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Protective Role of Grape Seed Proanthocyanidins Against CCl₄ Induced Acute Liver Injury in Mice

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Background: We investigated the effect of grape seed proanthocyanidins (GSPs) on carbon tetrachloride (CCl₄)-induced acute liver injury.

Material/Methods: Sixty SPF KM mice were randomly divided into 6 groups: the control group, CCl₄-model group, bifendate group (DDB group), and low-, moderate-, and high-dose GSP groups. The following parameters were measured: serum levels of alanine aminotransferase (ALT); aspartate aminotransferase (AST); tumor necrosis factor (TNF)-α; interleukin-6 (IL-6); high-mobility group box (HMGB)-1; body weight; liver, spleen, and thymus indexes; superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity; HMGB1 mRNA; malondialdehyde (MDA) content; hepatocyte proliferation; and changes in liver histology.

Results: Compared to the CCl₄-model group, decreases in liver index and increases in thymus index significantly increased SOD and GSH-Px activities and reduced MDA content, and higher hepatocyte proliferative activity was found in all GSP dose groups and the DDB group (all *P*<0.001). Compared with the CCl₄-model group, serum TNF-α and IL-6 levels and HMGB 1 mRNA and protein expressions decreased significantly in the high GSP dose group (all *P*<0.05).

Conclusions: Our results provide strong evidence that administration of GSPs might confer significant protection against CCl₄-induced acute liver injury in mice.

MeSH Keywords: **Aspartate Aminotransferase, Cytoplasmic • Drug-Induced Liver Injury • Glutathione Peroxidase**

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Background

Acute liver injury is caused by multiple factors, such as hepatic viruses, excessive alcohol consumption and hepatotoxins (for example, paracetamol, flutamide and carbon tetrachloride), and leads to down-regulation of enzymes that influence drug pharmacokinetics [1,2]. Carbon tetrachloride (CCl₄) is an industrial solvent and one of the most potent inducers of acute liver injury, often used in animal studies to replicate human liver injury model [3]. CCl₄-induced toxicity depends on the dose and duration of exposure. At low CCl₄ doses, transient effects occur, including the loss of Ca²⁺ sequestration, impaired lipid homeostasis and release of several cytokines. Longer CCl₄ exposures alter fatty acid metabolism, and induce fibrosis, cirrhosis and cancer [4]. CCl₄ induced hepatotoxicity is the result of reductive dehalogenation reactions catalyzed by hepatic cytochrome P-450, forming unstable trichloromethyl and trichloromethyl peroxy radicals capable of binding to proteins or lipids, initiating lipid peroxidation and liver damage [5]. Depending on the specific context, treatment of liver diseases involves antiviral drugs, vaccines, N-acetylcysteine, melatonin, 5-aminoisoquinolinone as well as bone marrow stromal stem cells [6–9]. However, these treatments carry adverse side effects, thus safer and more effective drugs are in urgent need [10]. In recent years, focus has shifted towards traditional Chinese medicinal products, such as lignins extracted from *Schisandra chinensis*, polyphenols in green tea with epigallocatechin-3-gallate and apple polyphenols, for their efficacy and high safety profile in treatment of liver diseases [10–12].

Proanthocyanidins occur naturally in fruits, vegetables, seeds and flowers, and are the major polyphenols present in red wine and grape seeds, with an already wide use as nutritional supplements [13]. Proanthocyanidins improve anti-inflammatory, antioxidant, and anti-carcinogenic activities in human [14]. In particular, grape seed proanthocyanidins (GSPs) mostly contain epicatechin and catechin, and their gallic acid esters [15]. GSPs contain multiple hydroxyl groups, which are important for the beneficial pharmacological and therapeutic properties of GSPs against oxygen free radicals and oxidative stress [16]. GSPs also promote production of nitric oxide (NO), an important signaling molecule involved in multiple physiological pathways, such as regulation of blood pressure and anti-inflammatory mechanisms [17]. Several other beneficial functions of GSPs have been demonstrated by multiple studies in the literature. GSPs play a protective role in high fructose diet induced insulin resistance and in maintaining healthy myocardial activity [15,18]. GSPs have also been demonstrated to be effective anticancer agents in various human cancers, such as colorectal cancer, non-small cell lung cancer, head and neck squamous cancer, cervical cancer and skin cancer [19–23]. Furthermore, GSPs alleviate oxidative damage and inflammatory cytokines release, and promote hypoxia tolerance in hepatic steatosis [17].

