Shigakusan extract attenuates enhanced hepatic neutrophil infiltration and oxidative stress with progression of α-naphthylisothiocyanate-induced liver injury in rats

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In the present study, we examined whether shigakusan extract, a traditional Kampo medicine, attenuates enhanced hepatic neutrophil infiltration and oxidative stress with the progression of α-naphthylisothiocyanate (ANIT)-induced liver injury in rats. Rats were treated once with ANIT (75 mg/kg body weight, i.p.). Liver injury with cholestasis occurred 48 h after ANIT treatment and progressed at 48 h. A spray-dried material of shigakusan extract (SGS) (0.15, 0.75 or 1.5 g/kg body weight, p.o.) administered at 24 h after ANIT treatment prevented the progression of ANIT-induced liver injury dose-dependently. At 24 h after ANIT treatment, the treated rats showed increases in hepatic lipid peroxide (LPO) and reduced glutathione (GSH) contents and myeloperoxidase (MPO) activity, an index of tissue neutrophil infiltration, and decreases in hepatic superoxide dismutase (SOD) and glutathione reductase (GSSG-R) activities. At 48 h after ANIT treatment, the treated rats showed enhanced changes in hepatic LPO content and MPO, SOD, and GSSG-R activities except GSH content and decreases in hepatic catalase, Se-glutathione peroxidase, and glucose-6-phosphate dehydrogenase activities. SGS administered at 24 h after ANIT treatment attenuated the ANIT-induced changes in hepatic LPO and GSH content and MPO, catalase, and Se-glutathione peroxidase activities except SOD, GSSG-R, and glucose-6-phosphate dehydrogenase activities at 48 h dose-dependently. These results indicate that orally administered SGS attenuates enhanced hepatic neutrophil infiltration and oxidative stress with liver injury progression and increased hepatic GSH content at the progressed stage of liver injury in rats treated with ANIT, which could contribute to its therapeutic effect on this liver injury.

Key words α-naphthylisothiocyanate, liver injury (rat), shigakusan extract, 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 acute liver injury have been proposed by Roth and Dahm2) but the mechanisms have not been entirely clarified. The proposed mechanisms of ANIT-induced acute liver injury in rats are as follows: 1) neutrophils, i.e., polymorphonuclear leukocytes, contribute to the development of ANIT-induced liver injury through a mechanism independent of the production of reactive oxygen species (ROS).3,4) Namely, ANIT causes neutrophils to release toxic proteases, which cause hepatocellular damage, and cause bile duct epithelial cells to release a factor(s) that attracts neutrophils and stimulates them to injure hepatocytes.2,4) 2) Reduced glutathione (GSH) contributes to the development of ANIT-induced liver injury by virtue of its ability to form a reversible S-conjugate with ANIT that is critical in tapping ANIT into the bile, where it is released in large and probably toxic concentrations.2) 3) ANIT produces hepatotoxicity indirectly by increasing the permeability of the gastrointestinal tract to endogenous endotoxin, which gains access to the liver when it is released into mesenteric vessels and ultimately into the portal venous circulation.2)

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.5) 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 (O2•−), and glutathione reductase (GSSG-R), an enzyme to regenerate GSH from oxidized...
glutathione (GSSG) using NADPH, decreased at the early and progressed stages of liver injury and the activity of glucose-6-phosphate dehydrogenase (G-6-PDH), an enzyme to produce NAPDH using glucose-6-phosphate decreased at the progressed stage of liver injury.\(^6\) In addition, the hepatic activities of catalase, an enzyme to decompose hydrogen peroxide (H\(_2\)O\(_2\)), and Se-glutathione peroxidase (Se-GSH-Px), an enzyme to metabolize H\(_2\)O\(_2\) and lipid hydroperoxides using GSH as a co-substrate, were transiently increased before the appearance of ANIT-induced liver injury, although hepatic catalase and Se-GSHPx activities decreased at the progressed stage of the injury.\(^6\) In contrast, a similar increased level of hepatic GSH was maintained during the formation and progression of ANIT-induced liver injury.\(^6\) Thus, we have suggested that lipid peroxidation mediated by infiltrated neutrophils and oxidative stress associated with the disruption of hepatic antioxidant defense system contribute to the development of ANIT-induced acute liver injury in rats.

