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Letter

Hepatoprotective effect of kampo formula "Juzen-taiho-to" on bromobenzene-induced toxicity in mice

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ABSTRACT — Acute liver disease may develop due to various causes and occur by different mechanisms. Carbon tetrachloride (CCl_4), a well-known hepatic toxicant, was selected as a model of alkylating agents that do not induce glutathione depletion. Our previous study indicated CCl_4-induced hepatotoxicity was decreased by pretreatment with the Japanese herbal medicine "Juzen-taiho-to" (JTX), suggesting that prophylaxis with JTX protects mice from CCl_4-induced acute hepatic toxicity. In contrast, bromobenzene (BB) is a known glutathione-depleting agent. Although BB-induced hepatotoxicity also promotes lipid peroxidation, the mechanism of hepatic injury is different from that of CCl_4. Hence, in this study, we investigated whether pretreatment with JTX ameliorated BB-induced hepatotoxicity. Mice injected with BB showed increased plasma levels of hepatic injury markers (alanine aminotransferase and aspartate aminotransferase) in addition to hepatic lipid peroxidation. Pretreatment with JTX decreased BB-induced plasma levels of hepatic injury markers. BB-induced hepatotoxicity is mainly caused by oxidative stress. JTX pretreatment also decreased BB-induced lipid peroxidation. Our results suggest that JTX has the potential to protect against BB-induced hepatotoxicity and modulate oxidative stress.

Key words: Bromobenzene, Liver, Juzen-taiho-to, Glutathione, Oxidative stress

INTRODUCTION

Liver injury induced by chemicals, drugs, and viruses is a well-recognized toxicological problem. The pathogenesis of the damage is multifactorial, ranging from oxidation and inflammation to immune reactions. Bromobenzene (BB), an industrial solvent and an additive in motor oils, causes necrosis in the liver. BB was selected as a model molecule of glutathione (GSH)-depleting agents. The metabolism and toxicity of BB in the liver has been studied in detail (Comporti, 1987; Heijne *et al*., 2004). BB is subjected to biotransformation in the liver, and the metabolites of BB are highly hepatotoxic. Glutathione-S-transferases (also referred to as phase-II drug-metabolizing enzymes) catalyze the sequestration of reactive epoxides through conjugation to GSH. Epoxides are also hydrolyzed by microsomal epoxide hydrolase and the cytochromes P450 (CYPs). At high doses of BB, conjugation to the metabolites depletes the hepatic GSH pool, impairing intracellular protection against reactive oxygen species (ROS) and hazardous xenobiotic metabolites. GSH depletion may lead to a number of secondary events that damage the cell including lipid peroxidation, ATP depletion, and changes in intracellular calcium levels (Casini *et al*., 1988; Locke and Brauer, 1991). Multiple compounds have been reported to show protective effects against BB-induced hepatotoxicity (Maellaro *et al*., 1990; Wang *et al*., 1999; El-Sharaky *et al*., 2009). These compounds mainly act as antioxidants.

The Japanese herbal medicine "Juzen-taiho-to" (JTX) is a Kampo medicinal prescription. It has been historically used in Japan for the treatment of cancers, rheumatoid arthritis, and atopic dermatitis (Saiki, 2000). JTX consists of ten medicinal herbs in the indicated amounts (grams per day in human intake): *Astragali Radix* (3.0), *Cinnamomi Cortex* (3.0), *Rehmanniae Radix* (3.0), *Paeoniae Radix* (3.0), *Cnidii Rhizoma* (3.0), *Atractylodis Lanceae Rhizoma* (3.0), *Angelicae Radix* (3.0), *Gin-*

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seng Radix (3.0)*, Poria Cocos* (3.0), *and Glycyrrhizae Radix* (1.5). Our previous study reported that JTX prevented carbon tetrachloride (CCl_4)-induced hepatotoxicity in mice (Yoshioka *et al*., 2016b). CCl_4 was selected as a model molecule of alkylating agents that do not induce GSH depletion. Metabolic activation of CCl_4 by CYP2E1 to free radicals is reported to enhance lipid peroxidation in liver, resulting in hepatocyte necrosis. Since the mechanism of BB-induced hepatic injury is similar to that of CCl_4, one hypothesis is that BB-induced lipid peroxidation is prevented by JTX. Therefore, to test this hypothesis, we investigated whether pretreatment with JTX was sufficient to attenuate BB-induced hepatic injury.

MATERIALS AND METHODS

Animal treatment

Male ddY mice were purchased from Japan SLC (Shizuoka, Japan). At our facility, the mice were maintained under standard conditions of controlled temperature (24 ± 1°C), humidity (55 ± 5%), and light (12:12-hr light/dark cycles) with free access to water and food. Experimental treatments were performed in 7-week-old animals. After the experiment, the surviving mice were sacrificed using pentobarbital. All experiments were approved by the Institutional Animal Care and Experiment Committee of Kinjo Gakuin University (No. 130).

