

Protective effect of tea polyphenols against paracetamol-induced hepatotoxicity in mice is significantly correlated with cytochrome P450 suppression

Xia Chen, Chang-Kai Sun, Guo-Zhu Han, Jin-Yong Peng, Ying Li, Yan-Xia Liu, Yuan-Yuan Lv, Ke-Xin Liu, Qin Zhou, Hui-Jun Sun

Xia Chen, Chang-Kai Sun, Guo-Zhu Han, Jin-Yong Peng, Ying Li, Yan-Xia Liu, Yuan-Yuan Lv, Ke-Xin Liu, Qin Zhou, Hui-Jun Sun, Department of Pharmacology, Dalian Medical University, Dalian 116044, Liaoning Province, China

Author contributions: Chen X, Sun CK contributed equally to this work; Han GZ, Sun HJ designed the research; Chen X, Liu YX, Lv YY performed the research; Peng JY, Li Y analyzed the data; Sun HJ, Liu KX wrote the paper; Zhou Q provided the vital reagents and analytical tools.

Supported by Grant from the Science Foundation of Educational Department of Liaoning Province, 05L117 and Dalian Science & Technology Bureau, 2007J22JH012

Correspondence to: Hui-Jun Sun, Professor, Department of Clinical Pharmacology, College of Pharmacy, Dalian Medical University, 9 west section, Lvshun South Road, Lvshunkou District, Dalian 116044, Liaoning Province, China. sunhuijun@hotmail.com

Telephone: +86-411-86110413 Fax: +86-411-86110407

Received: December 31, 2008 Revised: March 3, 2009

Accepted: March 10, 2009

Published online: April 21, 2009

Abstract

AIM: To investigate the hepatoprotective activity of tea polyphenols (TP) and its relation with cytochrome P450 (CYP450) expression in mice.

METHODS: Hepatic CYP450 and CYPb₅ levels were measured by UV-spectrophotometry in mice 2 d after intraperitoneal TP (25, 50 and 100 mg/kg per day). Then the mice were intragastrically pre-treated with TP (100, 200 and 400 mg/kg per day) for six days before paracetamol (1000 mg/kg) was given. Their acute mortality was compared with that of control mice. The mice were pre-treated with TP (100, 200, and 400 mg/kg per day) for five days before paracetamol (500 mg/kg) was given. Hepatic CYP2E1 and CYP1A2 protein and mRNA expression levels were evaluated by Western blotting, immunohistochemical staining and transcriptase-polymerase chain reaction.

RESULTS: The hepatic CYP450 and CYPb₅ levels in mice of TP-treated groups (100, 200 and 400 mg/kg per day) were decreased in a dose-dependent manner compared with those in the negative control mice.

TP significantly attenuated the paracetamol-induced hepatic injury and dramatically reduced the mortality of paracetamol-treated mice. Furthermore, TP reduced CYP2E1 and CYP1A2 expression at both protein and mRNA levels in a dose-dependent manner.

CONCLUSION: TP possess potential hepatoprotective properties and can suppress CYP450 expression.

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Key words: Tea polyphenols; Cytochrome P450; Paracetamol-induced hepatotoxicity

Peer reviewer: Vincent Lai, PhD, Derby NHS Foundation Trust, Utooxeter Road, Derby DE22 3NE, United Kingdom

Chen X, Sun CK, Han GZ, Peng JY, Li Y, Liu YX, Lv YY, Liu KX, Zhou Q, Sun HJ. Protective effect of tea polyphenols against paracetamol-induced hepatotoxicity in mice is significantly correlated with cytochrome P450 suppression. *World J Gastroenterol* 2009; 15(15): 1829-1835 Available from: URL: <http://www.wjgnet.com/1007-9327/15/1829.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.1829>

INTRODUCTION

Tea has been consumed in China to promote health and longevity since 3000 B.C. It is now a popular beverage all over the world. Tea polyphenols (TP) are a large and diverse class of compounds extracted from tea. The major compounds of TP are picatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin-3-gallate (EGCG). Recent studies indicate that TP can prevent oxidative stress-related diseases, including cancer, cardiovascular and degenerative diseases and have other bioactive properties^[1-4].

