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Original Article

The Kampo formula "Juzen-taiho-to" exerts protective effects on ethanol-induced liver injury in mice

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ABSTRACT — The aim of this study was to investigate whether the Japanese herbal medicine "Juzen-taiho-to (JTX)" has a protective effect on ethanol (EtOH)-induced liver injury. Seven-week-old male ICR mice were orally administered JTX or saline once a day for three days. Twenty-four hours after the last administration, the mice were intraperitoneally injected with EtOH (2 g/kg). The mice in each group were killed 24 hr after EtOH administration and were bled to obtain plasma. The mice injected with EtOH had high plasma levels of alanine aminotransferase and aspartate aminotransferase and lipid peroxidation. Histopathological examination of the liver of mice treated with EtOH revealed an abnormal outline around the central vein, glycogen depletion, and expression of prostaglandin-endoperoxide synthase 2. Pretreatment with JTX prevented the EtOH-induced increase in the levels of alanine aminotransferase and aspartate aminotransferase, lipid peroxidation, and histopathological changes. Our results suggest that JTX exerts protective effects against EtOH-induced liver disease by modulating oxidative stress and inflammatory response.

Key words: Juzen-taiho-to, Ethanol, Oxidative stress, Inflammation

INTRODUCTION

The liver is an organ vulnerable to attack by chemical toxic agents because it is one of the most important internal organs with multiple crucial functions such as protein synthesis and detoxification (Guo *et al*., 2017). Therefore, liver diseases are among the most serious health problems worldwide. The most common causes of hepatic damage include acute infection with the hepatitis viruses, drug-related liver injury, alcoholic liver disease (ALD), and the alcohol-acetaminophen syndrome. In particular, ALD continues to be the leading cause of mortality and morbidity around the world. ALD is characterized by various symptoms such as hepatocellular carcinoma, ranging from steatosis and steatohepatitis to fibrosis, and cirrhosis (Kanuri *et al*., 2009; Sugimoto and Takei, 2017). Moreover, acute alcohol poisoning by drinking a large amount of alcohol in a short time may lead to death.

Ethanol (EtOH) is metabolized to acetaldehyde via three main pathways (Sugimoto and Takei, 2017). EtOH is metabolized by alcohol dehydrogenase (ADH) in the cytoplasm of liver cells; this is the most important metabolic pathway. The second pathway involves metabolization via cytochrome P450 2E1 (CYP2E1); metabolization via this pathway produces reactive oxygen species (ROS). In addition, EtOH is metabolized by catalase. Acetaldehyde directly induces hepatotoxicity and lipid peroxidation. ROS-induced oxidative stress produces proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-6, which increase inflammation and lead to apoptosis, steatosis, and fibrosis, thereby inducing severe liver damage (Kim *et al*., 2008; Ishii *et al*., 2003; McKillop and Schrum, 2009; Sugimoto and Takei, 2017). Previous studies show that traditional herbal medicines such as cinnamon bark and Lysimachia christinae have protective effects against EtOH-induced liver disease

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(Kanuri *et al*., 2009; Wang *et al*., 2012).

Traditional Japanese herbal medicines such as the Kampo formula, which comprises hot water extracts 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 commonly prescribed Kampo formulations that consists of ten different medicinal herbs (Anjiki *et al*., 2005). JTX is widely prescribed for anemia, rheumatoid arthritis, chronic fatigue syndrome, and inflammatory bowel diseases. In addition, JTX is widely used for the prevention of cancer metastasis and infection in immunocompromised patients (Ohnishi *et al*., 1996; Ohnishi *et al*., 1998).

The results of our previous study showed that pretreatment with JTX prevents acute liver disease (Yoshioka *et al*., 2016a) induced by carbon tetrachloride, which is an established agent that induces liver. Thus, our results suggest that JTX, which contains various active ingredients, may attenuate several kinds of hepatic diseases. Therefore, in this study, we investigated whether pretreatment with JTX reduces EtOH-induced liver damage.

MATERIALS AND METHODS

Animals

Six-week-old male ICR mice (28-30 g) were purchased from Japan SLC (Shizuoka, Japan) and were maintained under standard conditions of controlled temperature (24 ± 1°C), humidity (55 ± 5%), and light (8:00/20:00 light/dark cycles) with free access to water and food. Experimental treatments were performed using 7-week-old mice. After the experiments, any surviving mice were killed using pentobarbital. All experimental protocols were approved by the Institutional Animal Care and Experiment Committee of Kinjo Gakuin University.

