Protective Effect of Salvianic Acid A on Acute Liver Injury Induced by Carbon Tetrachloride in Rats

Chao-Yun WANG,^{a,b} Fu-Lu MA,^b Jun-Tian LIU,^{*a} Jing-Wei TIAN,^b and Feng-Hua FU^b

^a Department of Pharmacology, Xi'an Jiaotong University School of Medicine; Xi'an, Shaanxi, 710061 P. R. China; and

^b Shandong Engineering Research Center for Nature Drug; Yantai, Shandong, 264005 P. R. China.

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Previous research has shown that salvianic acid A [2-(3,4-dihydroxyphenyl)-2-hydroxy-propanoic acid, SA] extracted from *Salvia miltiorrhiza* BGE (Danshen) markedly inhibits lipid peroxidation of mitochondrial membrane of hepatic cells *in vitro*. The present study was conducted to examine protective effect of SA on liver injury induced by carbon tetrachloride (CCl₄) and its possible mechanism *in vivo*. Male Sprague-Dowley rats weighing 180–200 g were used in the experiments. Five mmol/kg CCl₄ in olive oil was given to rats *i.p.* Spectrophotometric method was used to measure activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum, activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) as well as malondialdehyde (MDA) level in hepatic tissue and the rate of superoxide anion (O₂^{•-}) generation in hepatic submitochondrial particles. Hepatic histological structure was observed under light microscopy. CCl₄ caused significant changes of activities of the enzymes, MDA level, and the rate of O₂^{•-} generation and histopathological changes of acute hepatic injury were noted. SA reversed the significant changes induced by CCl₄. These results demonstrate that SA produces protective action on acute hepatic injury induced by CCl₄ *via* an antioxidative mechanism.

Key words salvianic acid A; aspartate aminotransferase; alanine aminotransferase; antioxidative enzyme; malondialdehyde; superoxide anion

Oxidative stress is implicated in the pathogenesis of age-related liver diseases. Hepatic damage caused by chronic ethanol ingestion,¹⁾ iron overload,²⁾ and carbon tetrachloride (CCl₄)^{3,4)} is attributed to heightened production of reactive oxygen species. Normally, the free radical level in the body is low because healthy organisms can neutralize, metabolize, or subtract the toxic effects by free radical scavengers such as superoxide dismutase (SOD), and catalase (CAT).⁵⁾ *S. miltiorrhiza* BGE (Danshen) is a well-known Chinese medicinal herb widely used to treat heart and cerebral diseases,⁶⁾ hepatitis, hemorrhage, and type 2 diabetes.^{7,8)} Salvianic acid A [2-(3,4-dihydroxyphenyl)-2-hydroxy-propanoic acid, SA] is one of the main active components of *S. miltiorrhiza* BGE. Previous studies have shown that SA is effectively able to protect myocardial mitochondrial membrane from ischemia-reperfusion injury,⁹⁾ inhibit lipid peroxidation of mitochondrial membrane of hepatic cells,¹⁰⁾ and prevent apoptosis of human neuroblastoma SH-SY5Y cells by antioxidant mechanism *in vitro*.¹¹⁾ So far, however, there has been little research reported on the hepatoprotective effect of SA. The present study was undertaken to evaluate whether SA elicits protective action against acute hepatic injury induced by CCl₄ and its mechanism in rats.

MATERIALS AND METHODS

Extraction and Isolation of SA *S. miltiorrhiza* BGE (Bozhou Crude Drug Market, Anhui, P. R. China) was identified by the authors and a voucher specimen (No. 045214) was kept at Shandong Engineering Research Center for Nature Drug. Fifty grams of *S. miltiorrhiza* BGE was extracted twice with 600 ml of boiling water containing 0.4% sodium hydroxide. The total water extract adjusted to pH 3–4 with hydrogen chloride was applied to macroporous adsorption resin columns D301 and AB-8 (Anhui Tianxing Resin Co.,

Ltd, P. R. China) and eluted with water containing 0.4% sodium hydroxide. Finally, 0.5 g of SA was obtained by condensing and evaporating of the collected extract. The active compound was identified by instrumental analysis with the following physical and chemical properties: white needle power (purity >98% by HPLC), $[\alpha]_D^{25} = +35^\circ$ (H₂O), mp 253–255 °C, MFC₉H₁₀O₅, MW 198.173 (Fig. 1)¹²⁾

Animals Experiments were carried out according to the National Institutes of Health Guidelines for the care and use of laboratory animals and were approved by the local authority. Sprague-Dowley rats weighing 180–200 g (Animal Center of Shandong Engineering Research Center for Nature Drug, Yantai, P. R. China) were housed at 22 ± 1 °C in a 12 h/12 h light/dark cycle with pellet food and tap water.

