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Sterically stable liposomes improve the therapeutic effect of hepatic stimulator substance on fulminant hepatic failure in rats

Feng Li [a,1], Jian-yong Sun [a,1], Min Liu [b], Wei-yue Lu [b], Ji-yao Wang [a,*], Jian-ying Shi [c]

[a] Department of Gastroenterology, Zhongshan Hospital Affiliated to Fudan University, 180 Fenglin Road, Shanghai, China
[b] Fudan-Pharmco Targeting Drug Research Center, Fudan University, Shanghai, China
[c] Department of Pathology, Fudan University, Shanghai, China

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ABSTRACT

Background and aims: Few drugs have been confirmed to be effective for fulminant hepatic failure (FHF). The purpose of this study was to prepare sterically stable liposomes (SSL) encapsulating hepatic stimulator substance (HSS) and determine their therapeutic effect on FHF.
Methods: HSS were encapsulated into SSL (HSS-SSL). FHF was induced in rats by thioacetamide (TAA) injection (400 mg/kg, three times with a 24-h interval). The agents, including HSS-SSL, SSL, HSS, and sodium chloride (NS), were each injected intravenously 2 h after the second and the third TAA injection.
Results: Freshly prepared HSS-SSL had a mean size of 93.59 nm and the average encapsulation efficiency was 37.20%. HSS encapsulated in SSL showed a longer half life and more potent target to injured livers than free HSS. Twenty-four hours after the third TAA-injection, the survival rate of HSS-SSL-treated rats (80%) was significantly higher than that of rats treated with NS (20%), SSL (25%), or HSS (50%). Histopathologic examination showed that there was the least necrosis and inflammation in the livers of HSS-SSL-treated rats. The incidence of stage 3 or 4 hepatic encephalopathy in HSS-SSL-treated rats was significantly lower than that in rats treated with other agents. The serum pro-inflammatory cytokine levels and hepatic lipid peroxidation levels were both markedly reduced, while hepatocyte proliferative rate was markedly increased after HSS-SSL treatment.
Conclusion: Encapsulation by SSL markedly improved the therapeutic effect of HSS on FHF in rats. Encapsulation by SSL may be an effective approach to enhance the therapeutic potency of drugs for FHF.

1. Introduction

Fulminant hepatic failure (FHF) is a rare but severe complication of acute hepatitis, which is characterized by massive hepatic necrosis and hepatic encephalopathy (HE) with a very high mortality [1]. The only treatment of proven efficacy for FHF is emergency liver transplantation [2]. Although liver transplantation results in decreased mortality, its availability is often limited by the chronic shortage of donor livers, particularly in developing countries, for the lack of medical resources [2]. Additionally, approximately 40% of patients die while waiting for liver transplantation [3], emphasizing the need to develop new strategies for the management and treatment of FHF. Although a wide variety of medical therapies as well as artificial and bioartificial liver support systems have been used for management of this ominous condition, very few therapies have been evaluated in controlled clinical trials. Unfortunately, the use of such strategies has so far yielded disappointing results [1,2].

Hepatic stimulator substance (HSS) is a heat-stable, alcohol-precipitable extract, first extracted from the cytosol of regenerating adult rat livers and normal livers of weanling rats. HSS has been shown to stimulate liver regeneration when injected intraperitoneally into 34% partially hepatectomized rats [4]. It is a 12–20-kDa polypeptide growth factor, with mitogenic effects on the liver in an organ-specific but species-nonspecific manner [4]. Mao-Hua et al. reported that HSS administration was able to protect the liver of mice against acute failure induced by CCl_4 poisoning, by affecting the stability of biomembranes and cellular enzymatic systems [5]. Additionally, the survival rate in rats with fulminant hepatic failure induced by D-galactosamine was improved after the administration of HSS [6]. Though HSS showed attractive therapeutic potential in several animal models with FHF, it has not shown

Abbreviations: AUC, area under the curve; BrdU, 5-bromo-2′-deoxyuridine; CC, carrying capacity; FHF, fulminant hepatic failure; HE, hepatic encephalopathy; HSS, hepatic stimulator substance; IL, interleukin; MDA, malondialdehyde; CL, serum clearance; NS, sodium chloride; SD, standard deviation; SSL, sterically stable liposomes; TAA, thioacetamide.

* Corresponding author. Tel.: +86 21 64041990x2117; fax: +86 21 64432583.
E-mail addresses: lifeng197881@yahoo.com.cn, wang.jiyao@zs-hospital.sh.cn, wang.jiyao@gmail.com (J.-y. Wang).

[1] The two authors contributed equally to this manuscript.

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the prospective therapeutic effect for patients with FHF in the clinic [1,2]. HSS has a short half-life and is predominantly eliminated though the renal route. When HSS is administered intravenously, it also rapidly accumulates in other organs and tissues besides liver. As a consequence, sufficient concentrations of HSS could not be accumulated or sustained in hepatocytes, which might result in the insufficient therapeutic effect of HSS in patients with FHF.

For decades, liposomes have been used as versatile delivery systems for biologically active compounds [7]. One evident drawback of 'classical' liposomes is their rapid elimination by cells of the reticuloendothelial system, primarily by liver and spleen [7,8]. An important breakthrough in this respect was the development of long-circulating liposomes, such as liposomes coated with polyethyleneglycol, known as sterically stabilized liposomes (SSL). SSL have a prolonged blood residence time due to the presence of the hydrophilic polymer polyethyleneglycol, which provides steric stabilization. This stabilization inhibits the penetration of plasma proteins that could destabilize the liposomes or could serve as opsonins, resulting in decreased recognition by the reticuloendothelial system [7,9,10]. Another important characteristic of SSL is their localization to infectious targets, which is also the general property of liposomes with a certain size range. In experimental and clinical studies, it has been demonstrated that SSL leave the blood in areas of inflammation as a result of locally increased vascular permeability and endothelial leakage, both developing at the infected site during the progression of the infection [7,10,11]. Thus, these characteristics allow SSL to increase the accumulation amount of biologically active compounds encapsulated in liposomes at infected areas, which may result in the improved efficacy of encapsulated compounds.

In the present study, we prepared SSL encapsulating HSS (HSS-SSL) and determined the therapeutic effect of HSS-SSL on FHF in rats induced by thioacetamide (TAA) poisoning.

2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources: HSS was provided by Sailuojin Pharmaceutic Company (Weihai, Shangdong, China), which was extracted and purified from the fresh livers of healthy Anthony pigs with a purity of above 98%; egg phosphatidylcholine, cholesterol, and methoxy-polyethylene glycol$_{2000}$-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine were from Avanti Polar Lipids (Alabaster, AL); epidermal growth factor (EGF) was from Promega (Beijing, China); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 5-bromo-2′-deoxyuridine (BrdU) and 1,1,3,3-tetraethoxypropane were from Sigma-Aldrich (Shanghai, China); Dulbecco's modified Eagle's medium and fetal bovine serum were from Invitrogen (Carlsbad, CA); iodogen was from Pierce (Rockford, IL); [^{125}I]NaI was from PerkinElmer (Hong Kong, China). TAA and all other chemicals were of analytical grade and from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

2.2. Animals

Eight-week-old inbred male Sprague-Dawley rats (body weight 200 ± 20 g) were obtained from the Laboratory Animal Research Center of Fudan University (Shanghai, China) and maintained on standard laboratory rat chow on a 12-h light/dark cycle with free access to water and food. All studies were approved by the Institutional Ethical Committee of Animal Experimentation, and all experiments were performed strictly according to governmental and international guidelines on animal experimentation.

