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Isolation of herpetin from *Herpetospermum* seed and hepatoprotective activity of liposomal herpetin against carbon tetrachloride-induced liver injury in mice

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Objective: The aims of this study were to demonstrate the hepatoprotective activity of herpetin (HPT) and the enhanced hepatoprotective efficiency of liposomal herpetin against carbon tetrachloride-induced liver injury in mice. *Methods:* Herpetin was isolated from Herpetospermum seed and identified by ESI-MS and NMR. To enhance liver targeting and improve solubility of HPT, liposomal HPT was prepared with optimal formulation. The intravenous injection safety of the liposomes was then evaluated. Further, the hepatoprotective effects of liposomal HPT on model mice were investigated by the comparison of different liver marker enzymes and histopathological examination. *Results:* The prepared HPT liposome showed spherical or ellipsoidal vesicles with the entrapment efficiency of 94.50 ± 2.15% and particle size of 119.2 ± 10.7 nm. After 4 days intravenous administration of liposomal herpetin, no obvious damage could be observed at the injection site of each group. The liposomal HPT has no destructive effect on erythrocytes and little influence on whole blood clotting time. Free HPT exhibited only a weak protective function to model mice, whereas an enhanced hepatoprotective activity was observed using liposomal herpetin for treatment. *Conclusion:* The hepatoprotective efficiency of herpetin is able to be promoted through pharmaceutical application of liposome and liposomal herpetin is a promising new medicine for hepatoprotection.

1. Introduction

Hepatitis is one of the most common liver diseases and characterized by the presence of inflammatory cells in the organ. It is mostly caused by immune cells in the body attacking the liver and causing autoimmune hepatitis under the condition of infections, hepatotoxic drugs, alcohol, etc. Hepatic inflammation is responsible for liver cells damage, fibrosis, and cirrhosis. Although there are many chemical drugs for treating these diseases, limited efficacy is obtained (Zhang et al. 2012). Therefore, the increasing demands for low toxicity drugs with good therapeutic performance are appealing to find new compounds in plants. Numerous positive results on significant hepatoprotective activity of plant drugs have been reported, which has gained momentum over the years (Li et al. 2013; Yin et al. 2014; Yu et al. 2014).

Herpetospermum seed, a common medication used for liver diseases as a folk medicine, is the dried ripe seed of *Herpetospermum caudigerum* Wall. It is distributed in south-west China, Nepal, and northeast India, at an altitude of 2300-3500 m. Ethanolic extracts of *Herpetospermum* seeds have been reported to have hepatoprotective potential. *Herpetospermum* seeds contain a large amount of lignans, such as herpetal, herpetriol, herpetrione and so on, which showed anti-liver injury and anti-hepatitis effects (Yuan et al. 2005). Herpetin (HPT) is a new bioactive lignan isolated firstly from *Herpetospermum* seed in 2005 (Fig. 1) and it exhibits it's effects in reducing the replication and expression of HbsAg and HbeAg, and has significant inhibitory effect on HBV-DNA *in vitro* (Yuan et al. 2005). However, it is unknown whether herpetin is protecting against liver injury.

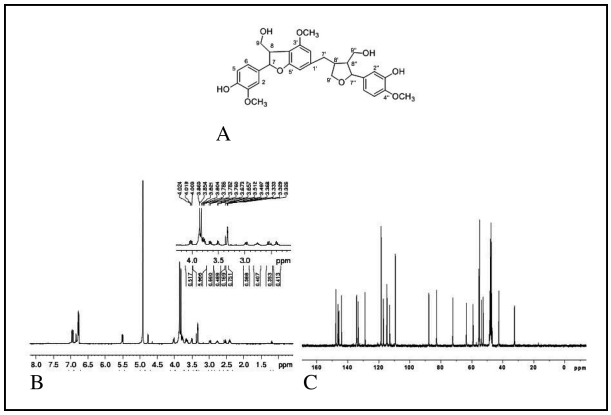

Fig. 1: The structure of herpetin(A) and NMR spectrum: ^{1}H NMR (B) and ^{13}C NMR (C). The corresponding data were consistent with those in reference.

Therefore, in the present work an attempt has been made to study the anti-liver injury function of HPT in CCl_4 induced-liver mice. Firstly, HPT was isolated and purified according to Yuan's method with some improvements (Yuan et al. 2005). The

Table 1: Effects of different preparation methods on the properties of liposomal HPT

Method	Stability	Particle size (nm)	EE(%)
Film dispersion method	No aggregation within 24 h	113.7 ± 19.8	90.78 ± 2.51%
Injection method	Aggregation within 24 h	107.6 ± 20.3	40.62 ± 6.18%
Anti-phase evaporation method	no obvious aggregation within 24 h	386.7 ± 36.7	84.65 ± 5.40%

Data were expressed as the mean ± SD (n = 3)

structure of HPT was elucidated by means of ESI-MS and NMR. Unfortunately, HPT is a poorly soluble drug with a solubility of less than 0.1 mg/mL in water and exhibited weak hepatoprotective efficiency. The similar negative results were reported by Tasaduq et al. (2003). Increasing the liver targeting profile of HPT maybe allow more effective incorporation for treatment. To achieve the therapeutic purpose, liposome as a strategy for pharmaceutical modification is adopted to improve the solubility and enhance the liver targeting profile of HPT.

With this consideration in mind, liposomal HPT was prepared with optimal formulation design. Then, lyophilization of liposomal HPT was adopted for long-time storage. Meanwhile, the intravenous injection safety of liposomal HPT with perfect entrapment efficiency was evaluated. Finally, the model mice of CCl_4-induced liver injury were employed with pretreatment of free HPT or liposomal HPT. The Liver marker enzymes, such as serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and malondialdehyde (MDA) and superoxide dismutase (SOD) were measured and compared. In addition, histopathological examination of the lives was also performed. The aims of the present study were to assess whether HPT or liposomal HPT possesses *in vivo* hepatoprotective effects against CCl_4-induced liver injury in mice, explore the possible mechanism and lay the basement for a promising new drug for hepatoprotection.

