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Saikokeishito Extract Exerts a Therapeutic Effect on α-Naphthylisothiocyanate-Induced Liver Injury in Rats through Attenuation of Enhanced Neutrophil Infiltration and Oxidative Stress in the Liver Tissue

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Summary **We examined whether Saikokeishito extract (TJ-10), a traditional Japanese herbal medicine, exerts a therapeutic effect on α-naphthylisothiocyanate (ANIT)-induced liver injury in rats through attenuation of enhanced neutrophil infiltration and oxidative stress in the liver tissue. In rats treated once with ANIT (75 mg/kg, i.p.), liver injury with cholestasis occurred 24 h after treatment and progressed at 48 h. When ANIT-treated rats orally received TJ-10 (0.26, 1.3 or 2.6 g/kg) at 24 h after the treatment, progressive liver injury with cholestasis was significantly attenuated at 48 h after the treatment at the dose of 1.3 or 2.6 g/kg. At 24 h after ANIT treatment, increases in hepatic lipid peroxide and reduced glutathione contents and myeloperoxidase activity occurred with decreases in hepatic superoxide dismutase and glutathione reductase activities. At 48 h after ANIT treatment, these changes except for reduced glutathione were enhanced with decreases in catalase, Se-glutathione peroxidase, and glucose-6-phosphate dehydrogenase activities. TJ-10 (1.3 or 2.6 g/kg) post-administered to ANIT-treated rats attenuated these changes found at 48 h after the treatment significantly. These results indicate that TJ-10 exerts a therapeutic effect on ANIT-induced liver injury in rats possibly through attenuation of enhanced neutrophil infiltration and oxidative stress in the liver tissue.**

Key Words**: α-naphthylisothiocyanate, liver injury (rat), Saikokeishito extract (TJ-10), neutrophil infiltration, oxidative stress**

Introduction

The hepatotoxicity of α-naphthylisothiocyanate (ANIT) in experimental animals is thought to be useful for studying the mechanisms of drug-induced cholestasis, because cholestasis and hepatic damage resulting from the administration of certain drugs (e.g., erythromycin estolate, chlorpromazine, and others) to humans are mimicked by ANIT administration to rats [*1*]. The mechanisms of ANIT-induced liver injury have been proposed but have not been entirely clarified [*2*]. It has been suggested the contribution of neutrophils, i.e.,

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polymorphonuclear leukocytes, to the development of ANIT-induced liver injury in rats through a mechanism independent of the production of reactive oxygen species (ROS) [*3*]. It has been shown in an *in vitro* experiment that ANIT causes neutrophils to release toxic proteases, which cause hepatocellular damage, and that ANIT causes bile duct epithelial cells to release a factor(s) that attracts neutrophils and stimulates them to injure hepatocytes [*4*, *5*]. In contrast, we have suggested that lipid peroxidation induced by ROS derived from infiltrated neutrophils might be closely associated with the formation and progression of ANIT-induced acute liver injury in rats [*6*]. Our previous reports showed that, in the liver of rats treated once with ANIT, the activities of superoxide dismutase (SOD), an enzyme to scavenge superoxide radical ($O_2^{\cdot -}$), and glutathione reductase (GSSG-R), an enzyme to regenerate reduced glutathione (GSH) from oxidized glutathione (GSSG) using NADPH, decreased with the formation and progression of liver injury [*7–9*]. In addition, the hepatic activities of catalase, an enzyme to decompose hydrogen peroxide (H_2O_2), and Se-glutathione peroxidase (Se-GSH-Px), an enzyme to metabolize H_2O_2 and lipid hydroperoxides using GSH as a co-substrate, transiently increased before the appearance of ANIT-induced liver injury, although hepatic catalase and Se-GSHpx activities decreased at a progressed stage of the injury [*7–9*]. In contrast, hepatic GSH content remained increased during the formation and progression of ANIT-induced liver injury [*7–9*].

Saikokeshito (Chai-Hu-Gui-Zhi-Tang) is a traditional Japanese herbal medicine, i.e., Kampo medicine, which is composed of 9 herbs such as *Bupleuri Radix*, *Pinelliae Tuber*, *Scutellariae Radix*, *Glycyrrhizae Radix*, *Cinnamomi Cortex*, *Paeonia Radix*, *Ziyphi Fructus*, *Ginseng Radix*, and *Zingibers Rhizoma*. This Kampo medicine is clinically used for treatments of duodenal ulcers, pancreatitis, and chronic liver diseases in Japan. Feeding a diet containing a spray-dried material of Saikokeishito extract (Tsumura TJ-10) is known to protect against liver injury induced by D-galactosamine-induced liver injury with reduction of increased hepatic lipid peroxide (LPO) level in mice [*10*] and gut ischemia/reperfusion-induced liver injury in rats through nitric oxide (NO)-mediated inhibition of neutrophil infiltration into the liver tissue [*11*]. Our previous report has shown that a single oral administration of TJ-10 to ANIT-treated rats prevents the progression of liver injury with cholestasis and attenuates an enhanced increase in serum LPO level [*12*]. TJ-10 is known to exert an antioxidant action by inhibiting lipid peroxidation *in vitro* [*13*]. TJ-10 is also known to possess an activity to scavenge ROS such as $O_2^{\cdot -}$ and hydroxyl radical ($^{\cdot}OH$) *in vitro* [*14*]. However, it is still unclear whether TJ-10 exerts a therapeutic effect on ANIT-induced liver injury in rats through attenuation of enhanced neutrophil infiltration and oxidative stress associated with disrupted antioxidant defense systems in the liver.

In order to clarify the mechanism for the therapeutic effect of TJ-10 on ANIT-induced liver injury with cholestasis, we, therefore, examined whether orally administered TJ-10 attenuates enhanced hepatic neutrophil infiltration and oxidative stress associated with disrupted antioxidant defense systems at a progressed stage of liver injury in rats treated once with ANIT. Namely, we examined the effect of orally administered TJ-10 on liver injury progression and changes in hepatic LPO and GSH contents and the hepatic activity of myeloperoxidase (MPO), an index of tissue neutrophil infiltration [*15–17*], with liver injury progression in rats treated once with ANIT. We also examined the effect of TJ-10 administration on the hepatic activities of antioxidant enzymes such as SOD, catalase, Se-GSHpx, GSSG-R, and glucose-6-phosphate dehydrogenase (G-6-PDH), an enzyme to produce NAPDH using glucose-6-phospahte, with liver injury progression in ANIT-treated rats.

