



Therapeutic detoxification of quercetin against carbon tetrachloride-induced acute liver injury in mice and its mechanism*

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Abstract: This study observes the therapeutic detoxification of quercetin, a well-known flavonoid, against carbon tetrachloride (CCl₄) induced acute liver injury *in vivo* and explores its mechanism. Quercetin decreased CCl₄-increased serum activities of alanine and aspartate aminotransferases (ALT/AST) when orally taken 30 min after CCl₄ intoxication. The results of a histological evaluation further evidenced the ability of quercetin to protect against CCl₄-induced liver injury. Quercetin decreased the CCl₄-increased malondialdehyde (MDA) and reduced the glutathione (GSH) amounts in the liver. It also reduced the enhanced immunohistochemical staining of the 4-hydroxynonenal (4-HNE) in the liver induced by CCl₄. Peroxiredoxin (Prx) 1, 2, 3, 5, 6, thioredoxin reductase 1 and 2 (TrxR1/2), thioredoxin 1 and 2 (Trx1/2), nuclear factor erythroid 2-related factor 2 (Nrf2), and heme oxygenase-1 (HO-1) all play critical roles in maintaining cellular redox homeostasis. Real-time polymerase chain reaction (PCR) results demonstrated that quercetin reversed the decreased mRNA expression of all those genes induced by CCl₄. In conclusion, our results demonstrate that quercetin ameliorates CCl₄-induced acute liver injury *in vivo* via alleviating oxidative stress injuries when orally taken after CCl₄ intoxication. This protection may be caused by the elevation of the antioxidant capacity induced by quercetin.

Key words: Hepatotoxicity, Oxidative stress, Peroxiredoxin (Prx), Nuclear factor erythroid 2-related factor 2 (Nrf2), TrxR, Trx, HO-1

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1 Introduction

Flavonoids are widely distributed through red wine, fruits, vegetables, herbal tea and medicines. As flavonoids have various pharmacological activities, such as antioxidant, anti-inflammation, and anti-

tumor, the nutritional value of flavonoids is well-known to people (Hollman and Katan, 1999; Park and Pezzuto, 2012). Some epidemiological surveys report that a negative correlation can be observed between dietary intake of flavonoids and cardiovascular disease or cancer (Muldoon and Kritchevsky, 1996; Yang *et al.*, 2001). However, nutraceuticals, including flavonoids, may also have toxic effects depending on the dose and the redox status of the cell, which is also called as hormesis (Brambilla *et al.*, 2008; Calabrese *et al.*, 2010). Quercetin is a well-known flavonoid, and it is widely distributed in various Chinese medicinal herbs, such as *Flos sophorae Immaturus*

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(Kelly, 2011; Wu *et al.*, 2012). Various reports show that quercetin has neuro-protection, anti-tumor, anti-inflammation, anti-oxidant, and anti-diabetic effects (Chirumbolo, 2010; Kelly, 2011; Dajas, 2012). Furthermore, some reports demonstrate that quercetin can ameliorate various toxin-induced liver injuries, such as ethanol and acetaminophen (Vicente-Sánchez *et al.*, 2008; Liu *et al.*, 2010; Yousef *et al.*, 2010; de David *et al.*, 2011; Domitrović *et al.*, 2012). However, the concrete-engaged signals in regulating the protection of quercetin against liver injuries are not very clear.

Carbon tetrachloride (CCl₄), a classic hepatotoxin, has been widely used in the industry. CCl₄ can enter the body of humans and animals primarily through the respiratory and digestive tracts. Weber *et al.* (2003) reported that CCl₄ could lead to a lipid peroxidation of unsaturated fatty membrane and organelle membrane after its metabolic activation by hepatic microsomal cytochrome P450, and thus lead to serious hepatocytes damage. CCl₄-induced liver injury is one of the most widely used experimental models for exogenous toxins-induced hepatotoxicity (Ratziu and Zelber-Sagi, 2009; Chen *et al.*, 2012).

