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Research Paper

Recombinant bovine pancreatic trypsin inhibitor protects the liver from carbon tetrachloride-induced acute injury in mice

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Abstract

Objectives Toxicity caused by pharmacological and chemical substances, including carbon tetrachloride (CCl_4), is a major pathological factor for liver injury. Therefore, strategies to prevent toxicity are needed for maintaining a healthy liver. This study was designed to determine whether recombinant bovine pancreatic trypsin inhibitor (rBPTI), a non-specific serine protease inhibitor, prevents CCl_4-induced liver injury in mice.

Methods Mice were treated with CCl_4 in the presence or absence of co-treatment with rBPTI. Liver sections were prepared for histopathological assessment. Liver function was evaluated by detecting serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and liver index. Liver oxidative stress and inflammation were examined by detecting the liver malondialdehyde level and glutathione and superoxide dismutase activity, and serum tumour necrosis factor-α level, respectively.

Key findings CCl_4 induced hepatocyte necrosis, inflammatory cell infiltration and fatty degeneration, which were ameliorated by co-treatment with rBPTI in a concentration-dependent manner. Furthermore, rBPTI prevented CCl_4-induced disruption of liver function. Importantly, rBPTI reduced CCl_4-induced liver oxidative stress response and pro-inflammatory cytokine production.

Conclusions These results indicated that rBPTI exerted a protective effect on CCl_4-induced liver injury in mice. Thus, rBPTI may have potential application for prevention of liver injury induced by metabolism of drugs and toxic substances.

Keywords bovine pancreatic trypsin inhibitor; carbon tetrachloride; liver injury

Introduction

The liver is a major organ responsible for metabolism of drugs and toxic substances.[1,2] Some pharmacological and chemical substances, including carbon tetrachloride (CCl_4), paracetamol (acetaminophen), D-galactosamine (GalN) and dimethylnitrosamine (DMN), have been found to induce liver injury. Therefore, one challenge of research into maintaining a healthy liver is to successfully develop new strategies for the treatment of liver injury.

Bovine pancreatic trypsin inhibitor (BPTI; aprotinin), a 58-amino-acid basic protein, is produced in several organs, such as the lung, pancreas or parotid glands.[3,4] Endogenous BPTI not only inhibits trypsin activity, but also acts as an inhibitor for chymotrypsin, plasmin and kallikreine.[5,6] BPTI has been reported to relieve liver ischaemia–reperfusion-induced injury in rats and reduce blood loss during surgery and blood transfusion in humans.[7,8] Thus, BPTI serves as a potential treatment for acute pancreatitis and coagulation disorders, and a preventive reagent for haemorrhage in surgery.[9,10] In addition, the role of protease inhibitors in inflammation have been reported. Ulinastatin, a human trypsin inhibitor, has been found to inhibit endotoxin-induced thromboxane B2 production in human monocytes.[11] Another protease inhibitor, urinary trypsin inhibitor (UTI), has been shown to protect against bacterial endotoxin-induced systemic inflammatory response and subsequent organ injury[12] and acute lung injury[13] through preventing expression of pro-inflammatory cytokines and chemokines. However, the anti-inflammatory effect of BPTI in patients with cardiopulmonary bypass surgery is controversial.[14–17]

We have generated recombinant BPTI (rBPTI) using the eukaryotic expression system *Pichia pastoris*.[18] We reported that rBPTI exerts the same activity as that prepared from

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natural sources, such as bovine lung.[18] In this study, we used a mouse model of CCl_4-induced liver injury to investigate the potential hepatoprotective effect of rBPTI on CCl_4-induced liver injury. CCl_4 is one of the best-studied hepatotoxicants, which induces liver injury in many species, including humans and non-human primates.[19] Thus mice treated with CCl_4 serve as an in-vivo halogenated hydrocarbon-induced liver injury model.[20] Our results showed that rBPTI exerted a protective effect on CCl_4-induced liver injury in mice, which supported rBPTI's broader application for prevention of liver injury induced by metabolism of drugs and toxic substances.