2.3. Preparation and properties of HSS-SSL

2.3.1. Preparation of HSS-SSL

Liposomes were prepared as previously described [12]. In brief, lipids composed of egg phosphatidylcholine/cholesterol/methoxy-polyethylene glycol$_{2000}$-1,2-dioleoyl-sn-glycero-3-phosphoethanolamine at a molar ratio of 2:1:0.1 were dissolved in chloroform and the solvent was evaporated to form a lipid film under reduced pressure. The lipid mixture was hydrated in phosphate buffer saline (pH 7.4) and sequentially extruded 15 times through a double layer of polycarbonate membrane (Whatman, Kent, UK) in 400-, 200-, 100-, and 50-nm open mesh using a mini-extruder (Avanti Polar Lipids) to obtain a homogeneous liposome suspension (SSL). To prepare HSS-SSL, the lipid mixture was hydrated in phosphate buffer saline containing HSS and extruded. The resulting liposomes were passed through a Sepharose CL-4B column (Pharmacia-LKB, Uppsala, Sweden) to remove unencapsulated HSS. The prepared liposomes were stored at 4 °C for further experiments.

2.3.2. Properties of HSS-SSL

The morphology of HSS-SSL was examined by a transmission electronic microscope (Hitachi 7000; Hitachi, Tokyo, Japan) after being stained with 2% phosphotungstic acid and dried on carbon-coated grids. The size and polydispersity index of the resultant liposomes were determined using dynamic light scattering (Nicomp 380 ZLS; Particle Sizing Systems, Santa Barbara, CA). After passing through the Sepharose CL-4B column, the concentration of unencapsulated HSS was measured with an ultraviolet spectrophotometer and the encapsulation efficiency of HSS was calculated by deduction of the unencapsulated dosage from the total dosage of HSS as previously described [13]. The lipid concentration was determined by Bartlett analysis. Then the carrying capacity (CC) of SSL was determined according to an equation: $CC = W_E/W_{LN} \times 100\%$. Here W_{LN} refers to total weight of lipids and W_E refers to the weight of the entrapped substance.

The encapsulation efficiency, size, and polydispersity index of HSS-SSL were respectively measured at days 1, 7, and 30 after preparation to evaluate the stability of the resultant liposomes. These properties were also compared between HSS-SSL and SSL. All measurements were repeated three times.

2.3.3. Effect of HSS-SSL on hepatocyte proliferation in vitro

Primary rat hepatocytes were isolated by a two-step collagenase perfusion [14] and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and antibiotics for 12 h. To evaluate the effect of HSS-SSL and HSS on hepatocyte proliferation, hepatocytes (4000/well) in 96-well plates were respectively incubated with HSS-SSL, HSS or SSL for 24 h. The concentration range of HSS in the medium was 2–1 500 ng/mL, and the concentration of SSL was the same as that of HSS-SSL. HSS alone did not affect the growth of primary cultured hepatocytes to a great extent, but allowed them to be significantly stimulated by EGF and other hepatic mitogenic factors [15]. Therefore, 10 nmol/L EGF was added to the medium at the same time. After incubated for 24 h, MTT assay was performed as described [16]. The absorbance rate of each well optical density (OD value) was measured at 595 nm by a spectrophotometer and the cell proliferation rate was calculated as (average OD value of wells with administered agent/average OD value of control wells) × 100. The assays were repeated thrice.

2.3.4. Pharmacokinetics and organ biodistribution of HSS-SSL in rats with liver injury

HSS was labeled with iodine-125 (^{125}I) using the iodogen method. Briefly, HSS was labeled with iodine-125 (^{125}I) by

dissolving it (30 μg) into [^{125}I]NaI (37 MBq) in a 1.5 mL polypropylene vial coated with 100 μg of iodogen as described [17]. The sample was purified by a Gilson high-performance liquid chromatography (HPLC) system on a Superdex 75 column (GE Healthcare). The fractions containing the radioactive peak were selected and the radiochemical purity of ^{125}I-labeled HSS (^{125}I-HSS) was always higher than 95%. Then the phosphate buffer saline containing ^{125}I-HSS were added to the lipid mixture to prepare the liposomes encapsulating ^{125}I-HSS (^{125}I-HSS-SSL).

Liver injury was induced in rats (n = 30) intraperitoneally injected with a single dose of TAA (400 mg/kg). Twenty-four hours after treated with TAA, rats were randomly divided into two groups (n = 15 per group) and respectively injected with ^{125}I-HSS (0.5 MBq, 0.5 μg) or ^{125}I-HSS-SSL containing the same dosage of ^{125}I-HSS via the tail vein under normal pressure. Then, three rats were respectively randomly selected from each group and blood samples were collected via the intraocular vein at various time points up to 24 h. And after 24 h, the rats were sacrificed under anesthesia, and the organs and tissues were collected, washed in saline, and weighed. Radioactivity in all of the samples was measured with a gamma-counter. The percentage of ^{125}I-HSS remaining in blood at various time points were calculated, and serum pharmacokinetic parameters were calculated using the DAS (Drug and Statistics for Windows) software package (version 2.0 PK software; China). The total radioactivity per organ was also calculated and corrected for the blood-derived radioactivity. The organ accumulation of ^{125}I-HSS was calculated as a percentage of the injected dose per gram of wet tissue mass (%ID/g). Additionally, the remaining rats in each group were respectively sacrificed at 1 h, 2 h, 4 h and 8 h (n = 3 per group at every time point) after ^{125}I-HSS or ^{125}I-HSS-SSL administration, and liver tissues were collected to determine the hepatic accumulation amount of HSS.

2.4. Effect of HSS-SSL on FHF

2.4.1. Induction of FHF

For induction of FHF, rats were given intraperitoneal injections of TAA (400 mg/kg, three times at 24-h intervals) [18,19]. Supportive therapy by subcutaneous administration of 5% dextrose (25 mL/kg) and 0.9% NaCl (NS) with potassium (20 mEq/L) every 12 h were administered to avoid weight loss, hypoglycemia, and renal failure, as previously described [19].

2.4.2. Experiment protocol

The TAA-treated rats (n = 80) were divided randomly into four groups according to therapeutic agent: NS, SSL, HSS-SSL, or HSS. The agents were injected via the penile vein 2 h after the second and third TAA injection. The injection dosage of HSS at each treatment was 60 μg/kg. Rats were examined and staged for neurological signs every 12 h by an investigator who was unaware of the treatment. At 24 h after the third TAA injection, the surviving rats were sacrificed, and blood samples and liver tissue were collected for further experiments. To evaluate the proliferation of hepatocytes, BrdU (100 mg/kg) was injected intraperitoneally 1 h before the surviving rats were sacrificed.

