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Letter

Protective effect of the Kampo formula "Juzen-taiho-to" on isoniazid- and rifampicin-induced hepatotoxicity in mice

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ABSTRACT — The aim of this study was to investigate whether the Japanese herbal medicine Juzen-taiho-to (JTX) showed attenuating effects on isoniazid- and rifampicin-induced liver injury. Seven-week-old male Institute of Cancer Research mice were orally administered JTX or saline once a day at 9:00 for 3 days. Additionally, the mice received a mixture of 80 mg/kg isoniazid and 160 mg/kg rifampicin (10 mL/kg) via intraperitoneal injection three times (at 19:30) per 24 hr period. Twenty-four hours after the last administration of isoniazid/rifampicin, the mice in each group were sacrificed and blood was removed to obtain the plasma and livers. Mice that had received isoniazid/rifampicin showed high plasma levels of alanine aminotransferase, aspartate aminotransferase, and interleukin-6. In addition, the mice injected with isoniazid/rifampicin displayed increased hepatic lipid peroxidation and receptor-interacting protein-1 and -3 levels. Treatment with JTX prevented an isoniazid/rifampicin-induced increase in levels of alanine aminotransferase and aspartate aminotransferase, lipid peroxidation, and receptor-interacting protein changes. Our results suggest that JTX protects against isoniazid/rifampicin-induced hepatic injury by modulating oxidative stress and inflammatory responses.

Key words: Isoniazid, Rifampicin, Hepatic injury, Anti tubercular drug

INTRODUCTION

In Japan, approximately 700 million medicines are prescribed each year (2018, Ministry of Health, Labor and Welfare). Because drug metabolism occurs primarily in the liver (Tostmann *et al.*, 2008), drug-induced side effects may occur in this organ. Therefore, it is important to protect the liver against drug-induced hepatotoxicity.

Isoniazid (INH) and rifampicin (RFP) are commonly used anti-tuberculosis (AT) drugs. An AT drug regimen comprising INH and RFP prescribed for prolonged periods (≥ 6 months) at high doses is a major clinical concern, because of the risk of hepatic toxicity. Hepatitis is reported to occur in 0.5% of patients receiving these AT drugs (Tostmann *et al*., 2008). Although various factors are responsible for AT drug-induced hepatotoxicity, such as cytochrome P450-mediated metabolism and INH and RFP interactions, there is little insight into the biochemical mechanisms responsible. Therefore, it is important to understand the toxicity of AT drugs at a cellular level. Additionally, it is necessary to prevent AT drug-induced hepatotoxicity through the use of natural products and Kampo medicine. Previous studies have shown that traditional herbal medicines such as *Curcuma longa*, *Ocimum sanctum*, *Tinospora cordifolia* and *Zizyphus mauritiana* exert protective effects against INH- and RFP-

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induced liver disease (Adhvaryu *et al*., 2007).

Traditional Japanese herbal medicines such as Kampo formulas, which comprise hot water extracts obtained from a mixture of medicinal plants, have been widely used in routine clinical practice and have been accepted into the modern medical system. Juzen-taiho-to (JTX) is one of the most commonly prescribed Kampo formulations and is composed of ten different medicinal herbs (Anjiki *et al.,* 2005). JTX is widely prescribed for arthritis, anemia, rheumatism, inflammatory bowel diseases, and chronic fatigue syndrome. Additionally, JTX is widely used for the prevention of cancer metastasis and infection in immunocompromised patients.

Our previous investigation showed that pretreatment with JTX protects from acute hepatic injury induced by carbon tetrachloride and ethanol (Fukaya *et al.*, 2018; Yoshioka *et al.*, 2016). Thus, this previous study suggests that JTX may prevent several kinds of hepatic diseases. Therefore, in this study, we investigated whether JTX reduces INH- and RFP-induced liver damage.

MATERIALS AND METHODS

Sample preparation

A decoction of JTX was prepared by boiling it in 800 mL water until it had reduced by half. The decoction was filtered and freeze-dried overnight to obtain a dry powder (7.35 g). The powder prepared using the JTX decoction (10 mg) was extracted with 2 mL pure water. The supernatant was filtered through a 0.45-μm membrane filter, and high-performance liquid chromatography analysis was then performed using 10 μL of the sample. The fingerprint of chart was previously reported (Fukaya *et al.*, 2018).

Animals

Male Institute of Cancer Research (ICR) mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and maintained under standard conditions of 24 ± 1°C, 55 ± 5% humidity, and 12-hr light/dark cycles, with *ad libitum* access to water and food. Experimental treatments were performed on 7-week-old mice. At the end of the experiment, surviving mice were euthanized using pentobarbital. All experiments were approved by the Institutional Animal Care and Experiment Committee of Kinjo Gakuin University (approval no. 157).

