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Original Article

Zinc sulfate pretreatment prevents carbon tetrachloride-induced lethal toxicity through metallothionein-mediated suppression of lipid peroxidation in mice

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ABSTRACT — Carbon tetrachloride (CCl_4) is a well-known hepatotoxic chemical. Exposure to CCl_4 produces free radicals, which induce oxidative stress and cause hepatic injury. We demonstrated previously that pretreatment with zinc (Zn), which induces metallothionein (MT) expression, prevents CCl_4-induced lethal toxicity in a dose-dependent manner. While MT has been suggested as a possible hepatoprotective protein, its mechanism of protection remains unknown. In the current study, we evaluated the protective mechanism of MT, an endogenous scavenger of free radicals, against CCl_4-induced toxicity through subcutaneous administration of 50 mg/kg Zn (as $ZnSO_4$) once daily for three consecutive days, prior to a single intraperitoneal injection of 4 g/kg CCl_4 in male ddY mice. Our results showed that Zn pretreatment significantly decreased aspartate aminotransferase and total cholesterol levels, 6-hr after CCl_4 injection, as well as lipid peroxidation. Moreover, CCl_4-induced hepatic calcium level was downregulated by pretreatment with Zn while Zn-induced MT expression decreased by more than 500 μg/g liver (43%) in the Zn + CCl_4-treated group, implying that MT was consumed by CCl_4-induced free radicals. These findings suggest that prophylaxis with Zn protects mice from CCl_4-induced acute hepatotoxicity, presumably by inducing the expression of free radical-scavenging MT.

Key words: Carbon tetrachloride, Liver, Metallothionein, Zinc sulfate, Radical scavenger

INTRODUCTION

The liver is one of the most important organs in the human body, performing multiple functions such as detoxification, protein synthesis, and the production of important digestive biochemicals. In addition, it is most vulnerable to the effects of xenobiotics (Bhondave *et al*., 2014; Ma *et al*., 2014). Acute liver injury, which is usually defined as the rapid development of hepatocellular dysfunction, has a poor prognosis and may be induced by certain drugs, viral infections, and toxic or hepatic ischemic-reperfusion injury (Patel *et al*., 2014).

Carbon tetrachloride (CCl_4) is a xenobiotic used to study hepatotoxicity in animal models. The mechanism underlying CCl_4 hepatotoxicity has been thoroughly studied since 1967, using *in vivo* models of acute and chronic CCl_4 poisoning, as well as perfused livers and isolated or cultured hepatocytes (Recknagel, 1967; Weber *et al*., 2003). There is a consensus that CCl_4 toxicity is a multifactorial process, involving the generation of CCl_4-derived trichloromethyl free radicals by CYP2E1. These free radicals react with sulfhydryl groups such as glutathione (GSH) and protein thiols and antioxidant enzymes. The overproduction of these free radicals enhances membrane lipid peroxidation and causes covalent binding to macromolecules, loss of calcium homeostasis, and nucleic acid hypomethylation (Recknagel *et al*., 1989; Wong *et al*., 1998; Weber *et al*., 2003; Manibusan *et al*., 2007).

Initial CCl_4 toxicity studies were mostly performed *in vitro* on isolated hepatocytes, and revealed effects such as swelling of hepatocytes, disorganization of endoplasmic reticulum, mitochondrial morphological injuries, and increased levels of free cytoplasmic calcium (Tyson *et al*., 1983; Brattin *et al*., 1984; Berger *et al*., 1987). Consequently, little is known about the early effects of this organic solvent *in vivo;* nevertheless, we previously reported that pretreatment with Zn completely prevented CCl_4-induced acute lethal toxicity (within 24-hr post

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CCl_4-injection) in mice (Yoshioka *et al*., 2016a).

Several studies revealed compounds having protective effects against CCl_4-induced hepatotoxicity (Ohnuma *et al*., 2011; Khan *et al*., 2012; Knockaert *et al*., 2012; Huang *et al*., 2013; Ma *et al*., 2014; Zhang *et al*., 2014), although those investigations examined lower CCl_4-intoxidant level than our previous report (Yoshioka *et al*., 2016a); the hepatoprotective mechanisms of these compounds could be different. In addition, although previous studies have shown that pretreatment with Zn prevents CCl_4-induced lethal toxicity, the precise protection mechanism has not been evaluated. Therefore, in the present study, we investigated the mechanism of Zn-induced protection against CCl_4-induced lethal toxicity.