Surprisingly, there were few published studies reporting the potential use of GSPs in CCl₄-induced acute liver injury. Dai et al. mainly studied the antioxidant properties of proanthocyanidins in reducing CCl₄-induced steatosis and liver injury in rats through regulation of CYP2E1 enzyme [24]. However, our study aimed to focus on the effects of GSPs in acute liver injury in the aspect of anti-inflammation, anti-oxidation and anti-lipid peroxidation. To address this issue, our study investigated the experimental outcomes of administration of GSPs in CCl₄-induced acute liver injury mouse model, with an aim of providing a novel approach for safe and effective therapy for acute chemical induced hepatic injury in humans.

Material and Methods

Subjects and grouping

Sixty SPE KM mice (average weight, 20±2 g) were obtained from the Laboratory animal center of Liaoning Medical University. Mice were randomly allocated into 6 groups, with 10 mice in each group. The 6 groups were: control group, CCl₄-model group, low GSP dose group (50 mg/kg), moderate GSP dose group (250 mg/kg), high GSP dose group (500 mg/kg) and bifendate group (DDB group) (150 mg/kg). CCl₄ was purchased from Shanghai Reagent Factory and DDB from Beijing Union Pharmaceutical Factory. This study was conducted in strict adherence with the procedures mentioned in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The ethics committee of laboratory animal center of Liaoning Medical University approved the study protocol, and was carried out in accordance with the Declaration of Helsinki [25].

Source of GSPs

GSPs extracted from red grape seeds were obtained from Jianfeng Natural Products R&D Inc. (Tianjin, China). Chemical composition of GSPs was analyzed by high-performance liquid chromatography (HPLC) and the results showed 89.79% total proanthocyanidins, 3.20% total monomeric flavonols and other ingredients.

CCl₄ induced acute liver injury

Bean oil (0.2 ml/10 g body weight) was administered to the mice in the control group and the mice in other experimental groups were given 2% CCl₄ dissolved in plant oil (0.2ml/10g) by gavage every morning. The administered dose of CCl₄ is well-known in the literature to induce characteristic liver injury in mice that functionally resembles the acute liver injury observed in humans [26] (http://d.wanfangdata.com.cn/Periodical_xjykdxxb200711006.aspx). Three hours after CCl₄ administration, the

low dose, moderate dose and high GSP dose groups were given GSPs by gavage suspended in distilled water at 50, 250, 500 mg/kg body weight, respectively. The dose of GSPs used in this study was selected on the basis of previous studies [27]. The DDB group was given DDB at 150 mg/kg body weight by gavage [28], while the control group and CCl₄-model group were given same volume of saline. As per standard protocols, the experimental procedures were continued for 10 days until significantly elevated serum alanine aminotransferase levels were detected [26].

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), tumor necrosis factor (TNF)- α , interleukin-6 (IL-6), high-mobility group box (HMGB)-1 levels measurement

Blood samples were collected from eye sockets of mice 8 hours after the last drug administration. Serum levels of ALT and AST were used as biomarkers for hepatic damage. ALT and AST levels were determined based on the Reitman-Frankel method using commercially available assay kits (ProDia international, Germany) [29]. ELISA was used to detect serum levels of tumor necrosis factor (TNF)- α , interleukin-6 (IL-6) and high-mobility group box (HMGB)-1. TNF- α and IL-6 kits were obtained from Neobioscience Technology company (Shenzhen, China) and HMGB-1 kit was from Elabscience Biotechnology Co., Ltd (Wuhan, China).

Organ index measurement

Mice were weighed 10 h after the last drug administration. Liver, spleen and thymus were removed and the residual blood was rinsed-off with 0.9% sodium chloride solution. After the tissues were dried and weighed, the liver, spleen and thymus index was measured. Liver index=liver weight (g)/mice weight (g) \times 100%; spleen index=spleen weight (g)/mice weight (g) \times 100%; thymus index=thymus weight (g)/mice weight (g) \times 100%.