2.4.3. HE and survival

The stage of HE and the survival of the rats were recorded every 12 h. The stage of HE was determined by the following neurobehavioral scale: stage 1, lethargy; stage 2, mild ataxia; stage 3, lack of spontaneous movement and loss of righting reflex, but still responsive; and stage 4, coma and lack of response to pain [4].

2.4.4. Histopathologic analysis

Liver tissues were processed for light microscopy by fixing the specimens in 10% neutral formalin solution for 24 h, embedding them in paraffin, slicing 5-μm-thick sections, and staining with hematoxylin–eosin. All specimens were examined by an experienced histologist who was unaware of the experimental protocol. The degree of inflammation and necrosis was expressed as the mean of 10 different fields (200×) within each section that has been classified on a scale of 0–3 (normal: 0, mild: 1, moderate: 2, severe: 3) [20].

2.4.5. Serum biochemical parameters

The blood samples were allowed to clot, and serum was removed by centrifugation at 1000 × g for 10 min. All serum samples were sterile, hemolysis-free, and were kept at 4 °C before the determination of the enzymatic activities of aspartate aminotransferase and alanine aminotransferase, International Neutralization Ratio of prothrombin time, and blood ammonia levels.

2.4.6. Hepatocyte proliferation

Cell proliferation in livers was assessed by BrdU incorporation into nuclei and immunohistochemical recognition as previously described [20,21]. The BrdU labeling index was determined by counting 4000 hepatocyte nuclei per section from consecutive light microscopy fields (400×). The immunolabeled sections were evaluated blindly to avoid bias, with the number of positively stained nuclei being expressed as a percentage of total nuclei.

2.4.7. Serum cytokine levels

Commercial enzyme-linked immunosorbant assay kits (Jianchen, Nanjing, China) were used according to the manufacturer's instructions to determine the serum levels of tumor necrosis factor-α, interleukin (IL)-2, and IL-6.

2.4.8. Hepatic lipid peroxidation levels

The steady-state level of malondialdehyde (MDA), a lipid peroxidation end product, was analyzed by measuring the level of thiobarbituric acid-reactive substances spectrophotometrically at a wavelength of 535 nm using 1,1,3,3-tetraethoxypropane as the standard, as previously described [19].

2.5. Statistical analysis

Continuous variables were presented as mean ± standard deviation (SD) and analyzed using a one-way analysis of variance followed by Post Hoc Tests. Survival was analyzed using Kaplan–Meier curves with differences evaluated by the log-rank test and the incidence of stage 3 or 4 HE was analyzed using the Fish's exact test. All statistics were calculated using SPSS 11.5 statistical software (Chicago, IL), and P values less than 0.05 were considered significant.

3. Results

3.1. Properties of HSS-SSL

The liposomes were unilamellar, round, and regular in size (Fig. 1A and B). The lipid concentration of freshly prepared SSL was 9.23 ± 0.31 μmol/mL, and the CC of SSL was 1.21 ± 0.03 μg HSS/μmol lipid. The encapsulation efficiency, polydispersity index, and size of HSS-SSL (n = 3) at different times after preparation are shown in Table 1. There was no significant difference in the properties of HSS-SSL between days 1 and 7 post-preparation ($P > 0.05$), but the properties of HSS-SSL showed marked variances 30 days after preparation ($P < 0.001$), which indicated the occurrence of the leakage of HSS and the fusion of liposomes after HSS-SSL were stored for 30 days at 4 °C. In addition, the polydispersity index and size of SSL were similar to those of HSS-SSL (data not shown).

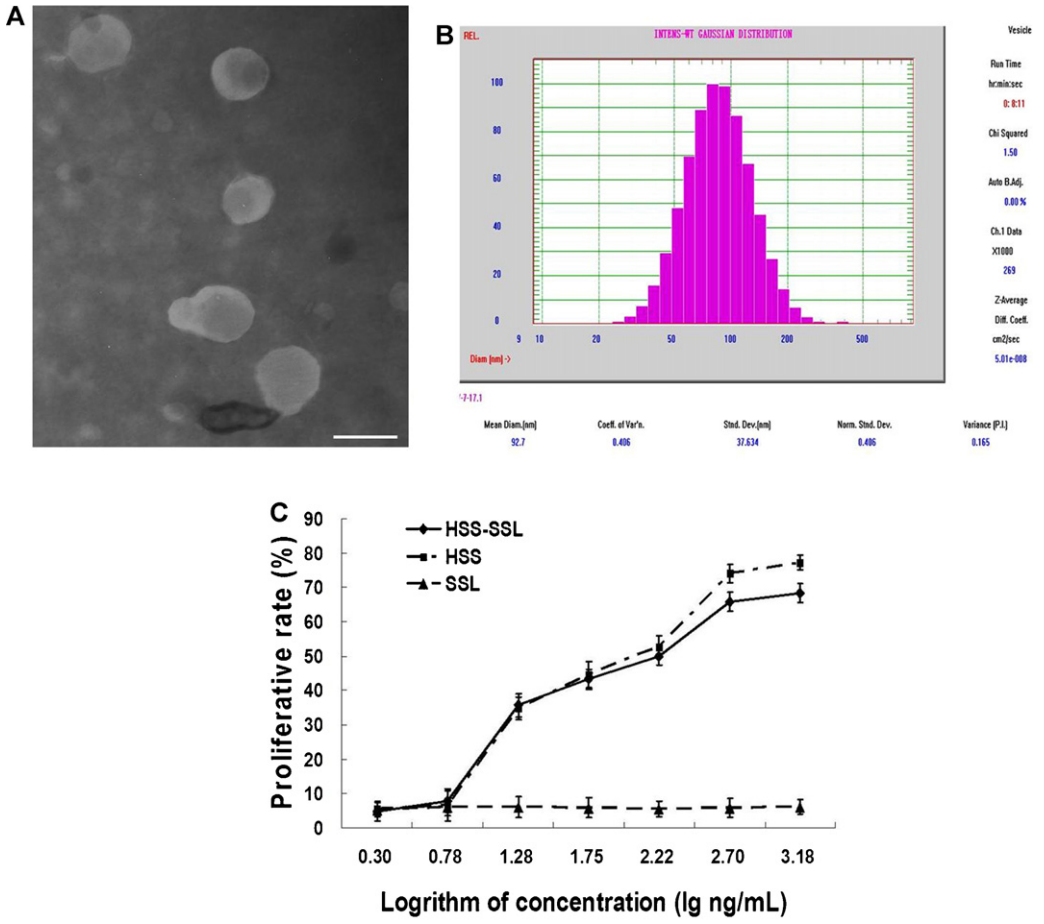

Fig. 1. Properties of HSS-SSL. (A) The morphology of HSS-SSL was examined by a transmission electronic microscope after being stained with 2% phosphotungstic acid and dried on carbon-coated grids. (B) The size and polydispersity index of the liposomes were determined using dynamic light scattering. (C) The effect of HSS-SSL, HSS and SSL on hepatocyte proliferation was evaluated by MTT assays. The absorbance rate of each well optical density (OD value) was measured at 595 nm by a spectrophotometer and the cell proliferation rate was calculated as (average OD value of wells with administered agent/average OD value of control wells) × 100. Every experiment was repeated thrice. Data were given as the mean ± SD.

