INTRODUCTION

Iron is a vital micronutrient for the human body, and the liver is the organ primarily responsible for maintaining body iron homeostasis. Clinically, iron overload is always present in patients with hereditary hemochromatosis and patients with repeatedly blood transfusion. In these patients, the risk of liver diseases, such as liver cirrhosis, was significantly increased. Laboratory studies have shown that large doses of iron dextran could induce liver injury and fibrosis in mice. Similarly, patients with liver diseases, such as alcoholic or nonalcoholic fatty liver disease or viral hepatitis, also accompanied by higher iron levels than healthy normal. These findings indicate that iron overload and liver disease are the interrelationships of cause and effect. Therefore, we believe that reducing hepatic iron deposition might be an effective way to relieve these diseases.

When iron uptake exceeds the capacity for export, large amounts of iron deposits in liver tissue. The excess iron induces widespread activation of oxidative stress, released of inflammatory cytokines, accelerated hepatocyte apoptosis, and eventually resulted in the damage of hepatic structure and function. Studies have suggested that hepatocyte uptake of iron is associated with several proteins. Transferrin/transferrin receptor (Tf/TfR)-mediated uptake of dfferic transferrin is a well-accepted process of iron uptake, and the (Fe₃Tf-TfR) enters hepatocytes through endocytosis. Divalent metal-ion transporter-1 (DMT-1) is ubiquitously expressed, but mainly in the proximal duodenum, and is responsible for intestinal iron absorption. Further research has indicated that DMT-1 protein is highly expressed in hepatocyte plasma membranes in the iron overloaded state. Besides, under iron overload conditions, the voltage-dependent L-type calcium channel might provide another entry point for iron. Also, αIC subunit (one of the channel protein subunits) is responsible for pore-forming, playing an important role in the channel gating and selectivity. So, the L-type calcium channel αIC, TfR, and DMT-1 proteins were identified as potential targets in this study.

Danshen (Salvia miltiorrhizae), a traditional Chinese herbal medicine, is mainly used for the treatment of cardiovascular diseases. Danshensu (3-(3,4-dihydroxyphenyl)-(2R)-lactic acid, Fig. 1), also known as salvianic acid A, is one of the water-soluble active ingredients of Danshen. Previous studies have indicated that Danshen Injection (a preparation commonly used in clinical practice) showed significant anti-iron overload ability, and it exhibited obvious protective effects on the heart, liver, and kidney. In these studies, we detected the concentration of Danshensu in the Danshen injection, and found that its content is higher than the other two ingredients: protocatechuic aldehyde and salvianolic acid B. Thus, we deduced that Danshensu might play an important role in the protection of organ injury induced by iron overload.

Therefore, the present study was carried out to explore the hepatic–protective effects of Danshensu on iron overload model mice induced by iron overload. Also, we focused on the potential mechanisms on iron uptake proteins, oxidative stress, inflammation, and apoptosis. Moreover, the iron-chelating agent desferrioxamine (DFO) was used as a positive control in this study.
**MATERIALS AND METHODS**

**Drugs and Reagents** Sodium Danshensu was purchased from Beijing SLF Chemical Research Institute (Beijing, China). Desferrioxamine mesylate (DFO) was obtained by Novartis Pharma AG (Basel, Switzerland). Unless otherwise stated, other reagents were obtained from Sigma (Shanghai, China).

**Animals and Experimental Protocol** All animal handling procedures were in accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics, National Health Department of China. And this study was approved by the Ethics Committee for Animal Experiments from the Committee of Medical Ethics, National Health Department of China. Six to eight-week-old male Kunming mice were purchased from Hebei Medical University (certificate of conformity no. 1602012). Mice were housed in a temperature- and humidity-controlled room (22°C, 50%) with a 12-h light and dark cycle with free access to food and water.

After 1 week of acclimation, these mice were randomly divided into five groups (n = 10 per group): control, iron overload (Fe), Fe + high dose Danshensu (H-DSS), Fe + low dose Danshensu (L-DSS), and Fe + DFO (DFO) groups. Mice in the control group were injected with saline (10 mL/kg) every day; mice in iron overload group were treated with iron dextran 50 mg/kg per day; Mice in H-DSS, L-DSS, and DFO groups were injected with the same dose of iron dextran in the morning and injected with 100 mg/kg/d Danshensu, 50 mg/kg/d Danshensu, and 100 mg/kg/d DFO in the afternoon, respectively. Food intake and activities of all mice were observed daily. The entire experimental period lasted for 2 weeks. When the animals were anesthetized (sodium pentobarbital, 50 mg/kg), the mice were sacrificed by cervical dislocation, and the liver of each mouse was rapidly removed and frozen in liquid nitrogen for the next analysis.