Liver homogenate preparation

Animals were sacrificed by cervical dislocation and immediately dissected. The root of the hepatic portal vein was clamped, and vessels and ligaments were cut. The operation was handled carefully to avoid liver damage. The liver was taken out and rinsed with saline pre-cooled to 4°C, and the organ was dried with filter paper and weighed after removing adipose and connective tissue. Approximately 1 g of liver tissue was placed into a 20 ml beaker and sliced into small pieces with eye scissors. A 2/3rd volume of saline was transferred into the beaker with a pipette (the total volume of saline at this point was 9 times the weight of the tissue, i.e., 9 ml ice-cold saline was added for 1 g liver tissue). Liver homogenates were obtained by high-speed tissue homogenization using FJ-200S (Shanghai Specimen and Model Factory) at 3000 r/min in an

ice bath (10s each time for 3–5 times and with a brief interval of 30 s). The homogenates were centrifuged at 3000 r/min for 15min at 4°C to obtain the supernatants.

Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) activity and malondialdehyde (MDA) determinations

The supernatants from liver homogenates were used for measurement of SOD and GSH-Px activities, and the MDA content. SOD activity was measured by xanthine oxidase method. GSH-Px activity was measured by 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) method. MDA was measured using thiobarbituric acid (TBA) colorimetric assay kit. All assay reagents were purchased from Nanjing Jiancheng Bioengineering Institute, Jiangsu, China.

RT-PCR detection

Total RNA of mice livers were extracted using Trizol reagent (Invitrogen, USA) following manufacturer's instructions. We used 12–18 oligo (dT) primer to obtain cDNA from 20 μ l of total RNA sample. HMGB1 fragments were amplified using cDNA as a template. HMGB1 amplification conditions were as follows: 95°C initial denaturation for 12 s, 40 cycles of 95°C denaturation for 30 s, 58 °C annealing for 30 s, 72 °C extension for 45 min, followed by 72°C extension for 12 min. PCR products conducted 1.2% agarose gel electrophoresis and BIO-Rad gel electrophoresis imaging system was used to analyze the area and absorbance value of each electrophoresis band. The β -actin was acted as an internal control. The relative expression level of HMGB-1 was calculated by the ratio of HMGB-1 and β -actin (Table 1).

MTT assay to detect hepatocyte proliferation

Freshly isolated hepatocytes were adjusted to 1 \times 10⁶/ml cell density in 10% FBS RPMI-1640 medium. Trypan blue staining to detect live hepatocytes showed more than 95% cell viability. The suspended hepatocytes were seeded into 96-well plates by distributing 100 μ l into each well. Each experimental group had five parallel samples and the cells were incubated at 37°C and 5% CO₂ under standard cell culture conditions. MTT solution (5 mg/ml) was added into the 96-well plates with 20 μ l per well and incubated for 4 h. After the supernatant was discarded, 100 μ l dimethyl sulfoxide was added to each well to dissolve blue-purple formazan crystals. The absorbance of each sample was measured by reading the results at 492 nm wavelength using a microplate reader. The absorbance detected can be used to represent the proliferation activity.

Histopathology examination

A small slice of tissue from left lobes of liver from each mouse was taken and fixed with 10% formaldehyde solution and

Table 1. Primer sequences and amplification lengths for HMGB1 and β-actin.

	Primer sequence	Amplification length
HMGB1	F: 5'-GATGGG CAAAGGAGATCCTAAG-3'	226bp
	R: 5'-TCACTTTTTGTCTC CCCTTTGGG-3'	
β-actin	F: 5'-CTGTCCCTGTATGCCTCT G-3'	289bp
	R: 5'-CATCGTACTCCTGCTTGTCT-3'	

HMGB1 – high-mobility group box-1; F – forward; R – reverse; bp – base pair.

Table 2. Serum ALT and AST levels in the control group, the CCl₄-model group, the low, middle, GSP dose group and the DDB group (±s).

Group	n	AST (U/L)	ALT (U/L)
Control group	10	26.27±6.85	27.56±14.72
CCl ₄ -model group	10	143.66±7.41 [#]	121.34±11.78 [#]
Low GSP dose group	10	85.91±6.63 ^{#,*}	113.46±12.14 [#]
Moderate GSP dose group	10	73.78±6.15 ^{#,*,§}	98.25±16.47 ^{#,*,§}
High GSP dose group	10	61.62±8.28 ^{#,*,§,&}	68.46±12.78 ^{#,*,§,&}
DDB group	10	60.32±6.19 ^{#,*}	63.56±14.32 ^{#,*,§,&}

CCl₄ – carbon tetrachloride; GSP – grape seed proanthocyanidins; DDB – bifendate; ALT – alanine aminotransferase; AST – aspartate aminotransferase; [#] *P*<0.01, compared with the control group; ^{*} *P*<0.05, compared with the CCl₄-model group; [§] *P*<0.05, compared with the low GSP dose group; [&] *P*<0.05, compared with the moderate GSP dose group.

embedded in paraffin wax. Thin sections of the embedded tissue were cut and hematoxylin-eosin stained for histopathological examinations. Images were captured under optical microscope (Olympus BH2, Japan) using Nikon coolpix990 camera at 250× magnification.