Materials and Methods

Reagents

Carbon tetrachloride (CCl_4) was purchased from Beijing Chemistry Plant (Beijing, China). Diammonium glycyrrhizinate was from Jiangsu Chia Tal-tianqing Pharmaceutical Company (Jiangsu, China). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) reagent kits were from the Beijing Chemistry Reagent Company (Beijing, China). The malondialdehyde (MDA) assay kit, superoxide dismutase (SOD) detection kit and glutathione (GSH) assay kit were from Jiancheng Institute of Biotechnology (Nanjing, China). The mouse tumour necrosis factor-α alpha (TNF-α) ELISA kit was from BD Bioscience (San Diego, USA).

Preparation of the recombinant BPTI

We generated rBPTI as described in our previously published paper.[18] Briefly, the bovine *bpti* gene was expressed in a eukaryotic expression system, *Pichia pastoris*, with human serum albumin as a signal peptide. Yeast was fermented for large-scale production of the rBPTI. After the fermentation, rBPTI was purified using cation exchange chromatography and reverse-phase chromatography. The purity of rBPTI was higher than 95% and the purified coefficient was over 60%. Purified rBPTI was evaporated using a rotary evaporator under reduced pressure at 50°C and the water extract was freeze-dried into powder.

Animals and treatments

Kunming mice, 19–23 g, were maintained in a controlled environment at 21 ± 2°C and 50 ± 5% relative humidity under a 12-h dark–light cycle and were acclimatized for at least one week before use. Experiments were performed according to protocols approved by the National Institute of Health Guidelines for the Care and Use of Laboratory Animals at Tianjin Medical University. Mice were randomly divided into seven groups with 50% male and 50% female in each group (10 mice per group): Group 1, normal control (without any treatment); Group 2, model control, mice treated with CCl_4 only; Groups 3–5, mice treated with CCl_4 and rBPTI at dosages of 3, 6 or 12×10^4 KIU/kg body weight, respectively; Group 6, mice treated with CCl_4 and BPTI (6×10^4 KIU/kg); Group 7, mice treated with diammonium glycyrrhizinate (25 mg/kg). rBPTI and BPTI were solublized in saline and were administered to mice intraperitoneally once a day for seven days. Saline was given to normal control and model control groups. One hour after final rBPTI or BPTI injection, mice were administered intraperitoneally with 0.1% CCl_4 in corn oil (20 ml/kg) twice with a 16 h interval, to induce acute liver injury. Mice in the control group were treated with corn oil only (20 ml/kg). Mice were fasted (water was allowed) for 1 h before sacrificing. Blood samples were collected immediately before mice were sacrificed.

Detection of serum alanine aminotransferase and aspartate aminotransferase and tumour necrosis factor-α

Blood samples were heparinized and centrifuged at 3000 rev/min for 10 min to obtain sera. All serum samples were saved at −80°C. Serum ALT and AST levels were detected using ALT and AST reagent kits, respectively, and measured using an autoanalyser (AU2700; Olympus, Japan). The serum TNF-α level was measured using a TNF-α ELISA kit, according to the manufacturer's instructions.

Detection of liver index, liver malondialdehyde level and glutathione and superoxide dismutase activity

Mouse body and liver weights were recorded to determine the liver index, which was calculated using the following formula: liver index = (liver weight /body weight) $\times$ 100%.

Liver tissues were homogenized in 0.15 M KCl. Homegenates were then centrifuged at 3000 rev/min for 20 min at 4°C and the supernatant was saved for analysis of MDA and GSH levels and SOD activity.[21] The level of MDA, a lipid peroxidation end product in the liver, was analysed by measuring the level of thiobarbituric acid reactive substance (TBARS).[22] The hepatic GSH level was detected by a colorimetric method using Ellman's reagent and glutathione reductase.[23] The assay to detect SOD activity was based on the ability of SOD to inhibit the oxidation of oxyamine by the xanthine–xanthine oxidase system.[24]

Histopathological examination

Liver tissues were fixed in 10% neutral formalin for 24 h for preparing paraffin-embedded tissue sections. Liver sections were stained with hematoxylin and eosin (H&E) and evaluated using light microscopy. The degree of portal inflammation, hepatocellular necrosis and inflammatory cell infiltration was evaluated semiquantitatively.[25,26] Injury was graded as follows: absent (0), minimal (I), mild (II), modest (III) and severe (IV).