Shigiyakusun (Si-Ni-San) is a traditional Japanese herbal medicine, i.e., Kampo medicine, which is composed of 4 herbs such as Bupleurum Radix, Paenonia Radix, Auranti Fructus Immaturus, and Glycyrrhizae Radix. This Kampo medicine is clinically used for treatments of cholecystitis, cholelithiasis, gastritis, gastric ulcers, and nervousness in Japan. It has been reported that oral administration of an aqueous extract of shigiyakusan three times after ANIT treatment prevents the development of ANIT-induced liver injury in rats,\(^9\) although the mechanism for this preventive effect has not been clarified yet. We have shown that a single oral administration of a spray-dried material of shigiyakusan extract (SGS) suspended in distilled water to ANIT-treated rats prevents the progression of liver injury with cholestasis with attenuation of an enhanced increase in serum lipid peroxide (LPO) level.\(^10\) SGS is known to possess an activity to scavenge ROS such as O\(_2^−\) and hydroxyl radical (•OH) \(in vitro\).\(^11\) However, it is still unclear whether SGS exerts a therapeutic effect on ANIT-induced liver injury in rats by attenuating enhancements of 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 SGS on ANIT-induced liver injury with cholestasis, we, therefore, examined whether orally administered SGS attenuates enhanced hepatic neutrophil infiltration and oxidative stress associated with disrupted antioxidant defense systems at the progressed stage of liver injury in rats treated once with ANIT. Namely, we examined the effect of orally administered SGS 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,\(^2\) with liver injury progression in rats with a single ANIT treatment. We also examined the effect of SGS administration on the changes in hepatic SOD, catalase, Se-GSHPx, GSSG-R, and G-6-PDH activities with liver injury progression in ANIT-treated rats.

### Materials and Methods

**Chemicals.** ANIT, 3,3′,5,5′-tetramethylbenzidine (TMB), and xanthine oxidase (XO) purified from cow milk 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.

SGS (Batch No. 910035992PO) was kindly provided by Tsumura & Co. (Tokyo, Japan). SGS was prepared from a boiled water extract of the following herbs: 5.0 g Bupleuri Radix (Bupleuri falcatum Linne), 4.0 g Paenonia Radix (Paenonia lactiflora Pallas), 2.0 g Aurantii Fructus Immaturus (Citrus aurantium Linne var.), and 1.5 g Glycyrrhizae Radix (Glycyrrhiza glabra Linne). The extracted amount of materials in the prepared SGS was 18.0% of the original amount. The main components present in the SGS preparation were confirmed by analysis using high-performance liquid chromatography (HPLC) with spectrophotometric detection as follows: SGS 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-GE\(L\) 80TS column (4.6×250 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 wavelength range between 200 and 400 nm, and the three-dimensional data was processed by a diode array detector, SPD-M10A (Shimadzu, Kyoto, Japan).

**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 23°C and 50% humidity with free access to rat chow (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and water for one week prior to the experiment. All animals received humane care in compliance with the guidelines of the Management of Laboratory Animals in Fujita Health University, Japan.

**ANIT and SGS treatments and sample collection.** Seven-week-old rats fasted for 15 h received an 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 (7.5 mg/mL) per 100 g BW, in order to induce liver injury, as described previously.\(^4\) The age-matched control rats were 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, SGS (0.15, 0.75 or 1.5 g/kg BW), suspended in 1 mL of distilled water, was orally administered to rats with and without ANIT injection. Rats without SGS administration received a single oral administration of the
same volume of distilled water at the same time point. All rats were fasted for 15 h before being terminated. They were euthanized under ether anesthesia at 24 or 48 h after the initial ANIT or vehicle injection at which time blood was collected from the inferior vena cava. The collected blood was separated into serum by centrifugation. Immediately after euthanasia, the livers were well perfused with ice-cold 0.15 KCl 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 latozyme TA-LQ (Dai-bataon 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 BI-Test Wako (Wako Pure Chemical Industry Ltd., Osaka, Japan). 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-cold 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., using the thiobarbituric acid reaction except that 1.0 mM EDTA was added to the reaction medium. The content of hepatic LPO is expressed as that of MDA equivalents. Hepatic GSH was assayed by the method of Sedlak and Lindsay using Ellman’s reagent and GSH as a standard.