MATERIALS AND METHODS

Animal treatment

Male ddY mice were purchased from Japan SLC Inc. (Shizuoka, Japan), and were maintained under standard conditions of controlled temperature (24 ± 1°C), humidity (55 ± 5%) and light (12:12hr light/dark cycles), with free access to water and food. Experimental treatments were performed on 8-week-old animals. After the experiment, any surviving mice were sacrificed using pentobarbital. All experiments were approved by the Institutional Animal Care and Experiment Committee of Kinjo Gakuin University (No. 110).

Experimental protocol

The mice were divided into four groups: group one (control), group two (Zn group), group three (CCl_4 group), and group four (Zn + CCl_4 group). At 24-, 48-, and 72-hr intervals, mice in groups two and four were injected subcutaneous (s.c.) with 50 mg/kg $ZnSO_4$ (as Zn) (Nacalai Tesque, Kyoto, Japan). Groups one and three were injected s.c. with saline as control. Twenty-four hours after the final Zn and saline injections, mice in CCl_4 and Zn + CCl_4 groups were injected i.p. with 4 g/kg (at 5 mL/kg) $CCl_{4,}$ while those in groups one and two were injected subcutaneous (i.p.) with same amount of olive oil. Six hours after the CCl_4 and olive oil injections, mice from each group were euthanized and bled for plasma. The resulting plasma samples were stored at -80°C, pending aspartate aminotransferase (AST) and total cholesterol assays. The liver was harvested from each of these animals, and separate samples from each liver were stored at -80°C, pending quantitative reverse transcription (qRT)-PCR assay, malondialdehyde (MDA), Ca, and MT determination.

Measurement of AST and total cholesterol

Plasma AST activities were measured using the Transaminase CII Test Wako (Wako Chemical, Tokyo, Japan) according to the manufacturer's instructions and as previously described (Yoshioka *et al*., 2016a).

Plasma total cholesterol levels were measured using the LabAssay Cholesterol (Wako Chemical) according to the manufacturer's instructions. Each plasma sample (2 µL) was mixed with a substrate mixture (200 µL) and incubated at 37°C for 5 min. The absorbance of the reaction mixture was measured at 600/700 nm. For relative quantification, calibration curves were prepared using a standard solution.

Isolation of total RNA and qRT-PCR assay

Total RNA was extracted from 0.1-g liver sections using ISOGEN II (Nippon Gene, Tokyo, Japan). qRT-PCR was performed with One Step SYBR PrimeScript PLUS RT-PCR kit (Perfect Real Time) (Takara Bio, Shiga, Japan) using an Applied Biosystems 7300 (Applied Biosystems, Foster City, CA, USA). PCR was performed in 20 µL of solution containing 0.4 µM primers, 0.4 µL ROX Dye, and sample RNA (30 ng) in 2 × One Step SYBR RT-PCR Buffer 4, TaKaRa Ex Taq HS Mix, and PrimeScript PLUS RTase Mix. PCR conditions were as follows: at 42°C for 5 min, at 95°C for 10 sec, and 40-cycles at 95°C for 5 sec and 60°C for 31 sec. Gene expression was normalized to *GAPDH* mRNA levels. The oligonucleotide sequences of the primers were as follows: sense, 5'-TGGTGAAGGTCGGTGTGAAC-3', and antisense, 5'-GTCGTTGATGGCAACAATCTCC-3' for the mouse GAPDH (NM_001289726); sense, 5'-CATTCCTGTGTTCCAGGAGTACAAG-3', and antisense, 5'-GATACTTAGGGAAAACCTCCGCAC-3' for the mouse CYP2E1 (NM_021282).

Measurement of malondialdehyde (MDA) levels and total antioxidant power in the liver

The total MDA levels and total antioxidant power levels were examined by colorimetric microplate assay (FR40, Oxford Biochemical Research, Oxford, MI, USA) according to the manufacturer's protocol and as previously described (Yoshioka *et al*., 2016b).