In recent years, the interest in understanding the metabolic benefits of TP has been increasing. Liver is the main organ responsible for the metabolism of TP. Liu *et al*^[5] reported that TP can markedly increase cytochrome P450 (CYP450) activity in rats. However, the effect of TP on CYP450 activity remains controversial^[6,7].

Until now, no one could give a clear explanation of the different results. CYP450 enzymes play a pivotal role not only in the metabolism of xenobiotics, but also in the biosynthesis and catabolism of endogenous substrates, such as vitamins, fatty acids, hormones and prostaglandins^[8]. Alteration in hepatic CYP450 enzyme expression would affect the pharmacokinetic profiles of clinically used drugs. Furthermore, both induction and suppression of several CYP450s may lead to cellular oxidative stress and tissue injury in response to xenobiotics^[9].

Paracetamol, one of the most widely used hepatotoxic drugs, is safe at therapeutic doses, but causes liver failure in overdoses^[10,11]. When a normal dose is used, paracetamol is extensively metabolized by conjugation with sulphate and glucuronic acid. A small fraction of the drug is subjected to oxidation reactions catalyzed by CYP450 enzymes in the liver, resulting in generation of N-acetyl-p-benzo-quinoneimine (NAPQI), a highly electrophilic metabolite that triggers ensuing liver damage. Exposure to high doses of paracetamol increases the NAPQI level. Normally, toxic oxidation metabolites generated in the liver are converted into non-toxic metabolites excreted in urine via conjugation with glutathione (GSH) containing sulphhydryl groups. However, high doses of paracetamol limit the ability of GSH to detoxify NAPQI, and result in the consumption of liver GSH stores^[12,13]. It was reported that oxidative stress constitutes a major mechanism underlying the pathogenesis of paracetamol-induced liver damage^[14,15].

There are three families of CYP450 isoforms, CYP1, CYP2, and CYP3, which are mainly involved in the biotransformation of xenobiotics^[16]. It has been shown that CYP2E1, CYP1A2, and intracellular GSH play an important role in the hepatotoxicity induced by paracetamol^[17-19]. The present study was to investigate the hepatoprotective activity of TP and its relation with CYP450 expression in mice.

MATERIALS AND METHODS

Animal treatment

TP obtained from Wuyuan Tea Plantation (Jiangxi Province, China) with a purity of > 98% containing 63.84% of EGCG and 7.30% of ECG were decaffeinated. Kunming male mice, weighing 18-22 g, provided by Experimental Animal Center of Dalian Medical University, were used in the study. The mice were randomly divided into high, medium, low TP dose groups, positive and negative control groups ($n = 10$). The mice in low, medium and high dose TP groups were intraperitoneally injected with 25, 50 and 100 mg/kg of TP per day for two days. The mice in positive control group were given 50 mg/kg chloramphenicol one hour before they were killed. The mice in the negative control group were given the same volume of saline solution for two days. On day 3, all the mice were killed by decapitation, with their livers removed immediately and cleaned with a cold saline solution. Specimens were stored at -80°C for preparation of liver microsomes.

Male mice in each group received intragastric TP at the dose of 100, 200, and 400 mg/kg, once a day for six days. The mice in positive control group were given 900 mg/kg N-acetylcysteine, once a day. The mice in negative control group were given the same volume of a saline solution. All the mice were given 1000 mg/kg paracetamol one hour later and the acute mortality of mice in both groups was recorded 72 h after paracetamol was given.

Male mice in each group received intragastric TP at the dose of 100, 200, and 400 mg/kg, once a day for five days. The mice in model and negative control groups were given the same volume of a saline solution. The mice were given 500 mg/kg paracetamol one hour later, except for the mice in negative control group. All the mice were killed by decapitation 24 h later with their livers removed immediately and cleaned with a cold saline solution. Specimens were fixed in formalin for histological examination or snap-frozen in liquid nitrogen and stored at -80°C for protein and RNA extraction.

Preparation of liver microsomes

Liver microsomes extracted from frozen samples were prepared as previously described^[20]. Microsomal protein level was measured as previously described^[21] with bovine serum albumin as a standard.

Measurement of microsome enzyme levels

The levels of CYP450 and CYPb₅ were measured as previously described^[22].