Materials and Methods

Chemicals

TJ-10 is a spray-dried material of Saikokeishito extract. This herbal medicine was kindly provided by Tsumura & Co. (Tokyo, Japan). TJ-10 was prepared from a boiled water extract of the following herbs: 7.0 g *Bupleuri Radix* (*Bupleuri falcatum* Linne), 4.0 g *Pinelliae Tuber* (*Pinellia ternate* Breitenbach), 2.0 g *Scutellariae Radix* (*Scutellaria baicalensis* Georgi), 2.0 g *Glycyrrhizae Radix* (*Glycyrrhiza glabra* Linne), 2.0 g *Cinnamomi Cortex* (*Cinnamomum cassia* Blume), 2.0 g *Paeonia Radix* (*Paeonia albiflora* Pallas) 2.0 g *Zizyphi Fructus* (*Zizyphus vulgaris* Lamark), 2.0 g *Ginseng Radix* (*Panax ginseng* C.A. Meyer), and 1.0 *Zingiberis Rhizoma* (*Zingiber officinale* Roscoe). The extraction percentage of the prepared TJ-10 was 18.2%. The main components present in the TJ-10 preparation were confirmed by analysis using high-performance liquid chromatography (HPLC) with spectrophotometric detection as follows: TJ-10 preparation (1.0 g) was extracted with methanol (20 ml) under ultrasonication for 30 min. The solution was filtrated and then submitted for HPLC analysis. HPLC equipment was controlled with a HPLC pump (LC-10AD, Shimadzu, Kyoto, Japan) using a TSK-GEL 80TS column (4.6 Ø × 250 cm) (TOSOH, Tokyo, Japan), eluting with solvents (A) 0.05 mM acetic acid-ammonium acetate buffer (pH 3.6) and (B) acetonitrile. A linear gradient of 90% A and 10% B changing over 60 min to 0% A and 100% B was used. The flow rate was 1.0 ml/min. The eluate from the column was monitored in a 200 and 400 nm wavelength range, and the three-dimensional data were processed by a diode array detector, SPD-M10A (Shimadzu, Kyoto, Japan). The three-dimensional HPLC chart of the methanol solution of TJ-10 preparation is shown in Fig. 1. This TJ-10

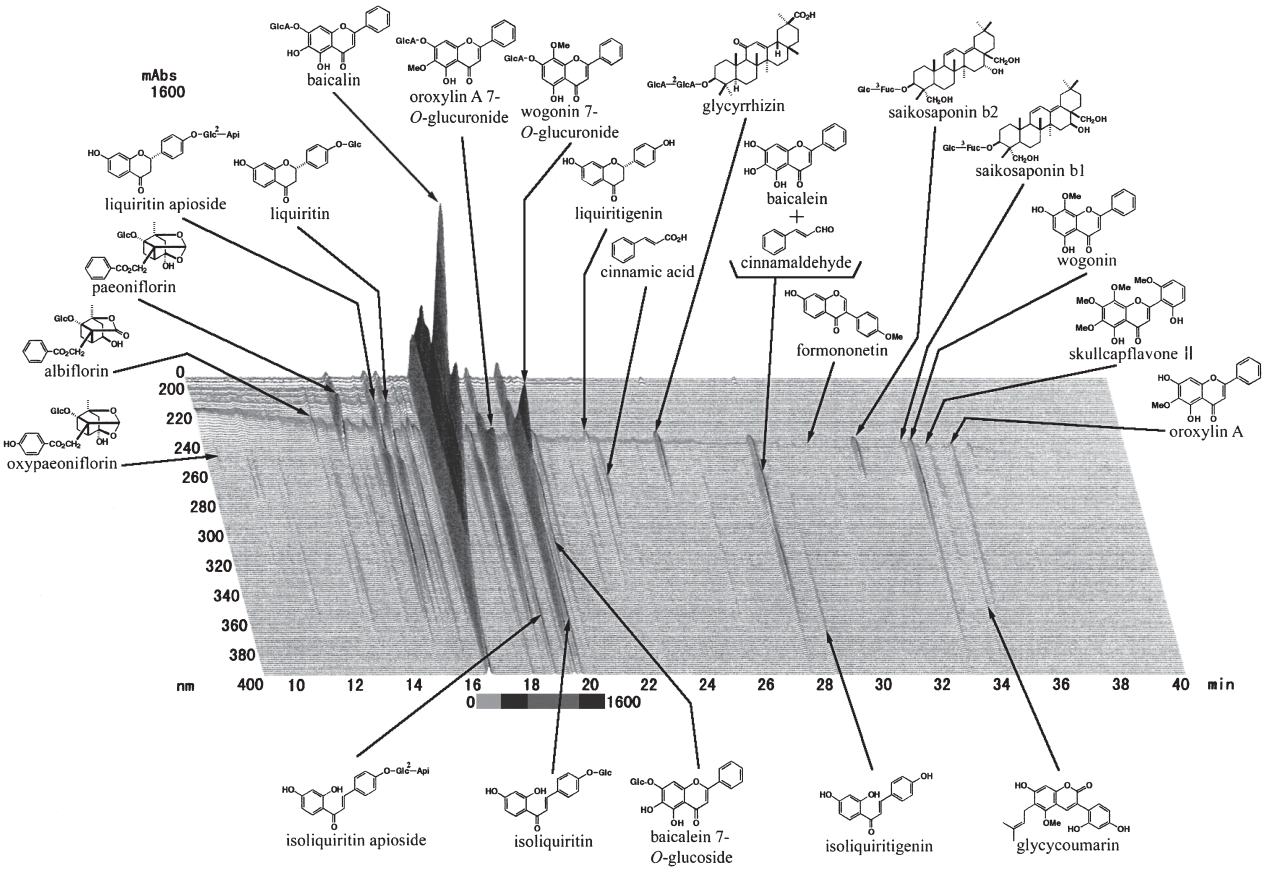

Fig. 1. Three-dimensional HPLC profile of main components in the methanol extract of TJ-10.

contained saikosaponin b1, saikosaponin b2 (derived from *Bupleuri Radix*), baicalin, oroxykin A, oroxykin A 7-O-glucuronide, wogonin, wogonin 7-O-glucuronide, baicalein, baicalein 7-O-glucoside, skullapflavone II (from *Scutellariae Radix*), liquirtin, liquiritin apioside, liquiritigenin, isoliquiritin, isoliqiritin apioside, isoliquiritigenin, glycyrrhizin, formomontetin, glycycoumarin (derived from *Glycyrrhizae Radix*), cinnamic acid, cinnamaldehyde (from *Cinnamomi Cortex*), oxypaeoniflorin, albiflorin, and paeoniflorin (from *Paeonia Radix*). ANIT, 3,3',5,5'-tetramethylbenzidne, and xanthine were purchased from Sigma Chemical Co. (St. Louis, MO); milk xanthine oxidase and SOD purified from bovine erythrocytes were from Roche-Diagnostics (Tokyo, Japan): *N*,*N*-dimethylformamide, ethylenediaminetetraacetic acid (EDTA), NADPH, $NADP^+$, GSH, GSSG, 2-thiobarbituric acid, yeast GSSG-R, and other chemicals were from Wako Pure Chemical Industry Ltd. (Osaka, Japan). These reagents were used without further purification.