Therefore, liposomes used for the following experiments were prepared and stored for no more than 7 days.

3.2. Effect of HSS-SSL on hepatocyte proliferation in vitro

MTT assay was used to determine hepatocyte proliferative rate *in vitro*. After incubation with EGF plus HSS-SSL or EGF plus HSS, hepatocyte proliferative rate were both increased along with the increasing concentration of HSS. But there was no significant change in the proliferative rate of hepatocyte after incubation with EGF plus SSL. The proliferative rate of hepatocytes incubated with EGF plus HSS-SSL or EGF plus HSS was significantly higher than that incubated with EGF plus SSL when the concentration of HSS was more than 20 ng/mL ($P < 0.001$ for all comparisons) (Fig. 1C).

Table 1
Properties of HSS-SSL at different time ($n = 3$).

Time	Size (nm)	PI	ee%
1 day	93.59 ± 0.95	0.165 ± 0.001	37.20 ± 0.36
7 days	99.34 ± 1.38	0.167 ± 0.001	36.11 ± 0.84
30 days	117.21 ± 0.88*	0.188 ± 0.002*	31.11 ± 0.71*

Polydispersity index (PI), encapsulated efficiency (ee).
* $P < 0.05$ versus liposomes in 1 day.

3.3. Pharmacokinetics and organ biodistribution of HSS-SSL in rats with liver injury

Encapsulation of HSS in SSL produced a significant change in drug pharmacokinetic parameters. In contrast to the rapid blood clearance observed for free ^{125}I-HSS, ^{125}I-HSS-SSL provided extended circulation lifetimes, with over 50% of the drug present 4 h after administration and 23.67 ± 2.08% remaining at 24 h (Fig. 2A). After intravenous administration, ^{125}I-HSS-SSL gave 10.21 ± 0.87 h as a terminal elimination half-life ($t_{1/2}$ β), compared with 1.51 ± 0.14 h for free drug, indicating a longer elimination half-life for HSS in SSL. It appears that HSS in SSL could be given intravenously with a long duration of action due to high drug and liposomal stability. The serum clearance (CL) and area under the curve (AUC) values confirmed this trend. ^{125}I-HSS-SSL resulted in approximately 6.06 times increase in AUC, significantly increased from 233.96 ± 22.81 ng h/L for the free drug to 1418.58 ± 167.17 ng h/L for the liposomes ($P < 0.05$), and 79.31% decrease in CL compared with ^{125}I-HSS ($P < 0.05$). More importantly, the encapsulation by SSL significantly increased the accumulation amount of HSS in injured livers. The liver accumulation of ^{125}I-HSS following administration of ^{125}I-HSS-SSL was significantly higher than that of free ^{125}I-HSS at the 1-, 2-, 4-, 8- and 24-h time points post injection (Fig. 2B). At 24 h post injection, most of free ^{125}I-HSS was observed to present in kidneys. In contrast, most of ^{125}I-HSS encapsulated in SSL was still accumulated in livers, which was

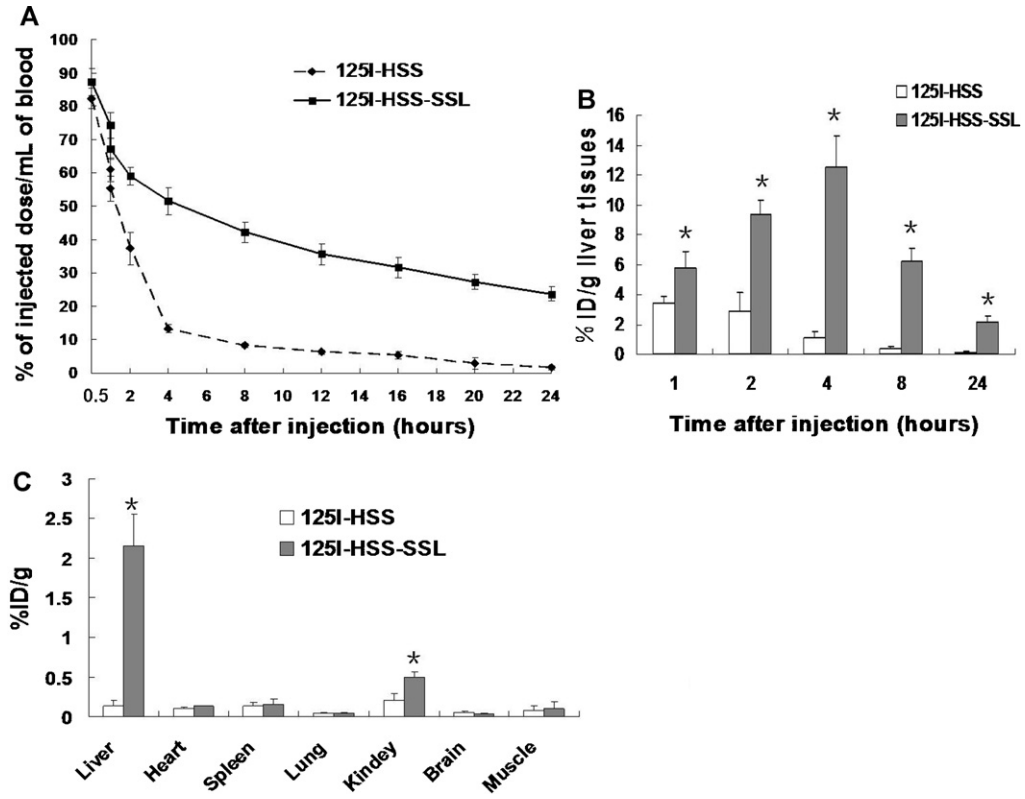

Fig. 2. Pharmacokinetics and organ biodistribution of HSS-SSL and free HSS in rats with liver injury. HSS was labeled with iodine-125 (^{125}I) using the iodogen method, and then the SSL encapsulating ^{125}I-HSS (^{125}I-HSS-SSL) was prepared. Liver injury was induced in rats intraperitoneally injected with a single dose of TAA (400 mg/kg). Twenty-four hours after treated with TAA, rats were randomly divided into two groups (n = 15 per group) and respectively injected with ^{125}I-HSS (0.5 MBq, 0.5 μg) or ^{125}I-HSS-SSL containing the same dosage of ^{125}I-HSS via the tail vein under normal pressure. Then, three rats were respectively randomly selected from each group and blood samples were collected via the intraocular vein at various time points up to 24 h. And after 24 h, the rats were sacrificed under anesthesia, and the organs and tissues were collected. Additionally, the remaining rats in each group were respectively sacrificed at 1 h, 2 h, 4 h and 8 h (n = 3 per group at every time point) after free ^{125}I-HSS or ^{125}I-HSS-SSL administration, and liver tissues were collected. Radioactivity in all of the samples was measured with a gamma-counter. (A) The percentage of ^{125}I-HSS remaining in blood at various time points after free ^{125}I-HSS or ^{125}I-HSS-SSL administration was calculated. (B) The total radioactivity in livers at 1 h, 2 h, 4 h, 8 h and 24 h post injection was determined and corrected for the blood-derived radioactivity. The hepatic accumulation of ^{125}I-HSS was calculated as a percentage of the injected dose per gram of wet liver tissue mass (%ID/g liver tissues). (C) The organ accumulation of ^{125}I-HSS at 24 h post injection was calculated as a percentage of the injected dose per gram of wet tissue mass (%ID/g). Data were given as the mean ± SD (n = 3 per group). $^{*}P < 0.05$ versus free ^{125}I-HSS.

nearly 20-fold compared to free ^{125}I-HSS (Fig. 2C). These results indicated that the encapsulation by SSL significantly prolonged the half-life of HSS and enhanced its target to injured livers.