Measurement of Ca concentrations

Liver (0.2-0.3 g) specimens were digested in 0.5 mL of concentrated nitric acid in glass test tubes. The temperatures were kept at 80°C for 1-hr, and increased subsequently by 10°C every hour until 130°C. The acid-digested specimens were diluted with distilled water up to 5 mL upon becoming transparent, and their Ca concentra-

tions were determined by atomic absorption using Z-2300 (Hitachi, Tokyo, Japan).

Determination of MT levels in the liver

The hepatic MT protein levels were determined by Cd saturation-hemolysate method (Cd-hem method). Liver tissue was homogenized with 5 vols. of 0.25 M sucrose. The homogenates were centrifuged at 18,000 *g* at 4°C for 20 min to separate the post-mitochondrial supernatant, suitable aliquots of which were used for MT assay by the Cd-hem method, as described previously (Onosaka and Cherian, 1981; Yoshioka *et al*., 2016a).

Statistical Analysis

All data from the control and treated groups were obtained from the same number of replicated experiments. All experiments were performed independently at least twice. Multiple comparisons were made by using post-hoc Tukey-Kramer's test. All statistical analyses were performed using SPSS 19.0J software (Chicago, IL, USA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Effect of Zn against CCl_4-induced acute toxicity on AST and total cholesterol

We examined the protective activity of Zn on hepatic biomarkers of CCl_4-induced toxicity. Administration of CCl_4 led to an increase in the plasma concentration of AST (Fig. 1A) and a decrease in plasma concentration of total cholesterol (Fig. 1B) as compared to that in the control group. Pretreatment with Zn was found to suppress AST activity by more than 50%. Zinc pretreatment led to the recovery of total cholesterol levels to control levels. There was no significant difference (AST and total cholesterol levels) between the control group and the Zn group.

Effect of Zn and CCl_4 on CYP2E1 expression

It is generally believed that CCl_4 toxicity results from the bioactivation of the CCl_4 molecule to the trichloromethyl free radical through CYP2E1. Further experiments were performed using real-time qRT-PCR to investigate whether Zn could affect *CYP2E1* mRNA expression. Compared with the controls, Zn treatment slightly reduced *CYP2E1* expression (17%). In contrast, CCl_4 and Zn + CCl_4 groups showed significantly lower expression (33%) of *CYP2E1* than the control group. No significant difference was observed between the CCl_4 and Zn + CCl_4 groups.

Effect of Zn against CCl_4-induced acute toxicity on MDA level and antioxidant status

To further investigate the protective activity of Zn against CCl_4, we studied oxidative stress in detail. CCl_4 is a prototypical lipid peroxidative agent that induces early lipid peroxidation in the liver. As a marker of lipid peroxidation, we measured MDA levels in all groups. CCl_4 treatment significantly increased MDA levels while pretreatment with Zn abolished MDA upregulation caused by CCl_4 injection (Fig. 3).

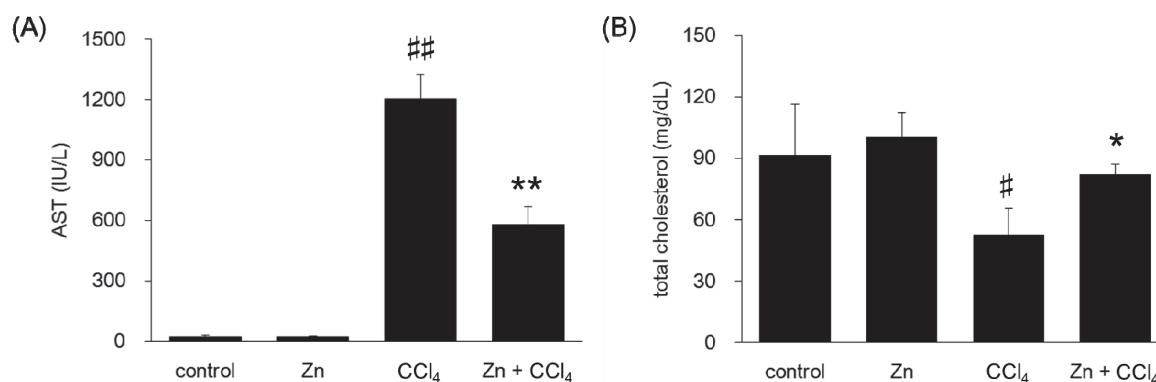

Fig. 1. Effect of pretreatment with Zn on AST and total cholesterol levels. Mice were injected s.c. with 50 mg/kg $ZnSO_4$ three times at 24-hr intervals. Twenty-four hours after the final pretreatment, the mice were injected i.p. with 4 g/kg CCl_4. The AST (A) and total cholesterol activity (B) in plasma were determined 6-hr after the injection. Data are representative of mean ± S.D. of five or six mice. $^{##}P < 0.01$ versus control group, and $^{*}P < 0.05$ versus CCl_4 group.