It has already demonstrated that pretreatment of mice or rats with quercetin for five consecutive days can prevent CCl₄-induced acute liver injury (Janbaz *et al.*, 2004; Domitrović *et al.*, 2012; Cui *et al.*, 2014). However, whether quercetin can also alleviate CCl₄-induced acute liver injury, when it is given after intraperitoneal (i.p.) injection of CCl₄, is unknown.

Peroxiredoxins (Prxs) and thioredoxins (Trxs) are important liver antioxidant proteins and play important roles in maintaining liver redox homeostasis (Watson *et al.*, 2004; Rhee *et al.*, 2005). The present study is designed to observe the therapeutic detoxification of quercetin against CCl₄-induced liver injury when it is orally given after CCl₄ intoxication, and to further investigate the underlying mechanisms via focusing on Prxs, Trxs, and other antioxidant signals.

2 Materials and methods

2.1 Drugs and reagents

Quercetin was purchased from Sigma (USA). Kits for analysis of alanine and aspartate aminotransferases (ALT/AST) and malondialdehyde

(MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Anti-4-hydroxynonenal (4-HNE) antibody was purchased from Abcam (Cambridge, UK). The DAKO EnVision™ detection system was purchased from DAKO Corporation (USA). The Trizol reagent was purchased from Life Technology (USA). PrimeScript RT master mix and SYBR Premix Ex Taq™ were all purchased from TaKaRa (Japan). Other reagents, unless noted, were purchased from Sigma (USA).

2.2 Experimental animals

Specific pathogen free male ICR mice (18–22 g) were purchased from the Shanghai Laboratory Animal Center of Chinese Academy of Science (Shanghai, China). Animals were fed with a standard laboratory diet and given free access to tap water. The animal room was maintained at (22±1) °C and (65±5)% humidity, with a 12 h:12 h light-dark cycle. The animals received humane care in compliance with the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine, China.

2.3 Treatment of animals

Quercetin was dissolved in a sodium hydroxide solution and kept in a dark storage area. The solution was adjusted to pH 9.0. CCl₄ was dissolved in olive oil to a 25% concentration.

Mice were randomly divided into four groups with eight mice each. The vehicle group (control) was first injected with olive oil i.p., and 30 min later was orally given a pH 9.0 vehicle solution. The CCl₄-treated group was injected with CCl₄ (15 ml/kg; i.p.), and 30 min later was orally given a pH 9.0 vehicle solution. The quercetin-treated groups were first injected with CCl₄ solution i.p., and 30 min later were orally administered with quercetin 20 or 80 mg/kg, respectively. Mice were sacrificed 24 h after CCl₄ injection, and plasma and livers were collected.

2.4 Analysis of ALT/AST activities

Fresh blood was obtained from mice in the four groups and put at room temperature for 60 min to clot. Serum was isolated following the centrifugation at 840g for 15 min. Serum ALT and AST activities were determined by kits according to the manufacturer's instructions.

2.5 Histological examination

Live slices were fixed in 10% phosphate-buffered saline (PBS)-formalin and embedded in paraffin. Liver samples were subsequently sectioned (5 μm), stained with hematoxylin and eosin (H&E), and examined under a microscope (Olympus, Japan) to evaluate liver injuries.

2.6 Determination of tissue lipid peroxidation (LPO)

Liver tissues were homogenized in cold PBS. LPO was determined according to the method described by Högberg *et al.* (1974). MDA was formed as the product of LPO and served as an index for LPO. MDA reacts with 2-thiobarbituric acid to generate a pink-colored product, which has an absorbance at 532 nm. The MDA level was expressed as $\mu\text{mol}/\text{mg}$ protein based on liver protein concentration.

2.7 Determination of liver glutathione (GSH)

The amounts of liver GSH and oxidative GSH (GSSG) were measured as described in our previous report (Liang *et al.*, 2011).