3.4. Effect of HSS-SSL on hepatic histopathology

In livers of rats treated with NS and SSL, the normal structure of the hepatic lobule was markedly damaged, along with extensive bridging necrosis and massive neutrophil infiltration. In contrast, rats treated with HSS-SSL showed preserved integrity of the hepatic lobule and less hepatocyte necrosis and neutrophil infiltration. In rats treated with HSS, the structure of the hepatic lobule was also preserved, but distinct evidence of necrosis with marked degenerative changes and infiltration of neutrophils was still visible (Fig. 3). Histopathologic examination of liver specimens of rats treated with HSS-SSL showed less necrosis and less inflammation than those treated with NS, SSL, or HSS (Table 2).

3.5. Survival and HE

Rat survival was evaluated every 12 h. After the second TAA injection, a varying number of rats died in the different treatment groups. At 24 h after the third TAA injection, the survival rate of rats treated with HSS-SSL (80%) was significantly higher than that of rats treated with NS (20%, $P < 0.001$), SSL (25%, $P = 0.001$), or HSS (50%, $P = 0.047$; Fig. 4A).

The incidence of stage 3 or 4 HE gradually increased as the treatment was prolonged. At 24 h after the third TAA-injection, the incidence of stage 3 or 4 HE in rats treated with HSS-SSL (25%) was significantly lower than that in rats treated with NS (100%, $P = 0.003$), SSL (100%, $P = 0.014$), or HSS (70%, $P = 0.032$; Fig. 4B).

3.6. Serum biochemical parameters

The serum hepatic enzyme levels, blood ammonia levels, and the International Neutralization Ratio of prothrombin time were

Table 2
Effect of HSS-SSL and other therapeutic agents on hepatic histology in rats with FHF.

Group	Inflammation (0–3)	Necrosis (0–3)
NS	2.75 ± 0.42	2.75 ± 0.50
SSL	2.60 ± 0.55	2.40 ± 0.89
HSS-SSL	1.17 ± 0.39$^{*,\#,\blacktriangle}$	0.67 ± 0.65$^{*,\#,\blacktriangle}$
HSS	1.86 ± 0.69$^{*,\#}$	1.14 ± 0.69$^{*,\#,\blacktriangle}$

0.9% NaCl (referred to as NS), sterically stabilized liposomes (SSL), sterically stabilized liposomes encapsuling hepatic stimulator substance (HSS-SSL), hepatic stimulator substance (HSS).
* $P < 0.05$ versus NS group.
$^{\#}$ $P < 0.05$ versus SSL group.
$^{\blacktriangle}$ $P < 0.05$ versus HSS group.

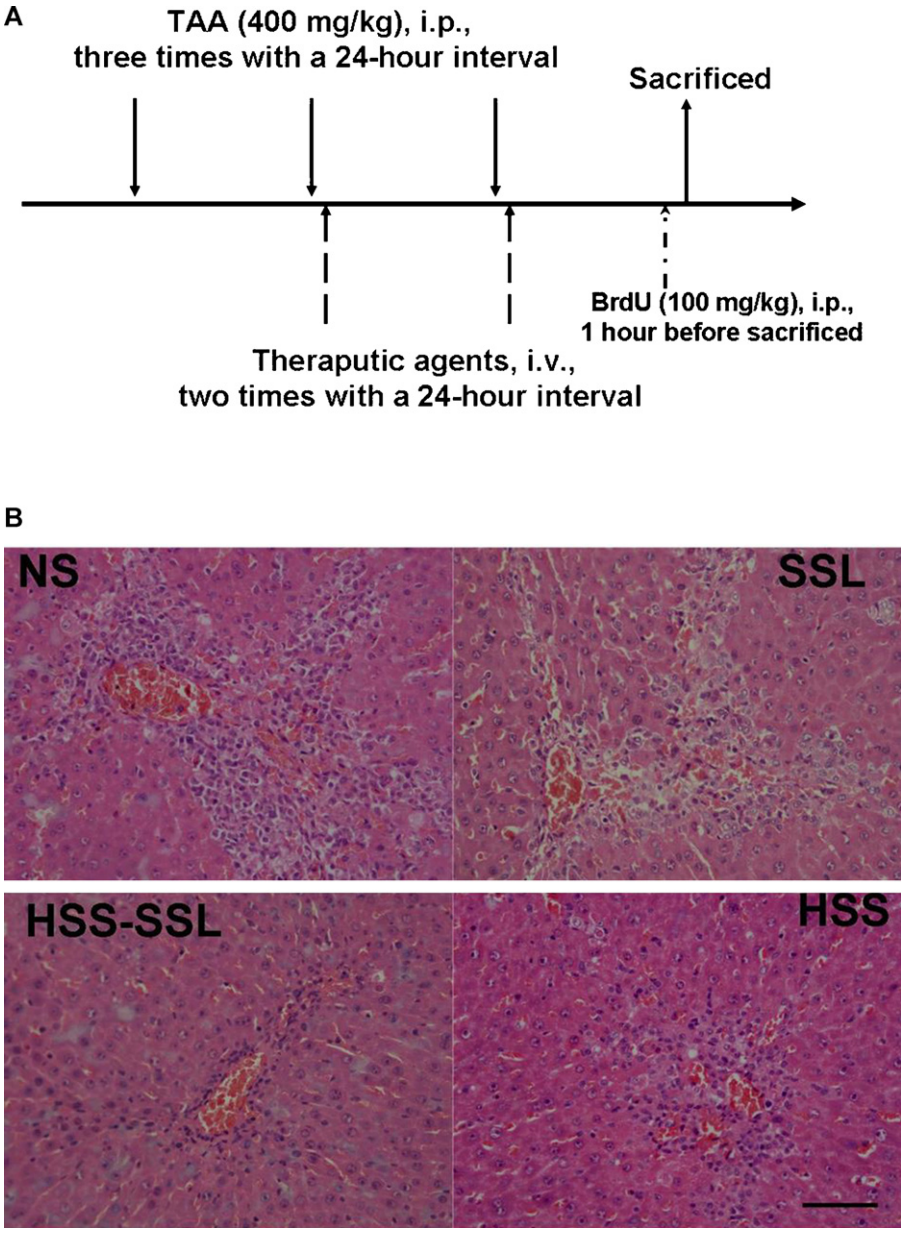

Fig. 3. Experimental protocol and representative micrographs of hepatic histology from rats treated with sodium chloride (referred to as NS), SSL, HSS-SSL, or HSS. (A) Experimental protocol. These rats were injected intraperitoneally with TAA (400 mg/kg, three times in a 24-h interval) to induce FHF, and the therapeutic agents were injected via the penile vein 2 h after the second and the third TAA dosage. At 24 h after the third TAA injection, surviving rats were sacrificed, and BrdU (100 mg/kg) was injected intraperitoneally 1 h before the surviving rats were sacrificed. (B) Livers were collected for hematoxylin–eosin staining after surviving rats were sacrificed. Images were taken at original magnification (200×). Scale bars, 100 μm.