Statistical analysis

SPSS 20.0 statistical software was used for data analysis. Measurement data were presented as mean ± standard deviation. The *t* test was applied to compare groups and *q* test compared the differences among multiple groups. A *P*<0.05 was considered as statistically significant.

Results

Serum ALT and AST levels

CCl₄-induced acute liver injury model was successfully established, as confirmed by elevated serum ALT and AST levels detected in the experimental groups, compared with the control group, following oral administration of 2% CCl₄, (all *P*<0.01). Serum AST levels in the low, moderate and high GSP dose group were remarkably lower than the CCl₄-model group (all *P*<0.05) and showed a

dose-dependent lowering in response to the increasing dose of GSPs, indicating the protective role of GSPs in CCl₄-induced acute liver injury. No significant difference in AST level was found between the GSP groups and the DDB group (all *P*>0.05). Serum ALT levels in the moderate and high GSP dose groups were lower than CCl₄-model group (all *P*<0.05), while the serum ALT level in the low GSP dose group showed no statistical differences compared to CCl₄-model group (*P*>0.05). A similar dose-dependent lowering in serum ALT levels was also observed across the three GSP dose groups, which further confirmed the beneficial role of GSPs in CCl₄-induced acute liver injury. In comparison with the DDB group, serum ALT levels were significantly higher in the low and moderate GSP dose group (both *P*<0.05), while it showed no difference in the high GSP dose group (*P*>0.05) (Table 2).

Serum TNF-α and IL-6 levels

Compared with the control group, serum TNF-α and IL-6 levels were significantly increased in other three groups (all *P*<0.05). Serum TNF-α and IL-6 levels in the high GSP dose group and the control group were lower than those in the model group (both *P*<0.05), significant difference in serum TNF-α level was found between the high GSP dose group and the SAA group (*P*<0.05), but no significant difference in serum IL-6 level was found between the two groups (*P*>0.05) (Table 3).

Table 3. Serum TNF- α and IL-6 levels in the control group, the CCl₄-model group, the low, middle, high GSP dose group and the DDB group (n=10).

Group	TNF- α (pg/mL)	IL-6 (pg/mL)
Control group	2132.26 \pm 258.69	32.58 \pm 7.58
CCl ₄ -model group	6315.26 \pm 742.54 [#]	215.58 \pm 24.68 [#]
High GSP dose group	3414.25 \pm 419.6 ^{#,*}	126.40 \pm 54.68 ^{#,*}
DDB group	3155.6 \pm 322.6 ^{#,*$\ddagger$$\S$}	116.24 \pm 34.93 ^{#,*}

TNF- α – tumor necrosis factor- α ; IL-6 – interleukin-6; GSP – grape seed proanthocyanidin; DDB – bifendate; [#] compared with the control group, $P < 0.05$; ^{*} compared with the model group, $P < 0.05$; ^{\S} compared with the GSP group, $P < 0.05$.

Table 4. Weight gain of the mice after 10 days of CCl₄ treatment (\pm s).

Group	n	Initial weight (g)	Final weight (g)	Gain in weight (g)
Control group	10	23.10 \pm 2.78	31.80 \pm 3.32	8.70 \pm 0.54
CCl ₄ -model group	10	24.10 \pm 2.69	33.10 \pm 2.65	9.00 \pm 0.04
Low GSP dose group	10	24.20 \pm 2.70	33.10 \pm 3.14	8.90 \pm 0.44
Moderate GSP dose group	10	24.30 \pm 2.80	33.3 \pm 2.81	9.00 \pm 0.01
High GSP dose group	10	23.50 \pm 2.74	32.4 \pm 2.92	8.90 \pm 0.18
DDB group	10	23.00 \pm 1.70	32.10 \pm 2.05	9.10 \pm 0.35
<i>F</i>				0.287
<i>P</i>				1.278

CCl₄ – carbon tetrachloride; GSP – grape seed proanthocyanidins; DDB – bifendate.

Body weight of mice

Initial weight and final weight of mice at 10 days of CCl₄ administration was measured in the six groups. Variance analysis showed no significant differences in weights among the six groups (all $P > 0.05$). Mice in each group showed normal behavior and no adverse reactions were observed (Table 4), indicating the status of health of the mice within the duration of the experiment.