Western blot assay

Aliquots (80 µg) of liver microsomes from each sample were loaded onto each lane of 10% SDS-PAGE gel electrophoresis and electroblotted onto nitrocellulose membranes (Millipore, Bedford, MA). The membranes were probed with rabbit antibody against mice CYP2E1 (Boster Biological Technology Co., Ltd, Wuhan, China) at a dilution of 1:400 or CYP1A2 (Santa Cruz Biotechnology, Inc., California, USA) at a dilution of 1:500 and peroxidase-conjugated affinity-pure goat anti-rabbit IgG (Zhongshan Golden Bridge Biotechnology Co., Ltd, Beijing, China) according to the manufacturer's constructions. The signals were visualized with a DAB assay kit (Zhongshan Golden Bridge Biotechnology Co., Ltd, Beijing, China) and analyzed with Quantity One software.

Histology and immunohistochemical assay

All specimens embedded in paraffin were cut into 4-µm thick serial sections. The specimens were mounted on slides and deparaffinized with graded concentrations of xylene and ethanol, and stained with hematoxylin and eosin (HE). For immunohistochemical staining, all sections were immersed in 3% H₂O₂ for 15 min at room temperature to block the endogenous peroxidase activity. To stain with CYP2E1 and CYP1A2, the sections were predigested with 0.4% proteinase K for 5 min at 37°C. The sections were then incubated with 10% normal goat serum for 15 min to reduce the nonspecific background

Table 1 Sequences of oligonucleotide primers

Primer	Sequence
CYP2E1 F	5'-CACCGTTGCCTTGCTTGCTCG-3'
CYP2E1 R	5'-CTCATGAGCTCCAGACACTTC-3'
CYP1A2 F	5'-AGTGTTCGGATGGTCAGAGC-3'
CYP1A2 R	5'-GCAAGAGGATGCTGACGTCG-3'
GAPDH F	5'-ATGGTGAAGGTCGGTGTGAAC-3'
GAPDH R	5'-GTCCTCTGGGTGGCAGTGATG-3'

Table 2 Effects of TP on CYP450 and CYPb₅ levels (mean \pm SD)

Group (<i>n</i> = 10)	CYP450 (nmol/g)	CYPb ₅ (nmol/g)
Negative control	0.354 \pm 0.024 ^b	0.160 \pm 0.011 ^b
Low dose	0.279 \pm 0.014 ^{a,b}	0.108 \pm 0.004 ^{a,b}
Medium dose	0.204 \pm 0.016 ^{a,b}	0.077 \pm 0.006 ^a
High dose	0.130 \pm 0.016 ^a	0.048 \pm 0.006 ^a
Positive control	0.147 \pm 0.019 ^a	0.061 \pm 0.009 ^a

^a*P* < 0.01 vs negative control group (NS group); ^b*P* < 0.01 vs positive control group (chloramphenicol group).

staining and reacted with polyclonal rabbit anti-mouse antibody (Ab) at 4°C overnight. The working dilution of Abs used for examining mouse CYP2E1 and CYP1A2 in the liver was diluted at 1:100 and 1:50. After rinsing with phosphate-buffered saline (pH 7.4), the sections were incubated with biotinylated anti-rabbit immunoglobulin secondary Abs at room temperature for 30 min. Horseradish peroxidase labeled streptomycin-avidin complex was used to detect the second antibody. Finally, the sections were counterstained with hematoxylin before examination under a light microscope. The brown or dark brown stained cells were considered positive cells.

Reverse transcription-polymerase chain reaction (RT-PCR) assay

Total RNA was extracted from liver tissue with the TRIquick reagent (Solarbio Science & Technology CO., Ltd, Beijing, China). RNA purity was assessed by the optical densities at 260 nm and 280 nm, and the integrity was verified by electrophoresis on a 1% agarose gel containing 0.5 μ g/mL of ethidium bromide. Reverse transcription was conducted for 30 min at 42°C from 500 ng of purified RNA in 10 μ L of reverse transcriptions system reaction mixture using AMV reverse transcriptase (TaKaRa, Japan), followed by 30 cycles of PCR (denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min). The amplified products were electrophoresed on a 1.5% agarose gel using 100 bp DNA ladder markers (TaKaRa, Japan) as a standard to determine the molecular size and visualized with ethidium bromide staining. Images of the ethidium bromide-stained agarose gel were acquired with a Gel Doc EQ system and quantified using Quantity One software. The sequences of oligonucleotide primers are provided in Table 1.