Sample preparation

A decoction of JTX was prepared by boiling it with 800 mL of water until it reduced by half. The decoction was filtered and freeze-dried overnight to obtain a dry powder (7.35 g) (Aburada *et al*., 1983). The JTX extract powder (Tsumura Corp. Inc., Tokyo, Japan) was used for comparison. The powder prepared using the decoction of JTX (10 mg) and JTX extract powder (10 mg) was extracted with pure water (2 mL), respectively. The supernatant was filtered using 0.45 μm membrane filter, and then, high-performance liquid chromatography (HPLC) analysis was performed using 10 μL of the sample.

3D-HPLC analysis

HPLC analysis was performed using the COSMOSIL Cholester packed column (4.6 mm i.d. × 250 mm, Nacalai Tesque, Kyoto, Japan) using a PU-2089 Plus pump (JASCO, Tokyo, Japan) and MD-2010 Plus photodiode array detector (JASCO) at 40°C. The mobile phase consisted of A: acetonitrile, B: H_2O (0.05 mol/L sodium dihydrogen phosphate) with a gradient program of 0 min, 10% A; 5-70 min, 10-90% A. The flow rate was 0.8 mL/min, and the elution was monitored at 200-600 nm and analyzed using ChromNAV Ver. 1.14 analysis system software (JASCO).

Experimental design

The animals were randomly divided into four groups. Animals in the JTX group and JTX + EtOH group were orally administered with 1.0 g/kg (5 mL/kg) of the JTX decoction for 3 days at 19:00. Animals in the control and EtOH groups were orally administered saline equivalent to the amount of JTX for 3 days. On the fourth day, intraperitoneal (i.p.) injection of EtOH (2.0 g/kg at 5 mL/kg) or saline (5 mL/kg) was administered at 10:00. At 24 hr after the injection of EtOH or saline, mice from each group were killed and bled to obtain plasma. The livers were stored at −80°C or fixed in 15% neutral buffered formalin (pH 7.4).

Biochemical analysis of plasma

We measured the plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using the Transaminase CII Test Wako (Wako Pure Chemical, Osaka, Japan) according to the manufacturer's instructions and as previously described (Yoshioka *et al*., 2016a).

Measurement of hepatic malondialdehyde levels

Total malondialdehyde (MDA) levels in the liver were examined using a colorimetric thiobarbituric acid reactive substances microplate assay kit according to the manufacturer's instructions as previously described (Yoshioka and Onosaka, 2016b).

Measurement of metallothionein concentrations

Hepatic metallothionein (MT) protein levels were determined using the Cd saturation-hemolysate method (Cd-hem method) as previously described (Yoshioka and Onosaka, 2016b).

Histopathological findings

Hematoxylin and eosin (H&E) or periodic acid Schiff (PAS) staining were performed using standard methodol-

ogies as previously described (Fukaya *et al*., 2017).

Immunohistochemical analysis was performed using paraffin-embedded sections that were deparaffinized and rehydrated in a graded ethanol series. After antigen retrieval using proteinase K (Wako Pure chemical) and quenching of endogenous peroxidase by hydrogen peroxide (Wako Pure chemical), the sections were incubated at 4°C overnight with mouse anti-cyclooxygenase 2 (anti-COX2) monoclonal antibody (Santacruz, CA, USA) as primary antibodies (1:160 dilution). The color reaction was developed using an anti-mouse IgG-FITC (MBL, Aichi, Japan) as secondary antibody (1:160 dilution). In addition, sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