Statistical analysis

Data were presented as the mean ± standard deviation. To analyse post-hoc multiple comparisons, one-way analysis of variance and Student–Newman–Keuls test were used. Statistical analysis was performed using the SPSS statistical software package (SPSS Inc., Chicago, USA). $P < 0.05$ was considered statistically significant.

Results

Recombinant bovine pancreatic trypsin inhibitor prevents carbon tetrachloride-induced liver injury

To determine the hepatoprotective effect of rBPTI, we chose the mouse model of CCl_4-induced liver injury. We chose the doses of BPTI for this study based on previously published studies to detect the protective effects of BPTI on haemorrhage in surgery.[27]

CCl_4 treatment induced various histological changes in the liver, including hepatocyte necrosis, inflammatory cell infiltration, fatty degeneration and hydropic degeneration (Figure 1). To assess these histopathological changes, liver injury and inflammation were scored by a pathologist blinded to the treatment (Table 1). CCl_4 induced mild to severe liver injury, including hepatocellular necrosis, fatty degeneration and inflammatory cell infiltration, in 100% of mice. Co-treatment of mice with 12×10^4 KIU/kg rBPTI resulted in 90% of mice with absence of, or minimal, hepatocellular necrosis and fatty degeneration, and 100% of mice with absence of, or minimal, inflammatory cell infiltration. We also found that the hepatoprotective effect of rBPTI was concentration dependent. Furthermore, we compared the effect of rBPTI on preventing liver injury with the effect of diammonium glycyrrhizinate injection. Diammonium glycyrrhizinate is a commonly used hepatoprotective medicine and is able to protect the liver cell membrane and improve liver function. In our study, 100% of mice showed absence of, or minimal, hepatocellular necrosis, fatty degeneration and inflammatory cell infiltration when they were co-treated with diammonium glycyrrhizinate. Thus, rBPTI and diammonium glycyrrhizinate exerted similar effects on reducing CCl_4-induced liver injury. In addition to diammonium glycyrrhizinate, we found that rBPTI had a similar hepatoprotective compared with natural BPTI.

Recombinant bovine pancreatic trypsin inhibitor ameliorates carbon tetrachloride-induced disruption of liver function

Next, the effect of rBPTI on preventing CCl_4-induced disruption of liver function was studied. We detected ALT and AST serum levels and the liver index as markers for liver function. CCl_4 disrupted liver function by increasing serum levels of ALT and AST and increasing the liver index compared with those in the normal control group ($P < 0.01$; Table 2). rBPTI significantly decreased serum levels of ALT and AST and the liver index in CCl_4-treated mice in a concentration-dependent manner (Table 2). These data indicate that rBPTI is able to preserve the liver function in mice treated with CCl_4.

Recombinant bovine pancreatic trypsin inhibitor reduces carbon tetrachloride-induced liver oxidative stress response and pro-inflammatory cytokine production

CCl_4 has been shown to induce oxidative stress, leading to lipid peroxidation and free radical production, which plays an important pathological role in liver injury.[28] To evaluate the oxidative status, we detected MDA, an end-product of lipid peroxidation. An elevated level of MDA is found in injured hepatocytes. We found that the CCl_4-induced increase in MDA level in the liver was significantly prevented by co-treatment with rBPTI (Table 3).

Furthermore, GSH levels and SOD activity were investigated in the liver. Both SOD[29] and GSH[30] can inhibit free radical-induced oxidative stress. The GSH concentration and SOD activity in the liver homogenate were significantly decreased in mice treated with CCl_4; this decrease was prevented by rBPTI co-treatment (Table 3).

Since an increase in TNF-α level is directly correlated with the histological evidence of hepatic necrosis, we investigated the effects of rBPTI on TNF-α production. The level of TNF-α was significantly increased in mice treated with CCl_4, which was decreased by rBPTI co-treatment (Table 4).