Statistical analysis

Data were calculated separately and expressed as mean

Table 3 Effect of TP on mortality induced by paracetamol in mice

Group (<i>n</i> = 10)	Dose (mg/kg)	Death (<i>n</i>)	Death rate (%)	Protection rate (%)
Negative control		9	90	10
Positive control		2	20	80
TP				
Low	100	7	70	30
Medium	200	4	40	60
High	400	2	20	80

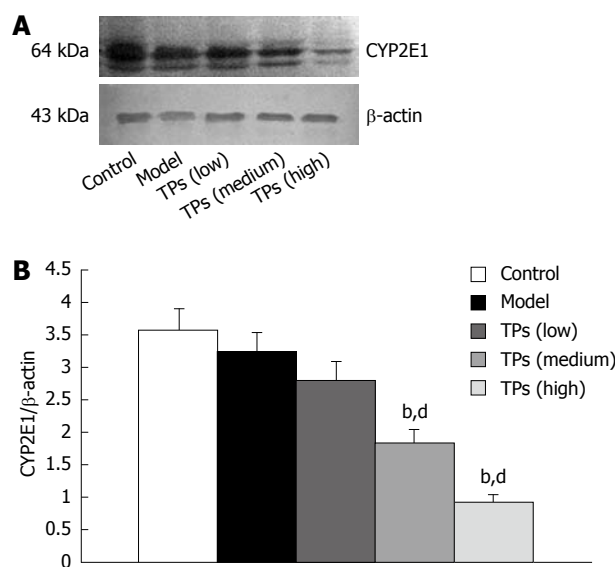


Figure 1 Western blot analysis of protein for CYP2E1 in liver tissue of mice showing representative bands of each group (A) and normalized densitometric ratios of CYP2E1 to β -actin (B). ^b*P* < 0.01 vs control group; ^d*P* < 0.01 vs model group.

\pm SD. Statistical significances were analyzed by one-way ANOVA followed by Student Newman-Keuls test using SPSS Version 11.5. The difference was considered significant at two-tailed. *P* < 0.05 was considered statistically significant.

RESULTS

Effect of TP on CYP450 and CYPb₅ levels

Two days after peritoneal injection of TP, the CYP450 and CYPb₅ levels in livers of mice were measured. The CYP450 and CYPb₅ levels were significantly lower in the high, medium and low dose TP groups than in the negative control group (*P* < 0.01), indicating that TP can reduce the CYP450 and CYPb₅ levels in liver of mice. The CYP450 level was higher in the medium and low dose groups than in the positive control group (*P* < 0.01); but, it was not significantly different between the high dose and positive control groups (*P* > 0.05). The CYPb₅ level was markedly different in the low dose group (*P* < 0.01); but it, was not significantly different in the high and medium dose groups (*P* > 0.05) compared the positive control group, indicating that TP can suppress both CYP450 and CYPb₅ in mice in a dose-dependent manner (Table 2).