Isolation of total RNA and qRT-PCR assay

Total RNA was extracted from 80 mg liver sections using the ISOGEN II kit (Nippon Gene, Tokyo, Japan). Aliquots (500 ng) of total RNA from each specimen were reverse-transcribed using ReverTraAce qPCR RT Master Mix (TOYOBO, Osaka, Japan) and were incubated for 15 min at 37°C; then, the reaction mixture containing synthesized cDNA was diluted three times with Tris-EDTA buffer. For the quantitative reverse transcription polymerase chain reaction (RT-PCR) assay, 2 μL aliquots of diluted reverse-transcription products were amplified using an Applied Biosystems 7300 system (Applied Biosystems, Foster City, CA) in a reaction mixture containing THUNDERBIRD SYBR qPCR Mix (TOYOBO) and 0.3 μM of each primer. PCR conditions were as follows: at 95°C for 60 sec, and 40 cycles at 95°C for 15 sec and 60°C for 31 sec. The amount of each target mRNA quantified was normalized against that of mRNA encoding β actin. The oligonucleotide sequences of the primers were as follows: sense, 5′- GCAACGAGCGGTTCCG-3′ and antisense, 5′- CCCAAGAAGGAAGGCTGGA-3′ for mouse β actin (NM_007393); sense, 5′-GGTTCTCAACTGGCTATGGC-3′ and antisense, 5′-CAGCCAATGATGACAGACAGAC-3′ for mouse adh1 (NM_007409); sense, 5′-CATTCCTGTGTTCCAGGAGTACAAG-3′ and antisense, 5′-GATACTTAGGGAAAACCTCCGCAC-3′ for mouse Cyp2el (NM_021282); sense, 5′-CAGATGAAGCAGTGGAAGGAG-3′ and antisense, 5′-ACCACATCCTGAACGAGGAG-3′ for mouse catalase (NM_009804); sense, 5′-AACCATCAGCCTGAGGTCTTC-3′ and antisense, 5′-ACCTGGCAGATGACCTCC-3′ for mouse alcohol dehydrogenase 2 (aldh2) (NM_009656); sense, 5′-CATTCCTGTGTTCCAGGAGTACAAG-3′ and antisense, 5′-AGGCTCAGTCCAGACAGGGATT-3′ for mouse H2A histone family (NM_010436); sense, 5′-GCTGCAGAATTGAAAGCCCTC-3′ and antisense, 5′-GCTCGGCTTCCAGTATTGAG-3′ for mouse prostaglandin-endoperoxide synthase 2 (ptgs2) (NM_011198); and sense, 5′-GAACTTCGGGGTGATCGGTC-3′ and antisense, 5′-GTGAGGGTCTGGGCCATAG-3′ for mouse TNF-α (NM_013693).

Statistical analysis

Multiple comparisons were made by one-way analysis of variance (ANOVA) with Tukey-Kramer's post-hoc test or two-way repeated-measures ANOVA. All statistical analyses were performed using the SPSS Statistics for Windows software (version 19.0; IBM Corp., Armonk, NY, USA); differences were considered statistically significant at p values of < 0.05.

RESULTS

3D-HPLC of JTX

We performed 3D-HPLC analysis to compare the powder obtained using the decoction of JTX with the JTX extract powder commercially available from Tsumura Corp. Inc. The fingerprint of 3D-HPLC is shown in Fig. 1. Several peaks were detected in the JTX decoction (Fig. 1A). Although partial peaks obtained using the JTX decoction were different from those of the commercially available powder, we identified major peaks (Fig. 1B). Therefore, we used the JTX decoction for subsequent animal experiments.

The effect of JTX treatment on the plasma levels of biochemical parameters and oxidative

We measured the plasma levels of ALT and AST (Fig. 2) which are known markers of liver injury and dysfunction. The control and JTX groups showed normal levels of ALT (Fig. 2A). Administration of EtOH increased the ALT levels ($p < 0.01$), whereas pretreatment with JTX suppressed the ALT levels ($p < 0.05$). Moreover, the results of AST levels, which is another indicator of hepatotoxicity, were similar to those observed with ALT levels (Fig. 2B).

In addition, we evaluated the hepatic MDA (Fig. 2C) and MT levels (Fig. 2D). MDA is a well-known indicator of lipid peroxidation and MT is an antioxidant. Treatment with EtOH significantly increased the hepatic MDA levels ($p < 0.01$), whereas pretreatment with JTX suppressed the EtOH-induced upregulation of hepatic MDA ($p < 0.05$). Treatment with EtOH significantly increased the hepatic MT levels ($p < 0.01$); however, the hepatic MT levels were similar to the control levels after admin-