Experimental protocol

Mice were divided into three groups. Twenty-four hours before BB injection, animals of Group-3 (JTX + BB group) were intraperitoneally (i.p.) administered a JTX extract powder (Tsumura, Tokyo, Japan) dissolved in saline (10% JTX solution) at 1 g/kg (10 mL/kg). This dose was according to that described by Anjiki *et al.* (2005). Animals in Group-1 (control group) and Group-2 (BB group) were injected i.p. with equivalent volumes of the saline vehicle. Twenty-four hours after the JTX or saline injection, both Group-2 and Group-3 were administered 300 mg/kg (at 10 mL/kg) BB i.p. Group-1 was injected i.p. with equivalent volumes of olive oil. Twenty-four hours after the BB or olive oil injection, mice from each group were euthanized and blood samples were collected for plasma analysis. The resulting plasma samples were stored at -80°C before conducting assays for alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The liver was harvested from each animal, and separate samples from each liver were stored at -80°C.

Measurement of ALT and AST activities

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

Measurement of malondialdehyde level in liver

Total malondialdehyde (MDA) levels in the liver samples were examined with a colorimetric thiobarbituric acid reactive substances (TBARS) microplate assay kit (FR40, Oxford Biochemical Research, Oxford, MI, USA), according to the manufacturer's protocol and as previously described (Yoshioka *et al*., 2016a).

Measurement of hepatic GSH level in liver

Hepatic GSH levels were measured using GSSG/GSH quantification kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions and as previously described (Miura *et al*., 2013).

Statistical analysis

Multiple comparisons were analyzed using one-way analysis of variance (ANOVA) with post-hoc Tukey-Kramer's test. All statistical analyses were performed using SPSS 19.0 software (Chicago, IL, USA). Values of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

First, we analyzed plasma ALT and AST levels (Fig. 1), as these are markers of liver injury and dysfunction. The control group showed normal levels of ALT (Fig. 1A) and AST (Fig. 1B). Administration of BB led to an increase in ALT and AST plasma levels, whereas pretreatment with JTX suppressed ALT and AST activities by 66 and 65%, respectively. There was no significant difference between the control and JTX-treated mice (data not shown) as previously reported (Yoshioka *et al*., 2016b).

Furthermore, to investigate the protective effect of JTX against BB, we calculated hepatic MDA content, which is a well-known marker for lipid peroxidation. BB treatment significantly increased MDA levels, whereas pretreatment with JTX eliminated BB-induced MDA upregulation (Fig. 2), suggesting that JTX itself and/or JTX-induced gene products may have an antioxidant effect. Our previous study had demonstrated that i.p. injected JTX increased hepatic metallothionein (MT) levels to more than 200 µg/g of liver (Yoshioka *et al*., 2016b). MT has antioxidant properties against

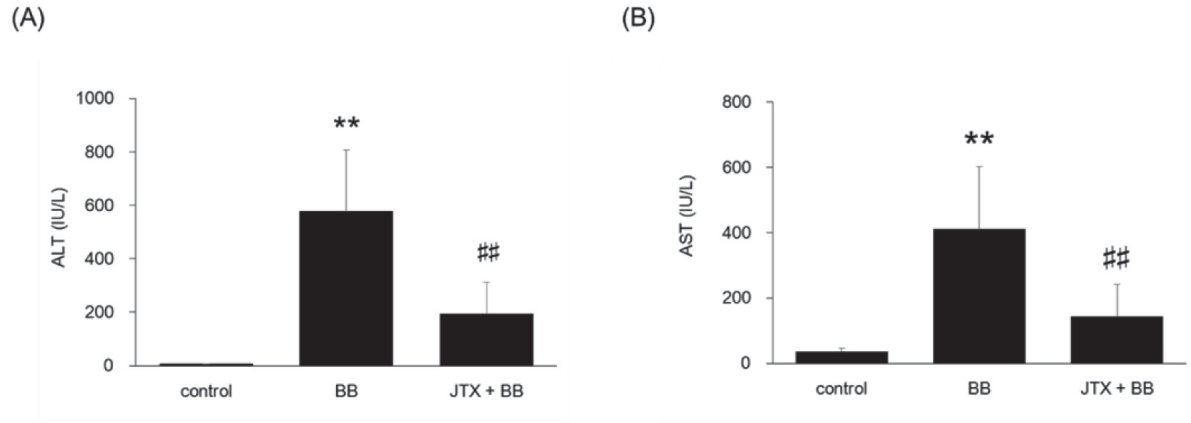

Fig. 1. Effect of pretreatment with JTX on ALT and AST levels. Mice were injected i.p. with 10% JTX solution (1 g/kg). Twenty-four hours after pretreatment, mice were injected i.p. with 300 mg/kg BB. ALT (A) and AST (B) levels in the plasma were determined 24 hr after i.p. injection of BB. Data are presented as mean ± S.D. values from six mice. $^{**}P < 0.01$ versus control group, and $^{\#\#}P < 0.01$ versus BB group.