These results indicate that the inhibitory effects of rBPTI on CCl_4-induced liver oxidative stress and increased pro-inflammatory cytokine production might mediate the hepatoprotective role of rBPTI in mice.

Discussion

These results showed that CCl_4 induced severe liver damage and disrupted liver function in mice, and that this was significant prevented by rBPTI co-treatment. Importantly, we found that there was no statistically significant difference between the effects of rBPTI and BPTI. These data indicated that the protection against acute liver injury of rBPTI was identical to that of natural BPTI.

CCl_4 stimulated oxidative stress, as indicated by the elevated MDA and GSH concentration and decreased SOD activity in the liver of mice, which would further induce lipid peroxidation, initiate free radical damage to the hepatocyte membrane, and eventually lead to liver injury.[28] MDA is one of the major lipid peroxidation products, and its elevated level could reflect the degree of injury in hepatocytes. Our data suggest that the hepatoprotective effect of rBPTI might be mediated by the suppression of lipid peroxidation since rBPTI can prevent the CCl_4-induced increased MDA level. SOD is a scavenger of peroxide anion radicals and could inhibit the initiation of lipid peroxidation by free radicals.[29] GSH plays a role in the antioxidant defence mechanism against the toxic effects of free radicals.[30] Our finding that rBPTI restored SOD and GSH levels indicates that rBPTI treatment reduced CCl_4-induced injury through its antioxidant activity. Therefore, inhibition of CCl_4-induced oxidative stress may serve as a mechanism of protection against acute liver injury by rBPTI.

TNF-α, a pleiotropic pro-inflammatory cytokine, is rapidly produced by macrophages in response to tissue damage.[31] While a low level of TNF-α may play a role in cell protection, an excessive amount causes cell impairment. An increase in the TNF-α level has been directly correlated with the histological evidence of hepatic necrosis and the increase in the serum aminotransferase levels.[32] Our study showed a significant increase in the serum TNF-α level by CCl_4, which was attenuated by rBPTI co-treatment. These results suggested that regulation of pro-inflammatory cytokine production might mediate the hepatoprotective effects of rBPTI.