2.8 Immunohistochemical staining of 4-HNE

Paraffin-embedded liver sections (5 μm) were first deparaffinized in xylene, and then rehydrated in a gradient of ethanol to distilled water. After quenching the endogenous peroxidase activity using 3% hydrogen peroxide, liver sections were further incubated with 5% bovine serum albumin to alleviate nonspecific binding, and then incubated with 4-HNE antibody at 4 $^{\circ}\text{C}$ overnight. The antigen-antibody reactions were detected using DAKO EnVision™ detection kits. All sections were counterstained with hematoxylin. Pictures were taken with a microscope (Nikon, Japan). Liver expression of 4-HNE was determined by image information object definition (IOD) values analyzed by Image-Pro Plus 6.0 (Media Cybernetics, USA).

2.9 Real-time polymerase chain reaction (PCR)

The total liver RNA was extracted by Trizol reagent as described by the manufacturer's protocol. Complementary DNA (cDNA) was synthesized according to the instruction of the PrimeScript RT

master mix kits. Real-time PCR was performed with STEPONE Plus (Carlsbad, CA) by a SYBR green premix according to the manufacturer's guide. Relative expressions of target genes were standardized to β -actin, evaluated by the $2^{-\Delta\Delta C_t}$ method and given as a ratio to control the experiments. The primer sequences used in this study are shown in Table 1.

Table 1 List of primers for real-time PCR

Target	Primer sequence (5'→3')
Prx1	F: CACCCAAGAAACAAGGAGGA R: AAAAAGGCCCTGAAAGAGA
Prx2	F: TGATGAGGGCATGCTTACAGG R: GAGCGTCCCACAGGTAGGTCAT
Prx3	F: TTCCCACTTCAGTCATCTTGCC R: ATGCCAGCACTTCCAACAGC
Prx4	F: TTCCTGTTGCGGACCGAAT R: TACACTTGTCACCAGCGTAGAA
Prx5	F: GGAAGGCGACAGACTTATTATTGG R: CCTTCACTATGCCGTTGTCTATCAC
Prx6	F: GCACCACAGAACTTGGCAGAG R: CCAGGCAAGATGATCCTCAACA
Trx1	F: TCCCTCCCCGCAACAGCCAA R: ACCGGAGAACTCCCCACCTTT
Trx2	F: TCGCCAAGCAGCACGGGAAG R: CGGTAGGCACAGCTGACACCTCA
TrxR1	F: TCGGCTCGCTGAACTGGGG R: GCGATGA GGAACCGCTCTGCTG
TrxR2	F: GATCGGTGGGGGATCCGGTG R: CCCACTTGGTGCCTCGGGGAG
HO-1	F: TCCCGAACATCGACAGCCCCA R: AGGGGCAGTATCTTGCACCAGG
Nrf2	F: TCTCCTCGCTGGAAAAAGAA R: AATGTGCTGGCTGTGCTTTA
Actin	F: TTCGTTGCCGGTCCACACCC R: GCTTTGCACATGCCGAGCC

F: forward primer; R: reverse primer

2.10 Statistical analysis

All experimental data were expressed as mean \pm standard error of the mean (SEM). Significant differences were determined by one-way analysis of variance (ANOVA). $P < 0.05$ was considered as a statistically significant difference.

3 Results

3.1 Quercetin ameliorates CCl₄-induced acute liver injury

Serum activities of ALT and AST were increased in the CCl₄ group compared with the control group ($P<0.001$; Fig. 1). After treatment with quercetin (80 mg/kg), CCl₄-increased ALT and AST activities were decreased ($P<0.05$ and $P<0.01$, respectively). Quercetin (20 mg/kg) had no effect on CCl₄-increased serum ALT or AST activity. In contrast to the control group, the mice in the CCl₄ group showed serious liver damage, indicated by obvious hepatocyte necrosis and shrinkage nuclei (Fig. 2b). After treatment with quercetin (20 and 80 mg/kg), these abnormal changes were all alleviated (Figs. 2c and 2d).

3.2 Quercetin alleviates CCl₄-induced liver oxidative injury

As the product of LPO, the MDA amount can reflect the liver LPO level (Högberg *et al.*, 1974).