**Hematoxylin and Eosin (H&E) and Prussian Blue Staining**

H&E staining was used for histopathological analysis, and the Prussian blue staining was used for analysis hepatic iron accumulation. The fixed liver tissues were embedded in paraffin and cut into 4-µm sections, and then the sections were deparaffinized, dehydrated, and stained with H&E or with Prussian blue according to the manufacturer’s protocol. Image-Pro Plus software was used for the analysis of Prussian blue-stained samples, and values were expressed as percentage of integral optical densities (IOD%) = positive IOD/sum IOD × 100%.

**Detection of Serum Glutamic-Pyruvic Transaminase (ALT) and Glutamic–Oxaloacetic Transaminase (AST)**

The activity of ALT and AST in serum were detected by spectrophotometry method using commercially available kits (Jian Cheng Biological Engineering Institute, China).

**Measurement of Iron Uptake Related Proteins**

Western blotting was used to detect the iron uptake related proteins (DMT1, TIR, L-type calcium channel α1C subunit (L-type Ca2+ CP α1C)) on hepatocytes. Frozen liver tissue samples were cleavaged and homogenized with 400 µL lysis buffer/20 mg tissue, and then centrifuged at 12000 r/min for 10 min, collecting the supernatant. The total proteins were loaded and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a nitrocellulose membrane. Blocking with 5% skimmed milk powder in phosphate buffer saline (PBS), then the membranes were incubated with primary antibodies overnight at 4°C. Uncombined primary antibodies were removed by three washes (10 min each), and then incubated with secondary antibodies (Santa Cruz, U.S.A.) at room temperature for 90 min. Another three washes with PBS were performed to remove the unbound secondary antibodies, the film was exposed and scanned by the Gel imaging analysis system (UVP, U.S.A.), and the gray value is automatically generated by the system. The primary antibodies included: anti-DMT-1 (ab55812, Abcam, China), anti-TfR (ab84036, Abcam), and anti-L-type Ca2+ CP α1C (sc-398433, Santa Cruz).

**Detection of Malondialdehyde (MDA) Content and Activities of Glutathione Peroxidase (GSH-Px) and Superoxide Dismutase (SOD)**

The antioxidant enzymes GSH-Px and SOD, as well as the product of oxidative stress MDA, were detected using a spectrophotometric method. Preparation of liver homogenate was done using a tissue homogenizer of liver homogenate was done using a tissue homogenizer (100 mg tissue per mL of 50 mM PBS). The homogenate was centrifuged, then the supernatant was used for biochemical analyses according to the manuals of commercially kits (Jian Cheng Biological Engineering Institute).

**Assessment of Interleukin-6 (IL-6) and Transforming Growth Factor β (TGF-β)**

The inflammatory markers IL-6 and TGF-β were detected using immunohistochemistry. The fixed liver tissues were made into 4-µm thick paraffin sections. Then, the sections were deparaffinized and hydrated with xylene and gradient ethanol. Rinsed with PBS, the sections incubated with 3% H2O2 for 30 min. After rinsing, the sections were subjected to microwave antigen retrieval with citrate buffer. After three washes with PBS (5 min each), sections were blocked for 30 min and then incubated with primary antibodies overnight at 4°C. The control group was treated with PBS instead of the primary antibody, then rinsed and incubated with secondary antibodies at 37°C for 30 min. After three washes with PBS (5 min each), 3,3’-diaminobenzidine (DAB) was added to the sections for color reaction, washed, and counterstained with hematoxylin. Image-Pro Plus software was used to analyze 20 randomly selected fields per slide at magnification (×400), and values were expressed as percentage positive area = positive area/sum area × 100%. The primary antibodies included: anti-TGF-β (A00892, Boster, China) and anti-IL-6 (BA4339, Boster).

**Analysis of Hepatocyte Apoptosis**

First, the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) staining was used to observe the degree of apoptosis. TUNEL was performed using an in situ cell death detection kit (Roche Applied Science, Mannheim, Germany), and the steps were similar to immunohistochemistry. Second, three apoptotic factors (Bax,
Bcl-2, and Caspase-3) were detected by Western blotting (related steps were described above). The primary antibodies included: anti-Bax (ab32503, Abcam), anti-Bcl-2 (ab32124, Abcam) and anti-Caspase-3 (ab44976, Abcam).