Animals

Male Wistar rats aged 6 weeks were purchased from Nippon SLC Co. (Hamamatsu, Japan). The animals were maintained under a daily controlled 12 h-light, 12 h-dark lighting cycle at 23ºC and 50% humidity with free access to rat chow (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and water for one week prior to the experiments. All animals received humane care in compliance with the guidelines of the Management of Laboratory Animals in Fujita Health University, Japan.

ANIT and TJ-10 treatments and sample collection

Seven-week-old rats fasted for 15 h received an intraperitoneal (i.p.) injection of ANIT, dissolved in olive oil, at a dose of 75 mg/kg body weight (BW), i.e., 1 ml of an ANIT solution in olive oil (75 mg/ml) per 100 g BW, in order to induce liver injury, as described previously [*7–9*]. The control rats were age-matched and also fasted for 15 h. They received an i.p. injection of the same volume of olive oil. At 24 h after the initial ANIT or vehicle injection, TJ-10 (0.26, 1.3 or 2.6 g/kg BW), suspended in 1 ml of distilled water, was orally administered to rats with and without ANIT injection. Rats without TJ-10 administration received a single oral administration of the same volume of distilled water at the same time point. Each rat fasted for 15 h was sacrificed under ether anesthesia at 24 or 48 h after the initial ANIT or vehicle injection at which time blood was collected

form the inferior vena cava. The collected blood was separated into serum by centrifugation. Immediately after sacrifice, the livers were well perfused with ice-cold 0.15 M NaCl and then isolated, washed well in ice-cold 0.15 M KCl, blotted on a filter, and weighed as soon as possible. The livers and sera obtained were stored at −80°C until use.

Assays of serum enzymes and components

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed using a commercial test kit of Iatrozyme TA-L_Q (Dai-Iatron Co., Tokyo, Japan). Serum γ-glutamyl transpeptidase (γ-GTP) was assayed using a commercial test kit of γ-GTP C-Test Wako (Wako Pure Chemical Industry Ltd., Osaka, Japan), respectively. These enzyme activities are expressed as an international unit (IU/l). Serum total bilirubin was assayed using commercial test kits of Bilirubin BII-Test Wako (Wako Pure Chemical Industry Ltd., Osaka, Japan), respectively. Serum ALT and AST were used as indices of hepatic cell damage and serum γ-GTP and total bilirubin as indices of biliary cell damage and cholestasis.

Assays of hepatic components and enzymes

Livers were homogenized in 9 volumes of ice-old 0.15 M KCl containing 1.0 mM EDTA using a glass homogenizer with a Teflon pestle. This homogenate was used for hepatic LPO and GSH assays. Hepatic LPO was assayed by the method of Ohkawa *et al*. [*18*] using the thiobarbituric acid reaction except that 1.0 mM EDTA was added to the reaction medium. The concentration of hepatic LPO is expressed as that of MDA equivalents. Hepatic GSH was assayed by the method of Sedlak and Lindsay [*19*] using Ellman's reagent. The prepared liver homogenate was sonicated on ice for 30 s × 2 times using a Handy Sonic model UR-20P (Tomy Seiko Co., Tokyo, Japan). The sonicated homogenate was centrifuged at 12,000 × g for 20 min and the resultant supernatant was dialyzed against 100 volumes of 0.05 M Tris-HCl buffer (pH 7.4) for 1 h using a micro-dialysis device (Molecular weight cut-off = 3,500) (Bio-Tec International Inc., Belleuve, WA). These treatments were performed at 4°C. The dialyzed supernatant was used for hepatic MPO, SOD, catalase, Se-GSH-Px, GSSG-R, and G-6-PDH assays. MPO present in liver tissues was assayed as follows: the dialyzed supernatant was incubated at 60°C for 2 h to increase the recovery of MPO in liver tissues according to the method of Schierwagen *et al*. [*16*]. MPO activity in the heat-treated liver tissue sample was assessed by measuring the H_2O_2-dependent oxidation of TMB (dissolved in dimethylsulfoxide) at 37°C according to the method of Suzuki *et al*. [*20*]. This TMB oxidation was measured spectrophotometrically at 655 nm. One unit of this enzyme activity is expressed as the amount of enzyme causing a change in absorbance of 1.0 min^{-1} at 655 nm. Hepatic SOD, catalase, Se-GSH-Px, GSSG-R, and G-6-PDH were assayed by the methods of Oyanagui [21]. Bergmeyer [*22*], Hochstein and Utley [*23*], Lopez-Barea and Lee [*24*], and Bergmeyer *et al*. [*25*], respectively. SOD activity was determined at 37°C by the XO-NH_2OH method using purified bovine erythrocyte SOD (5000 units/mg solid) as a standard. This enzyme activity is expressed as the amount of the erythrocyte SOD showing activity equivalent to the determined activity. Catalase activity was measured at 37°C by recording H_2O_2 decomposition at 240 nm. One unit (U) of this activity is defined as the amount of enzyme decomposing 1 μmol H_2O_2 as a substrate per min. Se-GSH-Px activity was determined at 37°C by recording the decrease in absorbance at 340 nm following the oxidation of NADPH in the presence of H_2O_2, GSH, yeast GSSG-R, and NaN_3 as a catalase inhibitor. One unit (U) of this activity is defined as the amount of enzyme oxidizing 1 μmol NADPH per min. GSSG-R activity was measured at 37°C by checking the oxidation of NADPH following the reduction of GSSG to GSH at 340 nm. One unit (U) of this activity is defined as the amount of enzyme oxidizing 1 μmol NADPH per min. G-6-PDH activity was measured at 37°C by checking the production of NADPH from $NADP^+$ in the presence of glucose-6-phosphate at 340 nm. One unit (U) of this activity is defined as the amount of enzyme producing 1 μmol NADPH per min. Protein in liver tissue samples was measured by the method of Lowry *et al*. [*26*] using bovine serum albumin as a standard.