Effect of Zn against CCl_4-induced acute toxicity on calcium level

It is known that exposure to CCl_4 elevates hepatocellular calcium levels. In the present study, CCl_4 injection upregulated hepatic calcium levels, while pretreatment with Zn decreased it (Fig. 4).

Effect of Zn against CCl_4-induced acute toxicity on MT level

The hepatoprotective effect of MT against CCl_4-induced toxicity was evaluated by determining MT protein level using the Cd-hem assay (Fig. 5). Pretreatment with Zn increased the amount of MT protein to 1,240 µg/g liver (29-times higher than that in the control). Moreover, CCl_4 (711 µg/g liver) injection decreased Zn-induced MT level in the liver by 57%.

DISCUSSION

Our current study demonstrated that pretreatment with Zn suppressed AST (a marker of liver injury) and total cholesterol (a marker of hepatic protein synthesis) levels, indicating that Zn inhibited liver injury induced by CCl_4 metabolites (Fig. 1).

In the present study, two possible mechanisms might be suggested for the protective effect of Zn against CCl_4-induced toxicity. One possible explanation is Zn-mediated inhibition of CYP2E1 expression, preventing toxic CYP-derived CCl_4 metabolites formation. Inhibition of CYP2E1 by Zn has been previously reported (Kang and Zhou, 2005; Liu *et al*., 2009) and was confirmed in the present study wherein a 17% suppression of *CYP2E1* mRNA expression by Zn was observed (Fig. 2). CCl_4 is known as a CYP2E1 suicide substrate and binding of trichloromethyl radical to the active site of the enzyme leads to its inactivation and degradation (Tierney *et al*., 1992; Dai and Cederbaum, 1995). Although CCl_4 alone reduced CYP2E1 expression by 33%, no significant change was observed between the Zn + CCl_4 and the CCl_4 groups. Zn is a powerful inhibitor of CYP2E1 and degradation of CYP2E1 in the Zn + CCl_4 group was attenuated compared to that in the CCl_4 group. Taken together, this does not explain the almost total reversal of CCl_4 toxicity by this concentration of Zn.

The second possible explanation is that Zn or Zn-induced protein reacts preferentially with CCl_4-derived radicals. Zn is well known to be a powerful inducer of MT, which shows antioxidative activity against reaitive oxygen species by scavenging of free radicals (Cagen and Klaassen, 1979; Sato and Bremner, 1993; Sato and Kondoh, 2002). The free radical scavenging activity of MT is 300 times higher than that of GSH (Thornalley and Vasak, 1985). In the current study, Zn-induced MT level decreased by 43% after CCl_4 was injected into the liv-

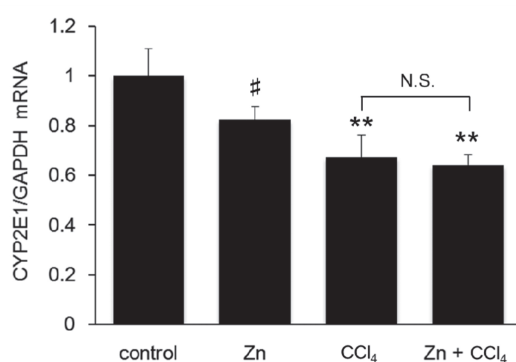

Fig. 2. Effect of Zn and CCl_4 on CYP2E1 expression in mouse liver. Mice were injected s.c. with 50 mg/kg $ZnSO_4$ three times at 24-hr intervals. Twenty-four hours after the final pretreatment, the mice were injected i.p. with 4 g/kg CCl_4. Total RNA was isolated 6-hr after injection and quantitative RT-PCR analysis was performed. The amount of *CYP2E1* mRNA was normalized by *GAPDH* mRNA. Data are representative of mean ± S.D. of five or six mice. $^{\#}P < 0.05$, versus control group and $^{**}P < 0.01$ versus control group. N.S. means no significant change.