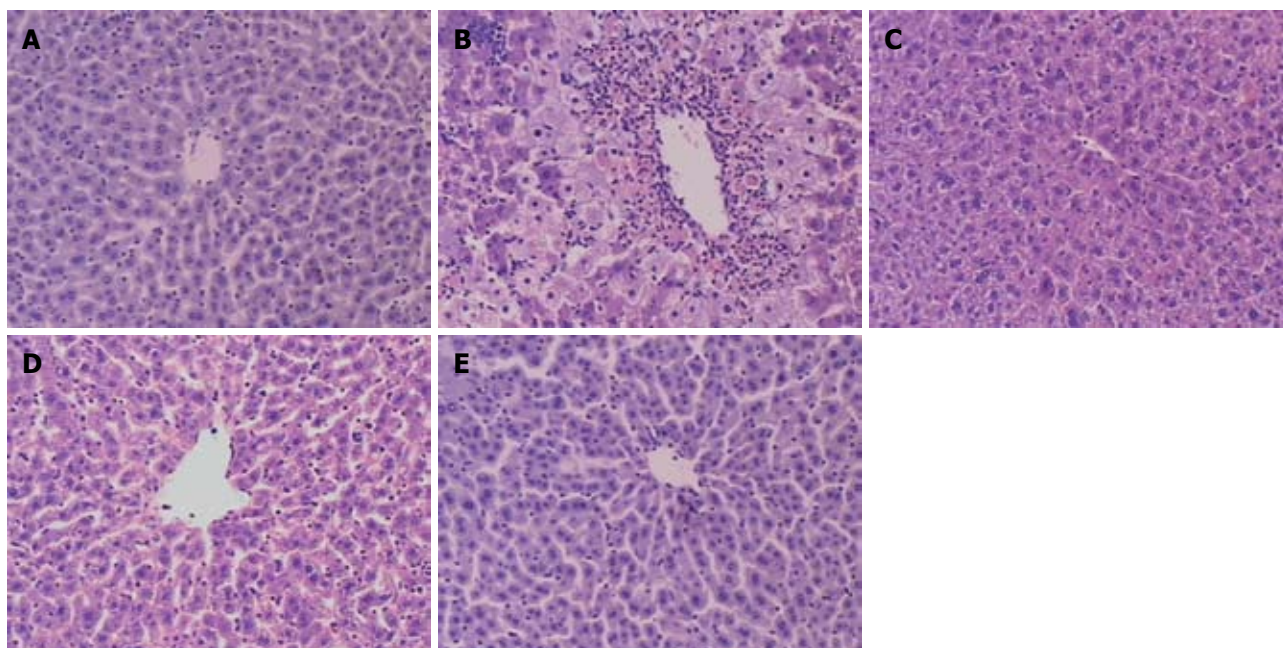


Figure 2 Changes of HE staining in liver tissue of mice 24 h after paracetamol administration ($\times 100$) in control group with a normal central lobular region (A), model group with some central lobular hepatocyte necrosis and microvesicular fatty change (B), low TP dose group (C), medium TP dose group (D) and high TP dose group (E). The pathological change of liver was much milder in different TP dose groups than in model group.

Effect of TP on mortality of paracetamol-treated mice

We examined the effect of TP on mortality of paracetamol-treated mice. The mortality rate of mice in the negative control group, low, medium and high dose TP groups was 90%, 70%, 40% and 20%, respectively, indicating that TP can significantly reduce the mortality of paracetamol-treated mice. The effect of high dose TP on mortality of paracetamol-treated mice was not different from that of mice in the positive control group (Table 3).

Western blot analysis

Western blot showed strong positive CYP2E1 signals in the control and model groups. The expression of CYP2E1 in the low dose group was not significantly different from that in the control and model groups. However, TP significantly decreased the CYP2E1 expression level in the medium and high dose groups in a dose-dependent manner (Figure 1). The expression of CYP1A2 and CYP2E1 protein was similar in the liver microsomes (Data not shown).

Histology analysis

Liver tissues from the control group showed a normal lobular architecture with central veins and radiating hepatic cords (Figure 2A). However, liver tissues from the model group showed formation of more fibrous tissues extending into the hepatic lobules, leading to their complete separation. A large number of inflammatory cells infiltrated the intralobular and interlobular regions. The liver structure was in disorder and there were more necrotic and fatty degenerated liver cells than in the controls (Figure 2B). In the three TP treatment groups, however, hepatocyte degeneration, necrosis and infiltration of inflammatory cells were all apparently ameliorated (Figure 2C-E). Compared with the model

group, the liver condition in TP treatment groups was significantly improved in a dose-dependent manner.

Immunohistochemical analysis of liver CYP2E1 and CYP1A2

The expression of CYP2E1 showed a deep brown immunostaining. No difference was found in the expression of CYP2E1 between the control and model groups. However, in medium and high dose groups, TP significantly suppressed the CYP2E1 expression in a dose-dependent manner (Figure 3A).

The expression level of CYP1A2 (showing a deep brown immunostaining) was markedly lower in the low, medium and high dose TP groups than in the control and model groups (Figure 3B).