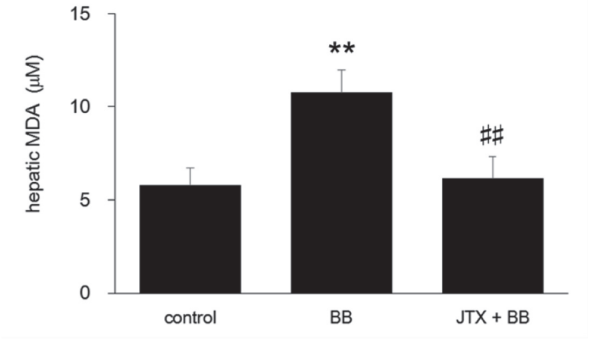

Fig. 2. Effect of pretreatment with JTX on acute BB toxicity, as measured by MDA levels. Mice were injected i.p. with 10% JTX solution (1 g/kg). Twenty-four hours after pretreatment, mice were injected i.p. with 300 mg/kg BB. Liver MDA levels were determined 24 hr after i.p. injection with BB. Data are presented as mean ± S.D. values from six mice. $^{**}P < 0.01$ versus control group, and $^{\#\#}P < 0.01$ versus BB group.

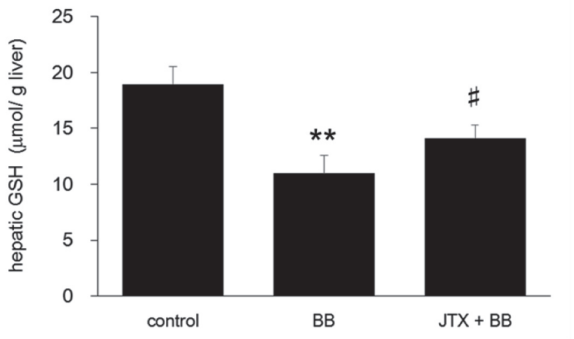

Fig. 3. Effect of pretreatment with JTX on acute BB toxicity, as measured by hepatic GSH levels. Mice were injected i.p. with 10% JTX solution (1 g/kg). Twenty-four hours after pretreatment, mice were injected i.p. with 300 mg/kg BB. Liver GSH levels were determined 24 hr after i.p. injection with BB. Data are presented as mean ± S.D. values from six mice. $^{**}P < 0.01$ versus control group, and $^{\#}P < 0.05$ versus BB group.

ROS. It also has free radical-scavenging abilities (Hamer, 1986; Kumari *et al*., 1998). We previously reported that Zn-induced MT expression has a protective effect on BB-induced hepatotoxicity and nephrotoxicity (Yoshioka *et al*., 2016a). Although the JTX-induced MT expression is lower than Zn-induced MT expression, our current results are sufficient to suggest attenuation of BB-induced hepatotoxicity because plasma hepatic injury markers and hepatic MDA levels were decreased by JTX pretreatment.

BB-induced toxicity is a multifactorial process. The first step is the metabolic activation of BB by CYPs. Subsequently, BB is converted to BB-3, 4-oxide, 2-bromohydroquinone, or 4-bromocatechol. The second step is radical binding. Free radicals are scavenged by antioxidant enzymes or react with sulfhydryl groups in substances such as GSH and protein thiols . The third step involves the overexpression of these free radicals to increase oxidative stress. This step is associated with alterations in

calcium homeostasis and initiation of signal transduction responses. The fourth step is ATP depletion and increase in cellular calcium levels. The fifth and final step is necrosis. Thornalley and Vasak reported that the radical scavenging activity of MT is 300-fold higher than that of GSH (1985), and JTX-induced MT counteracted BB-induced toxicity from the earliest stages (at least the second stage). We speculate that BB-derived metabolites preferentially react with MT, followed by consumption of GSH and other antioxidants after MT's depletion, disrupting calcium homeostasis and inducing cell death. Our present study indicates GSH depletion is prevented by pretreatment with JTX (Fig. 3). These data support our hypothesis.

In conclusion, we have demonstrated that pretreatment with JTX suppresses BB-induced hepatic injuries. We hypothesize that the hepatoprotective effect of JTX is attributable to its antioxidant properties. To our knowledge, this is the first evidence suggesting that JTX protects against BB-induced acute hepatotoxicity. Although further investigation is needed to clarify the active component of JTX, these findings have improved our understanding of the protective effect of this Japanese herbal medicine against free radical-induced organ injury and disease.

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

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