Liver, spleen and thymus indexes of mice

Compared with the control group, significantly increased liver index and decreased thymus index was found in CCl₄-model group (both $P < 0.05$), while the liver index and thymus index showed no significant differences within the GSP dose groups and the DDB group ($P > 0.05$). In comparison with the CCl₄-model group, a remarkably decreased liver index and increased thymus index was found in all the GSP dose groups and the DDB group (all $P < 0.001$), indicating the protective role of GSPs. However, no difference in spleen index was found among the six groups (all $P > 0.05$) (Table 5).

SOD, GSH-Px activities and MDA content in liver tissue

In comparison with the control group, decreased SOD and GSH-Px activity, and increased MDA content was found in the five experimental groups (all $P < 0.01$). On the other hand, in comparison with CCl₄-model group, the SOD and GSH-Px activities were significantly increased, and MDA content were significantly decreased in all the GSP dose groups and the DDB group (all $P < 0.05$). A clear dose-dependent increase in SOD activity, and decrease in MDA content was observed with increasing GSP dose (all $P < 0.05$), while GSH-Px activity showed a significant increase only in the high GSP dose group (both $P < 0.05$), and not in the low and moderate GSP dose groups ($P > 0.05$). Interestingly, higher SOD activity was observed in DDB group compared to the low GSP dose group ($P < 0.05$), but no such differences were found between the moderate and high GSP dose groups and the DDB group (all $P > 0.05$). GSH-Px activity in the DDB group was also higher than the low and moderate GSP dose groups (both $P < 0.05$), but its activity was comparable with high GSP dose group ($P > 0.05$). The MDA content in the DDB group was lower than low and moderate GSP dose groups (both $P < 0.05$), but no such difference was found between the DDB group and the high GSP dose group ($P > 0.05$) (Table 6).

Table 5. Comparison of liver, splenic and thymus index in the control group, the CCl₄-model group, the low, middle and high GSP dose group and the DDB group (±s).

Group	n	Liver index (×10 ⁻³)	Splenic index (×10 ⁻³)	Thymus index (×10 ⁻³)
Control group	10	55.96±6.41	3.45±0.77	4.78±0.66
CCl ₄ -model group	10	64.13±5.01*	3.83±0.83	3.03±0.47*
Low GSP dose group	10	53.32±3.71 [#]	3.80±0.65	4.35±0.28 [#]
Moderate dose GSP	10	54.97±5.65 [#]	3.72±0.63	4.55±0.28 [#]
High GSP dose group	10	55.34±3.94 [#]	3.52±0.59	4.53±0.28 [#]
DDB group	10	55.43±3.94 [#]	3.50±0.65	4.50±0.19 [#]

CCl₄ – carbon tetrachloride; GSP – grape seed proanthocyanidins; DDB – bifendate; * *P*<0.05 compared with the control group; [#] *P*<0.01 compared with the CCl₄-model group.

Table 6. SOD and GSH-Px activities and MDA content in liver tissue (±s).

Group	n	SOD (U/mg pro)	MDA (U/mg pro)	GSH-Px (U/mg pro)
Control group	10	267.43±33.52	3.74±1.74	203.92±35.01
CCl ₄ -model group	10	86.51±25.25 [#]	13.56±2.71 [#]	110.69±23.26 [#]
Low GSP dose group	10	134.74±44.82 ^{#,*}	10.73±1.41 ^{#,*}	140.29±25.41 ^{#,*}
Moderate dose GSP group	10	195.68±34.88 ^{#,*⁵}	9.03±1.64 ^{#,*⁵}	141.96±23.13 ^{#,*}
High GSP dose group	10	228.14±21.06 ^{#,*⁵,^{&}}	6.33±1.07 ^{#,*⁵,^{&}}	174.38±28.34 ^{#,*⁵,^{&}}
DDB group	10	213.47±25.12 ^{#,*⁵}	5.98±1.58 ^{#,*⁵,^{&}}	175.08±24.73 ^{#,*⁵,^{&}}

CCl₄ – carbon tetrachloride; GSP – grape seed proanthocyanidins; DDB – bifendate; SOD – Superoxide dismutase; MDA – malondialdehyde; GSH-Px – glutathione peroxidase; U/mg pro – nmol min⁻¹mg⁻¹protein; [#] *P*<0.01, compared with the control group; * *P*<0.05, compared with the CCl₄-model group; ⁵ *P*<0.05, compared with the low GSP dose group; [&] *P*<0.05, compared with the moderate GSP dose group.