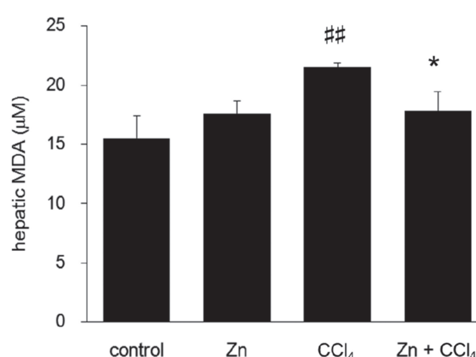

Fig. 3. Effect of pretreatment with Zn on MDA levels in acute CCl_4-induced toxicity. Mice were injected s.c. with 50 mg/kg $ZnSO_4$ three times at 24-hr intervals. Twenty-four hours after the final pretreatment, the mice were injected i.p. with 4g/kg CCl_4. MDA levels (A) and the antioxidant power (B) in the liver were determined 6 hr after the injection. Data are representative of mean ± S.D. of five or six mice. $^{\#\#}P < 0.01$ versus control group and $^{*}P < 0.05$ versus CCl_4 group.

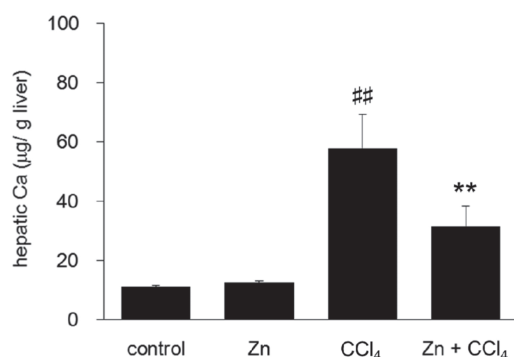

Fig. 4. Effect of pretreatment with Zn on hepatic Ca concentration in acute CCl_4-induced toxicity. Mice were injected s.c. with 50 mg/kg $ZnSO_4$ three times at 24-hr intervals. Twenty-four hours after the final pretreatment, the mice were injected i.p. with 4 g/kg CCl_4. Ca concentration in the liver was determined 6 hr after the injection. Data indicate mean ± S.D. of five or six mice. $^{##}P < 0.01$ versus control group and $^{**}P < 0.01$ versus CCl_4 group.

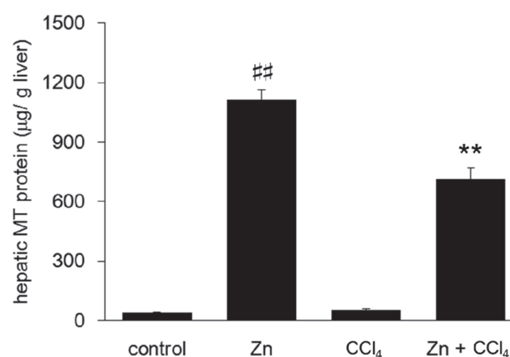

Fig. 5. Effect of pretreatment with Zn on hepatic MT protein levels in acute CCl_4-induced toxicity. Mice were injected s.c. with 50 mg/kg $ZnSO_4$ three times at 24-hr intervals. Twenty-four hours after the final pretreatment, the mice were injected i.p. with 4 g/kg CCl_4. The MT levels in the liver were determined 6 hr after the injection. Data indicate mean ± S.D. of five or six mice. $^{##}P < 0.01$ versus control group and $^{**}P < 0.01$ versus Zn group.

er (Fig. 5), suggesting that MT reacted preferentially with CCl_4-derived radicals and the reacted MT was consumed prior to GSH. Klaassen and Liu (1998) reported that radiolabeled CCl_4 was bound to MT in MT-induced animals than in controls, and this might support our hypothesis. In addition, Knockaert *et al.* (2012) reported that CCl_4 administration induced very early signs of hepatotoxicity (3-hr after injection) in mice and that these were mediated through lipid peroxidation-dependent and independent pathways. They noted that the lipid peroxidation-dependent pathway showed mitochondrial dysfunction while the independent pathway exhibited hepatocyte swelling. Our previous investigation revealed that CCl_4-induced hepatocyte swelling was recovered in some, but not all instances, by pretreatment with Zn (Yoshioka *et al.*, 2016a), suggesting that the hepatoprotective mechanism of Zn occurs through both lipid peroxidation-dependent and independent pathways. In sum, the major protective mechanism of Zn against CCl_4 toxicity might be attributable to the free radical scavenging property of MT.