2. Investigations and results

2.1. Isolation and identification of HPT

Herpetin was isolated according to the procedure described by Yuan et al. (2005). The structure was identified by MS, ^{1}H NMR, ^{13}C NMR.

2.2. Preparation of liposomal HPT

Three most common methods available for liposome preparation, film dispersion method, injection method and anti-phase evaporation method were applied in the experiments. As shown in Table 1, the film dispersion method is the best way to prepare HPT liposomes with small particle size, high stability and EE. To maximize the entrapment efficiency, an uniform design of mixture experiments with constraints was further applied to optimize the formulation. The factors, levels and experimental results are shown in Table 2. Using the soft analysis function, a polynomial equation was fitted as follows: $y = 67.2 - 16.5x_1x_1 + 184.96x_1x_2 + 506.25\ x_1x_3 - 436.81x_2x_3 - 2784.6x_3x_3$. The equation was evaluated for statistical significance ($p < 0.05$). Thus, the optimal formulation could be predicted, namely, 78.05% phospholipids, 19.51% cholesterol and 2.44% HPT. In addition, the concentration of F68 and sonic time were also evaluated by single-factor test. The data showed that EE of HPT liposome were 78.45 ± 3.60%, 94.71 ± 4.22%, 93.46 ± 3.13% and 95.48 ± 5.10%, when the concentrations of F68 were adopted for 0.2%, 0.5%, 1.0% and 2.0%, respectively. Indeed, EE increased with the increase of F68 usage and reached a plateau when more F68 was added. It's clear that

Table 2: Uniform design[a]

No.	x_1 (%)	x_2 (%)	x_3 (%)	EE (%)
1	84.9	12.3	2.71	88.3
2	80.8	12.9	6.28	80.3
3	79.1	19	1.92	90.5
4	77.5	11.8	10.7	76.8
5	75.9	18.3	5.76	82.3
6	73.2	16.5	10.4	72.4
7	70.7	13.7	15.7	62.3
8	67.3	18.2	14.6	50.1
9	62.3	18.7	19	42.5

[a] According to the rule of uniform design for constrained mixture experiment, the main ingredients should obey this equation: $x_1 + x_2 + x_3 = 100\%$. x_1: phospholipids concentration (w/w); x_2: cholesterol concentration (w/w); x_3: HPT concentration (w/w). The pre-experimental results suggested the concentration range should be set as $60\% \le x_1 \le 89\%$, $10\% \le x_2 \le 20\%$ and $1\% \le x_3 \le 20\%$. The polynomial equation: $y = 67.2\text{-}16.5x_1x_1 + 184.96x_1x_2 + 506.25x_1x_3\text{-}436.81x_2x_3\text{-}2784.6x_3x_3$.

0.5% F68 is enough to approach satisfactory results. With the increase of sonic time from 5 min to 20 min, EE of liposome rose from 82.65 ± 2.14% to 93.29 ± 3.26%. With further prolonged sonication treatment, no significant increase of EE was observed. Therefore, 0.5% F68 was finally adopted for liposome preparation with sonication treatment for 20 min

According to the results mentioned above, the optimized preparation could be defined. In brief, 24.4 mg HPT, 780.5 mg phospholipids and 195.1 mg cholesterol were dissolved in chloroform in a round-bottom flask. The lipid film was formed by eliminating the organic solvent under reduced pressure. Then, the thin film was swollen in 50 ml of 0.5% F68 solution for 30 min and sonicated in a water bath sonicator for 20 min. The obtained liposomes were filtered through a 0.22 μm filter. The prepared HPT liposomes showed spherical or ellipsoidal shape with good dispersity under TEM (Fig. 2A). The EE and particle size were 94.50 ± 2.15% and 119.2 ± 10.7 nm, respectively, with a PDI of 0.156 ± 0.075 (Fig. 2B). The release profile of liposomal HPT is presented in Fig. 2C. A fast release of HPT during the initial stage and consecutively slow release in the following stage were observed. The release data were modeled using Higuchi, first-order and zero-order model (Table 3), which indi-

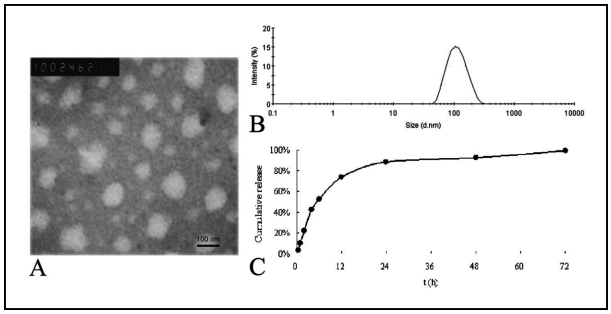

Fig. 2: Characterizations and *in vitro* drug release of HPT liposomes. A: TEM image of HPT liposomes; B: Particle size and polydispersity of HPT liposomes; C: The release curve of HPT liposomes in pH 7.0 PBS at 37 °C.

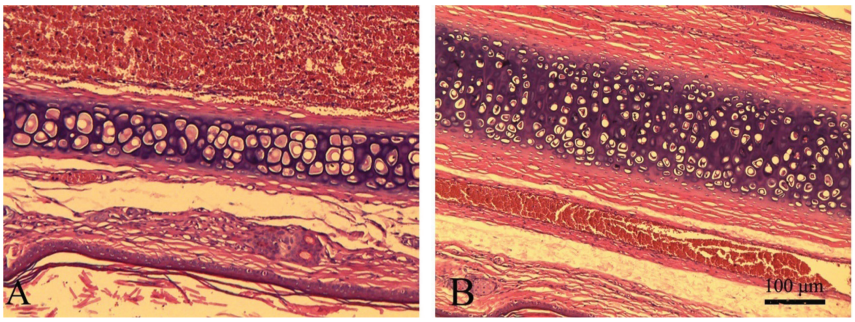

Fig. 3: The irritation test by intravenous administration. Histopathological examination of rabbit ears: A: saline group; B: HPT liposome group. Magnification: × 100.