Fig. 3a shows that the liver MDA amount increased significantly ($P<0.05$) in the CCl₄-treated mice when compared with the control group. Quercetin (80 mg/kg) reduced the increased MDA amount induced by CCl₄ ($P<0.05$). Quercetin (20 mg/kg) also reduced the increased MDA amount, but it showed no significant difference when compared with the CCl₄ group ($P>0.05$).

GSH is a cellular important antioxidant and it can protect cells against reactive oxygen species (ROS)-induced liver injury (Yuan and Kaplowitz, 2009). As shown in Fig. 3b, the liver GSH level in CCl₄-treated mice was enhanced compared with the control group ($P<0.05$). Quercetin (20 or 80 mg/kg) decreased the CCl₄-increased liver GSH level ($P<0.05$).

In addition, our results showed that the number of 4-HNE-stained hepatocytes increased in the CCl₄-treated mice (Fig. 4b). Quercetin (20 or 80 mg/kg) reduced the CCl₄-increased 4-HNE-stained cells (Figs. 4c and 4d). The statistical result in Fig. 4e

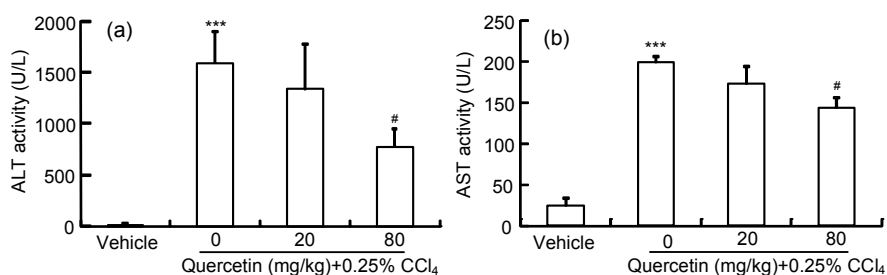


Fig. 1 Serum ALT (a) and AST (b) activities in mice
Data are shown as mean±SEM ($n=8$). *** $P<0.001$ vs. control; # $P<0.05$ vs. CCl₄-treated group

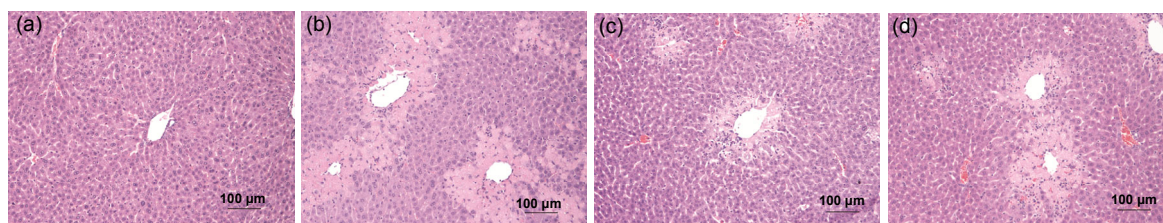


Fig. 2 Histological evaluation of liver by H&E staining

(a) Vehicle group; (b) CCl₄ group; (c) CCl₄+20 mg/kg quercetin group; (d) CCl₄+80 mg/kg quercetin group

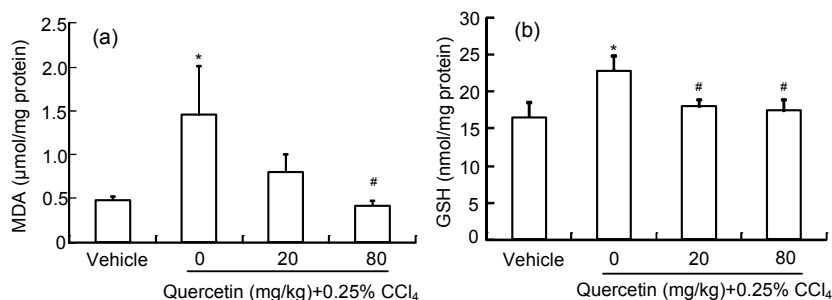


Fig. 3 Liver MDA (a) and GSH (b) amounts in mice
Data are shown as mean±SEM ($n=8$). * $P<0.05$ vs. control; # $P<0.05$ vs. CCl₄-treated group

