions with sinomenine. *Pharm Res* 23: 2768–2780.
- Luo Y, Chen D, Ren L, Zhao X, Qin J (2006) Solid lipid nanoparticles for enhancing vinpocetine's oral bioavailability. *J Control Release* 114: 53–59.
- Meca G, Ruiz MJ, Soriano JM, Ritieni A, Moretti A, Font G, Manes J (2010) Isolation and purification of enniatins A, A₁, B, B₁, produced by *Fusarium tricinctum* in solid culture, and cytotoxicity effects on Caco-2 cells. *Toxicol* 56: 418–424.
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55–63.
- Mu L, Feng SS (2002) Vitamin E TPGS used as emulsifier in the solvent evaporation/extraction technique for fabrication of polymeric nanospheres for controlled release of paclitaxel (Taxol). *J Control Release* 80: 129–144.
- Müller RH, Radtke M, Wissing SA (2002a) Nanostructured lipid matrices for improved microencapsulation of drugs. *Int J Pharm* 242: 121–128.
- Müller RH, Radtke M, Wissing SA (2002b) Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. *Adv Drug Delivery Rev* 54: 131–155.
- Müller RH, Runge S, Ravelli V, Mehnert W, Thunemann AF, Souto EB (2006) Oral bioavailability of cyclosporine: Solid lipid nanoparticles (SLN) versus drug nanocrystals. *Int J Pharm*, 317: 82–89.
- Naruse I, Ohmori T, Ao Y, Fukumoto H, Kuroki T, Mori M, Saijo N, Nishio K (2002) Antitumor activity of the selective epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) Iressa (ZD1839) in an EGFR-expressing multidrug-resistant cell line *in vitro* and *in vivo*. *Int J Cancer* 98: 310–315.
- Nassimi M, Schleh C, Lauenstein HD, Hussein R, Hoymann HG, Koch W, Pohlmann G, Krug N, Sewald K, Rittinghausen S, Braun A, Müller-Goymann C (2010) A toxicological evaluation of inhaled solid lipid nanoparticles used as a potential drug delivery system for the lung. *Eur J Pharm Biopharm* 75: 107–116.
- O'Driscoll CM, Griffin BT (2008) Biopharmaceutical challenges associated with drugs with low aqueous solubility—the potential impact of lipid-based formulations. *Adv Drug Delivery Rev* 60: 617–624.
- Plain KJ, Wilson CG (1984) The effect of different oils on the absorption of probucol in the rat. *J Pharm Pharmacol* 36: 641–643.
- Radhamani K, Muthu KS, Gautam S (2011) Molecular targets of celastrol derived from Thunder of God Vine: Potential role in the treatment of inflammatory disorders and cancer. *Cancer Lett* 303: 9–20.
- Ritger PL, Peppas NA (1987). A simple equation for description of solute release. I. Fickian and non-Fickian release from nonswellable device in the form of slabs, spheres, cylinders or discs. *J Control Release* 5: 23–26.
- Roger E, Lagarce F, Benoit JP (2009) The gastrointestinal stability of lipid nanocapsules. *Int J Pharm* 379: 260–265.
- Rouxhet L, Dinguizli M, Latere Dwan'isa JP, Ould-Ouali L, Twaddle P, Nathan A, Brewster ME, Rosenblatt J, Arien A, Preat V (2009) Monoglyceride-based self-assembling copolymers as carriers for poorly water-soluble drugs. *Int J Pharm* 382: 244–253.
- Silva AC, González-Mira E, García ML, Egea MA, Fonseca J, Silva R, Santos D, Souto EB and Ferreira D (2011) Preparation, characterization and biocompatibility studies on risperidone-loaded solid lipid nanoparticles (SLN): High pressure homogenization versus ultrasound. *Colloid Surface B*, 86: 158–165.
- Venkateswarlu V, Manjunath K (2004) Preparation, characterization and *in vitro* release kinetics of clozapine solid lipid nanoparticles. *J Control Release* 95: 627–638.
- Yong L, Yan L, Yang D (2003) Enteric disposition and recycling of flavonoids and ginkgo flavonoids. *J Altern Complement Med* 9: 631–640.
- Zhuang CY, Li N, Wang M, Zhang XN, Pan WS, Peng JJ, Pan YS, Tang X (2010) Preparation and characterization of vinpocetine loaded nanostructured lipid carriers (NLC) for improved oral bioavailability. *Int J Pharm* 394: 179–185.