Many researchers investigated the protective effects of various compounds against CCl_4-induced hepatotoxicity and reported their protective mechanisms (Ohnuma *et al.*, 2011; Khan *et al.*, 2012; Knockaert *et al.*, 2012; Huang *et al.*, 2013; Ma *et al.*, 2014; Zhang *et al.*, 2014). Although these mechanisms are in part common to our current study, the degree or level of protection differs greatly. In a previous study, one time pretreatment with Zn or Cd, metals known to powerfully induce MT expression, could not rescue all mice against 4 g/kg CCl_4-induced lethal toxicity until 24-hr. This is because MT does not reach optimal levels (optimal MT level after pretreatment with Zn and Cd is 629 and 585 µg/g liver, respectively) in spite of high level of MT induction and antioxidant activity compared to that observed in previous reports by other authors. We speculate that CCl_4-derived free radicals preferentially attack MT, followed by the consumption of GSH and other antioxidants after MT's depletion, disrupting calcium homeostasis and causing inducing cell death. In Fig. 5, more than 500 µg/g liver tissue of MT is depleted 6-hr post CCl_4-injection, which is consistent with our previous study in which mouse death in a group pretreated with Zn or Cd was observed around 9-hr post CCl_4-injection. In addition, more than 1,000 µg/g liver of MT-induction is required to protect against lethal toxicity due to CCl_4 under this experimental condition (i.p. injection with 4 g/kg CCl_4 for 24 hr). Thus, we need not only to research into elucidating the protective mechanisms of MT but also to search for novel protective agents, specifically when investigating lethal toxicity.

In conclusion, our current study demonstrated that pretreatment with Zn at three time intervals, repressed both the lipid peroxidation-dependent and independent pathways. This is most likely due to Zn-induced MT, which acts as a GSH-sparing agent through high radical scavenging activity. Although further investigations are warranted to clarify how MT suppresses CCl_4-induced toxicity, this research is expected to encourage the search for

other free radical-generating chemicals.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Berger, M.L., Reynolds, R.C. and Combes, B. (1987): Carbon tetrachloride-induced morphologic alterations in isolated rat hepatocytes. Exp. Mol. Pathol., **46**, 245-257.

Bhondave, P.D., Devarshi, P.P., Mahadik, K.R. and Harsulkar, A.M. (2014): 'Ashvagandharishta' prepared using yeast consortium from Woodfordia fruticosa flowers exhibit hepatoprotective effect on CCl_4 induced liver damage in Wistar rats. J. Ethnopharmacol., **151**, 183-190.

Brattin, W.J., Pencil, S.D., Waller, R.L., Glende, E.A.Jr. and Recknagel, R.O. (1984): Assessment of the role of calcium ion in halocarbon hepatotoxicity. Environ. Health Perspect., **57**, 321-323.

Cagen, S.Z. and Klaassen, C.D. (1979): Protection of carbon tetrachloride-induced hepatotoxicity by zinc: role of metallothionein. Toxicol. Appl. Pharmacol., **51**, 107-116.

Dai, Y. and Cederbaum, A.I. (1995): Inactivation and degradation of human cytochrome P4502E1 by CCl_4 in a transfected HepG2 cell line. J. Pharmacol. Exp. Ther., **275**, 1614-1622.

Huang, G.J., Deng, J.S., Huang, S.S., *et al*. (2013): Hepatoprotective effects of eburicoic acid and dehydroeburicoic acid from Antrodia camphorata in a mouse model of acute hepatic injury. Food Chem., **141**, 3020-3027.

Kang, Y.J. and Zhou, Z. (2005): Zinc prevention and treatment of alcoholic liver disease. Mol. Aspects. Med., **26**, 391-404.

Khan, R.A., Khan, M.R. and Sahreen, S. (2012): CCl_4-induced hepatotoxicity: protective effect of rutin on p53, CYP2E1 and the antioxidative status in rat. BMC Complement. Altern. Med., **12**, 178.