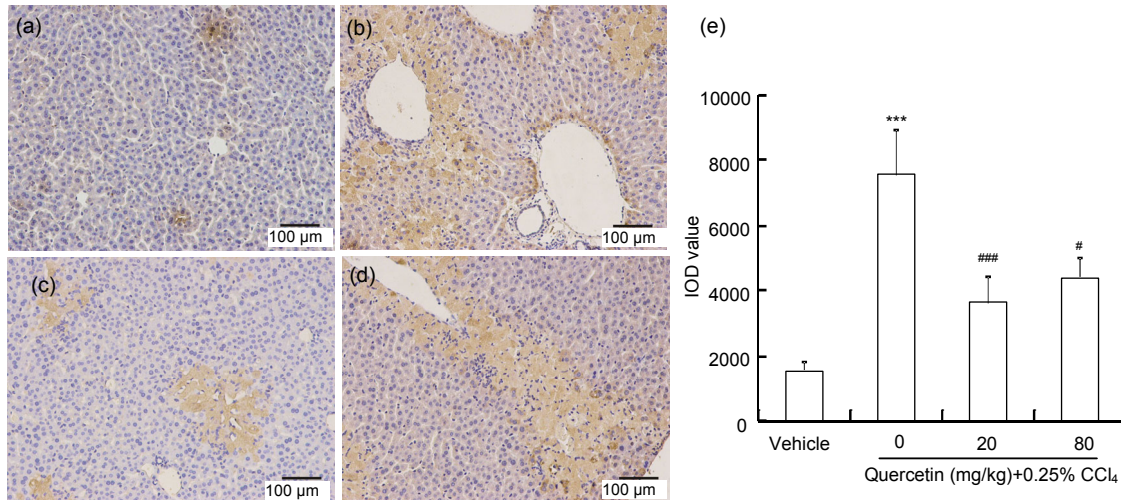


Fig. 4 Immunohistochemical staining of 4-HNE in mice livers and IOD values of the density of 4-HNE

Typical images selected from each group: (a) vehicle control; (b) CCl₄; (c) CCl₄+20 mg/kg quercetin; (d) CCl₄+80 mg/kg quercetin. (e) IOD values of the density of 4-HNE staining analyzed in every section of at least three random fields. Data are expressed as mean±SEM ($n=4$). *** $P<0.001$ vs. control; # $P<0.05$, ### $P<0.001$ vs. CCl₄-treated group

showed that CCl₄ enhanced the density of the immunohistochemical staining of 4-HNE ($P<0.001$), while quercetin (20 and 80 mg/kg) decreased this increase induced by CCl₄ ($P<0.001$ and $P<0.05$, respectively).

3.3 Effects of quercetin on Prx 1–6 mRNA expression

As shown in Fig. 5, the liver mRNA expressions of Prx 1–3, 5, 6 in CCl₄-treated mice all decreased remarkably compared with the control group ($P<0.001$). After treatment with quercetin (20 mg/kg), the mRNA expressions of Prx 1, Prx 2, Prx 3, Prx 5, and Prx 6 all increased when compared with the CCl₄-treated mice ($P<0.01$, $P<0.001$, $P<0.05$, $P<0.01$, and $P<0.01$, respectively). The mRNA expressions of Prx 1, Prx 2, and Prx 6 in 80 mg/kg quercetin-treated mice also increased when compared with the CCl₄-treated mice ($P<0.05$, $P<0.05$, and $P<0.001$, respectively).