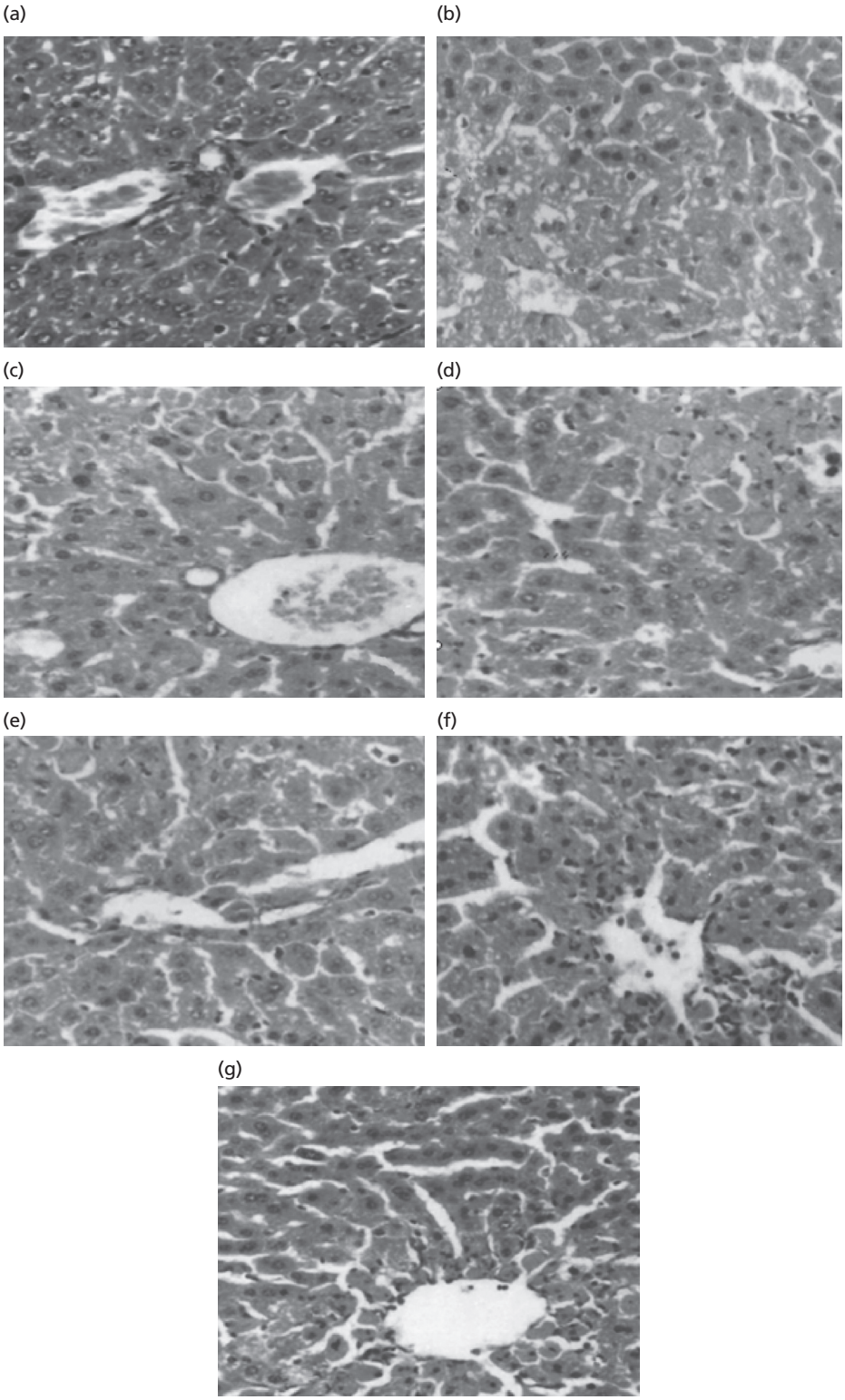

Figure 1 Recombinant bovine pancreatic trypsin inhibitor reduces carbon tetrachloride-induced liver damage in mice. Paraffin-embedded liver tissue sections were stained with H&E for light microscopic assessment of liver damage (200× magnification). Images shown are representative of ten mice in each group. (a) Normal control, no treatment. (b) Model control, CCl_4 only. (c) rBPTI (3×10^4 KIU/kg) + CCl_4. (d) rBPTI (6×10^4 KIU/kg) + CCl_4. (e) rBPTI (12×10^4 KIU/kg) + CCl_4. (f) BPTI (6×10^4 KIU/kg) + CCl_4. (g) Diammonium glycyrrhizinate (25 mg/kg) + CCl_4

Table 1 Recombinant bovine pancreatic trypsin inhibitor protects carbon tetrachloride-induced liver damage

Histopathological grade	Control	CCl_4 Model	rBPTI (3×10^4 KIU/kg)	rBPTI (6×10^4 KIU/kg)	rBPTI (12×10^4 KIU/kg)	BPTI (6×10^4 KIU/kg)	DGI (25 mg/kg)
Hepatocellular necrosis							
Grade 0	10	0	0	2	3	3	3
Grade I	0	0	2	7	6	7	7
Grade II	0	3	5	1	1	0	0
Grade III	0	5	3	0	0	0	0
Grade IV	0	2	0	0	0	0	0
Fatty degeneration							
Grade 0	10	0	0	1	2	1	4
Grade I	0	0	6	7	7	8	6
Grade II	0	5	2	2	1	1	0
Grade III	0	4	2	0	0	0	0
Grade IV	0	1	0	0	0	0	0
Inflammatory cell infiltration							
Grade 0	10	0	4	5	7	6	6
Grade I	0	4	5	5	3	4	4
Grade II	0	5	1	0	0	0	0
Grade III	0	1	0	0	0	0	0
Grade IV	0	0	0	0	0	0	0

rBPTI, recombinant bovine pancreatic trypsin inhibitor; BPTI, natural bovine pancreatic trypsin inhibitor; DGI, diammonium glycyrrhizinate. Histopathological grading: 0, absent; I, minimal; II, mild; III, modest; IV, severe. Data are presented as the number of mice in each histopathological grade.