Table 3: Fitting of release model of HPT liposomes *in vitro*

Model	Fitting equations	Correlation coefficient
Higuchi	$y = 0.123^{*}x^{1/2} + 0.107$	$r^2 = 0.8494$
first class	$y = 0.948^{*}(1\text{-}e^{-0.132x})$	$r^2 = 0.9945$
zero class	$y = 0.012x + 0.313$	$r^2 = 0.664$

cated that the release profile reasonably well fits the first-order model.

2.3. *Optimal lyophilization of HPT liposome*

Glucose, mannitol, lactose and sucrose as the common lyoprotectants were used alone or in combination for optimal lyophilization. As shown in Table 4, generally, lyoprotectants with lower concentration could cause higher HPT leakage, while lyoprotectants containing mannitol might remarkablely enlarge the particle size in spite of good appearance obtained. The process of freeze-drying had no effect the particle size of rehydrated liposomes containing 5% lactose combined with 5% sucrose and did not cause any drug leakage, which indicated that this combincation of lyoprotectants was much suitable for the preparation of HPT liposome lyophilization. The residual water content (w/w) in the samples of the optimal lyophilization liposome was $1.27 \pm 0.12\%$ (n = 3), which met the regulatory requirement.

2.4. *Safety of liposomal HPT for i.v. injection*

After 4 days intravenous administration, no obvious damage could be observed at the injection site of each group. There was no thrombus, angiectasia or vascular congestion in blood vessel at or beside the injection site under histopathologic examination (Fig. 3A-B). In addition, tissues surrounding the injection site were normal without any signs of dropsy, hemorrhage, inflammatory cell infiltrate or endothelial cell necrosis and degeneration. The results indicated that intravenous injection of liposomal HPT did not cause vascular irritation. As summarized in Fig. 4A, the hemolysis rates of 2%, 4% and 6% liposome were lower than the safe value of 5% according to ISO 10993-4:2002 (Zhang et al. 2009), suggesting that the liposomal HPT has no destructive effect on erythrocytes. The curves of whole blood clotting time of liposomes and physiological saline is drawn in Fig. 4B. It is obvious that there was no difference between liposome and saline in clotting time. Liposomal HPT showed little influence on whole blood clotting time. From the results mentioned above, we preliminarily ensured liposomal HPT is safe for intravenous injection.

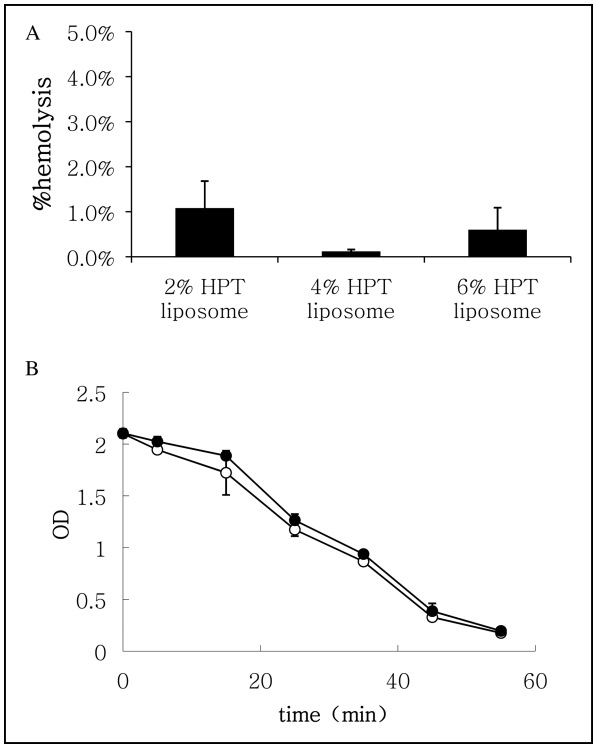

Fig. 4: The hemolysis test (A) and whole blood clotting time (B) of liposome. A: The hemolysis rates of 2%, 4% and 6% liposome were lower than the safe value of 5%. B: The curves of whole blood clotting time of HPT lyophilized liposomes and physiological saline. HPT lyophilized liposomes(●); physiological saline (◯). Data represent mean ± SD (n = 5).

2.5. *Hepotaprotective activity of liposomal HPT*

Carbon tetrachloride-induced acute liver injury can be identified by elevated serum ALT, AST and ALP levels in mice. As shown in Fig. 5 (A-C), the levels of serum ALT, AST and ALP in the normal group were very low, while those in the control group were dramatically higher due to the CCl_4 injury. However, pretreatment with free HPT, Qing Kai Ling injection and liposomal HPT in other groups for 7 consecutive days reduced the elevation of serum ALT, AST and ALP levels. Of the three groups, the HPT group showed a decrease trend, however, no obvious difference was observed with the control group ($p > 0.05$). Interestingly, the activities of serum ALT, AST and ALP in the liposomal HPT group were significantly lower than those in the HPT group ($p < 0.05$).

Histopathological observation of liver section for the normal group exhibited a normal cellular architecture with regular arrangement of hepatic cells, sinusoidal spaces and a central vein (Fig. 6A). In comparison, the control group showed severe degenerative changes and the hepatic cells presented fatty

Table 4: Optimized preparation of HPT lyophilized liposomes

Lyoprotectants	Appearance	Dispersion	Particle size(nm)	EE(%)
5% Glucose	bad	good	898.7 ± 9.1	93.37 ± 0.19
5% Mannitol	bad	good	2694 ± 25	97.25 ± 0.45
2.5% Glucose + 2.5%Mannitol	bad	good	1591 ± 16	91.17 ± 0.46
5%Lactose + 5% Sucrose	good	good	107.0 ± 1.2	99.70 ± 0.50
10% Sucrose	average	good	253.1 ± 0.9	67.10 ± 0.62
3% Glucose	good	good	493.8 ± 5.1	70.10 ± 0.35
3% Mannitol	excellent	good	4390 ± 39	58.61 ± 0.93
1.5% Glucose + 1.5% Mannitol	good	good	2530 ± 21	64.65 ± 0.21
1% Glucose + 1% Sucrose + 1%Lactose	good	good	119.6 ± 2.1	65.19 ± 0.38