3.4 Effects of quercetin on Trx1/2 and TrxR1/2 mRNA expression

The liver mRNA expressions of other antioxidant enzymes, such as Trx1/2 and TrxR1/2, all decreased in the CCl₄-treated mice when compared with the control group ($P<0.01$ and $P<0.001$, respectively) (Fig. 6). After treatment with 20 mg/kg quercetin, the mRNA expressions of Trx1/2 and TrxR1/2 were all

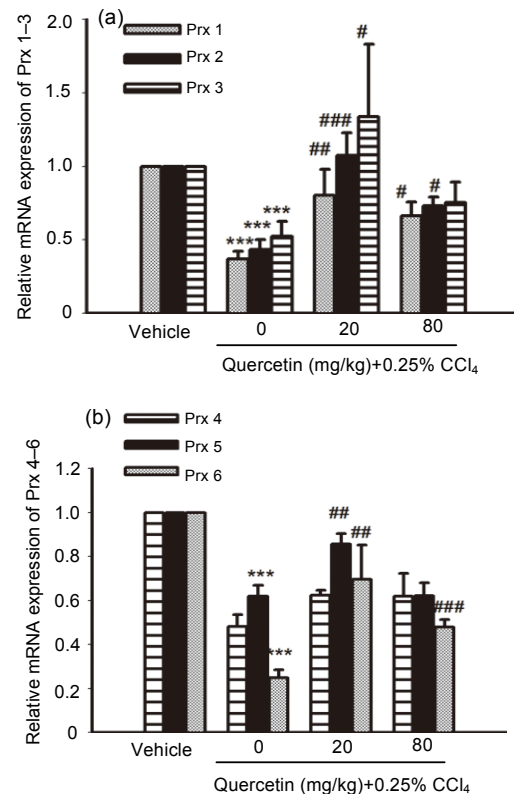


Fig. 5 mRNA expressions of Prx 1–3 (a) and Prx 4–6 (b) in mice livers

Data are shown as mean±SEM ($n=5-6$). *** $P<0.001$ vs. control; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ vs. CCl₄-treated mice

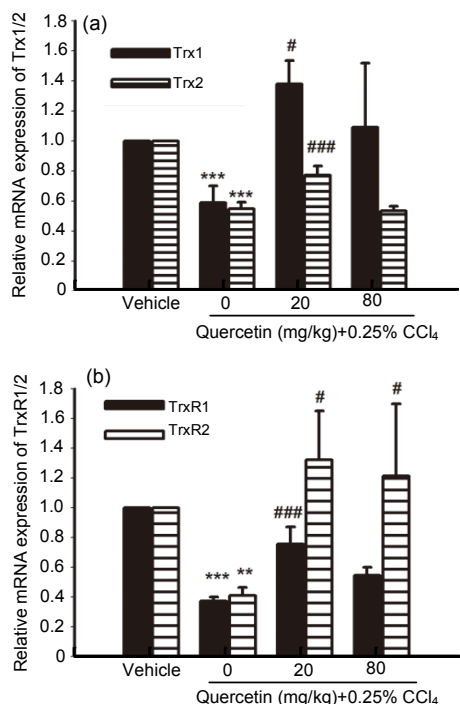


Fig. 6 mRNA expressions of Trx1/2 (a) and TrxR1/2 (b) in mice livers

Data are shown as mean \pm SEM ($n=5-6$). ** $P<0.01$, *** $P<0.001$ vs. control; # $P<0.05$, ### $P<0.001$ vs. CCl₄-treated mice

elevated when compared with the CCl₄-treated mice ($P<0.05$ and $P<0.001$, respectively). After treatment with 80 mg/kg quercetin, the mRNA expression of TrxR2 increased when compared with the CCl₄-treated mice ($P<0.05$).

3.5 Effects of quercetin on the mRNA expression of HO-1 and nuclear factor erythroid 2-related factor 2 (Nrf2)

The liver mRNA expressions of HO-1 and Nrf2 in CCl₄-treated mice decreased when compared with the control group ($P<0.001$ and $P<0.05$, respectively) (Fig. 7). After treatment with 20 mg/kg quercetin, the liver mRNA expressions of HO-1 and Nrf2 increased when compared with the CCl₄-treated mice ($P<0.01$ and $P<0.05$, respectively).