Expression levels of CYP2E1 and CYP1A2 mRNA

The expression levels of CYP2E1, CYP1A2 and GAPDH cDNAs in the mouse liver were assayed by RT-PCR (Figure 4A), and the ratio of CYP2E1 and CYP1A2/ GAPDH cDNAs was calculated (Figure 4B). TP significantly inhibited the CYP2E1 mRNA expression in medium and high dose groups ($P < 0.01$), compared to the control and model groups ($P > 0.05$). The expression of CYP1A2 mRNA was reduced in all TP groups compared with the control and model groups ($P < 0.05$), indicating that TP can suppress the expression of CYP2E1 and CYP1A2 mRNA in a dose-dependent manner.

DISCUSSION

Hepatic drug metabolizing enzyme is called mixed-function oxidase or monooxygenase containing many enzymes including phase I enzymes such as

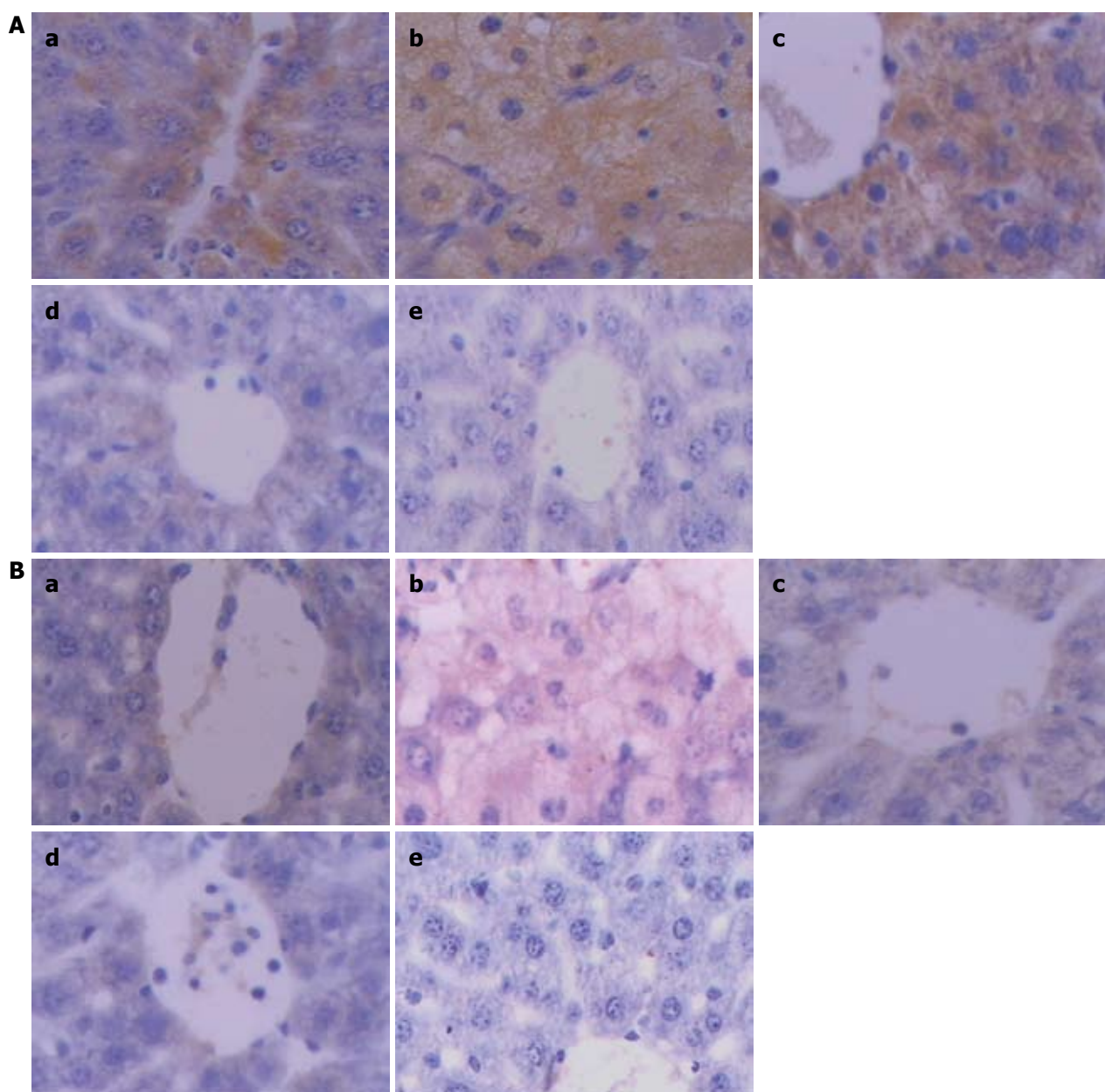


Figure 3 Immunohistochemical staining showing expression of CYP2E1 (A) and CYP1A2 (B) in liver tissue of mice 24 h after paracetamol administration ($\times 400$) in control group (a), model group (b), low TP dose group (c), medium TP dose group (d) and high TP dose group (e). The brown or dark brown stained cells were considered positive.

CYP450 and CYPb_s, *etc.*, and plays a prominent role in the metabolism of many pharmaceutical agents and activation or deactivation of potential carcinogens. Acquiring metabolic information and determining the effect of chemicals on hepatic drug metabolizing enzymes are important in developing clinically safe and efficient medications^[23].

TP have various pharmacological effects on cancer, cardiovascular and degenerative diseases and have other bioactive properties^[1-4]. In this experiment, TP significantly suppressed the CYP450 and CYPb_s levels in liver of mice, suggesting that TP can restrain the activity of drug metabolizing enzymes.