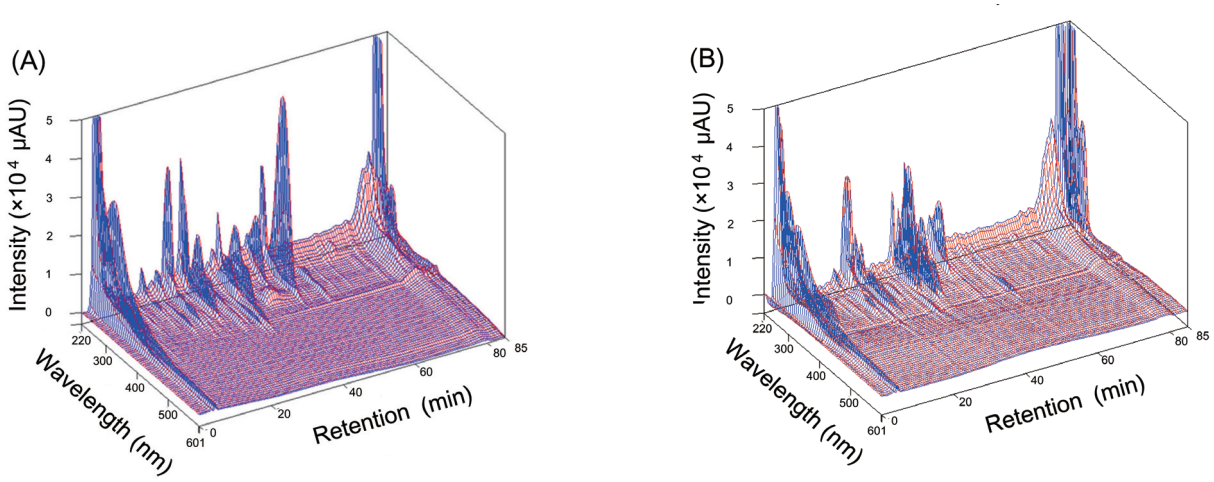

Fig. 1. 3D-HPLC fingerprint of the Juzen-taiho-to. (A) A decoction of Juzen-taiho-to (JTX) was prepared using 800 mL of water and was boiled until the amount reduced by half. The decocted solution was filtered and freeze-dried overnight to obtain dry decocted powder (7.35 g). (B) The JTX extract powder (Tsumura Corp Inc.,) was extracted with pure water. These two solution (5 mg/mL) was filtered and HPLC analysis was performed.