4 Discussion

CCl₄-induced acute liver injury in mice is widely used as an experimental animal model for screening

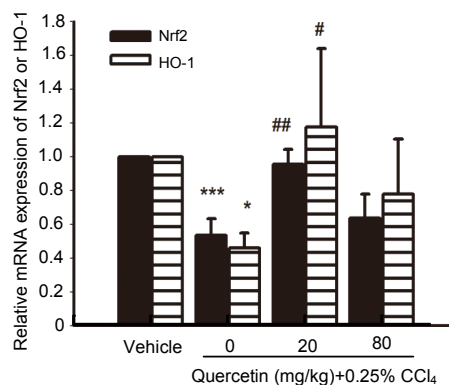


Fig. 7 Liver mRNA expressions of HO-1 and Nrf2

Data are shown as mean \pm SEM ($n=5-6$). * $P<0.05$, *** $P<0.001$ vs. control; # $P<0.05$, ## $P<0.01$ vs. CCl₄-treated mice

hepato-protective drugs (Morio *et al.*, 2001; Yuan *et al.*, 2008). Some previous studies have already demonstrated that quercetin can prevent CCl₄-induced acute liver injury when given several days before CCl₄ intoxication (Janbaz *et al.*, 2004; Domitrović *et al.*, 2012; Cui *et al.*, 2014). From the results of ALT/AST analysis and histological observations, our study demonstrates that quercetin can ameliorate CCl₄-induced acute liver injury when it is given 30 min after CCl₄ intoxication. Our study shows the potential therapeutic effects of quercetin against CCl₄-induced acute liver injury, and its scientific significance is far more important than indicated in previous reports.

Generally, oxidative stress liver injuries are the main reason for CCl₄-induced hepatotoxicity. 4-HNE and MDA are both the end-products of LPO (Högberg *et al.*, 1974; Vatsyayan *et al.*, 2011). Our results of liver MDA level and immunohistochemical staining of 4-HNE show that quercetin can decrease CCl₄-induced LPO, which indicates that quercetin can ameliorate CCl₄-induced liver oxidative injuries. GSH is the key antioxidant in liver, and its homeostasis is associated with various toxin-induced liver injuries (Kretzschmar and Klinger, 1990; Han *et al.*, 2006). Further results show that CCl₄ increased the cellular GSH level, which may contribute to the elevation of the body's defensive capacity to protect CCl₄-induced oxidative stress injuries. After treatment with quercetin, the increased liver GSH level was reduced, which suggests that oxidative stress injuries were ameliorated. There are already existing

reports that state that quercetin has a free radical scavenging activity (Pascoal *et al.*, 2011; di Meo *et al.*, 2013), so we can speculate that quercetin itself may have the capacity to scavenge free radicals, and thus substitute for the function of GSH, which leads to a decrease of GSH levels in quercetin-treated mice.

The Prx family contains Prx 1–6, which are all antioxidant enzymes and have the capacity of scavenging free radicals in cells (Rhee *et al.*, 2005). Previous reports have demonstrated the changed expression of the Prx family in exogenous toxin-induced liver injuries such as alcoholic liver injury and pyrazole-induced liver injury (Cohen *et al.*, 2011; Bae *et al.*, 2012). In addition, Hong *et al.* (2011) reported that an alkaline water named ENA actinimneral resource A (ENA-A) could prevent CCl₄-induced liver injury, and Prx1 is an increased component in the ENA-A group. Our results showed that the expressions of Prx 1–6 mRNAs in the CCl₄-treated group all decreased when compared with the vehicle group. Except Prx 4, the mRNA expressions of all the other members of the Prx family increased in quercetin-treated mice. Our study suggests that quercetin can ameliorate liver oxidative injuries via elevating the expressions of Prx 1, 2, 3, 5, 6.

Trxs, including Trx1 and Trx2, are cellular important antioxidant proteins (Watson *et al.*, 2004). Trx1/2 and Trx reductase 1/2 (TrxR1/2) constitute the Trx system, which plays a partially overlapping and highly complementary role to the GSH antioxidant system, and contributes to the maintenance of the cellular reducing environment (Williams *et al.*, 2000). Our results show that the mRNA expressions of Trx1/2 and TrxR1/2 decreased in the CCl₄-treated mice, but this decrease was reversed in the quercetin-treated mice. These results suggest that quercetin can ameliorate liver oxidative injuries via elevating the expression of the Trx system.