analyzed. Liver injury, as manifested by an elevation in serum alanine aminotransferase and aspartate aminotransferase, was less severe in the HSS-SSL group ($P < 0.05$) than in the other three groups (Fig. 4C). The lowest International Neutralization Ratio of prothrombin time and the lowest blood ammonia level were found in the HSS-SSL group ($P < 0.05$; Fig. 4D and E).

3.7. Hepatocyte proliferation

Cell proliferation in livers of rats treated with TAA was assessed by BrdU incorporation into nuclei. After immunohistochemical recognition, there were the most cells with nuclei positive for BrdU-staining in HSS-SSL treatment group. The highest proliferative rate of hepatocytes ($12.56 \pm 2.31\%$) was observed in the HSS-SSL group, which indicated that HSS-SSL stimulated hepatocyte proliferation more significantly than NS ($2.00 \pm 0.82\%$), SSL ($1.60 \pm 1.14\%$), or HSS ($5.40 \pm 1.24\%$, $P < 0.001$; Fig. 5).

3.8. Serum cytokines levels and hepatic MDA level

The serum levels of pro-inflammatory cytokines were measured. The serum levels of tumor necrosis factor-α, IL-2, and IL-6 in the HSS-SSL group were all significantly decreased ($P < 0.05$) compared to other groups (Fig. 6A–C).

Hepatic lipid peroxidation levels were measured by analyzing the steady-state level of MDA. After injection of TAA, the hepatic MDA level in rats treated with HSS-SSL (11.25 ± 3.13 nmol/g wet tissue) was significantly lower than that in rats treated with NS (43.32 ± 4.18 nmol/g wet tissue), SSL (42.56 ± 2.18 nmol/g wet tissue) or HSS (25.95 ± 5.00 nmol/g wet tissue, $P < 0.05$; Fig. 6D).

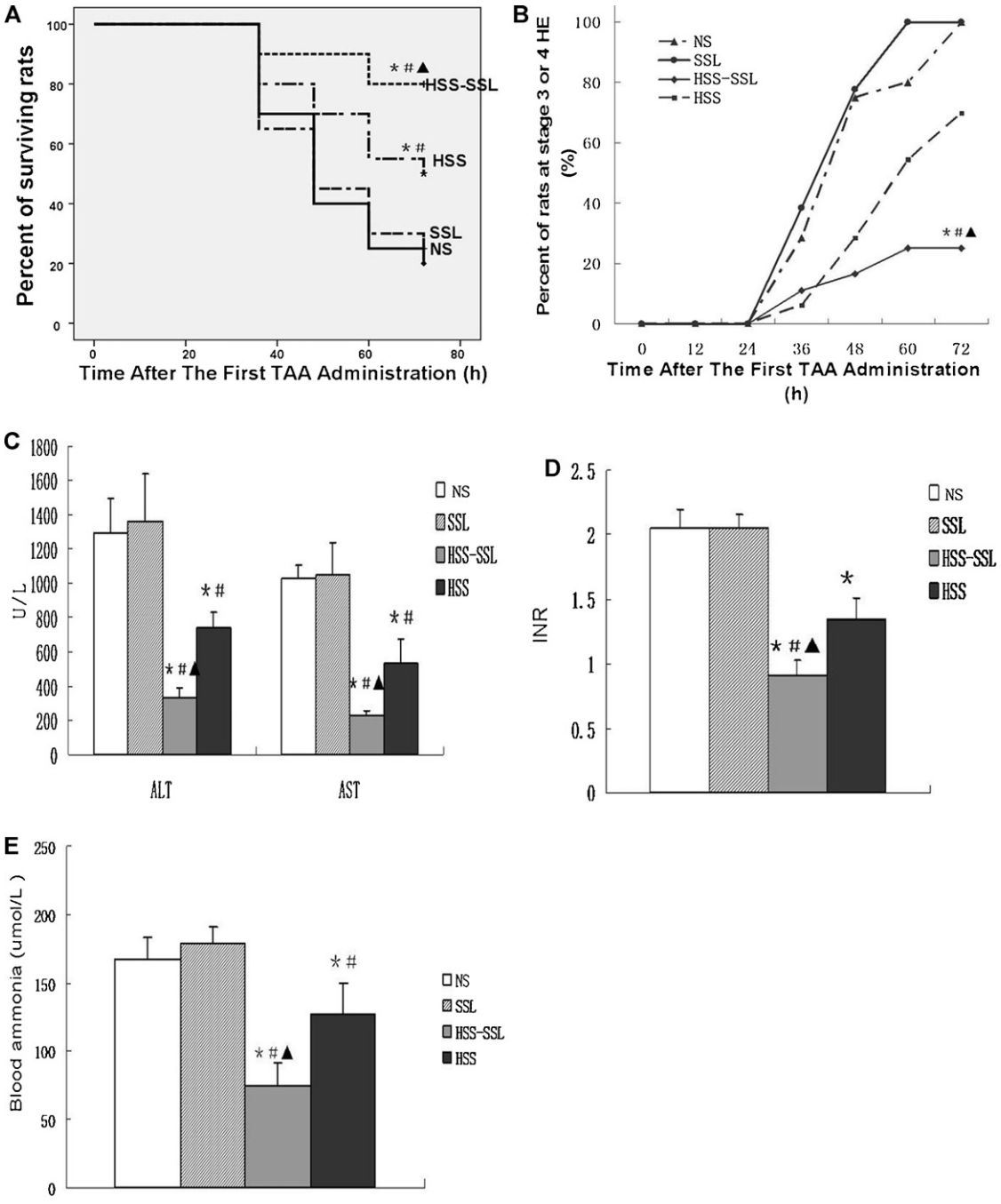

Fig. 4. Therapeutic effect of HSS-SSL and different agents on FHF. Animals from all groups were examined and staged for neurological signs every 12 h. (A) Survival was analyzed using Kaplan–Meier curves with differences evaluated by the log-rank test. (B) The stage of HE was determined by the neurobehavioral scale. (C–E) Blood samples were drawn via the inferior vena cava. The enzymatic activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (C), and the International Neutralization Ratio (INR) of prothrombin time (D), and blood ammonia levels (E) were compared. Data were given as the mean ± SD. $^{*}P < 0.05$ versus NS; $^{\#}P < 0.05$ versus SSL alone; $^{\blacktriangle}P < 0.05$ versus HSS alone.