Data were expressed as the mean ± SD (n = 3)

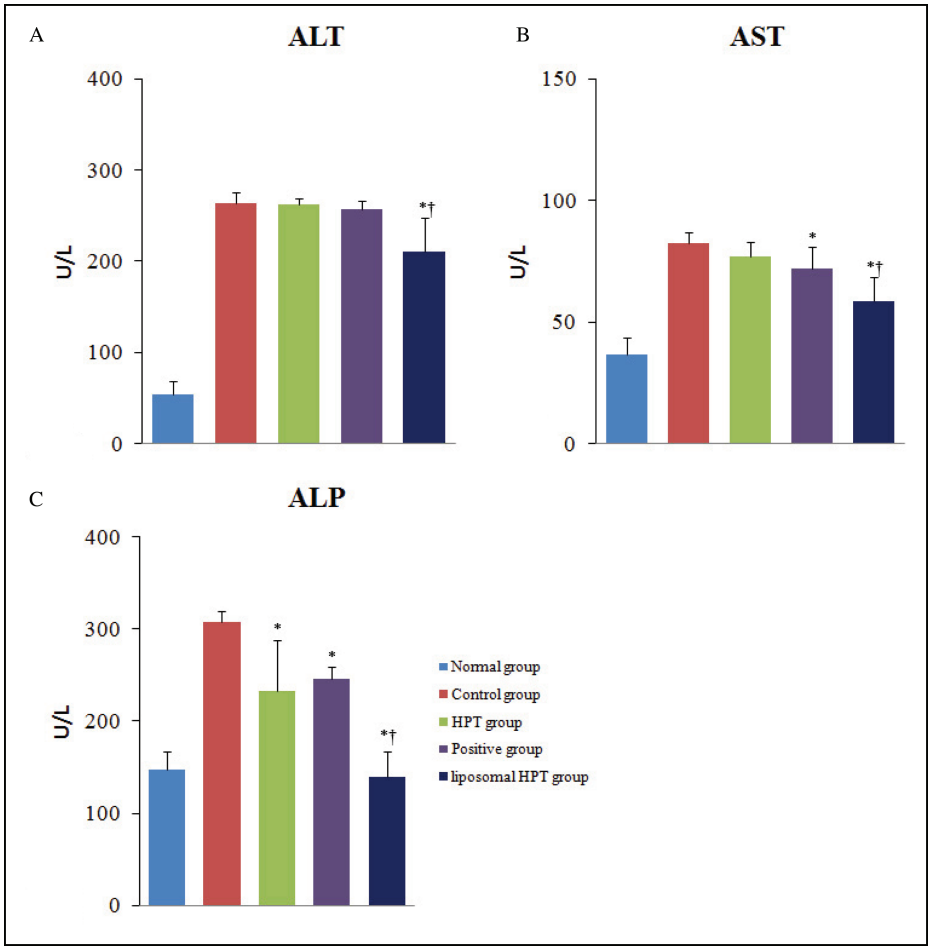

Fig. 5: Effect of free HPT, HPT liposome and Qing Kai Ling injection on mice intoxicated with carbon tetrachloride (CCl4). Levels of liver marker enzymes: ALT (A), AST (B) and ALP (C).Data were expressed as the mean ± SD. $^{*}p < 0.05$, *vs*. control group; †$p < 0.05$, *vs*. HPT group.

degeneration, necrosis and inflammation (Fig. 6B). After the 7 consecutive administrations in advance, a certain alleviation of pathological changes could be observed in the HPT group (Fig. 6C). In addition, a dramatic reduction of degeneration, necrosis and inflammation was achieved in the positive group and the liposomal HPT group (Fig. 6D-E). A further evaluation of MDA and SOD is shown in Fig. 6F. The production of MDA increased and SOD decreased remarkably in the control group, when compared with the normal group. Similarly, certain improvements were also observed in the HPT and the positive group with the comparison to the control group. Obviously, liver marker enzymes in the liposomal HPT group were improved sharply and its function for MDA and SOD improvement was more efficient than in the HPT group ($p < 0.05$). A similar trend can be seen, being consistent with the histopathological observation. The findings therefore supported the marker enzymes data in advocating the superior efficacy of HPT when it was loaded to liposomes.

3. Discussion

Yuan et al. first isolated HPT from *Herpetospermum caudigerum* using semi-preparative HPLC and proved inhibitory effects of HPT on HBV-DNA and the replication and expression of HBsAg and HBeAg (Yuan et al. 2006, 2005). In this paper, HPT was also prepared using a Sephadex LH-20 column. Data of MS, ^{1}H NMR and ^{13}C NMR spectra were used to identify the structure of obtained HPT, which were consistent with Yuan's report.