Experimental protocol

Animals were randomly divided into four groups. Animals in the JTX group and JTX + INH/RFP group were administered 1 g/kg (5 mL/kg) JTX orally (p.o.) at 9:00 for 3 days. Animals in the control group and INH/RFP group were administered equivalent volumes of vehicle (saline) orally at 9:00. Both the INH/RFP and JTX + INH/RFP groups received a mixture (10 mL/kg) containing 80 mg/kg INH and 160 mg/kg RFP via intraperitoneal (i.p.) injection at 19:30 for 3 days. The control and JTX groups received i.p. injections at 19:30 of equivalent volumes of 10% dimethyl sulfoxide (DMSO). Twenty-four hours after the final administration of INH/RFP or 10% DMSO, mice were euthanized and blood was collected to obtain plasma, which was stored at -80°C. The livers were rapidly removed, snap-frozen in liquid nitrogen, and subsequently stored at -80°C.

Plasma biochemical analysis

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using the Transaminase CII Test Wako (Fujifilm Wako Pure Chemical, Osaka, Japan) according to the manufacturer's instructions as previously described (Fukaya *et al.,* 2018). Plasma levels of interleukin (IL)-6 were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Thermo Fisher, Waltham, MA, USA). For relative quantification, calibration curves were prepared using standard solutions.

Determination of malondialdehyde (MDA) levels in the liver

Total MDA levels in the liver were determined via colorimetric microplate assay (Oxford Biomedical Research, Oxford, MI, USA) according to the manufacturer's protocol. Livers (80 mg) were homogenized with 720 μL ice-cold phosphate-buffered saline (PBS) containing protease inhibitors (Nacalai Tesque, Inc., Kyoto, Japan) and centrifuged at 18,000 × *g* for 20 min at 4°C. The supernatant was collected, and the protein levels were estimated using a bicinchoninic acid (BCA) protein assay kit (Nacalai Tesque, Inc.). The supernatant (40 μL) was deproteinized by the addition of trichloroacetic acid (2 μL) and centrifuged at 1,000 × *g* for 10 min at 4°C. The resulting supernatant (25 μL) and indicator solution (150 μL) were mixed and incubated at 65°C for 45 min, and the absorbance was measured at 532 nm (Yoshioka and Onosaka, 2016).

Western blot analysis

Protein samples (30 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel and transferred to polyvinylidene difluoride membranes. Mouse anti-receptor-interacting protein

(RIP)-1 monoclonal antibodies and anti-RIP-3 monoclonal antibodies obtained from Santa Cruz Biotechnology (Dallas, TX, USA), and mouse anti-β-actin monoclonal antibodies purchased from Medical & Biological Laboratories Co., LTD (Aichi, Japan) were used as primary antibodies (1:1500 dilution) for immunoblotting. A peroxidase-conjugated anti-mouse immunoglobulin G (IgG; Cell Signaling Technology, Danvers, MA, USA) was used as a secondary antibody (1:4000 dilution). Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) system.

Statistical analyses

Multiple comparisons were made via one-way analysis of variance (ANOVA) with Tukey-Kramer post-hoc test or two-way repeated-measures ANOVA. All statistical analyses were performed using the SPSS Statistics for Windows software (version 24.0; IBM Corporation, Armonk, NY, USA). Differences were considered statistically significant if $p < 0.05$.

RESULTS

First, we analyzed plasma ALT and AST levels (Fig. 1), as these are well-known liver injury and dysfunction markers. The control and JTX groups showed normal ALT and AST levels (Fig.1 A and B, respectively), but injection of INH/RFP led to an increase in ALT and AST plasma levels. In addition, co-injection of JTX and INH/RFP caused a greater decrease in ALT and AST levels than treatment with INH/RFP alone.

Next, we determined plasma levels of IL-6 (Fig. 2), as this is a representative inflammatory cytokine that is known to increase in response to INH and/or RFP exposure (Yuhas *et al*., 2009, 2011). Our results indicate that INH and RFP administration increased IL-6 levels and co-administration with JTX significantly decreased plasma IL-6 levels.

Furthermore, we measured hepatic MDA levels, as hepatic injury is known to elevate oxidative stress (Chowdhury *et al.*, 2006). Although INH/RFP administration increased hepatic MDA levels, co-administration of JTX and INH/RFP decreased hepatic MDA levels in comparison to those in the INH/RFP group (Fig. 3).