4. Discussion

To our knowledge, this is the first report about the application of SSL for the therapy of FHF. In the present study, the encapsulation by SSL markedly improved the therapeutic effect of HSS on FHF induced by TAA poisoning in rats. This positive effect was demonstrated by increased survival rate and reduced incidence of stage 3 or 4 HE by promoting hepatocyte proliferation, inhibition of pro-inflammatory cytokine release, and amelioration of hepatic lipid peroxidation. As mentioned previously, the important characteristics of SSL are their prolonged blood residence time and infectious target localization. Encapsulation by SSL endued the encapsulated drug with these characteristics, increasing the effective concentration of drug at the infectious site [7–11]. In the present study, the increased hepatic accumulation amount of HSS was confirmed by the pharmacokinetic study and the organ biodistribution study of HSS-SSL in rats with liver injury. Therefore, HSS-SSL showed a more potent therapeutic effect on FHF than free HSS.

The experimental animal model of FHF in rats induced by TAA is a reliable analog to human FHF and HE [18]. Following two to three consecutive doses of TAA, rats develop FHF characterized by

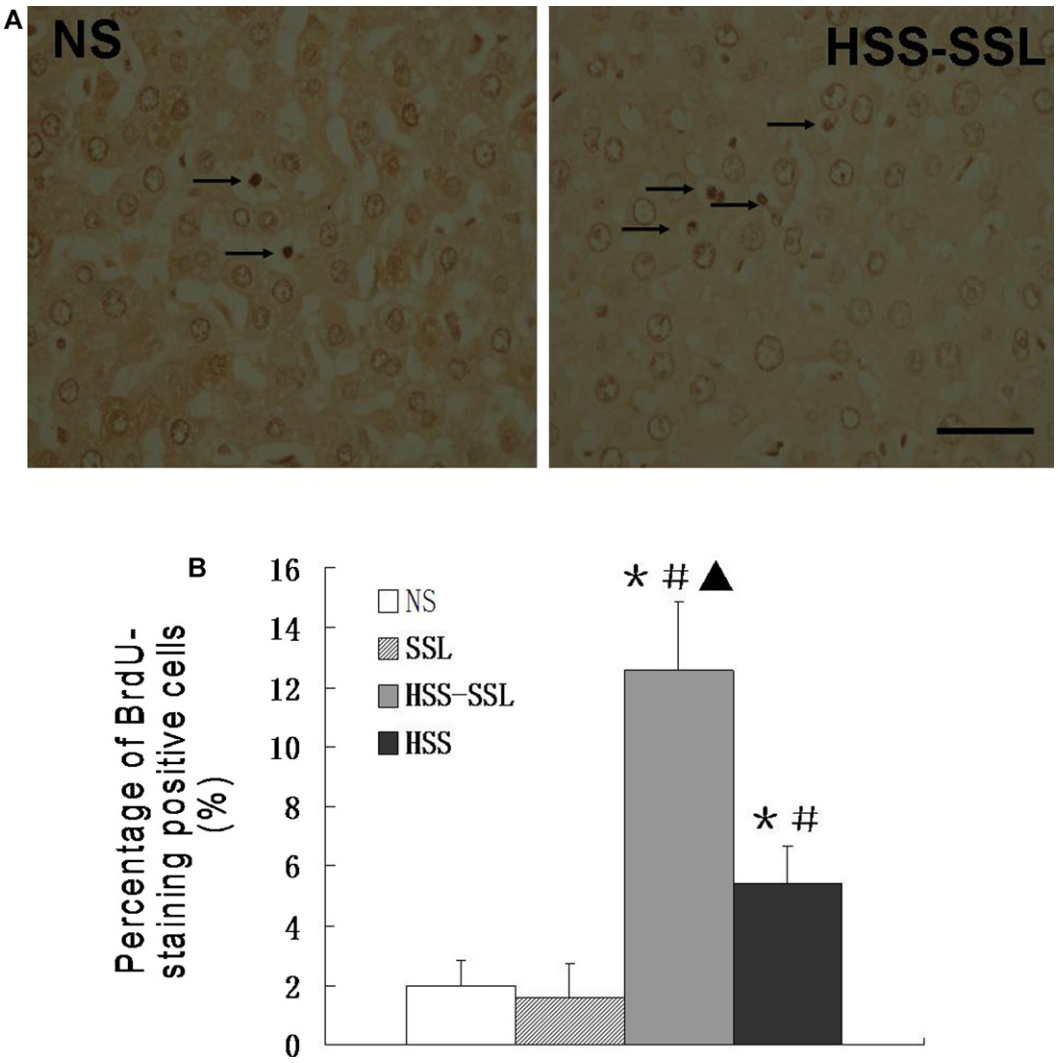

Fig. 5. Effect of HSS-SSL and different therapeutic agents on hepatocyte proliferation in rats with FHF. 5-bromo-2′-deoxyuridine (BrdU; 100 mg/kg) was injected intraperitoneally 1 h before the surviving rats were sacrificed and hepatocyte proliferation was assessed by BrdU incorporation into nuclei and immunohistochemical recognition. (A) Representative micrographs of hepatocyte proliferation from rats treated with NS and HSS-SSL. Liver sections were subjected to immunohistochemical staining for BrdU (400×). The arrows show positive BrdU-staining hepatocytes. Scale bars, 100 μm. (B) The percent of positive BrdU-staining cells among all treatment groups were compared. Data were given as the mean ± SD. $^{*}P < 0.05$ versus NS; $^{\#}P < 0.05$ versus SSL alone; $^{\blacktriangle}P < 0.05$ versus HSS alone.

massive liver necrosis, rapid neurologic deterioration, and death due to severe encephalopathy and brain edema [18,19]. In most experimental studies of FHF, the early therapeutic intervention commonly practiced (i.e., before or at the same time as the induction of FHF) is impractical under clinical conditions. In our preliminary study, we demonstrated that the obvious necrosis and inflammation of the liver, and the marked deterioration of liver function were present 24 h after the first TAA administration (data not shown). Accordingly, to simulate clinical conditions, therapeutic agents were administered to the TAA-treated rats after signs of hepatic failure appeared instead of being administered before or at the same time as TAA injection.

HSS is produced in regenerating and developing livers and affects hepatocyte proliferation. Increased HSS activity was found in rats with FHF and peaked 12 h after the third TAA injection, a reaction in response to repeated exposure to toxic TAA metabolites [4,22]. Despite the increase in endogenous HSS activity in the livers of rats with FHF, increased mortality was still observed, indicating that the increased endogenous HSS activity had not enhanced hepatocyte replicative capacity enough to improve liver histology and survival [19]. In contrast, exogenous HSS administration has been shown to enhance liver proliferative capacity and to improve survival in rats with FHF [5,6,23]. In the present study, HSS-SSL administration significantly improved the short-term survival rate of rats with FHF compared to HSS alone, SSL, and NS. Because of the high short-term mortality of FHF, it was impractical to compare the effects of different therapeutic drugs on the long-term survival rate. However, increased short-term survival would be crucial for patients with FHF, as it would increase the opportunity for liver transplantation to these patients.