Histological examination

Liver samples were taken from the central part of the right large lobe of ANIT-treated and untreated rats with and without post-TJ-10 administration at 48 h after the treatment. They were fixed with 10% formalin in phosphate buffered saline for 24 h and then washed with tap water, dehydrated in alcohols, and embedded in paraffin. Sections 6–7 μm thick were mounted in glass slides. Staining with hematoxylin and eosin (H-E) was performed in each slide and then histological examination was conducted under light microscopy.

Statistical analysis

All values obtained are expressed as the mean ± standard deviation (SD). All data were statistically analyzed by computerized statistical packages (StatView). Each mean value is compared by one-way analysis of variance and Fisher's protected significant difference for multicomparison as the post hoc test. The level of significance was set at $p<0.05$.

Results

Serum ALT and AST activities, indices of hepatic cell damage, in the ANIT-treated group significantly increased

24 h after treatment with further their increases at 48 h when compared with those in the control group (Fig. 2). TJ-10 administered orally to ANIT-treated rats at 24 h after the treatment significantly reduced the enhanced increases in serum ALT and AST activities at 48 h after the treatment at its dose of 1.3 or 2.6 g/kg BW, but not 0.26 g/kg BW (Fig. 2A and B). In the ANIT-treated group, serum γ-GTP activity and total bilirubin concentration, which are indices of biliary cell damage and cholestasis, significantly increased 24 h after treatment with further their increases at 48 h when compared with those in the control group (Fig. 2 C and D). The TJ-10 post-administered to ANIT-treated rats significantly reduced the enhanced increases in serum γ-GTP activity and bilirubin concentration at 48 h after the treatment at its dose of 1.3 or 2.6 g/kg BW, but not 0.26 g/kg BW (Fig. 2C and D). The increased serum ALT and AST activities in the ANIT-treated group given TJ-10 (2.6 g/kg BW) were near those in the ANIT-treated group found at 24 h after the treatment (Fig. 2A and B). The increased serum γ-GTP activity in the ANIT-treated group given TJ-10 (1.3 or 2.6 g/kg BW) was almost equal to that in the ANIT-treated group found at 24 h after treatment (Fig. 2C). The same doses of TJ-10 given to ANIT-untreated did not affect the serum ALT, AST, and γ-GTP activities and total bilirubin concentration (Fig. 2).

The liver sections stained by H-E in ANIT-treated rats post-administered with and without TJ-10 and untreated control rats were examined for necrosis and the extent of inflammation at 48 h after the treatment. Hepatocytes in the untreated control group showed little histological changes (Fig. 3A). Hepatocytes in the group treated with ANIT alone presented necrotic and degenerative changes with severe inflammatory cell infiltration (Fig. 3B). In contrast, hepatocytes in the ANIT-treated group post-administered with TJ-10 (2.6 g/kg BW) presented clearly less necrotic and degenerative changes and less inflammatory cell infiltration (Fig. 3C).

Hepatic MPO activity in the ANIT-treated group was significantly higher than that in the control group at 24 h after treatment and the increase in hepatic MPO activity in the ANIT-treated group was enhanced at 48 h (Fig. 4). TJ-10 (1.3 or 2.6 g/kg BW) post-administered to ANIT-treated rats significantly reduced the enhanced increase in hepatic MPO activity at 48 h after the treatment (Fig. 4). The hepatic MPO activity in the ANIT-treated group given TJ-10 (1.3 or 2.6 g/kg BW) was not significantly different from that in the ANIT-treated group found at 24 h after the treatment. The same doses of TJ-10 given to ANIT-untreated did not affect the hepatic MPO activity (Fig. 4).

Significant increases in hepatic LPO and GSH contents in the ANIT-treated group were found at 24 h after the treatment when compared with those in the control group and further increase in the hepatic LPO content occurred