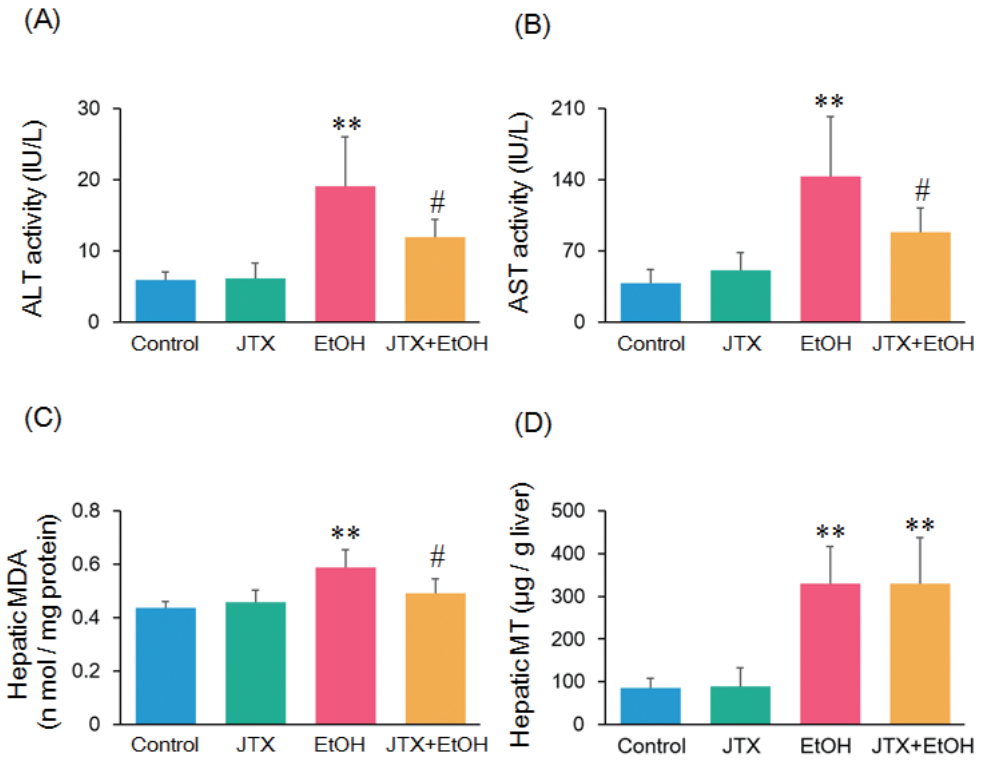

Fig. 2. Effect of pretreatment with Juzen-taiho-to on the levels of alanine aminotransferase and aspartate aminotransferase and hepatic malondialdehyde and metallothionein levels in a mouse model of ethanol-induced liver disease. Mice were orally administered a decoction Juzen-taiho-to (JTX) (1.0 g/kg) for 3 days. On the fourth day, the mice were intraperitoneally (i.p.) administered 2.0 g/kg ethanol (EtOH). After 24 hr, the mice in each group were killed and bled to obtain plasma and liver. The plasma levels of (A) alanine aminotransferase (ALT) and (B) aspartate aminotransferase (AST) and the hepatic levels of liver (C) malondialdehyde (MDA) and (D) metallothionein (MT) are shown in this figure. Data are presented as mean ± standard deviation (SD) of 4-6 mice. $^{**}p < 0.01$ versus control group. $^{\#}p < 0.05$ versus EtOH group.

istration of JTX.

Histopathological analysis of the liver

In addition to the measurement of functional markers, we performed histopathological examination using H&E and PAS staining. Results of H&E staining showed that liver sections obtained from the control group and JTX group showed normal nucleus and cytoplasm (Fig. 3A and 3B). Although obvious liver injury was not observed in the sections obtained from the mice injected with EtOH, we observed an abnormal outline around the central vein (Fig. 3C). The liver section of mice in the JTX + EtOH group showed normal histology of the liver (Fig. 3D). The liver sections from the control group stained with PAS showed many PAS-positive granules within the hepatocytes (Fig. 3E). In addition, the results of the JTX group were similar to those observed in the control group (Fig. 3F). We observed depletion of intrahepatic glycogen in the liver tissue of EtOH group (Fig. 3G), whereas preservation of glycogen was observed in the JTX-treated group (Fig. 3H).

Effect of JTX on EtOH-induced liver toxicity as assessed by qRT-PCR and immunohistochemical analysis

To further investigate the protective effect of JTX against EtOH-induced liver injury, we measured the mRNA levels by qRT-PCR. We compared these levels with middle value. We measured the levels of adh1 (Fig. 4A), Cyp2e1 (Fig. 4B), catalase (Fig. 4C), and aldh2 (Fig. 4D). JTX administration induced a slight but not a significant change in the levels of adh1, Cyp2e1, and catalase. The levels of aldh2 were comparable in all groups. We measured the level of histone 2A X (H2AX) as an indicator of DNA injury since EtOH-induced hepatotoxicity causes the formation of DNA adducts (Yin *et al*., 1999). However, no significant difference was observed in all groups (Fig. 4E). We measured the levels of inflammatory markers ptgs2 (Fig. 4F) and TNF-α (Fig. 4G). While the EtOH group showed high ptgs2 levels (middle value, 0.045), the levels of ptgs2 decreased in the group pretreated with JTX (middle value of control, 0.0027 and JTX + EtOH, 0.006). The results for TNF-α levels were similar to those for ptgs2.

We performed fluorescent immunostaining of the liver sections (Fig. 5) whether to reflect the mRNA levels. The control and JTX groups did not show expression of COX2. The EtOH group showed COX2 expression in the nucleus and cytoplasm. The COX2 expression level decreased in the JTX + EtOH group although a COX2-positive cell was observed in the cytoplasm.

In addition, we determined the localization of H2AX localization in the liver cell using fluorescent immunostaining. None of the groups showed H2AX-positive cells, which indicated that the results of qRT-PCR were similar to those of immunofluorescent staining (data not shown).