**Determination of Interaction between Danshensu and Iron Ions in Vitro**  In vitro, the iron chelation effects of Danshensu were detected by UV-visible spectrophotometry method, and the experimental process is briefly described below. Accurately transfer 0.5 mL Danshensu control solution (1 mM/mL) into a 10mL volumetric bottle, diluted to 2mL with deionized water, added 2mL HAc-NaAc buffer solution (1 mM/mL, pH = 6.5), and then added 1mL ferrous ammonium sulfate (FAS, 1 mM/mL) reference solution (Danshensu + Fe²⁺) or 1mL ferric chloride reference solution (Danshensu + Fe³⁺) or added 1mL deionized water as a control (Danshensu + dH₂O), diluted to the scale (10mL) with deionized water, shake well, and let it stand for 10min in the dark. The three working solutions were detected by double-beam UV-visible spectrophotometer (UV-9000, Shanghai Metash instruments Co., Ltd, Shanghai, China), and the absorption spectra were recorded at 300–900nm.

**Data Analysis** Data are expressed as the mean ± standard error of the mean (S.E.M.). Statistically significant differences were identified using a one-way ANOVA. Differences were considered statistically significant at \( p < 0.05 \). The statistical analysis software, Origin 7.5 was used for all analyses.

**RESULTS**

**Morphological Analysis** H&E staining in Fig. 2 shows the morphological changes among different groups. The sections of the iron overload group showed disruption of hepatic architecture, and plenty of iron particles accumulated in the hepatic mesenchyma, forming a mass and surrounded by a large number of inflammatory cells around. In the Danshensu and DFO treatment groups, the hepatic architecture was more orderly than that of the iron overload group, and there were no visible clumps of iron particles and inflammatory cells, but the hepatocytes were exhibiting obvious swelling in DFO treatment mice.

**Biochemical Markers of Liver Function** Serum ALT and AST were detected to assess the changes of liver function. As shown in Fig. 2B, the activities of serum ALT and AST significantly increased in the iron overload group compared with the control group. In the treatment groups, the activities of serum ALT and AST were lower than that of the iron overload group.

**Iron Deposition and Iron Uptake Proteins** Prussian

![Fig. 3. Effects of Danshensu on Iron Deposition Changes in Mouse Liver](image-url)

The blue reactions on the sections represent the deposited iron (magnification 400×, scale bar = 50µm). The percentage of integral optical densities (IOD%) of Prussian blue staining in each group were quantified. Values are mean ± S.E.M. \(^{**}p < 0.01\) vs. control group; \(^{**}p < 0.01\) vs. Fe group. (Color figure can be accessed in the online version.)
blue staining showed that there was extensive iron accumulation (mainly in the nonparenchymal cells) in the iron overload group (Fig. 3). Correspondingly, Fig. 4 indicated that the DMT1, TfR, and L-type Ca^{2+} CP α1C proteins were all up-regulated in the conditions of iron overload. After the treatment of Danshensu and DFO, the iron deposition was decreased (Fig. 3). At the same time, Western blot detection found that the expression of these proteins was significantly down-regulated in the Danshensu treatment groups (Fig. 4). The regulatory effect of DFO on these proteins was not better than that of Danshensu. Therefore, the three proteins might be important targets for Danshensu to regulate iron overload.

**Oxidative Stress** Figure 5 shows that, in the iron overload group, the activities of antioxidant enzymes SOD and GSH-Px were inhibited, and the concentration of peroxidation product MDA was increased compared with the control group. In contrast, the activities of SOD and GSH-Px were strengthened, and the level of MDA was decreased under the effect of Danshensu. We found that the degree of oxidation in the Danshensu group was decreased, and the antioxidant activity of DFO is between the high dose Danshensu and low dose Danshensu.

**Inflammation** IL-6 and TGF-β were detected as inflammatory factors in this study. The immunohistochemistry results (Fig. 6) demonstrated that the percentages of the positive area of IL-6 and TGF-β were increased in the iron overload group. However, the expression levels in the Danshensu and DFO treatment groups were significantly decreased compared with iron overload group. These results show that Danshensu
decreased the degree of inflammatory response.

**Apoptosis** The TUNEL results in Fig. 7A show that the percentage of positive areas in the iron overload model group was higher than that in the control group. However, the positive hepatocytes were significantly decreased in the Danshensu and DFO-treated groups. Moreover, three apoptosis-related proteins (Bax, Bcl-2, and Caspase-3) were detected using Western blotting. As shown in Figs. 7B and C, Danshensu treatment obviously down-regulated the expression levels of the pro-apoptotic protein Bax and Caspase-3, and up-regulated the level of the anti-apoptotic protein Bcl-2. These results show that Danshensu can reduce iron overload-induced hepatocyte apoptosis by regulating the expression of apoptosis-related proteins.

**The Chelating Effect of Danshensu in Vitro** The results in Fig. 8 showed that, in vitro, Danshensu showed a weak chelating effect with Fe$^{2+}$ (the maximum value of absorption ($A_{\text{max}}$) is approximately 0.02), but it showed a little stronger chelating effect with Fe$^{3+}$ ($A_{\text{max}}$ is approximately 0.08).