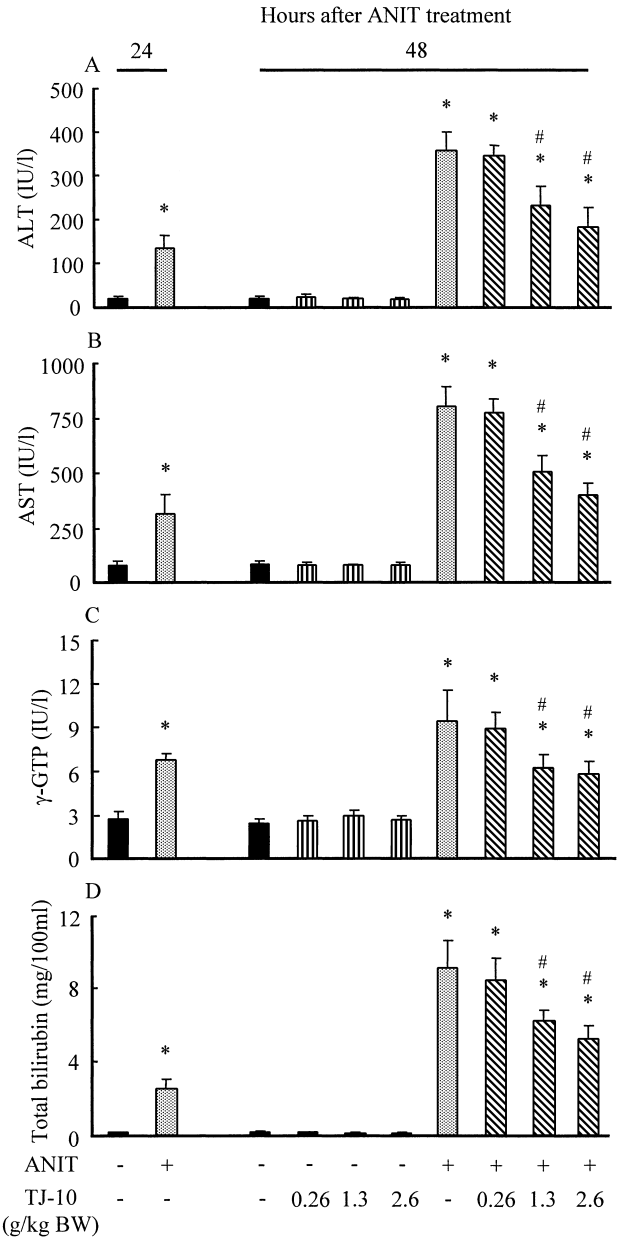

Fig. 2. Effect of orally post-Administered TJ-10 on serum AST (A), ALT (B), and γ-GTP (C) activities and total blirubin concentration (D) in rats treated with and without ANIT. TJ-10 (0.26, 1.3 or 2.6 g/kg BW) was orally administered to rats treated with and without ANIT (75 mg/kg BW, i.p.) 24 h after the treatment. Rats not given TJ-10 received vehicle at the same time point. AST, ALT, γ-GTP, and total bilirubin in the serum of each rat were assayed 24 or 48 h after ANIT treatment as described in Materials and Methods. Each value is a mean ± SD (n = 5 for ANIT-untreated rats with and without TJ-10 administration; n = 8 for ANIT-treated rats with and without TJ-10 administration). *Significantly different from control rats without any treatment, $p<0.05$. #Significantly different from ANIT-treated rats without TJ-10 administration, $p<0.05$.

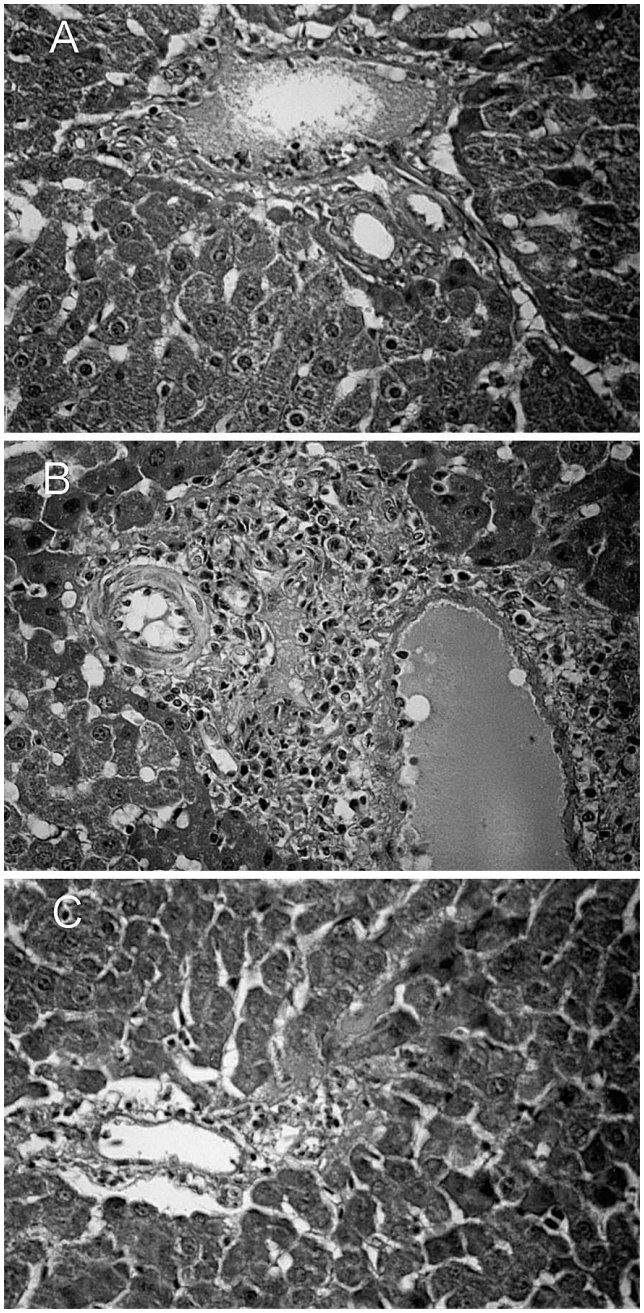

Fig. 3. Histological figures of liver cells from control rats and ANIT-treated rats with and without TJ-10 post-administration. A, untreated control group: little histological change was observed; B, ANIT-treated group: necrotic and degenerative changes with severe inflammatory cell infiltration were observed; C, ANIT-treated group with oral administration ofTJ-10 (2.6 g/kg BW) which was conducted 24 h after the treatment: dramatic decreases in necrotic and degenerative changes and inflammatory cell infiltration were seen. (H-E, original magnification × 100).

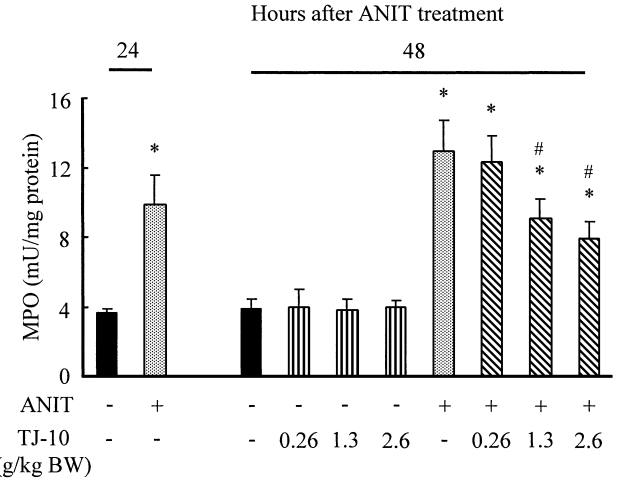

Fig. 4. Effects of orally post-administered TJ-10 on hepatic MPO activity in rats treated with and without ANIT. Experimental condition and explanation are the same as described in the legend for Fig. 2 except that hepatic MPO was assayed as described in Materials and Methods.

with a slight decrease in the increased hepatic GSH content at 48 h (Fig. 5). The post-administration of TJ-10 to ANIT-treated rats at a dose of 1.3 or 2.6 g/kg BW, but not 0.26 g/kg BW, significantly reduced the enhanced increase in hepatic LPO content at 48 h after the treatment (Fig. 5A). TJ-10 post-administered at a dose of 2.6 g/kg BW significantly attenuated the increased hepatic GSH content at 48 h after ANIT treatment (Fig. 5B). The hepatic LPO content in the ANIT-treated group given TJ-10 (1.3 or 2.6 g/kg BW) was not significantly different from that in the ANIT-treated group found at 24 h after the treatment (Fig. 5A). The hepatic GSH content in the ANIT-treated group given TJ-10 (2.6 g/kg BW) was not significantly different from that in the control group (Fig. 5B). The same doses of TJ-10 given to ANIT-untreated rats did not affect the hepatic LPO and GSH contents (Fig. 5).

