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• *BASIC RESEARCH* •

Anti-lipid peroxidation and protection of liver mitochondria against injuries by picroside II

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Abstract

AIM: To investigate the anti-lipid peroxidation and protection of liver mitochondria against injuries in mice with liver damage by picroside II.

METHODS: Three animal models of liver damage induced by carbon tetrachloride (CCl_4: 0.1 mL/10 g, *ip*), D-galactosamine (D-GalN: 500 mg/kg, *ip*) and acetaminophen (AP: 0.15 g/kg, *ip*) were respectively treated with various concentrations of picroside II (5, 10, 20 mg/kg, *ig*). Then we chose the continuously monitoring method (recommended by International Clinical Chemistry League) to analyze serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) values, Marland method to detect the activity of manganese-superoxide dismutase (SOD) in liver mitochondria, TBA colorimetry to determine the content of malonicdialdehyde (MDA) in liver tissue, DTNB method to evaluate the activity of glutathioneperoxidase (GSH-Px) and Lowry method to detect protein level in liver tissue. Meanwhile, effects of picroside II on the activity of ATPase and swelling extent of mitochondria in hepatocytes damaged by AP were also evaluated.

RESULTS: Picroside II could significantly prevent liver toxicity in the three models of liver damage. It decreased the high levels of ALT and AST in serum induced by the administration of CCl_4, D-GalN and AP, reduced the cellular damage of liver markedly, and appeared to be even more potent than the positive control drug of biphenyl dimethyl dicarboxylate pilules (DDB). In groups treated with different doses of picroside II, compared to the model group, the content of MDA in serum decreased evidently, whereas the content of SOD and GSH-Px increased in a dose-dependent manner, and the difference was statistically significant. Further, in the study of AP model, picroside II inhibited AP-induced liver toxicity in mice, enhanced the activity of ATPase, improved the swelling extent of mitochondria and helped to maintain a normal balance of energy metabolism.

CONCLUSION: Picroside II can evidently relieve hepatocyte injuries induced by CCl_4, D-GalN and AP, help scavenge free radicals, protect normal constructions of mitochondria membrane and enhance the activity of ATPase in mitochondria, thereby modulating the balance of liver energy metabolism, which might be part of the mechanisms of hepatoprotective effects of picroside II.

Key words: Anti-lipid peroxidation; Liver mitochondria

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INTRODUCTION

Picrorhiza scrophulariflora Pennell belongs to the crophularia plants, whose active medicinal constituents are obtained from its dried root and rhizomes. It has been traditionally used to treat disorders of the liver, upper respiratory tract diseases, fevers, dyspepsia, chronic diarrhea and scorpion sting. Current researches on picroside II are focused on its hepatoprotective, anticholestatic, antioxidant and immune-modulating activities[1]. However little is known about the mechanisms of its pharmacological pathway. Picroside II is the active constituent extracted from *Picrorhiza scrophulariflora Pennell*.

MATERIALS AND METHODS

Animals and materials

Mice of Kunming strain (weighing 20-22 g) were supplied by the Animal Center of Academy of Military Medical Sciences, among which males and females accounted for 50% of the total mice.

Picroside II ($C_{23}H_{28}O_{13}$, molecular weight 512) was supplied by Bescholor Research Center of Peking University. Biphenyl dimethyl dicarboxylate pilules (DDB) and CCl_4 were obtained from Beijing Union Pharmaceutical Factory. D-GalN and AP were purchased from Sigma Chemical Co. Detection kits for serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), MDA, SOD, glutathioneperoxidase (GSH-Px) and inorganic phosphorus were all domestic products.

Model establishment and treatment

Three kinds of mice models of acute liver damage were

induced by CCl_4, D-GalN and AP, respectively. Sixty mice were divided randomly into six groups, including normal group, model group, positive control group and groups treated with picroside II 5, 10, 20 mg/mL. Normal and model groups were treated with 0.9% NaCl, the others were intragastrically (*ig*) administrated with various concentrations of picroside II or the positive drug DDB 0.2 mL/10 g (200 mg/kg), once a day for 7 d. On the 6th d of drug administration, except for the normal control group, the others were all intraperitoneally (*ip*) injected with 0.1% CCl_4 peanut oil solution 0.1 mL/10 g, AP 0.15 g/kg or D-GalN 500 mg/kg to establish experimental models of acute liver damage and to observe effects of picroside II and DDB on these models.

Fifteen hours post-model establishment, picroside II and DDB were administrated the last time. One hour later, blood of each mouse was sampled through eye orbital veins. Serum ALT and AST were then monitored. Liver tissue slices from each mouse were prepared for pathology evaluation. Meanwhile liver homogenates were prepared for detection of protein level and suspensions of liver mitochondria were obtained to analyze the activities of ATPase and the swelling extent of mitochondria.