Table 2 Effect of recombinant bovine pancreatic trypsin inhibitor on regulating liver enzyme levels and liver index in mice treated with carbon tetrachloride

Group	Dose	ALT (U/l)	AST (U/l)	Liver index
Normal control	n.a	68.52 ± 3.61	96.04 ± 7.62	1.15 ± 0.03
Model control	n.a.	145.16 ± 15.8$^{\#\#}$	145.14 ± 13.28$^{\#\#}$	1.40 ± 0.12$^{\#\#}$
rBPTI	3×10^4 KIU/kg	124.35 ± 11.57**	135.39 ± 12.42	1.29 ± 0.25
rBPTI	6×10^4 KIU/kg	120.94 ± 12.65**	130.26 ± 11.76*	1.24 ± 0.07*
rBPTI	12×10^4 KIU/kg	112.64 ± 15.36**	128.42 ± 13.42*	1.23 ± 0.05*
BPTI	6×10^4 KIU/kg	113.42 ± 13.28**	129.68 ± 11.42*	1.20 ± 0.08**
DGI	25 mg/kg	111.35 ± 12.56**	128.62 ± 12.43*	1.22 ± 0.08*

ALT alanine aminotransferase; AST, aspartate aminotransferase; n.a., not applicable; rBPTI, recombinant bovine pancreatic trypsin inhibitor; BPTI, natural bovine pancreatic trypsin inhibitor; DGI, diammonium glycyrrhizinate. Ten mice were used in each group. Data are shown as mean ± SEM. $^{\#\#}P < 0.01$ vs control group; $^{**}P < 0.01$ and $^{*}P < 0.05$ vs model group.

Table 3 Effect of recombinant bovine pancreatic trypsin inhibitor on regulating levels of malondialdehyde and glutathione and superoxide dismutase activity in mice treated with carbon tetrachloride

Group	Dose	MDA (nmol/g)	SOD (U/mg protein)	GSH (U/mg protein)
Normal control	n.a.	3.36 ± 0.86	25.23 ± 2.36	81.54 ± 8.36
Model control	n.a.	12.33 ± 1.53$^{\#\#}$	8.65 ± 3.58$^{\#\#}$	56.65 ± 7.53$^{\#\#}$
rBPTI	3×10^4 KIU/kg	5.98 ± 0.46**	13.65 ± 3.87	70.16 ± 6.87**
rBPTI	6×10^4 KIU/kg	5.27 ± 1.43**	17.16 ± 4.02**	75.32 ± 7.52**
rBPTI	12×10^4 KIU/kg	4.54 ± 1.21**	21.08 ± 5.21**	78.65 ± 8.86**
BPTI	6×10^4 KIU/kg	4.32 ± 1.17**	20.25 ± 4.56**	80.24 ± 8.72**
DGI	25 mg/kg	4.33 ± 1.23**	21.75 ± 3.86**	79.31 ± 9.75**

MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; n.a., not applicable; rBPTI, recombinant bovine pancreatic trypsin inhibitor; BPTI, natural bovine pancreatic trypsin inhibitor; DGI, diammonium glycyrrhizinate. Ten mice were used in each group. Data are shown as mean ± SEM. $^{\#\#}P < 0.01$ vs control group; $^{**}P < 0.01$ and $^{*}P < 0.05$ vs model group.

Table 4 Effect of recombinant bovine pancreatic trypsin inhibitor on regulating tumour necrosis factor-α level in mice treated with carbon tetrachloride

Group	Dose	TNF-α (pg/ml)
Normal control	n.a.	21.86 ± 5.4
Model control	n.a.	193.56 ± 46.73$^{\#\#}$
rBPTI	3 × 10^4 KIU/kg	65.98 ± 19.47**
rBPTI	6 × 10^4 KIU/kg	75.27 ± 23.63**
rBPTI	12 × 10^4 KIU/kg	81.43 ± 21.81**
BPTI	6 × 10^4 KIU/kg	80.32 ± 23.54**
DGI	25 mg/kg	84.36 ± 24.64**

TNF-α, tumour necrosis factor-α; n.a., not applicable; rBPTI, recombinant bovine pancreatic trypsin inhibitor; BPTI, natural bovine pancreatic trypsin inhibitor; DGI, diammonium glycyrrhizinate. Ten mice were used in each group. Data are shown as mean ± SEM. $^{\#\#}P < 0.01$ vs control group; $^{**}P < 0.01$ and $^{*}P < 0.05$ vs model group.