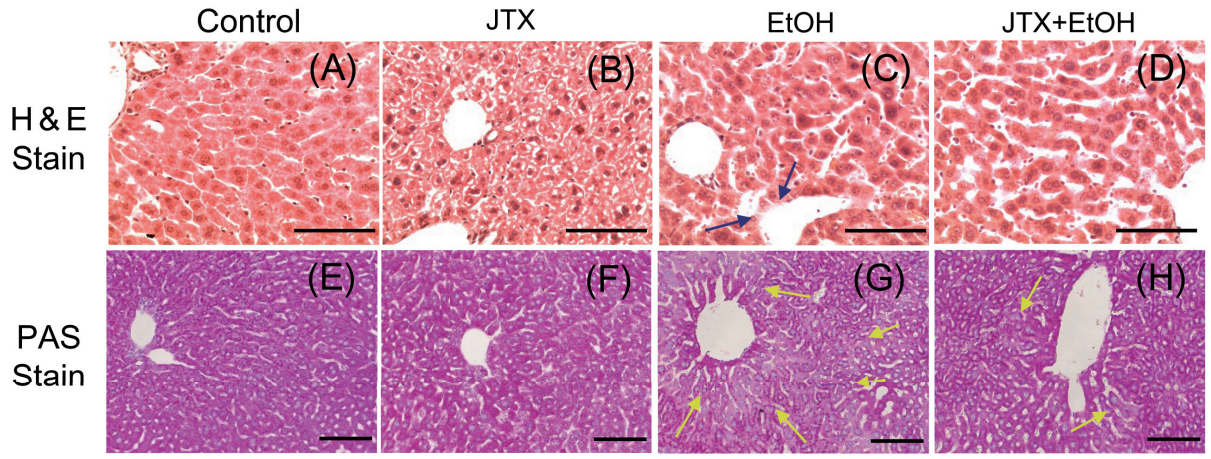

Fig. 3. Histopathological examination of the effect of pretreatment with Juzen-taiho-to on the liver. Mice were treated as described in legend for Fig. 2. The liver specimens were removed, fixed, and processed by standard methods, and sections were stained with hematoxylin and eosin (H&E) (A-D) and periodic acid Schiff (PAS) (E-F) stains. Panels (A) and (E), (B) and (F), (C) and (G), and (D) and (H) indicate liver tissues of control, Juzen-taiho-to (JTX), ethanol (EtOH), and JTX + EtOH groups, respectively. Scale bar indicates 100 μm. Blue and yellow arrows indicate the position of injury and glycogen depletion, respectively.