Finally, we performed western blotting. We determined RIP1 and RIP3 levels in the liver (Fig. 4), as these are well-known parameters involved in necrosis (Vandenabeele *et al.*, 2010). Additionally, expression of these proteins is reported to increase following administration of INH and RFP (Sarich *et al.*, 1996). In the control and JTX groups, no RIP1 expression was observed, although RIP1 was detected after administration of INH/RFP. In contrast, RIP1 expression was attenuated after co-administration of JTX. This pattern was also observed

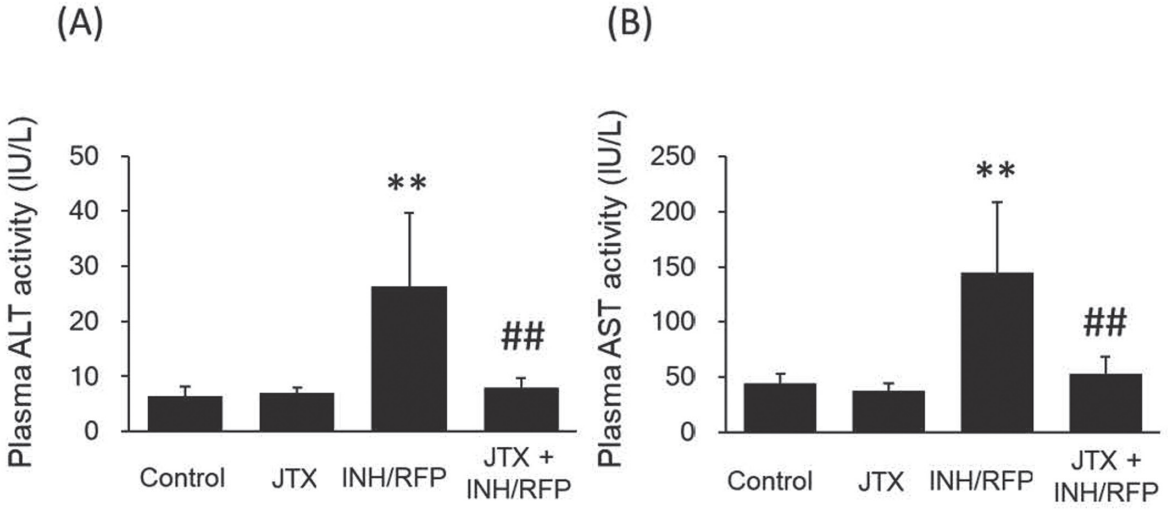

Fig. 1. Effect of Juzen-taiho-to (JTX) on alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in a mouse model of isoniazid (INH)/rifampicin (RFP)-induced hepatotoxicity. Male Institute of Cancer Research (ICR) mice were orally administered 1 g/kg JTX at 9:00 for 3 days. The mice also received 80 mg/kg INH and 160 mg/kg RFP JTX via intraperitoneal (i.p.) injection at 19:30 for 3 days. ALT (A) and AST (B) levels were determined using plasma samples 24 hr after INH/RFP administration. Data are presented as means ± standard deviations (SD) of 4-7 mice. $^{**}p < 0.01$ versus the control group. $^{\#\#}p < 0.01$ versus the INH/RFP group.

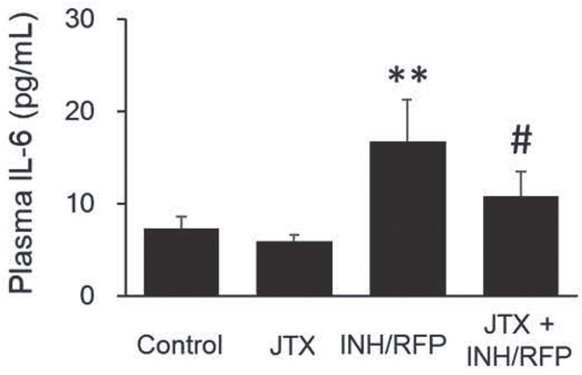

Fig. 2. Effect of JTX on plasma interleukin (IL)-6 levels in a mouse model of isoniazid (INH)/rifampicin (RFP)-induced hepatotoxicity. Mice were treated according to the methods described in Fig. 1. Plasma IL-6 levels were measured 24 hr after the final INH/RFP injection. Data are presented as means ± SD of 4-7 mice. $^{**}p < 0.01$ versus the control group, $^{\#}p < 0.05$ versus the INH/RFP group.