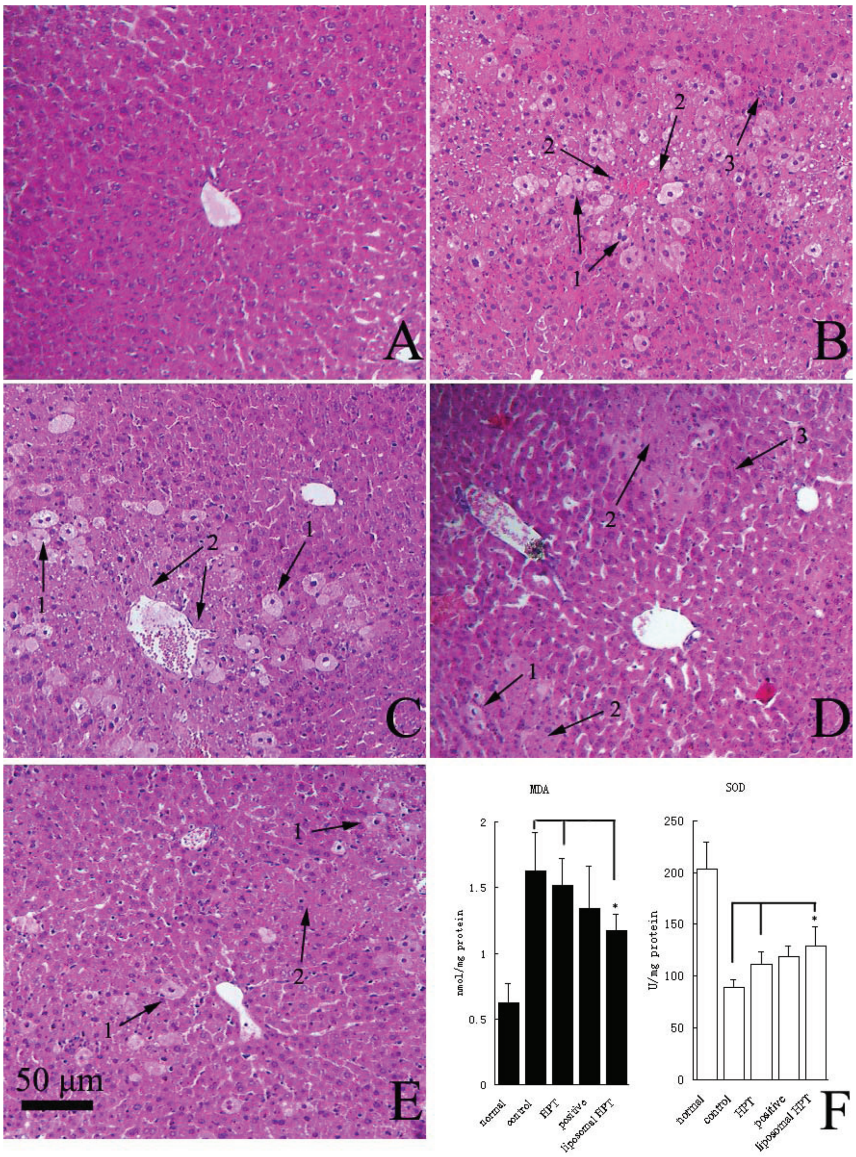

Fig. 6: Histological examination of liver section stained by HE (A-E) and determination of MDA and SOD level in liver (F). 1: degeneration; 2: necrosis; 3: inflammation. Magnification: × 200. Histopathological observation of liver section for normal group exhibited the normal cellular architecture with regular arrangement of hepatic cells, sinusoidal spaces and a central vein (A), whereas control group showed severe degenerative changes and the hepatic cells were fatty degeneration, necrosis and inflammation (B). A certain alleviation of pathological changes were observed in the HPT group (C).A dramatic reduction of degeneration, necrosis and inflammation was achieved in the positive group and liposomal HPT group (D-E). The production of MDA and SOD in liposomal HPT group were improved sharply and its function for MDA and SOD improvement was more efficient than HPT group (F, $^{*}p < 0.05$).

To investigate whether herpetinimproves resistance to liver injury, three doses of HPT (1 mg/kg, 10 mg/kg and 20 mg/kg) were given to mice prior to CCl_4 injection in our preliminary experiment. However, even at the high dose of 20 mg/kg, HPT still exhibited a weak protective function to the model mice. Additionally, owing to the poor solubility of HPT (less than 0.1 mg/mL in water), we found it difficult to solubilize HPT using surfactant or organic solvents to safely increase treatment dose. In order to solve the problem, liposomes as carriers came under our consideration. Liposomes are accepted as potent carriers not only for their biocompatible nature but also for increasing the dissolution of poorly soluble drugs. Furthermore, because of the presence of efficient capture by liver, liposomes are effective in the site-specific drug delivery to hepatic tissue. It is reported that liposomes are largely taken up by Kupffer cells whose high endocytic activity makes them most competent to internalize colloidal particles like liposomes (Mandal et al. 2007).

With the constrained mixture design, the optimal ratio of main ingredients in the liposome formulation was determined. Mixture designs are common in pharmaceutical applications for natural reasons and are applied both for screening and optimization studies (Gabrielsson et al. 2002; Shen et al. 2014; Tan et al. 2011). Compared with other design methods, the uniform design cannot only greatly reduce the test points but also obtain a better result because that test point is more evenly distributed in the test range (Cao et al. 2013). It is very suitable for resolving the formulation problem with multiple factors and levels. In this study, the entrapment efficiency achieved for optimal liposomes using uniform design was found to be $94.50 \pm 2.15\%$ with excellent homogeneity and sustained release.

Due to physical and chemical instabilities (e.g. hydrolysis and oxidation of HPT and phospholipids, HPT leakage and liposomes aggregation) of liposomal HPT in aqueous dispersions, lyophilization should be used extend the shelf-life of HPT liposomes. The protective effect during liposome lyophilization is

mainly determined by formulation factors, such as the nature of the drug, the lipid bilayer composition, the choice of lyoprotectants. Optimization of these parameters is an effective way to improve the stability of lyophilized liposomes (Chen et al. 2010). Our results show that the HPT liposomes with 3% lyoprotectants might be more liable to become leaky, which might be ascribed to the greater curvature or packing defects. When the concentration of lyoprotectant increased to 5%∼10%, no obvious effect on the HPT leakage could be observed on the HPT leakage. It seems that the amount of lyoprotectants play a key role to control the leakage. In addition, the combination of lyoprotectants can effectively prevent HPT leakage and liposome aggregation, whereas a single protective agent is ineffective for the HPT liposomes.

It is necessary to evaluate the intravenous injection safety before administration of particle solutions in animals (Gao et al. 2011; Sharma et al. 2013; Zhao et al. 2010). Liposomal HPT never caused irritation at the injection site and surrounding tissues for a consecutive administration and had no negative influence on erythrocyte and whole blood clotting time. In contrast having solubilized free HPT by adding excess surfactant or organic solvent to increase administration dose, damage of free HPT solution to erythrocytes could be observed. Additionally, deep respiration of rabbits occurred immediately following injection of free HPT solution. Thus, repeated injection of the solution cannot be tolerated by the rabbit. This reason makes the preparation of liposomal HPT amenable for intravenous injection.