In the present study, 100, 200 and 400 mg/kg TP could reduce the mortality of mice after treatment with paracetamol (Table 2). The immunohistochemical study

showed that TP markedly alleviated paracetamol- induced hepatotoxicity, thus improving alterations in liver tissue pathology. Especially marked hepatoprotective effects of TP were observed in the high dose TP group with its diffuse hemorrhage and necrosis changed into focal hemorrhage and necrosis (Figure 2) compared to the model group that received a single dose of 500 mg/kg paracetamol, indicating that TP can protect liver of mice against paracetamol injury, which is consistent with the reported data^[24].

It was reported that many mechanisms are involved in paracetamol hepatotoxicity^[25], showing that the toxicity is mediated by CYP450 metabolism of paracetamol to NAPQI which covalently binds to critical proteins leading to inactivation of these proteins, especially after GSH depletion. CYP2E1 is usually assumed to be the

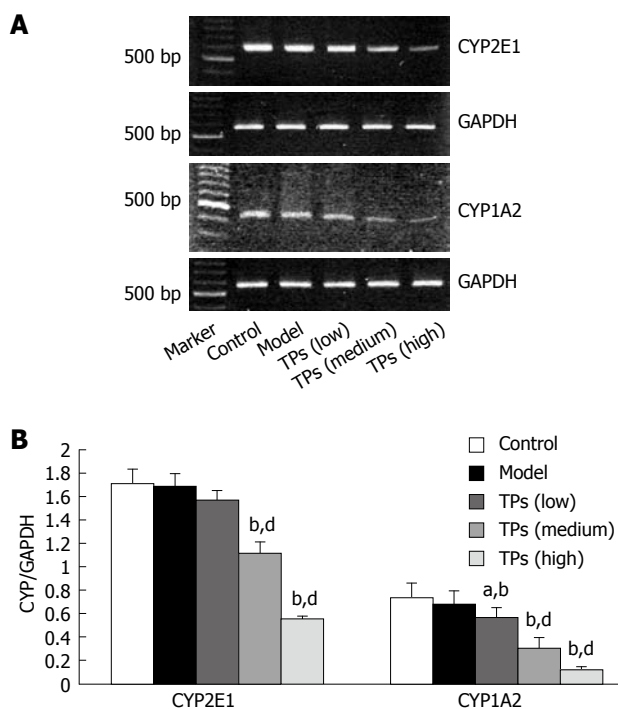


Figure 4 RT-PCR analysis of mRNA for CYP2E1 and CYP1A2 expression in liver tissue of mice showing representative bands of each group (A) and normalized densitometric ratio of CYP2E and CYP1A2 to GAPDH (B). ^a $P < 0.05$ vs model group; ^b $P < 0.01$ vs control group; ^d $P < 0.01$ vs model group.

most active CYP450 in catalyzing the metabolism of paracetamol to hepatotoxic NAPQI^[15,18]. Studies with knockout mice showed that CYP2E1 and CYP1A2 play an important role in the metabolism of paracetamol^[26,27]. To confirm whether the protective effects of TP on liver are correlated with the inhibition of CYP2E1 and CYP1A2, we investigated the effect of TP on the expression of CYP2E1 and CYP1A2 mRNA and protein.