For the future clinical application of rBPTI, we chose the doses of BPTI based on the previously published studies to detect the protective effects of BPTI on haemorrhage in surgery.[27] However, since this study was designed to test the preventive effects of rBPTI on CCl_4-induced liver injury, studies regarding the treatment effects of rBPTI are needed to further its clinical application. Therefore, we are performing experiments to detect the effect of rBPTI on chronic liver injury induced by CCl_4. These data will provide information about the treatment effects of rBPTI on liver injury.

In addition, we should consider the side effects of rBPTI in its clinical application. For example, BPTI has been reported to induce anaphylactic reactions and anaphylactic shock in patients.[33,34] To limit the side effects of rBPTI, future clinical trials are needed to asses the correct dosage, combination with other medications, and patients' physiological conditions, which may improve the efficacy of this treatment for liver injury and other diseases.

In summary, our results support the involvement of rBPTI in suppression of the oxidative responses in liver cells and inhibition of systemic inflammatory cytokine production as a mechanism of rBPTI preventing liver inflammation and injury. Therefore, rBPTI may serve as a potential reagent for injury and inflammation-related liver diseases.

Conclusions

In summary, our studies demonstrate that rBPTI exerts a hepatoprotective effect on acute liver injury induced by CCl_4. Thus rBPTI may serve a potential role in the prevention of liver injury induced by metabolism of drugs and toxic substances.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Lili Yang and Wen Dong contributed equally to this paper.

References

1. Bissell DM *et al*. Drug-induced liver injury: mechanisms and test systems. *Hepatology* 2001; 33: 1009–1013.
2. Lee WM. Drug-induced hepatotoxicity. *N Engl J Med* 2003; 349: 474–485.
3. Antuch W *et al*. The NMR solution structure of a Kunitz-type proteinase inhibitor from the sea anemone Stichodactyla helianthus. *Eur J Biochem* 1993; 212: 675–684.
4. Pritchard L, Dufton MJ. Evolutionary trace analysis of the Kunitz/BPTI family of proteins: functional divergence may have been based on conformational adjustment. *J Mol Biol* 1999; 285: 1589–1607.
5. Hagihara Y *et al*. Screening for stable mutants with amino acid pairs substituted for the disulfide bond between residues 14 and 38 of bovine pancreatic trypsin inhibitor (BPTI). *J Biol Chem* 2002; 277: 51043–51048.
6. Barbar E *et al*. NMR-detected order in core residues of denatured bovine pancreatic trypsin inhibitor. *Biochemistry* 2001; 40: 9734–9742.
7. Polat KY *et al*. The protective effect of aprotinin and alpha-tocopherol on ischemia-reperfusion injury of the rat liver. *Transplant Proc* 2008; 40: 63–68.
8. Pereboom IT *et al*. Aprotinin and nafamostat mesilate in liver surgery: effect on blood loss. *Dig Surg* 2007; 24: 282–287.
9. Bull DA *et al*. Aprotinin preserves myocardial biochemical function during cold storage through suppression of tumor necrosis factor. *J Thorac Cardiovasc Surg* 2000; 119: 242–250.
10. Molenaar IQ *et al*. The effect of aprotinin on renal function in orthotopic liver transplantation. *Transplantation* 2001; 71: 247–252.
11. Aibiki M, Cook JA. Ulinastatin, a human trypsin inhibitor, inhibits endotoxin-induced thromboxane B2 production in human monocytes. *Crit Care Med* 1997; 25: 430–434.
12. Inoue K *et al*. Urinary trypsin inhibitor protects against systemic inflammation induced by lipopolysaccharide. *Mol Pharmacol* 2005; 67: 673–680.
13. Inoue K *et al*. Protective role of urinary trypsin inhibitor in acute lung injury induced by lipopolysaccharide. *Exp Biol Med (Maywood)* 2005; 230: 281–287.
14. Mojcik CF, Levy JH. Aprotinin and the systemic inflammatory response after cardiopulmonary bypass. *Ann Thorac Surg* 2001; 71: 745–754.
15. Tassani P *et al*. High-dose aprotinin modulates the balance between proinflammatory and anti-inflammatory responses during coronary artery bypass graft surgery. *J Cardiothorac Vasc Anesth* 2000; 14: 682–686.
16. Englberger L *et al*. Aprotinin in coronary operation with cardiopulmonary bypass: does 'low-dose' aprotinin inhibit the inflammatory response? *Ann Thorac Surg* 2002; 73: 1897–1904.