Carbon tetrachloride forming the highly reactive trichloro free radical has been reported to attack polyunsaturated fatty acids, which can produce hepatotoxicity by altering liver microsomal membranes in experimental animals (Mandal and Das 2005). Therefore, CCl_4 has been extensively used in the establishment of animal models of liver injury for evaluating the hepatoprotective activity of drugs (Dhanasekaran et al. 2009; Jia et al. 2011). The extent of liver damage is normally assessed by the level of ALT, AST and ALP in circulation as reliable markers for liver function. Dramatically increased serum levels of AST and ALT have been attributed to damaged structural integrity of the liver. It has been established that AST and ALT are released into circulation when cell membrane permeability increases after the damage of hepatocytes (Recknagel et al. 1989). ALP excreted *via* bile by the liver is a membrane bound enzyme. The increase of ALP level also indicates the injury of liver tissue (Nemesanszky 1996). In our study, administration of CCl_4 induced liver injury in ICR mice with obvious increase in ALT, AST and ALP. Qing Kai Ling injection as a positive drug is used extensively by Chinese doctors to detoxify and protect the liver. Indications for its use include upper respiratory inflammation, viral encephalitis, hepatitis, stroke, cerebral thrombosis, tonsillitis, tracheitis and high fever (Chen et al. 2002). Published literature reported that Qing Kai Ling injection has therapeutic effects on experimental hepatic lesions (Qi et al. 1983; Wang et al. 1987; Ye 1981). In this paper, Qing Kai Ling injection also showed hepatoprotective effects, which was consistent with previous reports. Importantly, pretreatment of free HPT for 7 consecutive days, led to a decrease trend in the levels of ALT, AST and ALP, however, the liposomal HPT significantly reduced these indexes. This is probably attributed to to the intrinsic liver targeting specificity of liposomal HPT. In parallel with the alteration of reliable markers for liver function, histopathological examination also showed the advance of liposomal HPT in alleviating the degeneration and necrosis and decreased inflammation of hepatocyte.

Lipid peroxidation is a major cause of CCl_4 mediated liver injury. CCl_4 can elicit severe hepatocellular damage for its highly toxic metabolite trichloromethyl free radical by the action of the cytochrome P540 system. In the presence of oxygen, it is converted into a more reactive radical and initiates the process of lipid peroxidation (Zhang et al. 2012). MDA level is the most commonly used index for the measurement of lipid peroxidation(Kalender et al. 2005). It has been reported to be an important biochemical component to detect harmfulness of CCl_4. SOD as antioxidant enzyme is also easily inactivated by lipid peroxidation, which results in decreased activities of the enzymes after CCl_4 administration. Similar changes of MDA and SOD were also substantiated by our experimental data in model mice. Fortunately, pretreatment for 7 consecutive days, liposomal HPT showed a remarkable improvement through a decrease in MDA production and an increase in SOD level of mice liver. A similar trend was also observed in the free HPT group, indicating direct anti-oxidative stress mechanisms of HPT.

Various lines of evidence in the present study suggest that liposomal HPT firstly exhibits dramatical hepatoprotective capability, which attenuates hepatic oxidative stress and inhibits inflammation in CCl_4 induced liver-injury in mice. It is clarified for the first time that free HPT compound possesses certain hepatoprotective activity, however, it is less efficient than liposomal HPT. There are several possible reasons for this discrepancy: (1) Liposomes not only increases HPT circulating time in plasma, but enhance the accumulation of HPT in the liver, especially in hepatocytes, to easily maintain therapeutic concentration. (2) Liposome loaded compounds interact with cells at a much faster rate than free drug (Mandal and Das 2005). (3) Due to the physicochemical properties of HPT, it's half life *in vivo* is short. Repeated treatment with a higher dose could be useful for maintaining efficient drug concentrations.

In conclusion, liposomal HPT exhibited more hepatoprotective efficiency in model mice, than free HPT including the decrease of ALT, AST and ALP level in circulation, the improvement of MDA and SOD indexes in liver and the protection of liver histological structure. Thus, the hepatoprotective efficiency of herpetin can be promoted through pharmaceutical application of liposomes and liposomal herpetin is a promising new medicine for hepatoprotection.

4. Experimental

4.1. Chemicals

Phospholipid was supplied by Lipoid Company (Ludwigshafen, Germany). Cholesterol, silica gel and Sephadex LH-20 were obtained from Sigma-Aldrich Company Ltd. (Gillingham, UK). Qing-Kai-Ling injection was manufactured by Guanzhou Mingxing Pharmaceuticals (Guanzhou, China). AST, ALT and ALP kits were purchased from Changchun Hui Li Biotechnology Company Ltd. (Changchun, China). The commercial MDA and SOD kits were from Jiancheng Institute of Biotechnology (Nanjing, China). All other chemical reagents and solvents used were analytical grade.

4.2. Animals

ICR mice with an initial body weight 18–22 g were obtained from the Da-Shuo Experimental Animal Ltd. (Beijing, China). These animals were allowed to acclimatize in environmentally controlled quarters (24 ± 1 °C and 12 h light/dark cycle). Unless specified otherwise, water and food were given throughout the experiment *ad libitum*. All procedures of the animal studies were approved by Animal Care and Use Committee of Southwest University for Nationalities, and were performed according to the requirements of the People's Republic of China National Act on the use of experimental animals.