Acute CCl₄-Induced Liver Injury In Rats A total of 50 rats were divided into 5 groups: normal control group with *i.g.* saline, model control group with CCl₄ treatment and *i.g.* saline, silymarin (Madaus AG, German) group with CCl₄ treatment and *i.g.* 25 mg/kg silymarin and SA groups with CCl₄ treatment and *i.g.* 10 or 20 mg/kg SA. All rats were treated once daily for 3 d. At 60 min after the last pretreatment, 20% CCl₄ diluted with olive oil (5 mmol/kg) was given to rats *i.p.* (olive oil only for normal control group). Sixteen hours after administration of CCl₄, rats were anaesthetized with 20% urethane (*i.p.*, 1 ml/kg) to collect blood for measuring activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum in accordance with

Fig. 1. Chemical Structure of Salvianic Acid A

* To whom correspondence should be addressed. e-mail: lj@mail.xjtu.edu.cn

methods provided by the diagnostic kits (Nanjing Jiancheng Bioengineering Institute, P. R. China), and liver for measuring activities of SOD, CAT, glutathione peroxidase (GPx), and malondialdehyde (MDA) and superoxide anion ($O_2^{\cdot-}$) levels with the following methods.

Measurement of Lipid Peroxidation MDA, production of lipid peroxidation (LPO), was assayed by the method of Buege *et al.*¹³⁾ Reaction mixture corresponding to a total volume of 10 ml of tissue homogenate was heated at 95 °C for 60 min. The pink-colored chromogen formed by the reaction of 2-thiobarbituric acid with MDA was spectrophotometrically measured at 535 nm. The results were expressed as MDA nmol/mg protein.

Isolation of Mitochondria and Preparation of Submitochondrial Particles All steps in the isolation of mitochondria and preparation of submitochondrial particles were performed at ice-cold temperature. Hepatic mitochondria were isolated according to the method of Sohal *et al.*¹⁴⁾ Liver was homogenized in 10 volumes (w/v) of isolation buffer containing 0.22 M mannitol, 0.07 M sucrose, 3 mM EDTA, and 1 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 280 *g* for 20 min. Then, the supernatant was recentrifuged at 1200 *g* for 20 min and at 3000 *g* for 10 min. Finally, the pellet was resuspended in 30 mM phosphate buffer (pH 7.0).

Hepatic submitochondrial particles were isolated by resuspending the mitochondrial pellet in 4 volumes of phosphate buffer. The suspended solution was sonicated 3 times for 30 s each at 1 min intervals. The sonicated mitochondria were centrifuged at 8000 *g* for 10 min to sediment the unfragmented mitochondria, and the supernatant was recentrifuged at 80000 *g* for 40 min to pellet the submitochondrial particles. The collected pellet was resuspended in phosphate buffer for further assay or storage at -80 °C.¹⁵⁾

Determination of Superoxide Generation The rate of superoxide anion ($O_2^{\cdot-}$) generation by submitochondrial particles was measured according to the method of Boveris.¹⁶⁾ Both the test and reference cuvettes contained 20 to 40 μ l of the submitochondrial suspension, 0.1 M phosphate buffer (pH 7.4), 7.2 μ M cytochrome C, 0.6 μ M antimycin A, and 7 mM succinate. Two hundred U/ml SOD (Sigma, U.S.A.) was added into the reference cuvette. The reduction of cytochrome C was spectrophotometrically monitored at 550 nm. Since both the test and reference cuvettes contained identical ingredients except SOD, the measured rate of cytochrome C reduction was specific because of its interaction with $O_2^{\cdot-}$.

Detection of SOD, CAT, and GPx Activities Hepatic tissue was homogenized in 10 volumes (w/v) of 0.1% Triton X. The homogenate was centrifuged at 3000 *g* for 5 min, then the supernatant recentrifuged at 50000 *g* for 30 min. The remaining supernatant was used for measurement of activities of SOD, CAT, and GPx. The method for the SOD assay was a slight modification of the indirect inhibition assay developed by Sun¹⁷⁾ In the method, xanthine-xanthine oxidase was used to generate superoxide, which catalyzed the reduction of nitro-tetrazolium blue tetrazolium (NBT) to blue formazon. The absorbance was determined at 560 nm. SOD in the sample competed for superoxide and inhibited the reaction rate of superoxide with NBT. One unit of SOD was defined as the amount of protein that inhibited the rate of NBT reduction by 50%. The results were given in U/mg protein.

CAT activity was assayed by the method of Takahara *et al.*¹⁸⁾ After 0.2 ml of the homogenate was added to 1.2 ml of 50 mM phosphate buffer (pH 7.0), the reaction was started by addition of 1.0 ml of 30 mM H_2O_2 solution. The decrease in absorbance was measured at 240 nm in 30 s intervals for 3 min. The reaction in the blank was run simultaneously with distilled water instead of H_2O_2 . The enzyme activity was expressed as μ mol of decomposed H_2O_2 /min/mg protein.