The incidence of stage 3 or 4 HE in the surviving rats was significantly reduced after HSS-SSL treatment compared to NS, SSL and HSS treatment. HE is an important prognostic factor for FHF, because as higher grades of encephalopathy develop, the prognosis becomes worse and more difficult to predict [24]. The HSS-SSL-treated rats showed the highest short-term survival rate and the lowest incidence of stage 3 or 4 HE compared to the other three treatment groups, which would predict a better long-term prognosis. Thus, the encapsulation by SSL significantly improved the therapeutic potency of exogenous HSS for FHF. When the therapeutic effects of HSS-SSL on FHF induced by TAA were evaluated, the direct effect of SSL or HSS on the metabolism of TAA

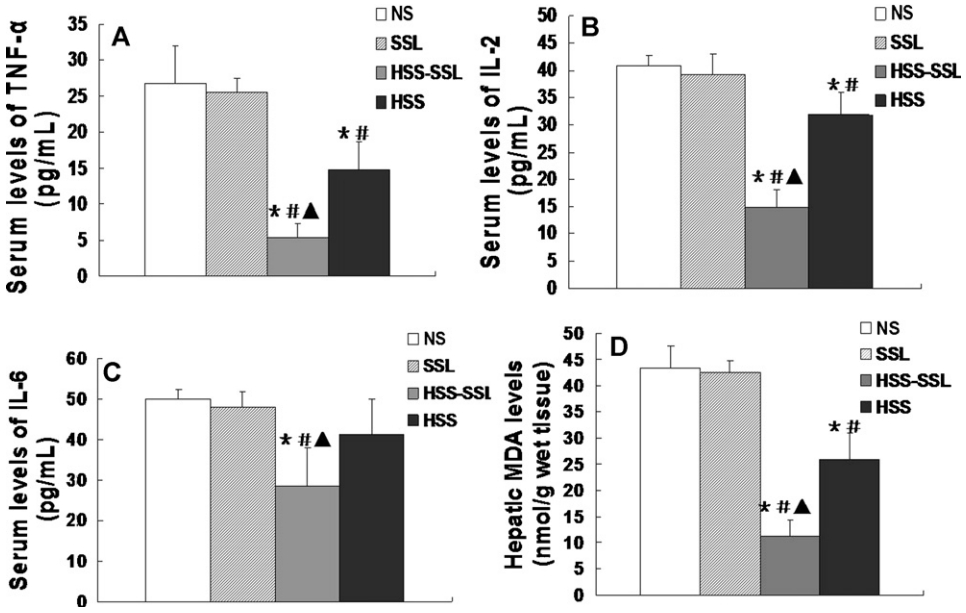

Fig. 6. Effect of HSS-SSL and different therapeutic agents on serum cytokine levels and hepatic lipid peroxidation levels of rats with FHF. (A–C) After blood samples were drawn from the surviving rats, the serum levels of tumor necrosis factor-α (TNF-α) (A), interleukin (IL)-2 (B), and IL-6 (C) were compared. (D) The steady-state level of malondialdehyde (MDA), a lipid peroxidation end product, was analyzed by measuring the level of thiobarbituric acid reactive substances. Data were given as the mean ± SD. $^{*}P < 0.05$ versus NS; $^{\#}P < 0.05$ versus SSL alone; $^{\blacktriangle}P < 0.05$ versus HSS alone.

had to be considered for potential interference with the development of TAA-induced FHF. However, the survival and the incidence of stage 3 or 4 HE showed no marked difference between the SSL-alone group and the control group. Additionally, both of HSS-SSL and HSS treatment significantly promoted hepatocyte proliferation compared to NS treatment, which did not relate with the metabolism of TAA. Hence, in the present study, the therapeutic effect of HSS-SSL on FHF was not resulted from the direct effect of SSL or HSS on the metabolism of TAA.

Hepatocyte proliferation has a crucial effect in the recovery from FHF [23,25]. In the present *in vivo* study, the hepatocyte proliferative rate in the HSS-SSL and HSS groups was significantly higher than that in the NS or SSL groups, and was the highest in the HSS-SSL group. These results indicated that the encapsulation by SSL significantly enhanced the potency of exogenous HSS to promote hepatocyte proliferation.

TAA inflicts tissue injury by producing reactive oxygen species, thereby directly promoting the peroxidation of membrane lipids [26,27]. It has been reported that antioxidants decrease the levels of lipid peroxidation end products and improve liver histology in TAA-induced FHF animal models [28]. Previous studies had revealed that besides promoting hepatocytes proliferation, HSS exerted its hepatoprotective actions by increasing mitochondrial respiratory activity, restoring the mitochondrial respiratory function and antioxidant defense, as well as decreasing lipid peroxidation in the liver [29,30]. In the present study, the hepatic level of MDA, a lipid peroxidation end product, was compared among the different treatment groups. After administration of TAA, HSS-SSL significantly decreased the hepatic MDA level compared to NS, SSL, and HSS, suggesting that HSS-SSL administration ameliorated oxidative stress in the livers of rats with FHF.

Clinically, viral hepatitis, drugs, and chemically induced liver injury account for most cases of FHF [1]. In addition to the primary damage caused by the noxious agent on the liver cells, there is also secondary damage due to a vicious circle set up by the release of pro-inflammatory cytokines (e.g., tumor necrosis factor-α, IL-2, and IL-6) and cytotoxic mediators from activated cells (e.g., Kupffer cells and neutrophil granulocytes) [30]. In the present study, HSS-SSL treatment showed better therapeutic potency for FHF and coincided with decreased levels of the pro-inflammatory cytokines mentioned above. However, decreased levels of pro-inflammatory cytokines potentially can inhibit regeneration of the diseased liver, for these cytokines have been considered priming factors (e.g., tumor necrosis factor-α and IL-6) for hepatocyte proliferation, allowing hepatocytes to become more strongly sensitized to the influence of growth factors (e.g., epidermal growth factor) [31,32]. Although HSS-SSL significantly inhibited the release of pro-inflammatory cytokines, it still promoted hepatocyte proliferation *in vivo*, likely due to the direct effect of HSS itself on enhancing hepatocyte proliferative capacity.

In conclusion, encapsulation by SSL significantly enhances the therapeutic potency of HSS for FHF in rats induced by TAA. After encapsulated in SSL, HSS markedly increases the short-term survival for FHF. This is crucial for patients with FHF who is waiting for liver transplantation, because it will increase their opportunity for liver transplantation. HSS-SSL also markedly decreases the incidence of HE, promotes hepatocyte proliferation and interrupts the vicious circle set up by oxidative stress and pro-inflammatory cytokines. All of these will significantly improve the long-term prognosis of patients with FHF. Thus, HSS-SSL is a potential therapeutic agent for FHF and the encapsulation by SSL may be an effective general approach to enhance the therapeutic potency of drugs for FHF.

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