4.3. Separation and purification of HPT

Separation and purification of HPT was carried out according to Yuan et al. (2005). Dried powder of *Herpetospermum caudigerum* seeds (1.0 kg) was extracted twice with 6L 80% ethanol solution for 2 h followed by evaporation of ethanol at 70 °C. The extract was then partitioned into petroleum ether, ethyl acetate and *n*-butanol parts, respectively. The ethyl acetate fraction was concentrated and separated into 18 fractions (Fr.1-Fr18) with silica gel column chromatography using a methanol-chloroform gradient system. Of fractions, Fr 10 was further isolated to obtain crude HPT by silica gel col-

umn chromatography eluted with a petroleum: chloroform: acetone (1:1:1) system. After that, the crude HPT was purified on a Sephadex LH-20 column with methanol to yield HPT compound identified using NMR (Bruker Avance III 400 MHz spectrometer) and MS (Bruker amaZon SL, Bremen, Germany) analysis.

The amounts of petroleum ether, ethyl acetate and n-buthanol fractions of ethanol extract of *Herpetospermum caudigerum* were 31.0 g, 29.7 g and 15.3 g, respectively. In order to achieve an efficient isolation of target compound, silica gel column chromatography using methanol-chloroform gradient system was used to separate the ethyl acetate fraction into 18 sub-fractions. According to pre-experimental results, the 10^{th} sub-fraction was further isolated to yield 170 mg HPT powder as a white amorphous solid. The structural identification of HPT was carried out by MS, ^{1}H NMR and ^{13}C NMR spectra (Fig. 1) as follows: ESI-MS m/z: 538.2$[M]^+$, ^{1}H-NMR (CD_3OD,400 MHz) δ:6.97(1H, d, J = 2.0 Hz, H-2), 6.93(1H, s, H-2"), 6.85(1H, dd, J = 8.0 Hz, 2.0 Hz, H-6), 6.79(1H, s, H-6"), 6.78(2H, d, J = 8.0 Hz, H-5, 5'), 6.78(2H, s, H-2', 6'), 5.13(1H, d, J = 6.4 Hz, H-7), 4.76(1H, d, J = 7.2 Hz, H-7"), 4.02(1H, m, H-9'), 3.86(3H, s, -OCH_3), 3.85(3H, s, -OCH_3), 3.84(1H, m, H-9), 3.82(3H, s, -OCH_3), 3.80(1H, m, H-9), 3.78(1H, m, H-9'), 3.76(1H, m, H-9"), 3.65(1H, m, H-9"), 3.50(1H, m, H-8), 2.97(1H, m, H-7'), 2.76(1H, m, H-8'), 2.54(1H, m, H-7'), 2.40(1H, m, H-8"); ^{13}C-NMR(CD_3OD, 100MHZ) δ:133.7(C-1), 109.7(C-2), 148.0(C-3), 146.5(C-4), 115.1(C-5), 118.83(C-6), 88.0(C-7), 54.4(C-8), 63.9(C-9), 134.5(C-1'), 117.3(C-2'), 146.8(C-3'), 144.3(C-4'), 124.7(C-5'), 124.4(C-6'), 87.5(C-7'), 55.3(C-8'), 72.6(C-9'), 133.3(C-1"), 109.4(C-2"),149.0(C-3"), 147.5(C-4"), 130.0(C-5"), 115.8(C-6"), 87.3(C-7"), 55.3(C-8"), 72.5(C-9"), 134.4(C-1"), 111.9(C-2"), 148.7(C-3"), 145.4(C-4"), 116.1(C-5"), 118.5(C-6"), 89.2(C-7"), 55.1(C-8", 64.7(C-9"), 56.5(3, -OCH_3), 56.7(-OCH_3). The corresponding data were consistent with those in reference (Yuan et al. 2006).

4.4. Preparation and formulation optimization of liposomal HPT

Liposomal HPT was prepared with phospholipids, cholesterol and HPT by three methods, such as injection method, film dispersion method and anti-phase evaporation method. With the higher entrapment efficiency (EE) of HPT, film dispersion method was chosen. Briefly, phospholipids, cholesterol and HPT were dissolved into chloroform in a round-bottom flask, in which the lipid film was formed by eliminating the organic solvent under reduced pressure. After the thin film was swollen in F68 solution with a certain concentration for 30 min, the dispersion was sonicated in a water bath sonicator for 20 min. The obtained liposomes were filtered through a 0.22 μm filter.

To prepare liposomal HPT with high EE, the influence of main experimental factors on EE was evaluated by a single factor experiment, such as the concentration of F68 and sonic time. After the factors influencing EE have been investigated, the formulation optimization was assessed by uniform design, including the range of main ingredients and HPT. A U9 (9^3) factorial design was established by uniform design software (version2.0, China), among which 3 is the number of independent variables and 9 stands for the number of levels for each factor. According to the preliminary experimental results, the factors were identified with certain restrictions as follows: phospholipids ($60\% \le x_1 \le 89\%$, w/w); cholesterol ($10\% \le x_2 \le 20\%$, w/w); HPT ($1\% \le x_3 \le 20\%$, w/w) and $x_1 + x_2 + x_3 = 100\%$. The designed experimental can result in a mathematical equation like: $y = k + a\ x_1 x_1 + b\ x_1 x_2 + c\ x_1 x_3 \ldots\ldots$, in which y is dependent variable and k is a constant and x_1, x_2, x_3 are the independent variable while $a, b, c, \ldots\ldots$ are the coefficients. The equation can show the effect of the different independent variables on EE and the optimal formulation can be obtained.

4.5. Determination of EE

The concentration of HPT was measured by a ACQUITY UPLC system (Waters, MA, USA) equipped with a tunable UV detector and a conditional autosampler. According to the previous method with improvement (Xie et al. 2006), the separation of HPT was established using BEH C_{18} column (50 × 2.1 mm, 1.7 μm) at 30 °C with detection wavelength of 280 nm. The determination was carried out using the mobile phase of acetonitrile: 2% acetate acid solution (24:76) at a flow rate of 0.6 mL/min. The injection volume was 5 μl and the total run time of each sample was 5 min. The regressive equation of peak area *versus* concentration for HPT standard solutions in methanol (2.0∼100.0 μg/ml) was y = 10976x-22391 ($r^2 = 0.9999$).