Table 7. HMGB 1 mRNA and protein in the control group, the CCl₄-model group, high GSP dose group and the DDB group (n=10).

Group	HMGB 1 mRNA	HMGB 1 protein (pg/mL)
Control group	0.62±0.09	4.11±0.74
CCl ₄ -model group	1.38±0.31 [#]	32.26±12.15 [#]
High GSP dose group	0.93±0.12 ^{#,*}	16.40±4.67 ^{#,*}
DDB group	0.94±0.13 ^{#,*}	15.24±4.23 ^{#,*}

HMGB 1 – high-mobility group box-1; CCl₄ – carbon tetrachloride; GSP – grape seed proanthocyanidin; DDB – bifendate; [#] compared with the control group, *P*<0.05; * compared with the model group, *P*<0.05.

HMGB 1 mRNA and protein expression

Compared with the control group, HMGB 1 mRNA and protein expression increased significantly in the model group (both *P*<0.05); compared with the model group, HMGB 1 mRNA and protein expression decreased significantly in the high GSP dose group (both *P*<0.05), while no differences in HMGB 1 mRNA expression and protein was found between the high GSP dose group and SAA group (both *P*>0.05) (Table 7).

Hepatocyte proliferative activity

Hepatocyte proliferative activities in the control group, the CCl₄-model group, the low, moderate and high GSP dose groups and the DDB group and positive control group DDB were 0.399±0.019, 0.293±0.011, 0.300±0.008, 0.337±0.025, 0.357±0.010 and 0.351±0.009, respectively. Compared with the control group, a significantly reduced hepatocyte proliferation was observed in the five experimental groups (all *P*<0.01). Compared with the CCl₄-model group, all GSP dose groups and