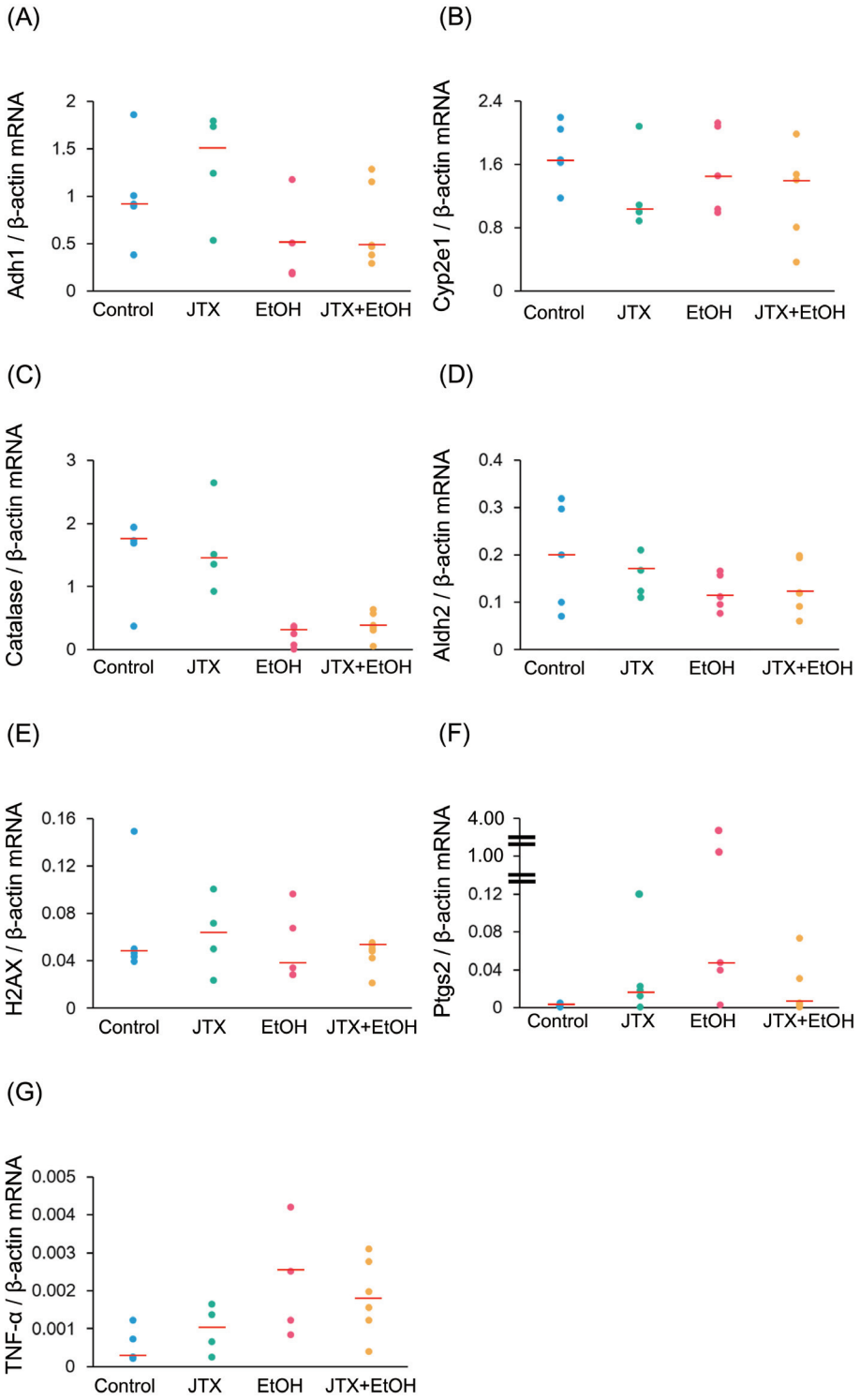

Fig. 4. Effect of pretreatment with Juzen-taiho-to on the levels of total RNA levels. Mice were treated as described in legend for Fig. 2. Panels (A), (B), (C), (D), (E), (F), and (G) indicate the hepatic mRNA levels of *adh1,* cytochrome P450 2E1 (*Cyp2e1*), *catalase, aldh2,* histone 2A X (*H2AX*), prostaglandin-endoperoxide synthase 2 (*ptgs2*), and tumor necrosis factor α (*TNF-α*), respectively. We measured the levels of β-actin as an internal control. Red line indicates central value.