Preparation of suspension of liver mitochondria[2]

Murine liver was rinsed with ice cold 0.9% NaCl, then mitochondria separating medium (0.25 mol/L saccharose, EDTA 2 mmol/L, MOPS 5 mmol/L, KH_2PO_4 5 mmol/L, bovine serum albumin 1 g/L, pH 7.4) was added to make 10% homogenates, centrifuged at 10 000 *g* (Beckman Avanti J-25) for 20 min. Supernatant was centrifuged at 10 000 *g* (Beckman Avanti J-25) for another 20 min, the pellets were resuspended with pH 7.4 mitochondria separating medium to make suspension containing mitochondria proteins 5 mg/mL and then stored at -20 ℃.

Preparation of homogenates of murine liver

Murine liver was rinsed to get rid of the residual blood, and 0.9 mL 1.5 g/L KCl solution was added to 100 mg liver tissue to make 100 g/L homogenates of liver, then stored at -20 ℃.

Biochemical observation

ALT and AST were detected according to the instructions of the detection kits. TBA[3] colorimetry was used to detect the content of MDA in liver tissue, o-phenyltriphenol self-oxidation method was used to analyze the activities of SOD[4], DTNB method[5] was used to evaluate the activities of GSH-Px (an active unit of enzyme decreases the concentration of GSH 1 μmol/mg protein within 1 min) and Lowry method was used to analyze the protein level in hepatocyte suspension.

Detection of ATPase in liver mitochondria of mice[6]

Two hundred microgram protein of mitochondria was pre-incubated with the reaction buffer (50 mmol/L Tris-HCl, pH 7.4, 75 mmol/L KCl, 0.4 mmol/L EDTA, 6 mmol/L $MgCl_2$) at 37 ℃ for 5 min. ATP at 6 mmol/L was added to start the reaction. Fifteen minutes later, 50 g/L SDS was added to stop the reaction. Then it was centrifuged at 3 000 r/min for 10 min at 4 ℃. The content of pi in 100 μL reaction supernatant was detected by an inorganic phosphorus detection kit. The quantity of pi produced represented the activities of ATPase, with the active unit μmoL (pi)/(h·mg) (protein).

Detection of swelling extent of liver mitochondria of mice[7]

$CaCl_2$ 3×10^{-4} mol/L was mixed with 1 mL swelling determination buffer containing 0.5 mg mitochondria protein (0.25 mol/L saccharose, 5×10^{-3} mol/L KH_2PO_4, 3×10^{-3} mol/L sodium succinate), then 520 nm absorption (A) of the mitochondria suspension was recorded continuously at 25 ℃ for 10 min. The swelling extent of mitochondria was evaluated according to the decreased values of 520 nm absorption.

Statistical analysis

All data were expressed as mean±SD. Data were assessed by using *t* test, and $P<0.05$ was considered statistically significant.

RESULTS

Effects of picroside II on serum ALT and AST of mice with acute liver damage induced by CCl_4, AP and D-GalN

Picroside II decreased the high serum ALT and AST levels induced by the administration of CCl_4, D-GalN and AP, as well as the cellular damage of liver, and appeared to be even more potent than the positive control drug of DDB (Tables 1-3).

Table 1 Effects of picroside II on serum ALT and AST of mice with acute liver injury induced by CCl_4 (mean±SD)

Group	Mice (*n*)	ALT (U/L)	AST (U/L)
Normal	10	47.68±15.23[b]	130.10±74.17[b]
Model control	10	511.86±255.86	562.93±183.84
Positive control	10	269.86±137.86[b]	378.42±189.63[a]
Picroside II (5 mg/kg)	10	404.15±21.10	416.28±230.48
Picroside II (10 mg/kg)	10	293.69±177.40[a]	402.14±181.09[a]
Picroside II (20 mg/kg)	10	242.01±163.96[b]	277.46±84.64[b]

[a]$P<0.05$, [b]$P<0.01$ *vs* model control.

Table 2 Effects of picroside II on serum ALT and AST of mice with acute liver injury induced by AP (mean±SD)

Group	Mice (*n*)	ALT (U/L)	AST (U/L)
Normal	10	56.53±11.52[b]	151.22±15.87[b]
Model control	10	508.91±220.93	711.77±151.27
Positive control	10	328.67±143.06[a]	335.84±257.02[b]
Picroside II (5 mg/kg)	10	342.52±186.33	411.23±190.89[b]
Picroside II (10 mg/kg)	10	225.89±75.28[b]	318.17±161.20[b]
Picroside II (20 mg/kg)	10	197.87±46.11[b]	283.40±140.76[b]

[a]$P<0.05$, [b]$P<0.01$ *vs* model control.