GPx activity was assayed by the method of Rotruck *et al.*¹⁹⁾ The reaction mixture consisted of 0.2 ml of 0.8 mM EDTA, 0.1 ml of 10 mM sodium azide, 0.1 ml of 2.5 mM H_2O_2 , 0.2 ml of the reduced glutathione, 0.4 ml of 0.4 M phosphate buffer (pH 7.0), and 0.2 ml of homogenate, and was incubated at 37 °C for 10 min. The reaction was arrested by addition of 0.5 ml of 10% trichloroacetic acid and tubes were centrifuged at 2000 rpm for 15 min. Three ml of 0.3 mM disodium hydrogen phosphate and 1.0 ml of 0.04% DTNB [5,5-dithiobis(2-nitrobenzoic acid)] were added to the supernatant and the developed colour was detected at 420 nm immediately. GPx activity was expressed as μ mol of the oxidized glutathione/min/mg protein.

Protein was detected according to the method described by Lowry *et al.*²⁰⁾

Histopathological Observation Few millimeter-thick midsection of the left lobe of liver from each animal was processed for observation by light microscopy. The process involved fixing the tissue specimen in 10% neutral buffered formalin solution, preparing the block in paraffin, cutting into 5–6 μ m thick sections, and staining the sections with haematoxylin-eosin stain. The sections were scanned and analyzed by pathologist who was blinded to the different treatments in the experiment.

Statistical Analysis Data from experiments were expressed as mean $\pm$ S.E.M. with 10 rats in each group. Statistical analysis was conducted by one-way ANOVA with Newman-Keuls multiple comparison test. $p < 0.05$ was considered statistically significant.

RESULTS

Table 1 shows levels of AST and ALT in serum. AST and ALT levels were elevated from 132.28 and 51.02 U/ml in the normal control group to 323.89 and 270.89 U/ml in model control group, respectively, and significantly reduced to 226.10 and 196.09 U/ml, respectively, in the SA-treated group (20 mg/kg) and to 281.78 and 199.10 U/ml, respectively, in the silymarin-treated group compared with model control group ($p < 0.05$ or 0.01). These findings suggest that

Table 1. Effect of SA on AST and ALT Activities in Serum of Rats

Group	Dose (mg/kg)	AST (U/ml)	ALT (U/ml)
Normal control		132.28 $\pm$ 22.14**	51.02 $\pm$ 18.64**
Model control		323.89 $\pm$ 35.67	270.89 $\pm$ 66.95
SA	10	317.42 $\pm$ 51.03	256.14 $\pm$ 86.98
SA	20	226.10 $\pm$ 44.52**	196.09 $\pm$ 53.07*
Silymarin	25	281.78 $\pm$ 50.02*	199.10 $\pm$ 58.67*

Animals were pretreated with SA (10, 20 mg/kg, i.g.), silymarin (25 mg/kg, i.g.) or vehicle. After 16 h of i.p. administration of CCl_4 , AST and ALT in serum were measured in accordance with the methods of the diagnostic kits. Data are represented as mean $\pm$ S.E.M. $n = 10$ in each group. SA: Salvianic acid A; AST: aspartate aminotransferase; ALT: alanine aminotransferase. * $p < 0.05$, ** $p < 0.01$ vs. model control.

Fig. 2. Effect of Salvianic Acid A (SA) on Malondialdehyde (MDA) Content in Liver

Animals were pretreated with SA (10, 20 mg/kg, i.g.), silymarin (25 mg/kg, i.g.), or vehicle. MDA in liver was measured after 16 h of i.p. administration of CCl_4 . Data are represented as mean $\pm$ S.E.M. $n=10$ in each group. *Significantly different from model control group, ** $p<0.01$.

Fig. 3. Effect of Salvianic Acid A (SA) on Rate of Superoxide Anion ($\text{O}_2^{\cdot-}$) Generation by Submitochondrial Particles

Animals were pretreated with SA (10, 20 mg/kg, i.g.), silymarin (25 mg/kg, i.g.), or vehicle. After 16 h of i.p. administration of CCl_4 , the rate of $\text{O}_2^{\cdot-}$ generation was monitored according to Boveris's method. $n=10$ in each group. Data are represented as mean $\pm$ S.E.M. *Significantly different from model control group, * $p<0.05$; ** $p<0.01$.

SA and silymarin protect the liver from injury induced by CCl_4 .

Figure 2 shows extent of MDA in liver. MDA was increased from 2.93 nmol/mg protein in the normal control group to 8.00 nmol/mg protein in the model control group. SA (10, 20 mg/kg) decreased MDA level in liver to 5.33 and 5.15 nmol/mg protein, respectively, and silymarin to 5.69 nmol/mg protein from 8.00 nmol/mg protein in the model control group ($p<0.01$). The results indicate that SA and silymarin are effectively able to inhibit production of MDA in CCl_4 -injured liver.