In the present study, 200 mg/kg and 400 mg/kg TP significantly down-regulated the CYP2E1 expression either at gene level (Figure 4) or at protein level (Figures 1 and 3) as shown by RT-PCR, Western blot and immunohistochemistry. The effects of TP on the expression of CYP1A2 and CYP2E1 gene and protein were similar. It was reported that the CYP1A2 level in increased in rats after drinking a 2% solution of green or black tea^[28]. Caffeine, a component of tea, is responsible for the induction of CYP1A2^[29]. In our study, however, different doses of TP (100, 200 and 400 mg/kg) significantly suppressed the expression of CYP1A2 protein and mRNA, partly because TP used in our experiment did not contain caffeine.

The above findings indicate that the dose-dependent liver-protective effects of TP are likely related to the suppression of CYP2E1 and CYP1A2 expression, which reduces NAPQI associated with the paracetamol hepatotoxicity, and effectively protects liver against paracetamol hepatotoxicity.

In conclusion, TP restrain the activity of drug metabolizing enzymes, CYP450 and CYPb_s, and protect liver against paracetamol-induced injury. The protective

effects of TP are associated with the expression of CYP2E1 and CYP1A2 gene and protein.

ACKNOWLEDGMENTS

The authors thank Professor Zhong-Gun Yan (Center for Molecular Medicine, Karolinska Institute, Stockholm, Sweden) and Jiang-Lin Fan (Department of Pathology, University of Yamanashi, Japan) for their technical assistance.

COMMENTS

Background

Paracetamol is one of the most widely used hepatotoxic drugs, which is safe at therapeutic doses, but causes liver failure at overdoses. High doses of paracetamol limit the ability of glutathione (GSH) to detoxify N-acetyl-p-benzoquinonimine (NAPQI), and results in consumption of liver GSH stores. There are three families of cytochrome P450 (CYP450) isoforms, CYP1, CYP2, and CYP3, which are mainly involved in the biotransformation of xenobiotics. It has been shown that CYP2E1, CYP1A2 and intracellular GSH play an important role in paracetamol-induced hepatotoxicity. Tea polyphenols (TP) are a large and diverse class of compounds extracted from tea. Liver is the main organ responsible for the metabolism of TP. Some work has been done in the field of TP modulation or interaction with drug metabolizing enzymes. However, until now, no one could give a clear explanation for this.

Research frontiers

TP are a large and diverse class of compounds extracted from tea. Liver is the main organ responsible for the metabolism of TP. Some work has been done in the field of TP modulation or interaction with drug metabolizing enzymes. However, until now, no one could give a clear explanation for this. The research hotspot is to investigate the effects of TP on CYP450 and CYPb_s expression in livers of male mice. Whether the protective effect of TP on paracetamol-induced hepatotoxicity in mice is related with their effect on CYP450 was studied.

Innovations and breakthroughs

The protective effect of TP on paracetamol-induced hepatotoxicity was found to be related with their effect on CYP450.

Applications

TP possess potential hepatoprotective properties, which are closely related with their suppressive effect on CYP450 expression.

Terminology

Paracetamol-induced hepatotoxicity: When a normal dose is used, paracetamol is metabolized extensively by conjugation with sulphate and glucuronic acid. A small fraction of paracetamol is subjected to oxidation reactions catalyzed by CYP450 enzymes in the liver, resulting in generation of NAPQI, a highly electrophilic metabolite that triggers the ensuing liver damage.

Peer review

This is a good descriptive study in which the authors gave a clear explanation of the effect of TP on the expression of CYP450, CYP2E1 and CYP1A2 in liver of mice at both protein and mRNA levels. The interesting results suggest that TP can significantly attenuate paracetamol-induced hepatic injury, which is closely related with their suppressive effect on CYP450 expression.

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