17. Schmartz D *et al*. Does aprotinin influence the inflammatory response to cardiopulmonary bypass in patients? *J Thorac Cardiovasc Surg* 2003; 125: 184–190.
18. Yang L *et al*. Expression and purification of natural N-terminal recombinant bovine pancreatic trypsin inhibitor from Pichia pastoris. *Biol Pharm Bull* 2008; 31: 1680–1685.
19. Williams AT, Burk RF. Carbon tetrachloride hepatotoxicity: an example of free radical-mediated injury. *Semin Liver Dis* 1990; 10: 279–284.
20. Weber LW *et al*. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit Rev Toxicol* 2003; 33: 105–136.
21. Wen T *et al*. Dynamic changes of heme oxygenase-1 and carbon monoxide production in acute liver injury induced by carbon tetrachloride in rats. *Toxicology* 2006; 228: 51–57.
22. Draper HH, Hadley M. Malondialdehyde determination as index of lipid peroxidation. *Methods Enzymol* 1990; 186: 421–431.
23. Lee KJ, Jeong HG. Protective effect of Platycodi radix on carbon tetrachloride-induced hepatotoxicity. *Food Chem Toxicol* 2002; 40: 517–525.
24. Sarban S *et al*. Plasma total antioxidant capacity, lipid peroxidation, and erythrocyte antioxidant enzyme activities in patients with rheumatoid arthritis and osteoarthritis. *Clin Biochem* 2005; 38: 981–986.
25. Frei A *et al*. The N-terminal propeptide of collagen type III in serum reflects activity and degree of fibrosis in patients with chronic liver disease. *Hepatology* 1984; 4: 830–834.
26. Lee KJ *et al*. Protective effect of caffeic acid phenethyl ester against carbon tetrachloride-induced hepatotoxicity in mice. *Toxicology* 2008; 248: 18–24.
27. Bull DA *et al*. Aprotinin preserves myocardial biochemical function during cold storage through suppression of tumor necrosis factor. *J Thorac Cardiovasc Surg* 2000; 119: 242–250.
28. Korabiowska M *et al*. Analysis of adenomatous polyposis coli gene expression, APC locus-microsatellite instability and APC promoter methylation in the progression of melanocytic tumours. *Mod Pathol* 2004; 17: 1539–1544.
29. Hsu CT. The role of the autonomic nervous system in chemically induced liver damage and repair – using the essential hypertensive animal model (SHR). *J Auton Nerv Syst* 1995; 51: 135–142.
30. Griffith OW, Mulcahy RT. The enzymes of glutathione synthesis: gamma-glutamylcysteine synthetase. *Adv Enzymol Relat Areas Mol Biol* 1999; 73: 209–267.
31. Brouckaert P, Fiers W. Tumor necrosis factor and the systemic inflammatory response syndrome. *Curr Top Microbiol Immunol* 1996; 216: 167–187.
32. Bruccoleri A *et al*. Induction of early-immediate genes by tumor necrosis factor alpha contribute to liver repair following chemical-induced hepatotoxicity. *Hepatology* 1997; 25: 133–141.
33. Scheule AM *et al*. Repeated anaphylactic reactions to aprotinin in fibrin sealant. *Gastrointest Endosc* 1998; 48: 83–85.
34. Laxenaire MC *et al*. Allergic risk of aprotinin. *Ann Fr Anesth Reanim* 2000; 19: 96–104.