Hepatic SOD and GSSG-R activities in the ANIT-treated group were significantly lower than those in the control group at 24 h after treatment, while there were no significant differences in hepatic catalase, Se-GSHpx, and G-6-PDH activities between the ANIT-treated and untreated control rats at 24 h (Figs. 6 and 7). In the ANIT-treated group, further decreases in hepatic SOD and GSSG-R activities occurred with decreases in hepatic catalase, Se-GSHpx, and G-6-PDH activities at 48 h after the treatment (Figs. 6 and 7). TJ-10 post-administered to ANIT-treated rats at a dose of 1.3 or 2.6 g/kg BW, but not 0.26 g/kg BW, significantly reduced the enhanced decreases in hepatic SOD and GSSG-R activities and the decreases in hepatic catalase, Se-GSHpx, and G-6-PDH activities at 48 h after the treatment (Figs. 6 and 7). The hepatic SOD activity in the ANIT-treated group given TJ-10 (1.3 or 2.6 g/kg BW) was not significantly

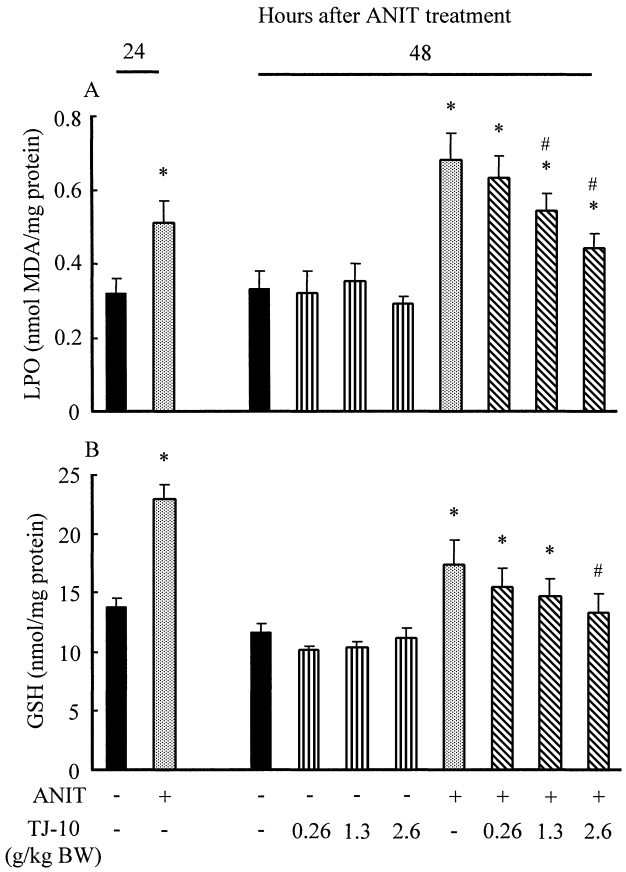

Fig. 5. Effect of orally post-administered TJ-10 on hepatic LPO (A) and GSH (B) contents in rats treated with and without ANIT. Experimental condition and explanation are the same as described in the legend for Fig. 2 except that hepatic LPO and GSH were assayed as described in Materials and Methods.

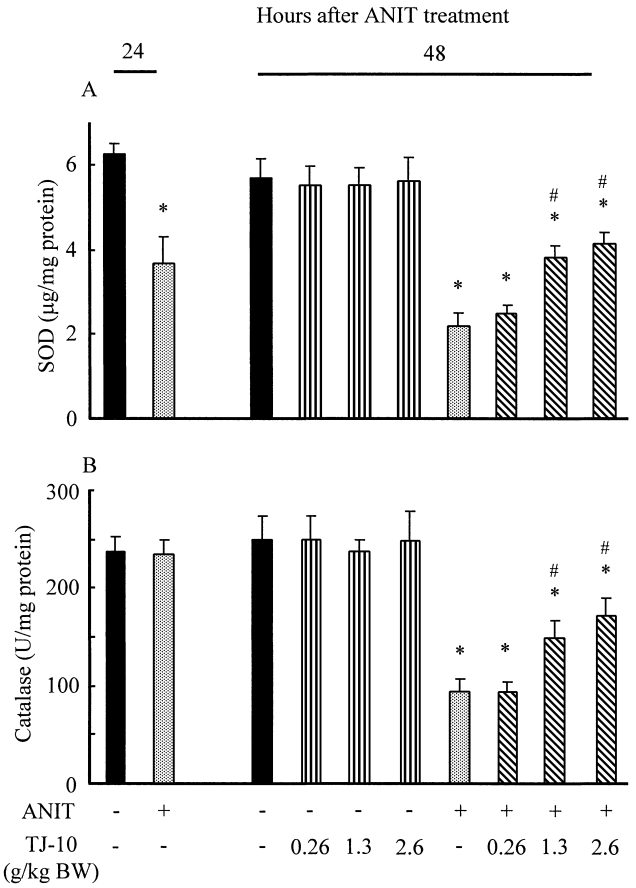

Fig. 6. Effect of orally post-administered TJ-10 on hepatic SOD (A) and catalase (B) activities in rats treated with and without ANIT. Experimental condition and explanation are the same as described in the legend for Fig. 2 except that hepatic SOD and catalse were assayed as described in Materials and Methods.

different from that in the ANIT-treated group found at 24 h after the treatment (Fig. 6A) In addition, the ANIT-treated group given TJ-10 (2.6 g/kg BW) had as much hepatic G-6-PDH activity as the control group (Fig. 7C). The same doses of TJ-10 given to ANIT-untreated rats did not affect the hepatic SOD, GSSG-R, catalase, and Se-GSHpx activities but the hepatic G-6-PDH activity in ANIT-untreated group given TJ-10 at a dose of 2.6 g/kg BW, but not 0.26 or 1.3 g/kg BW, was significantly higher than that in untreated control rats (Figs. 6 and 7).