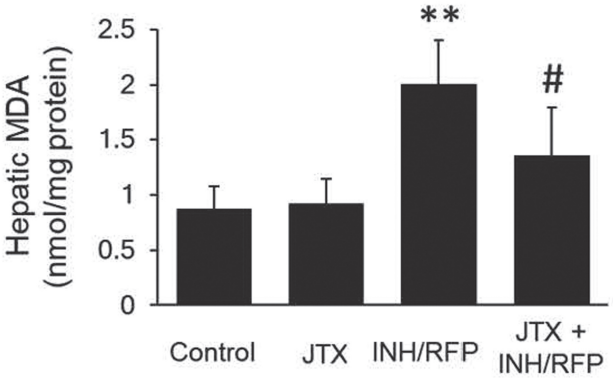

Fig. 3. Effect of JTX on hepatic malonaldehyde (MDA) levels in a mouse model of isoniazid (INH)/rifampicin (RFP)-induced hepatotoxicity. Mice were treated according to the methods described in Fig. 1. MDA levels in the liver were determined 24 hr after the final INH/RFP injection. Data are presented as means ± SD of 4-7 mice. $^{**}p < 0.01$ versus the control group, $^{\#}p < 0.05$ versus the INH/RFP group.

with regard to RIP3 expression. These data suggest that combination treatment of JTX and INH/RFP decreased RIP-induced necrosis.

DISCUSSION

AT drugs are considered potentially hepatotoxic and can induce liver injury. The co-injection of INH and RFP generates metabolic and morphological changes in the liver tissue, as the liver is the primary detoxifying organ for AT drugs. In the current study, a hepatic injury mouse model was developed via i.p. injection of INF/RFP for 3 days. Results indicated that plasma ALT, AST, and IL-6, as well as hepatic MDA and RIP, were increased compared to those in the control mice. JTX shows beneficial effects on human health and has been researched extensively as a potential candidate to prevent various diseases (Anjiki *et al*., 2005; Saiki, 2000). Previous investigations have shown the protective effects of JTX against CCl_4- and ethanol-induced hepatic injury via the attenuation of oxidative stress (Fukaya *et al.*, 2018; Yoshioka *et al.*, 2016). In addition, JTX is reported to decrease inflammation through inhibition of the nuclear factor-kappa B (NF-κB) signaling pathway (Kawamata *et al.*, 2000).

In this study, we explored the effects of JTX on INH/RFP-induced hepatic injury and the possible underlying mechanisms in mice. JTX administration significantly decreased INH/RFP-induced elevation of ALT and AST levels. These results indicate the hepatoprotective effects

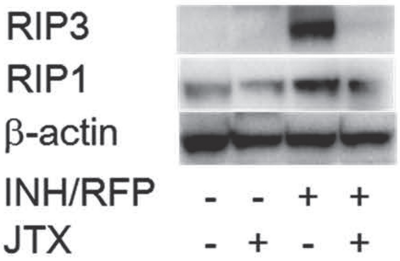

Fig. 4. Effect of JTX on INH/RFP-induced receptor-interacting protein (RIP) expression. Mice were treated according to the methods described in Fig. 1. Mice were sacrificed 24 hr after the final INH/RFP injection. The livers were harvested and proteins were collected. Results represent hepatic RIP1 and RIP3 levels, and β-actin served as an internal control.

of JTX against AT drug-induced liver injury.

Inflammatory cytokines produced during INH/RFP-induced hepatic injury promote tissue damage (Yuhas *et al.*, 2009, 2011). The current study revealed a significant increase in plasma levels of IL-6 in INH/RFP-treated mice. Several studies have reported that inflammatory cytokines such as TNF-α, IL-6, and IL-1β play a critical pathological role in liver necrosis and mediate inflammatory cell activation (Mohammed *et al.*, 2004; Tarantino *et al.*, 2010). Elevated plasma levels of inflammatory mediators in INH/RFP-treated mice may be attributed

to upregulation of liver NF-κB by reactive oxygen species (Czaja, 2007). NF-κB is a transcription factor that is involved in controlling the expression of various genes (Gilmore, 2006). Oral administration of JTX potentially decreased plasma IL-6 levels, and these results suggest that the hepatoprotective effects of JTX against AT drug-induced liver injury may be modulated by inflammatory responses.

Several studies linked INH/RFP-induced hepatic injury to oxidative stress (Chowdhury *et al.*, 2006). In this study, we observed that levels of hepatic MDA, a marker of lipid peroxidation, were markedly increased by INH/RFP administration. JTX also exhibits antioxidant activity against oxidative stress damage. A recent study showed that JTX attenuated oxidative stress by decreasing lipid peroxide levels in CCl_4-exposed livers (Yoshioka *et al.*, 2016). In the current study, JTX treatment attenuated INH/RFP-induced elevation of MDA. The results of the present investigation are in accordance with previous studies showing that JTX exerts significant hepatoprotective effects via inhibition of oxidative stress.

In conclusion, our results show that JTX protects against INH/RFP-induced hepatic injury through inhibition of lipid peroxidation and inflammatory responses.

As we could not identify the specific active ingredient among the 10 different medicinal herbs present in the Kampo formula JTX, further investigations are needed and are currently being performed to identify the active component of JTX. INH/RFP-induced hepatic injury is a common occurrence in patients with tuberculosis and the results from our study are therefore expected to protect against INH/RFP-induced liver disease.

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

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