HO-1 is the inducible isoform of heme oxygenase, and it is reported to protect acute liver injuries (Sass *et al.*, 2012). Our results showed that the mRNA expression of HO-1 decreased in the CCl₄-treated mice, but this decrease was reversed in the quercetin-treated mice. This result suggests that the increased HO-1 expression may contribute to the alleviation of quercetin against CCl₄-induced liver injury. It is well-known that HO-1/biliverdin reductase axis catalyzes the degradation of heme, and it can also reduce

biliverdin to bilirubin, which is a free radical scavenger and exerts cytoprotective effects (Barone *et al.*, 2009; Mancuso and Barone, 2009; Mancuso *et al.*, 2012). Whether quercetin will affect the amounts of liver bilirubin needs further investigation.

Nrf2 is a critical transcription factor for regulating the expression of some antioxidant enzymes, such as HO-1, Prxs, and Trx1 (Young *et al.*, 2010; Yang *et al.*, 2011; Ke *et al.*, 2013). Our results show that the mRNA expression of Nrf2 decreased in the CCl₄-treated mice, but this decrease was reversed in the quercetin-treated mice. The above results suggest that CCl₄ can regulate the expression of Nrf2 and quercetin can ameliorate the liver oxidative injuries via elevating the expression of Nrf2, which may contribute to the increased expression of its downstream antioxidant enzymes. Moreover, the increased expressions of these antioxidant enzymes by quercetin may not provide the appropriate regulation through Nrf2, which needs further investigation.

In conclusion, the present study demonstrates the therapeutic detoxification of quercetin against CCl₄-induced acute liver injury *in vivo*. Our results show that quercetin can ameliorate CCl₄-induced liver injury through inhibiting oxidative stress injury via elevating the expressions of the antioxidant transcription factor Nrf2 and some antioxidant enzymes, such as HO-1, Prx 1–3, Prx 5–6, Trx1/2, and TrxR1/2.

Compliance with ethics guidelines

Jia-qi ZHANG, Liang SHI, Xi-ning XU, Si-chong HUANG, Bin LU, Li-li JI, and Zheng-tao WANG declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要:

本文题目: 槲皮素对四氯化碳引起的小鼠急性肝损伤治疗作用及其机理

Therapeutic detoxification of quercetin against carbon tetrachloride-induced acute liver injury in mice and its mechanism

研究目的: 本研究旨在观察槲皮素对四氯化碳 (CCl₄) 诱导的肝损伤的治疗解毒作用及其机理。

创新要点: 首次发现槲皮素对 CCl₄ 诱导的肝损伤有治疗作用, 并且首次发现 Prx 和 Trx 家族参与其中。

研究方法: 检测小鼠血清转氨酶含量, 并检测肝组织中丙二醛 (MDA)、谷胱甘肽 (GSH) 和 4-羟基壬烯醛 (4-HNE) 含量, 并用实时聚合酶链式反应 (PCR) 检测肝组织中 Prx 1-6、TrxR1/2、Trx1/2、Nrf2 和 HO-1 的 mRNA 表达情况。

重要结论: CCl₄ 造模成功后口服槲皮素对其造成的急性肝损伤有治疗作用, 给药组小鼠血清中的转氨酶与模型组相比均有显著下降, 通过 MDA 和免疫组化分析其机理可能和保护氧化应激损伤有关, 通过实时 PCR 分析发现 CCl₄ 抑制了抗氧化酶 Prx 家族、TrxRd1、TrxRd2、Trx1、Trx2 和 Nrf2 及其下游 HO-1 的基因表达, 而槲皮素可以逆转 CCl₄ 降低的这些基因的表达。

关键词组: 肝毒性; 氧应激损伤; Prx; Nrf2; TrxRd; Trx; HO-1