Discussion

In the present study, rats treated with ANIT exhibited apparent liver injury with cholestasis at 24 h after treatment and progressed liver injury at 48 h, judging from the serum levels of ALT, AST, γ-GTP, and bilirubin, as shown in our previous reports [*6–9*, *12*]. When TJ-10 (0.26, 1.3 or 2.6 g/kg BW) was orally administered to the ANIT-treated rats at 24 h after the treatment at which time liver injury had appeared, progressive liver injury associated with liver cell damage and biliary cell damage with cholestasis was significantly attenuated at its does of 1.3 or 2.6 g/kg BW, judging from the serum levels of hepatobiliary markers, as shown in our previous report [*12*]. In addition, the post-administration of TJ-10 (2.6 g/kg BW) prevented the progression of ANIT-induced liver injury almost completely. This preventive effect of TJ-10 on ANIT-induced liver injury was confirmed by histological observation. Thus, it has been shown clearly that orally administered TJ-10 exerts a therapeutic effect on ANIT-induced liver injury with cholestasis in rats.

It is generally accepted that infiltrating neutrophils play an important role in the development of ANIT-induced liver injury [*2–5*]. In the present study, an enhanced increase in the hepatic activity of MPO, an index of tissue neutrophil infiltration [*15–17*], occurred at a progressed stage of ANIT-induced liver injury, as shown previously [*6–9*]. TJ-10 (1.3 or 2.6 g/kg BW) administered orally to ANIT-treated rats

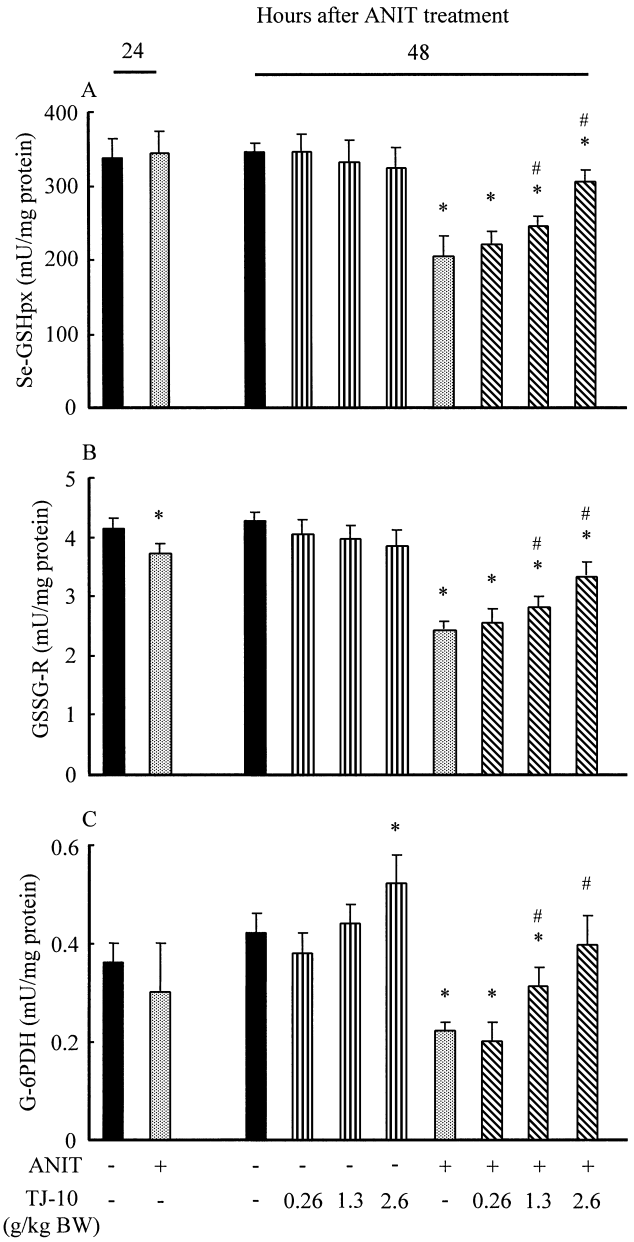

Fig. 7. Effect of orally post-administered TJ-10 on hepatic Se-GSHpx (A), GSSG-R (B), and G-6-PDH (C) activities in rats treated with and without ANIT. Experimental condition and explanation are the same as described in the legend for Fig. 1 except that hepatic Se-GSHpx, GSSG-R, and G-6-PDH were assayed as described in Materials and Methods.

after the appearance of liver injury significantly reduced the enhanced increase in hepatic MPO activity found at a progressed stage of liver injury. In addition, both doses of the herbal medicine attenuated the enhanced increase in hepatic MPO activity completely. These results suggest that orally administered TJ-10 exerts a therapeutic effect on ANIT-induced liver injury in rats by inhibiting enhanced neutrophil infiltration into the liver tissue. TJ-10 is known to protect against gut ischemia/reperfusion-induced liver injury in rats by NO-mediated inhibition of neutrophil infiltration into the liver [*11*]. Accordingly, there may be a possibility that post-administered TJ-10 inhibits enhanced neutrophil infiltration into the liver of ANIT-treated rats by the NO-mediated mechanism. This possibility should be clarified in the future research.

Our previous reports suggested that lipid peroxdation might be closely associated with the development of ANIT-induced liver injury in rats [*6*, *7*, *9*]. TJ-10 is known to inhibit lipid peroxidation *in vitro* [*13*] and to scavenge ROS such as $O_2^{\cdot -}$ and $^{\cdot}OH$, which are generated by the reaction between $O_2^{\cdot -}$ and H_2O_2 (Haber-Weiss reaction) or by the reaction between H_2O_2 and tradition metals (Fenton reaction), *in vitro* [*14*]. Gao *et al*. [*27*] have reported that baicalin, baicalein, and wogonin in *Scuttellariae Radix*, one of the components of Saikokeishito, possess an activity to scavenge $^{\cdot}OH$ and alkyl radical in vitro and exert an inhibitory action on lipid peroxidation induced *in vitro* in different extents. Yokozawa *et al*. [*28*] have shown that Saikokeishito and its constituent herbs such as *Bupleuri Radix*, *Cinnamoni Cortex*, *Ginseng Radix, Glycyrrhizae Radix*, *Paeoniae Radix*, *Pinelliae Tuber*, *Scuttellariae Radix*, and *Zingiberis Rhizoma* inhibit lipid peroxidation induced by H_2O_2 in rat liver homogenates and that *Cinnamoni Cortex*, *Ginseng Radix*, *Glycyrrhizae Radix*, *Paeoniae Radix*, *Pinelliae Tuber*, and *Scuttellariae Radix* inhibit lipid peroxidation induced by $H_2O_2 + FeSO_4$ (the so-called Fenton reaction), i.e., $^{\cdot}OH$, in rat liver homogenates. In the present study, an enhanced increase in hepatic LPO content occurred at a progressed stage of ANIT-induced liver injury, as shown previously [*6*, *7*, *9*]. TJ-10 (1.3 or 2.6 g/kg BW) administered orally to ANIT-treated rats after the appearance of ANIT-induced induced liver injury was found to attenuate the enhanced increase in hepatic LPO content observed at a progressed stage of liver injury significantly. In addition, both doses of the herbal medicine attenuated the enhanced increase in hepatic LPO content completely. The same doses of TJ-10 did not affect hepatic LPO content in ANIT-untreated rats.