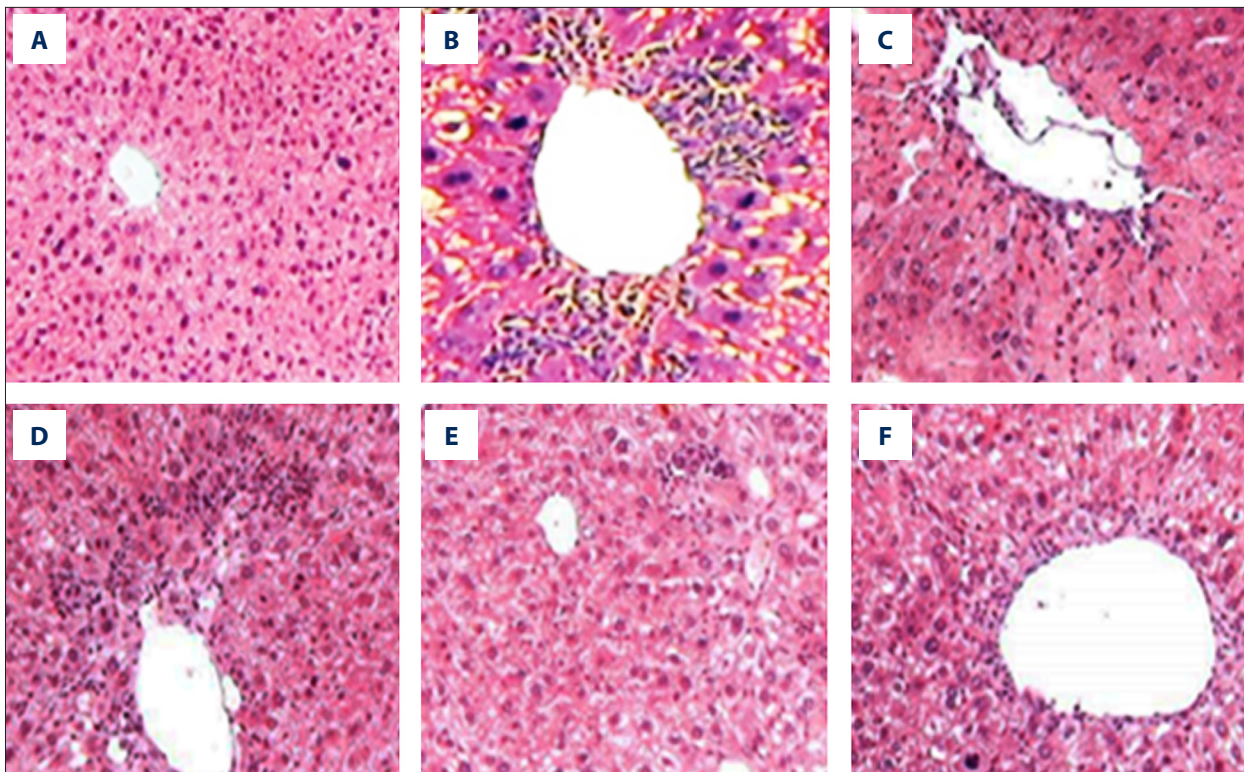


Figure 1. Representative images of histological examination results of mice liver tissues stained with hematoxylin and eosin ($\times 250$) (A the control Group; B the CCl₄-model group; C the low GSP dose group; D the DDB group; E the moderate GSP dose group; F the high GSP dose group). Note: CCl₄ – carbon tetrachloride; GSP – grape seed proanthocyanidins; DDB – bifendate.

DDB group showed significantly higher hepatocyte proliferation activity (all $P < 0.05$). Hepatocyte proliferative activity increased with increasing GSP in a dose-dependent manner. The hepatocyte proliferative activity in DDB group was higher than the low and moderate GSP dose groups (both $P < 0.05$), but showed no significant difference with high GSP dose group ($P > 0.05$).

Histopathological observations

Histological assessment of liver tissue was used to verify the protective role of GSP in CCl₄ induced acute liver injury. Control mice showed normal tissue structure with complete hepatic lobules, orderly arranged hepatic cords, with no abnormality in hepatic sinusoid and portal area, no inflammatory cell infiltration and normal hepatocyte morphology (Figure 1A). In the CCl₄-model group, swollen hepatocytes, cytoplasmic relaxation, dark stained nuclei and obvious punctate or focal necrosis were observed, and neutrophil and lymphocyte infiltration was found in portal area (Figure 1B). Compared with the CCl₄-model group, reduced tissue damage and decreased punctate and focal necrosis was observed in the low GSP dose group and the DDB group (Figure 1C, 1D). Importantly, the tissue structure appeared normal in the moderate GSP dose group and the high GSP dose group, with rare necrotic hepatocytes and markedly decreased infiltration of inflammatory cell types. (Figure 1E, 1F).

Discussion

CCl₄-induced liver injury simulates acute liver injury in humans and is used as an experimental model to investigate hepatoprotective role of natural products and drugs [10]. Epigallocatechin-3-gallate from green tea significantly reduces the severity of CCl₄-induced liver injury and prevents progression of liver fibrosis by decreasing oxidative stress and pro-inflammatory responses [12]. Dioscin also protects against chemically induced acute liver injury due to its inhibitory effects on lipid peroxidation, necrosis and apoptosis, and the suppression of inflammatory cytokine production [11]. *Schisandra chinensis* pollen extract protects against acute hepatotoxicity by strongly increasing antioxidant activity and inhibiting lipid peroxidation [5]. Our study investigated the protective effect of GSPs in CCl₄ induced acute liver injury model in mice. A previous study showed that GSPs protect against reperfusion-induced injury in cardiomyocytes via reducing or scavenging free radicals [30]. GSPs have remarkable beneficial effects in cancers. In head and neck squamous cell carcinoma, GSPs down-regulated epidermal growth factor receptor expression, suppressed NF- κ B activation and inhibited epithelial-to-mesenchymal transition [31]. Further, GSPs showed a significant therapeutic potential in preventing pancreatic beta-cell death in type 2 diabetes mellitus by regulating endoplasmic reticulum stress [13].

Therefore, GSPs appear to have extraordinarily beneficial activities in diverse disease settings.

In this study, we investigated the potential of GSPs to protect against chemically induced liver injury. In order to achieve this, CCl₄ induced acute liver injury model in mice was successfully established, as shown by the elevated serum ALT and AST levels after CCl₄ treatment [32]. Histopathological observations showing swollen hepatocyte morphology, cytoplasmic relaxation, dark stained nuclei, punctate or focal necrosis, and neutrophil and lymphocyte infiltration in the CCl₄-model group further confirmed that the liver injury was successfully induced by CCl₄. Serum AST and ALT levels decreased in the GSP groups compared with the CCl₄-model group, indicating the active protective effects of daily doses of GSPs. The protective effects of GSPs were further confirmed by significant reductions in liver tissue damage and decreased punctate and focal necrosis in the GSP groups. MTT assay to detect hepatocyte proliferation also showed that compared to the CCl₄-model group, all GSP dose groups and DDB group showed significantly higher hepatocyte proliferative activity, which increased in a dose-dependent manner with increasing GSP dose. Consistent with our results, Zhong et al. found that GSPs inhibit hepatocyte damage induced by ethanol and CCl₄, and stimulate normal hepatocyte proliferation [33].