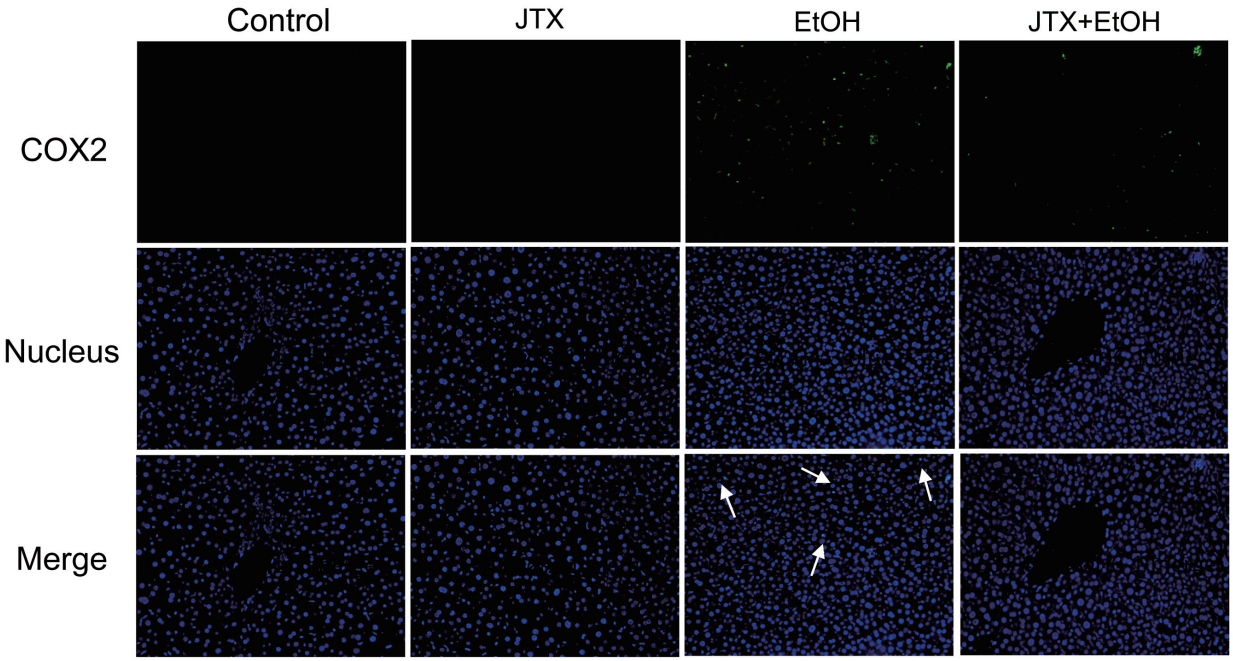

Fig. 5. Effect of pretreatment with Juzen-taiho-to on the liver cyclooxygenase 2 levels determined using fluorescent immunostaining. Mice were treated as described in legend for Figure 2. Liver specimens were removed, fixed, and processed using standard methods. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). The expression and localization of cyclooxygenase 2 (COX2) (green) were analyzed using mouse anti-COX2 monoclonal antibody and anti-mouse IgG-FITC.

DISCUSSION

The established methods for inducing liver injury using EtOH are liquid EtOH diet, long-term administration of EtOH, and administration of a single i.p injection of EtOH (Ki *et al*., 2010; Sugimoto and Takei, 2017; Tokumoto *et al*., 2017; Yin *et al*., 1999). In this study, we induced liver injury by administering a single i.p. injection of EtOH as reported by Tokumoto *et al*., since this is an easy method to induce liver injury (Tokumoto *et al*., 2017).

Administration of EtOH induced an increase in the levels of TNF-α and ptgs2 by increasing oxidative stress and inflammatory response (Sugimoto and Takei, 2017). Our results showed that pretreatment with JTX decreased the levels of *TNF-α* and *ptgs2*. Moreover, the levels of ALT and AST, which are markers of hepatic injury, were decreased by treatment with JTX. Thus, our results indicate that pretreatment with JTX suppresses EtOH-induced liver disease.

EtOH-induced hepatotoxicity involves many factors, including lipid peroxidation and antioxidation (Sugimoto and Takei, 2017; Tokumoto *et al*., 2017). MDA is widely used as a major marker of lipid peroxidation. In addition, EtOH-induced liver disease is associated with an increase lipid peroxidation and free radical production. Our results showed that i.p. administration of EtOH increased hepatic MDA levels, whereas pretreatment with JTX decreased the EtOH-induced increase in MDA levels. These finding suggest that JTX improves EtOH-induced liver disease by preventing lipid peroxidation.

MT is a low-molecular-weight protein with a high cysteine content that has been proposed to play a protective role against oxidative stress. MT I/II knockout mice are more sensitive to EtOH-induced hepatotoxicity (Tokumoto *et al*., 2017). Moreover, i.p. administration of JTX induces an increase in hepatic MT (Yoshioka *et al*., 2016a). Our results showed that administration of JTX did not increase in the hepatic MT levels, which suggests that MT does not involve a protective role in the present investigation.

Production of acetaldehyde is factor underlying EtOH-induced liver disease. EtOH is metabolized to acetaldehyde by adh, Cyp2e1, and catalase. A previous study shows that Cyp2e1 knockout mice inhibit EtOH-induced liver disease (Abdelmegeed *et al*., 2013). Further, aldh2 is

important factor that converts acetaldehyde to acetic acid. Kwon *et al*. reported increased levels of inflammatory cytokines in aldh2 knockout mice (Kwon *et al*., 2014). Our results show that pretreatment with JTX did not induce a significant decrease in the levels of Cyp2e1 and aldh2. In addition, no significant change was observed in the levels of adh1 and catalase, which are important enzymes governing acetaldehyde production. Therefore, acetaldehyde-associated factors are not involved in the mechanism underlying suppression of liver disease by pretreatment with JTX.

In conclusion, our results show that pretreatment with JTX suppresses EtOH-induced liver disease through inhibition of lipid peroxidation and inflammatory response.

We were unable to identify the specific active ingredient among the 10 different medicinal herbs present in the Kampo formula JTX. Further studies are currently being performed to identify the active component of JTX. EtOH-induced liver disease is a common disease that affects a large number of patients. The results from our study are expected to protect against EtOH-induced liver disease.

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

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