Table 3 Effects of picroside II on serum ALT and AST of mice with acute liver injury induced by D-GalN (mean±SD)

Group	Mice (*n*)	ALT (U/L)	AST (U/L)
Normal	10	46.59±14.18[b]	140.77±11.85[b]
Model control	10	1 036.67±588.34	800.82±418.75
Positive control	10	548.32±254.35[a]	502.42±192.43[a]
Picroside II (5 mg/kg)	10	635.93±266.90[a]	523.56±184.12
Picroside II (10 mg/kg)	10	495.3±260.70[a]	491.90±116.35[a]
Picroside II (20 mg/kg)	10	250.69±142.19[b]	368.61±121.79[b]

[a]$P<0.05$, [b]$P<0.01$ *vs* model control.

Effects of picroside II on MDA, SOD and GSH-Px of mice with acute liver damage induced by CCl_4, AP and D-GalN

Picroside II could reverse the increase of MDA and the decrease of SOD resulted from CCl_4, AP and D-GalN-induced liver injuries. The content of serum MDA was markedly decreased, whereas SOD and GSH-Px were increased in groups treated with different concentrations of picroside II in a dose-dependent manner compared with model group (Tables 4-6).

Table 4 Effects of picroside II on MDA, SOD, and GSH-Px of mice with acute liver damage induced by CCl_4 (mean±SD)

Group	M (*n*)	MDA (nmol/L)	SOD (NU/mL)	GSH-Px (μmol/g)
Normal	10	4.24±0.46[b]	264.82±14.8[b]	96.42±8.2[b]
Model control	10	8.48±0.56	218.41±14.4	64.22±5.4
Positive control	10	5.46±1.06[b]	244.54±12.6[a]	80.63±7.2[b]
Picroside II (5 mg/kg)	10	6.88±1.12[b]	238.63±14.6[a]	66.46±6.2
Picroside II (10 mg/kg)	10	5.22±0.38[b]	250.22±15.2[b]	70.82±8.4[a]
Picroside II (20 mg/kg)	10	4.46±0.88[b]	254.26±16.6[b]	88.66±9.8[b]

[a]P<0.05, [b]P<0.01 *vs* model control.

Table 5 Effects of picroside II on MDA, SOD, and GSH-Px of mice with acute liver damage induced by AP (mean±SD)

Group	M (*n*)	MDA (nmol/L)	SOD (NU/mL)	GSH-Px (μmol/g)
Normal	10	1.74±0.32[b]	288.61±11.8[b]	86.42±4.2[b]
Model control	10	5.48±0.24	202.42±10.4	54.67±3.4
Positive control	10	2.46±0.36[b]	236.35±14.6[b]	70.63±3.2[a]
Picroside II (5 mg/kg)	10	2.88±0.42[b]	216.62±11.6[a]	59.64±2.2
Picroside II (10 mg/kg)	10	2.24±0.28[b]	250.21±15.2[b]	68.82±2.4[a]
Picroside II (20 mg/kg)	10	2.02±0.12[b]	278.23±14.2[b]	81.62±6.8[b]

[a]P<0.05, [b]P<0.01 *vs* model control.

Table 6 Effects of picroside II on MDA, SOD, and GSH-Px of mice with acute liver damage induced by D-GalN (mean±SD)

Group	M (*n*)	MDA (nmol/L)	SOD (NU/mL)	GSH-Px (μmol/g)
Normal	10	2.24±0.22[b]	302.28±16.8[b]	82.24±3.22[b]
Model control	10	6.64±0.32	246.62±15.2	58.32±2.22
Positive control	10	3.48±0.16[b]	284.25±12.2[b]	72.28±3.01[a]
Picroside II (5 mg/kg)	10	4.88±0.42[a]	254.64±14.4	61.24±2.64
Picroside II (10 mg/kg)	10	3.22±0.26[b]	268.28±14.2[a]	70.26±2.86[a]
Picroside II (20 mg/kg)	10	3.02±0.24[b]	286.24±16.2[b]	78.24±3.12[b]

[a]P<0.05, [b]P<0.01 *vs* model control.

Effects of picroside II on activities of ATPase and swelling extent of mitochondria of mice with acute liver damage induced by AP

Picroside II could markedly inhibit the activities of ATPase in mitochondria and could decrease the swelling extent of mitochondria of mice with liver damage induced by AP in a dose-dependent manner, thus helping maintain a normal energy metabolism (Table 7).