**DISCUSSION**

Danshen (*Salvia miltiorrhiza*) is a traditional Chinese medicinal herb, and it has been used for promoting blood circulation and to treat cardiovascular diseases.$^{16-18}$ Danshensu is a major water-soluble component of Danshen, and it has been reported that Danshensu possesses extensive pharmacological activities on cardiovascular and cerebral diseases, such as myocardial ischemia and reperfusion, hypertension, cerebral ischemia, and cognitive impairments.$^{19}$ Moreover, one of the protective mechanisms of Danshensu on the isolated heart against ischemia-reperfusion may be associated with activating nuclear factor erythroid-2-related factor 2 (Nrf2) signaling pathway.$^{20}$ Recently, Danshensu has been reported to have liver-protective function against hepatic fibrosis induced by carbon tetrachloride through inhibition of Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway or against liver injury induced by omethoate through inhibition of tumor necrosis factor-alpha and cyclooxygenase-2.$^{21-23}$

Our previous studies demonstrated that chronic (7–9 weeks)
Fig. 7. Effects of Danshensu on Hepatocyte Apoptosis

A: TUNEL staining. Positive expression of TUNEL (arrows) is indicated (magnification 400×; scale bar = 50 µm), and percent of the TUNEL-positive area were quantified; B: The expression levels of Bax, Bcl-2, and Caspase-3 by Western blot analysis. Relative intensities were calculated by normalization to that of β-actin in each group. Values are mean ± S.E.M. *p < 0.01 vs. control group; *p < 0.05 and **p < 0.01 vs. Fe group. (Color figure can be accessed in the online version.)

Fig. 8. The Chelating Effect of Danshensu with Iron Ions

The black line represents the absorption of Danshensu chelating with deionized water (dH₂O), which is a control in this experiment; The red line represents the absorption of Danshensu chelating with ferric iron (Fe³⁺); The blue line represents the absorption of Danshensu chelating with ferrous iron (Fe²⁺). Values (the maximum value of absorption) are mean ± S.E.M. *p < 0.01 vs. Danshensu + dH₂O. (Color figure can be accessed in the online version.)
or acute (2 weeks) iron overload could induce hepatic fibrosis or injury, and Danshen injection presented notable hepatoprotective activity.\(^{2,3,24}\)

Danshen injection is a water extract of roots and rhizoma of *Salvia miltiorrhiza* (Danshen), and it is a mixed solution of many kinds of active ingredients with extensive pharmacological effects.\(^{25,26}\) However, it is reported that high-dosage of Danshen injection can cause adverse reactions.\(^{27,28}\)

Therefore, study a single active ingredient is of great clinical significance. Danshensu is one of main active ingredients of Danshen injection, in the present study, we have found that Dnshensu also showed obvious liver protective effect. Some of the mechanisms (such as decrease the iron deposition, anti-oxidation, and anti-apoptosis) are similar to that of Danshen injection. However, in addition to these mechanisms, we mainly focused on the mechanisms of reducing the hepatic iron deposition, that is, the regulative effects of Danshensu on some of the iron uptake related proteins.

The H&E staining and the biochemical analysis on AST and ALT show that Danshensu can obviously improve the structure and the function of the liver, and these results illustrate that Danshensu could protect hepatocytes (parenchymal and nonparenchymal cells) against iron overload. Moreover, researchers have found that the nonparenchymal cells also participate in the iron store,\(^{29}\) immune response,\(^{30,31}\) oxidant stress response,\(^{32}\) and other physiological responses.\(^{33}\)

To explore the underlying mechanisms, we noticed that Danshensu had been recognized as the most significant anti-oxidant marker among Danshen water-extracts and that it also has anti-apoptotic properties.\(^{19,22,34}\)

Furthermore, the anti-inflammation benefits of Danshensu have also been studied in various models, such as omethoate-induced liver injury, UVB-induced corneal damage, and lipo polysaccharide-stimulated THP-1 macrophages.\(^{23,35,36}\)

Similarly, we found that the activities of Danshensu have also been studied in various diseases. DFO has been used to treat thalassemia patients by reducing iron accumulation with chelating mechanisms,\(^{37}\) and it needs more studies to confirm.

In conclusion, the present results show that Danshensu has a protective effect on the acute liver injury induced by iron overload, and the underlying mechanisms at least partly involve anti-oxidation, anti-inflammation, anti-apoptosis, and decreasing hepatic iron deposition through down-regulating the expression of iron uptake proteins, which suggests that the iron regulate-proteins, such as DMT1, TFR, and L-type Ca\(^{2+}\) CP \(\alpha\)C proteins, are potential targets for cure of iron overload diseases in the development of new drugs.

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### Conflict of Interest

The authors declare no conflict of interest.

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