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. The resultant supernatant was dialyzed against 100 volumes of 0.05 M Tris-HCl buffer (pH 7.4) for 1 h using a microdialysis device (Molecular weight cut-off = 3,500) (Bio-Tec International Inc., Belleuve, WA, U.S.A.). The centrifugation and dialysis were performed at 4°C. The dialyzed supernatant was used for the assays of hepatic MPO, SOD, catalase, Se-GSH-Px, GSSG-R, and G-6-PDH. Before the assay of hepatic MPO, 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. MPO in the heat-treated liver tissue sample was assayed at 37°C by the method of Suzuki et al. This enzyme activity was assessed by measuring the H2O2-dependent oxidation of TMB (dissolved in dimethylsulfoxide) at 655 nm. One unit (U) of this activity is expressed as the amount of enzyme causing a change in absorbance of 1.0 min⁻¹ at 655 nm. Hepatic SOD activity was determined at 37°C by the XO-NH2OH method of Oyanagi using O2•− generation in the xanthine-XO system and purified bovine erythrocyte SOD (5000 units/mg solid) as a standard. This activity is expressed as the amount of the erythrocyte SOD showing activity equivalent to the determined activity. Hepatic catalase activity was measured at 37°C by recording H2O2 decomposition at 240 nm according to the method of Bergmeyer. One unit (U) of this activity is defined as the amount of enzyme decomposing 1 μmol H2O2 as a substrate per min. Hepatic Se-GSH-Px was assayed by the method of Hochstein and Utley. This enzyme activity was determined at 37°C by recording the decrease in absorbance at 340 nm following the oxidation of NADPH in the presence of H2O2, GSH, yeast GSSG-R, and NaN3 (as a catalase inhibitor). One unit (U) of this activity is defined as the amount of enzyme oxidizing 1 μmol NADPH per min. Hepatic GSSG-R was assayed by the method of Lopez-Barea and Lee. This enzyme 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. Hepatic G-6-PDH was assayed by the method of Bergmeyer et al. This enzyme 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. using bovine serum albumin as a standard.

**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 multicomparsion as the post hoc test. The level of significance was set at p < 0.05.

**Results**

The three-dimensional HPLC chart of the methanol solution of SGS used in the present study is shown in Fig. 1. This SGS contained saikosaponin b1, saikosaponin b2, saikosaponin h (derived from Bupleuri Radix), oxyaenoniflorin, (+)-catechin, albtiflorin, paoniflorin, benzoic acid, benzyoxaenoniflorin (from Paononia Radix), narirutin, narinigin, hesperidin, neohesperidin, poncirin (from Aurantii Fructus Immaturus), liquiritin, liquiritin apioside, isoliquiritin apioside, isoliquiritin, glycyrrhizin, formononetin, forononemodin, 7-O-glucoside, isoliquiritigenin, and glycycomarin (derived from Glycyrrhize Radix).

Serum ALT and AST activities, indices of hepatic cell damage, in the ANIT-treated group significantly increased 24 h after treatment with further increases at 48 h when compared with those in the control group (Fig. 2A and B). SGS 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 0.75 or 1.5 g/kg BW, but not 0.15 g/kg BW, although its dose of 1.5 g/kg BW was more effective than its dose of 0.75 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 increases at 48 h when compared with those in the control group (Fig. 2C and D). The post-administered SGS significantly lowered the enhanced increases in serum γ-GTP activity and bilirubin concentration at 48 h after ANIT treatment at its dose of 1.5 g/kg BW, but not 0.15 or 0.75 g/kg BW (Fig. 2C and D). The same doses of SGS given to ANIT-untreated did not affect the serum ALT, AST, and γ-GTP activities and total bilirubin concentration (Fig. 2).

Hepatic MPO activity in the ANIT-treated group was significantly higher than 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. 3A). SGS (0.75 or 1.5 g/kg BW) administered at 24 h after ANIT-treatment significantly reduced the enhanced increase in hepatic MPO activity at 48 h after the treatment, although its higher dose was more effective than its lower dose (Fig. 3A). 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 (Fig. 3B and C). Further increase in the hepatic LPO content occurred without any significant changes in the increased hepatic GSH content at 48 h (Fig. 3B and C). The post-administered SGS at a dose of 0.75 or 1.5 g/kg BW, but not 0.15 g/kg BW, significantly lowered the enhanced increases in hepatic LPO and GSH contents at 48 h after ANIT treatment, although its dose of 1.5 g/kg BW was more effective than its dose of 0.75 g/kg BW (Fig. 3B and C). The same doses of SGS given to ANIT-untreated rats did not affect the hepatic MPO activity and LPO and GSH contents (Fig. 3).