Figures 3–6 show the effects of SA on rate of $\text{O}_2^{\cdot-}$ generation in submitochondrial particles, and total SOD, CAT, and GPx activities in liver. The rate of $\text{O}_2^{\cdot-}$ generation in submitochondrial particles was increased and SOD, CAT, and GPx activities were decreased in livers of model control group animals in comparison with the normal control group ($p<0.05$ or 0.01). SA (20 mg/kg) and silymarin remarkably increased SOD, CAT, and GPx activities in liver and decreased the rate of $\text{O}_2^{\cdot-}$ generation in submitochondrial particles of liver ($p<0.05$ or 0.01).

Histological observation of the liver provided further evidence to support the hepatoprotective role of SA and silymarin. Figure 7B shows necrosis of hepatocytes with col-

Fig. 4. Effect of Salvianic Acid A (SA) on Superoxide Dismutase (SOD) Activity in Liver

Animals were pretreated with SA (10, 20 mg/kg, i.g.), silymarin (25 mg/kg, i.g.), or vehicle. After 16 h of i.p. administration of CCl_4 , SOD activity in liver was quantitated with the method of Sun. $n=10$ in each group. Data are represented as mean $\pm$ S.E.M. *Significantly different from model group, * $p<0.05$; ** $p<0.01$.

Fig. 5. Effect of Salvianic Acid A (SA) on Catalase (CAT) Activity in Liver

Animals were pretreated with SA (10, 20 mg/kg, i.g.), silymarin (25 mg/kg, i.g.), or vehicle. After 16 h of i.p. administration of CCl_4 , CAT activity in liver was surveyed on basis of the method of Takahara *et al.* $n=10$ in each group. Data are represented as mean $\pm$ S.E.M. *Significantly different from model control group, * $p<0.05$; ** $p<0.01$.

Fig. 6. Effect of Salvianic Acid A (SA) on Glutathione Peroxidase (GPx) Activity in Liver

Animals were pretreated with SA (10, 20 mg/kg, i.g.), silymarin (25 mg/kg, i.g.), or vehicle. After 16 h of i.p. administration of CCl_4 , GPx activity was detected by the method of Rotruck *et al.* $n=10$ in each group. Data are represented as mean $\pm$ S.E.M. *Significantly different from model control group, * $p<0.05$; ** $p<0.01$.

lapse of hepatic architecture, hemorrhage, and so on in CCl_4 -treated rats alone. Figures 7C and 7D indicate that SA (20 mg/kg) and silymarin were effectively able to relieve the histopathological changes of hepatic cells injured by CCl_4 .

Fig. 7. Effect of Salvianic Acid (SA) on the Histological Structure of Liver

Animals were pretreated with SA (10, 20 mg/kg, i.g.), silymarin (25 mg/kg, i.g.), or vehicle. Figures 7A—D represent typical histological changes from normal control group, model control group, 20 mg/kg SA-treated group, and silymarin-treated group, respectively.

DISCUSSION

CCl_4 hepatotoxicity depends on its reductive dehalogenation catalyzed by Cyt P450 in the endoplasmic reticulum of hepatic cells leading to the generation of an unstable complex trichloromethyl radical. The superoxide anion $\text{O}_2^{\cdot-}$, H_2O_2 , and the hydroxyl radical ($\text{OH}^{\cdot}$) are reactive oxygen species (ROS) mainly produced in mitochondria.²¹⁾ Cells have a number of mechanisms to protect themselves from the toxic effects of ROS including free radical scavengers and

chain reaction terminators such as SOD, CAT, and GPx systems.²²⁾ SOD removes $\text{O}_2^{\cdot-}$ by converting it to H_2O_2 that can be rapidly converted to water by CAT and GPx.²³⁾ Cellular injury occurs when ROS generation exceeds the cellular capacity of removal.

In the present study, increased production of MDA and $\text{O}_2^{\cdot-}$ as well as decreased activities of SOD, CAT, and GPx occurred in liver injury induced by CCl_4 , implying downregulation of numerous enzymatic oxidation reactions in the cytosolic compartments and mitochondria. Furthermore, liver damage *via* ROS pathway caused a remarkable increase of ALT and AST levels in serum and histological changes of hepatic cells. SA had a protective effect on liver injury in response to CCl_4 by producing significant elevation of SOD, CAT, and GPx activities to increase the scavenging of ROS in mitochondria, leading to decreased activities of AST and ALT in serum and relieved changes of hepatic histopathology. These results are consistent with the report from Wang *et al.* (2005a) that SA inhibits lipid peroxidation of mitochondrial membrane of hepatic cells *in vitro*. In conclusion, SA elicits a protective effect on hepatic injury by antioxidative action *in vivo*.

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