Free HPT was determined in the ultrafiltrate after separation of liposomes by ultrafiltration/centrifugation technique with a Milipore Ultrafree-MC 10k MW. An aliquot of 0.5 ml liposome solution was added into Milipore Ultrafree-MC followed by centrifugation for 10 min at 4000 rpm. The amount of free HPT in the ultrafiltrate was determined by UPLC method mentioned above. Another aliquot of 0.5 ml liposome solution was diluted with 10 time volume methanol and sonicated for 10 min. Thus, the amount of total HPT was still measured using UPLC. The HPT associated to liposomes was calculated from the difference between the total and free HPT.

4.6. Freeze-drying of liposomal HPT

To ensure long-term stability of liposomes, various lyoprotectants, such as glucose, mannitol, lactose, and so on, were, alone as in combination added to the freshly prepared liposomal HPT for optimizing the freeze-drying formulation. The dispersion was freeze-dried in aliquots of 2 ml in 10 ml glass vials. After freezing at −40 °C for 3 h, the vials were placed into MODULY-230 freeze dryer (Thermo Scientific, MA, USA) for 24 h under the pressure less than 1 Pa. all vials were sealed with rubber stopper and aluminum seals after lyophilization and stored at 4 °C until further treatment. Prior to the measurement of particle size and EE, the freeze-drying powder was resuspended with distilled water to the original volume.

4.7. Particle size and morphology of liposomal HPT

The mean particle size and size distribution of liposomal HPT were measured by a Zetasizer Nano ZS90 (Malvern, Worcs, UK). EE was evaluated by the method in section 2.4. Transmission Electron Microscopy (TEM) image of liposomal HPT was obtained using a JEM-100CX TEM microscope (Tokyo, Japan). A staining technique with 0.5% uranyl acetate was adopted to observe the liposomal HPT.

4.8. Release profile in vitro

HPT release from liposomes *in vitro* was also studied. 10 ml of liposome solution was poured on dialysis membrane (MWCO: 3500 Da) and dialyzed against phosphate buffer solution (PBS, pH 7.0, 100 ml) with stirring speed of 100 rpm at 37°C. At specified time intervals, 1 ml of receptor phase was removed and replaced with an equal volume of prewarmed fresh PBS. The released HPT was determined using UPLC and the release data were processed mathematically to deduce the mechanism of HPT release from liposomes.

4.9. Intravenous injection safety

To preliminarily clarify the intravenous injection safety, ear vein irritation, hemolysis and coagulant activity were studied. Rabbits weighing 1.8∼2.2 kg were involved in the assays. HPT liposomes were injected into the right ear marginal vein with a daily dose of 20 mg/kg. As a control, an equivalent volume of physiological saline was administrated into the left ear marginal. After injection for 4 days repeatedly, the rabbits were sacrificed and the vascular tissues of injection site were cut down and preserved in 10% formalin solution to prepare pathological sections for histopathological examination (Zhao et al. 2010).

Fresh rabbit blood with acid-citrate dextrose as anticoagulant agent was used to test the hemolysis effect of liposomal HPT. The erythrocytes were collected and washed with physiological saline for three times after centrifugation of whole blood at 2000 rpm for 10 min. Erythrocytes were redispersed by adding saline to obtain 2% hematocrit erythrocyte suspension without fibrinogen. Different volumes of liposomal HPT were mixed with 1 ml of erythrocyte suspension to achieve final concentrations of 2%, 4% and 6% (w/w). Physiological saline served as normal control and distilled water was used as 100% hemolysis control, respectively. All the samples were kept at 37 °C for 30 min. After that, the samples were centrifuged at 2000 rpm for 10 min to obtain the supernatant, which was determined at 542 nm OD using a Varioskan Flash microplate reader. The percentage hemolysis was calculated using the following equation (Zhang et al. 2008).

$$\%\text{hemolysis} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{normal control}}}{\text{OD}_{100\%\text{ hemolysis control}} - \text{OD}_{\text{normal contrl}}} \times 100\% \quad (1)$$

The effects of liposomal HPT on whole blood clotting time were determined as follows: freshly collected citrate rabbit blood was mixed 10:1 (v/v) with liposome solution or saline (control) in the glass vials. Then the 100 mM calcium chloride solution was added into the mixture with the ratio of 1:10 (v/v). At the arranged time point, 3 ml of preheated distilled water at 37 °C was infused into the vials (Koziara et al. 2005). Fire minutes min later, the samples were centrifuged at 2000 rpm for 10 min and the supernatant was obtained and measured at 542 nm in a Cary50 UV-spectrophotometer (Varian, CA). The whole blood clotting OD/time curve was drawn.

4.10. Animals and experimental design

Fifty male ICR mice were randomly divided into five groups with ten mice in each group. The normal group received a single dose of olive oil (20 ml/kg) intraperitoneally (i.p.) without any treatment or CCl_4 injury. Before CCl_4

treatment, mice in the control group received saline by i.v. injection; the HPT control group was treated with free HPT (dissolved in 10% glycerol saline solution) of 20 mg/kg, the positive group with Qing Kai Ling injection of 20 mg/kg (a traditional Chinese medicine with known anti-inflammatory properties) and the liposomal HPT group with liposome dispersion at an equivalent dose of HPT. The administration in these three groups was done for 7 consecutive days. One hour after the last administration, all mice were injected i.p. with 0.1% CCl_4 in olive oil (v/v, 20 ml/kg) except the mice in the normal group. 16 h later, all animals were sacrificed and the blood was collected for the test of serum ALT, AST and ALP. The liver was removed immediately and a part of the organ was processed for histopathological examination. Another portion of the liver was homogenized with corresponding buffer of kits followed by centrifugation at 2000 rpm for 10 min. The supernatant was then collected for the measurement of MDA and SOD levels.

4.11. Data analysis

The data were analyzed with SPSS 15.0 statistical package (SPSS Inc., IL). Multiple comparisons were performed by ANOVA. A value of $p < 0.05$ was considered statistically significant. The release profile was modeled by Origin 8.0 software (OriginLab Corporation, MA).

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