Klaassen, C.D. and Liu, J. (1998): Induction of metallothionein as an adaptive mechanism affecting the magnitude and progression of toxicological injury. Environ. Health Perspect., **106 Suppl. 1**, 297-300.

Knockaert, L., Berson, A., Ribault, C., *et al*. (2012): Carbon tetrachloride-mediated lipid peroxidation induces early mitochondrial alterations in mouse liver. Lab. Invest., **92**, 396-410.

Liu, J., Zhou, Z.X., Zhang, W., Bell, M.W. and Waalkes, M.P. (2009): Changes in hepatic gene expression in response to hepatoprotective levels of zinc. Liver Int., **29**, 1222-1229.

Ma, J.Q., Ding, J., Zhang, L. and Liu, C.M. (2014): Hepatoprotective properties of sesamin against CCl_4 induced oxidative stress-mediated apoptosis in mice via JNK pathway. Food Chem. Toxicol., **64**, 41-48.

Manibusan, M.K., Odin, M. and Eastmond, D.A. (2007): Postulated carbon tetrachloride mode of action: a review. J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev., **25**, 185-209.

Ohnuma, T., Anan, E., Hoashi, R., *et al*. (2011): Dietary diacetylene falcarindiol induces phase 2 drug-metabolizing enzymes and blocks carbon tetrachloride-induced hepatotoxicity in mice through suppression of lipid peroxidation. Biol. Pharm. Bull., **34**, 371-378.

Onosaka, S. and Cherian, M.G. (1981): The induced synthesis of metallothionein in various tissues of rat in response to metals. I. Effect of repeated injection of cadmium salts. Toxicology, **22**, 91-101.

Patel, R.P., Lang, J.D., Smith, A.B. and Crawford, J.H. (2014): Redox therapeutics in hepatic ischemia reperfusion injury. World J. Hepatol., **6**, 1-8.

Recknagel, R.O. (1967): Carbon tetrachloride hepatotoxicity. Pharmacol. Rev., **19**, 145-208.

Recknagel, R.O., Glende, E.A., Jr., Dolak, J.A. and Waller, R.L. (1989): Mechanisms of carbon tetrachloride toxicity. Pharmacol. Ther., **43**, 139-154.

Sato, M. and Bremner, I. (1993): Oxygen free radicals and metallothionein. Free Radic. Biol. Med., **14**, 325-337.

Sato, M. and Kondoh, M. (2002): Recent studies on metallothionein: protection against toxicity of heavy metals and oxygen free radicals. Tohoku J. Exp. Med., **196**, 9-22.

Thornalley, P.J. and Vasak, M. (1985): Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. Biochim. Biophys. Acta, **827**, 36-44.

Tierney, D.J., Haas, A.L. and Koop, D.R. (1992): Degradation of cytochrome P450 2E1: selective loss after labilization of the enzyme. Arch. Biochem. Biophys., **293**, 9-16.

Tyson, C.A., Story, D.L. and Stephens, R.J. (1983): Ultrastructural changes in isolated rat hepatocytes exposed to different CCl4 concentrations. Biochem. Biophys. Res. Commun., **114**, 511-517.

Weber, L.W., Boll, M. and Stampfl, A. (2003): Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. Crit. Rev. Toxicol., **33**, 105-136.

Wong, F.W., Chan, W.Y. and Lee, S.S. (1998): Resistance to carbon tetrachloride-induced hepatotoxicity in mice which lack CYP2E1 expression. Toxicol. Appl. Pharmacol., **153**, 109-118.

Yoshioka, H., Usuda, H., Nonogaki, T. and Onosaka, S. (2016a): Carbon tetrachloride-induced lethality in mouse is prevented by multiple pretreatment with zinc sulfate. J. Toxicol. Sci., **41**, 55-63.

Yoshioka, H., Usuda, H., Fukuishi, N., Nonogaki, T. and Onosaka, S. (2016b): Carbon tetrachloride-induced nephrotoxicity in mouse is prevented by pretreatment with zinc sulfate. Biol. Pharm. Bull. *In press*.

Zhang, F., Wang, X., Qiu, X., *et al*. (2014): The protective effect of esculentoside a on experimental acute liver injury in mice. PLoS One, **9**, e113107.