Hepatic SOD and GSSG-R activities in the ANIT-treated group were significantly lower than those in the control group at 24 h after the 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. 4 and 5). 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. 4 and 5). SGS (0.75 or 1.5 g/kg BW) administered at 24 h after ANIT treatment significantly reduced the decreases in hepatic catalase and Se-GSHpx activities at 48 h after the treatment, although none of the doses of post-administered SGS had any significant effects on the enhanced decreases in hepatic SOD and GSSG-R activities and the decreased hepatic G-6-PDH activity at 48 h after the treatment (Figs. 4 and 5). The same doses of SGS given to ANIT-untreated rats did not affect the hepatic SOD, GSSG-R, catalase, and Se-GSHpx, and G-6-PDH activities (Figs. 4 and 5).
Fig. 2 Effect of orally post-administered SGS on serum ALT (A), AST (B), and γ-GTP (C) activities and total bilirubin concentration (D) in rats treated with and without ANIT. SGS (0.15, 0.75 or 1.5 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 SGS received vehicle at the same time point. ALT, AST, 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 ± S.D. (n = 5 for ANIT-untreated rats with and without SGS administration; n = 8 for ANIT-treated rats with and without SGS administration). *Significantly different from control rats without any treatment, p < 0.05. †Significantly different from ANIT-treated rats without TJ-35 administration, p < 0.05.

Fig. 3 Effect of orally post-administered SGS on hepatic MPO activity (A) and LPO (B) and GSH (C) 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 MPO and LPO were assayed as described in Materials and Methods.

Fig. 4 Effect of orally post-administered SGS 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 catalase were assayed as described in Materials and Methods.
induced liver injury mainly associated with liver cell damage in rats.

It is generally accepted that infiltrated neutrophils play an important role in the development of ANIT-induced liver injury. In the present study, an enhanced increase in hepatic MPO activity, an index of tissue neutrophil infiltration, occurred at the progressed stage of ANIT-induced liver injury, as shown previously. SGS (0.75 or 1.5 g/kg BW, p.o.) administered to ANIT-treated rats after the appearance of liver injury significantly lowered the enhanced increase in hepatic MPO activity found at the progressed stage of liver injury. Thus, SGS administered orally after the increase in hepatic neutrophil infiltration was found to be able to prevent the enhanced increase in hepatic neutrophil infiltration observed thereafter in ANIT-treated rats.

Our previous reports suggested that lipid peroxidation might be closely associated with the development of ANIT-induced liver injury in rats. SGS is known to scavenge ROS such as O₂⁻ and ·OH, which are generated by the reaction between O₂⁻ and H₂O₂ (Haber-Weiss reaction) or by the reaction between H₂O₂ and transition metals (Fenton reaction), in vitro. Yokozawa et al. have shown in rat liver homogenates that Bupleurum Radix, Glycyrrhiza Radix, and Aurantii Fructus Immaturus inhibit lipid peroxidation induced by H₂O₂, that Bupleuri Radix and Glycyrrhiza Radix inhibit lipid peroxidation induced by Fe²⁺, and that Glycyrrhiza Radix and Paonieae Radix inhibit lipid peroxidation induced by H₂O₂ + FeSO₄ (the so-called Fenton reaction), i.e., ·OH. In addition, Tsai et al. have reported that when an aqueous extract of shigyakusan is orally administered to carbon tetrachloride-treated rats three times after the toxicant treatment, liver injury development is prevented with attenuation of increased hepatic LPO content. In the present study, an enhanced increase in hepatic LPO content occurred at the progressed stage of ANIT-induced liver injury, as shown previously. SGS (0.75 or 1.5 g/kg BW, p.o.) administered to ANIT-treated rats after the appearance of ANIT-induced liver injury was found to attenuate the enhanced increase in hepatic LPO content observed at the progressed stage of the injury significantly, although the same doses of SGS did not affect hepatic LPO content in ANIT-untreated rats.

It has been shown that neutrophils mediate lipid peroxidation through the production of O₂⁻ an H₂O₂ via activated NAPDH oxidase in the cells. It has also been shown that MPO mediates lipid peroxidation in the presence of H₂O₂ with halide ions. We have suggested that lipid peroxidation mediated by neutrophil-derived ROS might be associated with the formation and progression of ANIT-induced liver injury in rats. Accordingly, these findings suggest that orally administered SGS exerts a therapeutic effect on ANIT-induced liver injury in rats by attenuating an enhanced increase in hepatic lipid peroxidation mediated by ROS generated by infiltrated neutrophils in the liver tissue.