Compared with the control group, serum TNF- α and IL-6 levels were significantly increased in other three groups. Serum TNF- α and IL-6 levels in the GSP group were lower than those in the model group, suggesting the anti-inflammatory role of GSPs on the ALI. TNF- α was identified as an endogenous pyrogen and a key component of an inflammatory response and one of the most potent pro-inflammatory cytokines released by innate immune cells [34]. IL-6 is secreted by the white blood cells, such as macrophages and endothelial cells and any inflammatory stimulus can increase the IL-6 concentrations [35].

Interestingly, apple polyphenols prevented increase in body weight [10]. However, we did not detect any significant changes in mouse body weight within the experimental period in our study after CCl₄ and GSP treatments. Compared with the control group, a significantly increased liver index was observed in the CCl₄-model group, with swollen and deep colored liver, indicating liver injury. Compared to the CCl₄-model group, a remarkable decreased in liver index and increased thymus index were found in all the GSP groups. GSPs also displayed protective effects in organs important in immune function through its beneficial effects on the thymus. Thymus is an important organ that facilitates the maturation of T lymphocytes, macrophages and lymphocytes in preparation for their critical roles in immune system [36]. We did not observe differences in spleen index among the six groups.

Our study also obtained preliminary evidence of the potential mechanisms involved in the protection conferred by GSPs against the liver injury induced by CCl₄. Our result showed significantly elevated SOD and GSH-Px activities and decreased MDA content after administration of GSPs. Liver contains high endogenous expression of various antioxidant enzymes, such as SOD and GSH-Px, which are the primary defense mechanisms against reactive oxygen species [37]. SOD removes superoxides and protects cells from oxidative damage [38], and GSH-Px protects the structural integrity of cell membranes [39]. GSPs increased SOD and GSH-Px activities after CCl₄ treatment, suggesting that GSPs might augment the expression of antioxidant enzymes and increase the antioxidant capacity. Increased MDA content in cells leads to the deactivation of many lipids and proteins, which may further lead to DNA damage [40]. By down-regulating MDA, GSPs prevents lipid peroxidation, which is the main cause of CCl₄-induced liver injury.

Another result of our study showed HMGB1 mRNA and protein expression increased significantly in the model group compared with the control group, suggesting the cell inflammation caused by D-Gal in mice livers; compared with the model group, HMGB1 mRNA and protein expression decreased significantly in the GSP group, further indicating the anti-inflammatory role of GSPs on the ALI. HMGB1 is a highly conserved non-histone nuclear protein and constitutively expressed by nucleated cells and facilitates gene transcription, repair and recombination, and the biological activity of the HMGB1 depends on its location and post-translational modification [41]. Intracellular HMGB1 binds DNA and modulates chromosomal architecture; and once cells were activated and injured, HMGB1 can translocate outside of the cells [42]. Once released from the cell, HMGB1 binds to the transmembrane receptors, cytokines, and other exogenous molecules, and is known as one of the key endogenous damage-associated molecular pattern molecules and can trigger an array of inflammatory responses [43]. HMGB1 is an important pro-inflammatory molecule and has been reported to play a role in response to pro-inflammatory diseases such as acute lung injury, epithelial barrier dysfunction and arthritis in mice and arthritis, sepsis, disseminated intravascular coagulation, acute liver failure, acute appendicitis and other inflammatory disorders in human [44].

Conclusions

Our results showed that GSP protects against acute oxidative hepatotoxicity induced by CCl₄ in mice. We also obtained preliminary evidence that this protective effect of GSPs may mainly involve free radical scavenging, preserving the organs important for immune function, and increasing the antioxidant capacity and inhibition of lipid peroxidation. However, the obtained GSPs in our study contain other ingredients, such as

monomeric flavonols, which might also affect the injured liver. Thus, we need to exclude their effects in our further studies. Taken together, our results show that GSPs are an abundant source of polyphenolic antioxidants and they are safe and effective for treatment of acute chemically-induced liver injury.

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Competing interests

All authors in our study have no conflict of interest.

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