It has been shown that neutrophils mediate lipid peroxidation through the production of superoxide radical via activated NADPH oxidase in the cells [*29*]. It has also been shown that MPO mediates lipid peroxidation in the presence of H_2O_2 with halide ions [*30*]. We have suggested that neutrophil-derived ROS-related lipid peroxidation might be associated with the formation and progression of ANIT-induced liver injury in rats [*6*, *7*, *9*]. Accordingly, these findings suggest that orally administered TJ-10 exerts a therapeutic effect on ANIT-induced liver injury in rats by inhibiting an enhancement of hepatic lipid peroxidation meditated by infiltrated neutrophils in the liver tissue.

In the present study, the increased hepatic GSH content

found at an early stage of liver injury was slightly reduced at a progressed stage of injury in rats treated with ANIT, as shown previously [*7*, *9*]. TJ-10 administered to ANIT-treated rats at an early stage of liver injury reduced the increase in hepatic GSH concentration found at a progressed stage of injury at a dose of 2.6 g/kg BW. Dahm and Roth [*31*] reported that depletion of hepatic GSH by pretreatment with a glutathione synthesis inhibitor, buthionine sulfoximine, or a glutathione depletor, diethylmaleate or phorone, protected against ANIT-induced acute liver injury in rats, and suggested that GSH played a causal or permissive role in the ANIT-induced liver injury. It has been suggested that ANIT secreted as a reversible GSH conjugate into bile damages bile duct epithelial cells and induces cholestasis in rats treated with the hepatotoxin [*32–34*]. Thus, the highest dose of TJ-10 administered after the appearance of liver injury may reduce the cytotoxicity of ANIT secreted as a GSH conjugate into bile against bile duct epithelial cells in rats treated with the hepatotoxin.

Our previous reports have shown that, in rats treated once with ANIT, hepatic SOD and GSSG-R activities decrease with injury formation and progression, while catalase, and Se-GSHpx activities decrease with liver injury progression [*7–9*]. In the present study, not only hepatic SOD, catalase, Se-GSHpx, and GSSG-R activities but also hepatic G-6-PDH activity was found to decrease at a progressed stage of ANIT-induced acute liver injury in rats. Oral administration of TJ-10 (1.3 or 2.6 g/kg BW) to ANIT-treated rats at an early stage of liver injury significantly attenuated the decreases in hepatic SOD, catalase, Se-GSHpx, GSSG-R, and G-6-PDH activities found at a progressed stage of liver injury. In addition, TJ-10 administered to ANIT-treated rats at a dose of 1.3 or 2.6 g/kg BW attenuated the enhanced decrease in hepatic SOD activity completely. TJ-10 administered to ANIT-treated rats at a dose of 2.6 g/kg BW attenuated the decreased hepatic G-6-PDH activity completely. The same doses of TJ-10 administered to ANIT-untreated rats did not affect hepatic SOD, catalase, Se-GSHpx, and GSSG-R activities but the herbal medicine administered at a dose 2.6 g/kg BW caused a significant increase in hepatic G-6-PDH activity. However, the mechanism for the action of TJ-10 to increase hepatic G-6-PDH activity in ANIT-untreated rats is unknown at present. These results suggest that orally administered TJ-10 exerts a therapeutic effect on ANIT-induced liver injury in rats by attenuating an enhanced disruption of antioxidant defense systems in the liver tissue.

The mechanism by which hepatic SOD, catalase, Se-GSHpx, GSSG-R, and G-6-PDH activities are reduced with liver injury development in rats treated with ANIT has not been elucidated. However, Cu,Zn-SOD is inactivated *in vitro* by H_2O_2 [*35*]. Catalase and G-6-PDH are inactivated *in vitro* by $O_2^{\cdot -}$ [*36*, *37*]. Se-GSHpx and GSSG-R are inactivated *in vitro* by $^{\cdot}OH$ [*38*]. Cu,Zn-SOD, catalase, and Se-GSHpx are inactivated by hypochlorous acid *in vitro* [*39*]. Cu,Zn-SOD is inactivated by the MPO-H_2O_2-Cl^- system *in vitro* [*40*]. We have shown that the activity of Cu,Zn-SOD, which is localized in the cytosol of cells, but not the activity of Mn-SOD, which is localized in the mitochondira of cells, in the liver of ANIT-treated rats decreases with liver injury development [*8*, *9*]. As described above, ANIT-treated rats showed an increase in hepatic MPO activity with decreases in hepatic SOD, catalase, and Se-GSHpx activities at a progressed stage of liver injury and these changes were attenuated by post-administered TJ-10. Nuetrophils generate $O_2^{\cdot -}$ and H_2O_2 via NADPH oxidase and hypochlorous acid via MPO in the presence of H_2O_2 and Cl^-. It is known that TJ-10 scavenges $O_2^{\cdot -}$ and $^{\cdot}OH$ *in vitro* [*14*]. It is also known that *Scuttellariae Radix*, one of the constituents of Saikokeishito, scavenges ROS such as $O_2^{\cdot -}$ and $^{\cdot}OH$ *in vitro* [*41*]. Therefore, it seems likely that the enhanced decreases in hepatic SOD and GSSG-R activities and the decrease in hepatic catalase, Se-GSHpx, and G-6-PDH activities found at a progressed stage of liver injury in ANIT-treated rats are due to ROS derived from neutrophils accumulating in the tissue.

In conclusion, the results of the present study indicate that orally administered TJ-10 exerts a therapeutic effect on ANIT-induced liver injury with cholestasis in rats, possibly through attenuation of enhanced neutrophil infiltration and oxidative stress associated with disrupted antioxidant defense systems in the liver tissue, by its anti-inflammatory and antioxidant actions.

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