DISCUSSION

There is a certain quantity of oxygen free radicals in normal state in human body. Excess free radicals could be scavenged by endogenous enzymes, such as SOD, GSH-Px, which help maintain a normal oxidation-reduction balance. Tissues and cells would be subjected to oxidative injuries when large quantities of inner free radicals are generated or the activities of antioxidant system deteriorate. Mitochondria have membranes rich in polyunsaturated fatty acids, and several kinds of electron transport systems and are sensitive to the attack of free radicals. Polyunsaturated fatty acid is ready to be oxidized by free radicals to generate products of lipid peroxidation such as MDA, meanwhile, the quantity of polyunsaturated fatty acid reduces during the procedure of oxidation, thereby lowering the membrane fluidity. In addition, lipid peroxidation could impair the normal membrane constructions of mitochondria, increase its permeability and thus swell it[8]. CCl_4-induced hepatotoxicity is mainly caused by its active product CCl_3, which could induce unsaturated fatty acid to undertake lipid oxidation[9], thereby decreasing the content of cellular GSH, altering protein thio, disordering the transport and storage of Ca^{2+} in mitochondria, endoplasmic reticulum and cell membrane, increasing the content of plasmic Ca^{2+}, and ultimately causing death of cell[10]. ALT and AST in plasma are then released into the blood. D-galactosamine (D-GalN) is an indirect hepatotoxicity-inducing chemical, whose act might be related to its metabolism in liver and the subsequent effects on nucleic acid synthesis. It was reported that the liver function and morphological changes of liver tissue induced by D-GalN were similar to those of viral hepatitis[11]. AP is a widely used antipyretic and anodyne. It could cause liver poisoning and hepatocyte necrosis[12], which resulted from the products of transformed AP-semiquinone free radicals (NAPQI)[13,14]. Because the power of oxidation-respiration in mitochondria could be inhibited by many quinone chemicals, AP's metabolite NAPQI could affect the function of liver mitochondria

Table 7 Effects of picroside II on activities of ATPase and swelling extent of mitochondria of mice with acute liver damage induced by AP (mean±SD)

Group	Mice (*n*)	Activities of ATPase μmol (pi)/(h mg) (protein)	Swelling extent of mitochondria 0-5 min A_{520}	Swelling extent of mitochondria 5-10 min A_{520}
Normal	10	24.24±0.26[a]	0.528±0.004[b]	0.520±0.002[b]
Model control	10	20.18±0.16	0.462±0.002	0.432±0.001
Positive control	10	23.48±0.16[a]	0.495±0.001[b]	0.476±0.001[a]
Picroside II (5 mg/kg)	10	21.01±0.42	0.474±0.002	0.450±0.002[b]
Picroside II (10 mg/kg)	10	23.22±0.26[a]	0.496±0.001[a]	0.484±0.002[a]
Picroside II (20 mg/kg)	10	25.02±0.24[a]	0.512±0.006[b]	0.502±0.004[b]

[a]P<0.05, [b]P<0.01 *vs* model control.

and lead to mitochondria injuries[15]. Energy utilization in mitochondria could be evaluated by total ATPase activities in it. When mitochondria are damaged, energy generation in them is inevitably inhibited, ATPase activities and the energy ready to be utilized in cells simultaneously are decreased, and a disorder of liver energy metabolism and morphological changes of mitochondria ultimately happen.

According to Chinese traditional medicine theory, *Picrorhiza scrophulariflora Pennell* is of Indian and Sitsang origins. They have similar chemical constituents and virtues[16-18]. Researches revealed[19] that picroside I and picroside II were the key elements accounting for the effects of antitoxicity on hepatocytes. Picroside II used in the present study was extracted from *Picrorhiza scrophulariflora Pennell* of Sitsang origin, with a purity of over 94% determined by HPLC. It showed that picroside II significantly prevented the liver from toxicity in the three models of liver damage. It decreased the high levels of serum ALT and AST induced by the administration of CCl_4, D-GalN and AP, as well as cellular pathological damage of liver markedly, and appeared to be even more potent than the positive control drug-DDB. Values of SOD decreased, while MDA increased in model group compared to normal group, with a significant difference ($P<0.01$). In groups treated with different doses of picroside II, compared to the model group, the content of serum MDA decreased evidently, with significant differences ($P<0.01$). Whereas the content of SOD and GSH-Px increased in a dose-dependent manner, and the differences still had a markedly statistical significance ($P<0.05$ or $P<0.01$). All of the above suggested that picroside II could protect normal constructions of mitochondria membranes and enhance the activity of ATPase in mitochondria, thereby modulating the balance of liver energy metabolism, which might be part of the mechanisms of hepatoprotective effects of picroside II.

In a word, picroside II can relieve hepatocyte injuries induced by CCl_4, D-GalN and AP, help scavenge free radicals and protect normal constructions of mitochondria membranes. Our study provides theoretic bases of hepatoprotective effects of picroside II. Other mechanisms concerning the effect of picroside II remain to be investigated in the future.

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