In the present study, the increased hepatic GSH content found at the early stage of liver injury was maintained at the progressed stage of injury in rats treated with ANIT, as

Discussion

In the present study, rats with a single ANIT treatment 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. SGS used in the present study contained various components derived from four herbs used, i.e., Bupleurum Radix, Paonia Radix, Aurantii Fructus Immaturus, and Glycyrrhiza Radix. When SGS (0.15, 0.75 or 1.5 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 cell damage was significantly attenuated at its doses of 0.75 or 1.5 g/kg BW and progressive biliary cell damage with cholestasis was significantly attenuated at a dose of 1.5 g/kg BW, judging from the serum levels of hepatobiliary markers, as shown in our previous report. Thus, it has been shown that orally administered SGS exerts a therapeutic effect on ANIT-induced liver injury mainly associated with liver cell damage in rats.

It is generally accepted that infiltrated neutrophils play an important role in the development of ANIT-induced liver injury. In the present study, an enhanced increase in hepatic MPO activity, an index of tissue neutrophil infiltration, occurred at the progressed stage of ANIT-induced liver injury, as shown previously. SGS (0.75 or 1.5 g/kg BW, p.o.) administered to ANIT-treated rats after the appearance of liver injury significantly lowered the enhanced increase in hepatic MPO activity found at the progressed stage of liver injury. Thus, SGS administered orally after the increase in hepatic neutrophil infiltration was found to be able to prevent the enhanced increase in hepatic neutrophil infiltration observed thereafter in ANIT-treated rats.

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It has been shown that neutrophils mediate lipid peroxidation through the production of O₂⁻ an H₂O₂ via activated NAPDH oxidase in the cells. It has also been shown that MPO mediates lipid peroxidation in the presence of H₂O₂ with halide ions. We have suggested that lipid peroxidation mediated by neutrophil-derived ROS might be associated with the formation and progression of ANIT-induced liver injury in rats. Accordingly, these findings suggest that orally administered SGS exerts a therapeutic effect on ANIT-induced liver injury in rats by attenuating an enhanced increase in hepatic lipid peroxidation mediated by ROS generated by infiltrated neutrophils in the liver tissue.

In the present study, the increased hepatic GSH content found at the early stage of liver injury was maintained at the progressed stage of injury in rats treated with ANIT, as
shown previously.\(^6,9\) SGS administered to ANIT-treated rats at the early stage of liver injury reduced the increase in hepatic GSH content found at the progressed stage of injury at a dose of 0.75 or 1.5 g/kg BW. Dahm and Roth\(^6,9\) 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. Jean and Roth\(^7\) have 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. Therefore, SGS (1.5 g/kg BW) administration 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 decreased with injury formation and progression, while catalase, Se-GSHp, and G-6-PDH activities decreased with liver injury progression.\(^6,9\) In the present study, hepatic SOD, catalase, Se-GSHp, GSSG-R, and G-6-PDH activities were found to decrease at the progressed stage of ANIT-induced acute liver injury in rats. Oral administration of SGS (0.75 or 1.5 g/kg BW) to ANIT-treated rats at the early stage of liver injury significantly attenuated the decreases in hepatic catalase and Se-GSHp activities, but not the decreases in hepatic SOD, GSSG-R, and G-6-PDH activities, found at the progressed stage of liver injury. The same doses of SGS administered to ANIT-untreated rats did not affect hepatic SOD, catalase, Se-GSHp, GSSG-R, and G-6-PDH activities. These results suggest that orally administered SGS exerts a therapeutic effect on ANIT-induced liver injury in rats by attenuating an enhanced disruption of hepatic antioxidant defense system.

The mechanism by which hepatic SOD, catalase, Se-GSHp, GSSG-R, and G-6-PDH activities are reduced with liver injury development in rats treated with ANIT has not been elucidated. However, catalase is inactivated in vitro by \(O_3^-\).\(^9\) Se-GSHp is inactivated in vitro by \(OH\).\(^9\) Catalase and Se-GSHp are inactivated by hypochlorous acid in vitro.\(^9\) As described above, ANIT-treated rats showed an increase in hepatic MPO activity with decreases in hepatic catalase and Se-GSHp activities at the progressed stage of liver injury and these changes were attenuated by SGS administered at the early stage of the injury. Neutrophils generate both \(O_3^-\) 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 SGS scavenges \(O_3^-\) and \(-OH\) in vitro.\(^11\) Therefore, it seems likely that the decreases in hepatic catalase and Se-GSHp activities found at the 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 SGS attenuates enhanced hepatic neutrophil infiltration and oxidative stress, which is associated with an enhanced disruption of hepatic antioxidant defense system, with the progression of ANIT-induced liver injury and increased hepatic GSH content at the progressed stage of the injury in rats, which could contribute to its therapeutic effect on this liver injury.

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