Preventive Effects of a Traditional Chinese Medicine (Sho-saiko-To) against Oxygen Toxicity and Membrane Damage during Endotoxemia

Shuhei SAKAGUCHI,* Eiji TSUTSUMI, and Katsushi YOKOTA

First Department of Hygienic Chemistry, Tohoku College of Pharmacy, 4-4-1 Komatsushima, Aoba-ku, Sendai 981, Japan. Received January 18, 1993

The preventive effects of a traditional Chinese medicine Sho-saiko-to (Kampo prescription, TJ-9) were determined from oxygen toxicity and membrane damage in liver during endotoxemia. The liver lipid peroxide level and xanthine oxidase activity 18 h after administration of endotoxin (6 mg/kg, i.p.) to TJ-9 (500 mg/kg/d, p.o.)-pretreated mice were markedly lower than that in endotoxin-treated mice, whereas the administration of TJ-9 significantly increased superoxide dismutase and glutathione peroxide activities in liver of endotoxin-injected mice. In the mice pretreated with a TJ-9, the levels of α-tocopherol and nonprotein SH in liver tissue 18 h after endotoxin injection were remarkably increased as compared to those in endotoxin-treated mice. Leakages of acid phosphatase and lactate dehydrogenase isozyme in serum were markedly lower in endotoxin-TJ-9-treated mice than those in mice given endotoxin. The administration of TJ-9 clearly prevented the membrane protein damage arising from endotoxin challenge. Kampo prescription Sho-saiko-to thus appears to protect the liver plasma membrane from injury by free radicals which occur in a tissue ischemic state during endotoxemia.

Keywords kampo medicine Sho-saiko-to; endotoxin; preventive effect; oxygen free radical; membrane damage

The liver is known to be one of the main target organs to respond when animals are shocked by endotoxin. Endotoxin is believed to be initially detoxified in the reticuloendothelial system (RES), particularly in the Kupffer cells, in the liver, during endotoxemia.1,2 It has been recognized that ischemia causes functional and structural damage to tissues or organs by active oxygen generation. The modification induced in the apolar side residues of the membrane phospholipides by active oxygen generation are thought to bring about structural alterations in the membrane. In previous reports,3,4 we observed that endotoxin injection resulted in lipid peroxide formation and membrane damage in experimental animals, causing decreased levels of scavengers or quenchers of free radicals.

Sho-saiko-to is one of the most frequently prescribed Kampo medicines, and has primarily been used to treat chronic liver disease. The action of Sho-saiko-to has been extensively reported,5–9 for example, protection against liver cell injury by carbon tetrachloride or α-galactosamine, stimulation of RES, antiinflammatory action, weak antitumor activity, glucocorticoid-like action and inhibition of free radical generations. Kampo (Japanese herbal) medicines generally contain many components, and their actions are additive, synergistic, and often contradictory.

Based on our series of studies on metabolic response in endotoxemia,2–4,10–12 we focused on the pharmacological response of Sho-saiko-to to improve endotoxin shock. The present study was carried out to observe the effect of Sho-saiko-to in defending the mouse liver from damage during endotoxemia caused by oxygen toxicity.

Materials and Methods

Animals and Treatment Male ddY mice, 4 weeks old, weighing 18 to 20 g, were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and maintained in Tohoku College of Pharmacy Experimental Animal Center. Salmonella typhimurium lipopolysaccharide (endotoxin) (Westphal) preparation obtained from Difco Laboratories, Detroit, Mich., U.S.A.) was used throughout this study. The traditional Chinese preparation Sho-saiko-to was obtained from Tsumura Co., Tokyo. Tsumura-Sho-saiko-to (kampo prescription, TJ-9) contains spray-dried aqueous extracts of seven crude drugs in the following proportions: 7.0 g Bupleuri Radix, 5.0 g Pinelliae Tuber, 3.0 g Scutellariae Radix, 3.0 g Zizyphi Fructus, 3.0 g Ginseng Radix, 2.0 g Glycyrrhizae Radix, and 1.0 g Zingiberis Rhizoma. TJ-9 was suspended in distilled water and given to mice at a dose of 500 mg/kg by means of a stomach catheter once a day for 5 days. On the 6th day, endotoxin was injected at a dose of 6 mg/kg i.p. into the TJ-9-treated mice. Control mice were injected with 0.2 ml of saline alone. Mice were killed and bled by decapitation at the indicated times after endotoxin injection, and thereafter the liver or blood was collected for chemical analysis. Data are expressed as the mean ± S.E. Statistical significance was evaluated according to Student’s t-test.

Enzyme Assays Liver tissues were quickly removed, rinsed in cold saline, blotted dry, weighed, and immediately homogenized at 10% concentration in ice-cold 10 mm phosphate buffer (pH 7.4) solution, then centrifuged at 10500×g for 60 min. The supernatant fractions obtained were analyzed to estimate the activity of each enzyme’s Xanthine oxidase (XOD) activity was estimated according to the method of Fried and Fried.13 Glutathione peroxidase (GSH-Px) activity was assayed by the method of Stults et al.14 Superoxide dismutase (SOD) activity was calculated from the inhibited reduction of nitro blue tetrazolium (NBT) according to the method of Beauchamp and Fridovich.15 Proteins were determined by the method of Lowry et al.16 Each enzyme’s activity was expressed as units/mg protein.

Estimation of Tissue Vitamin E (α-Tocopherol) The livers were homogenized in cold water, and the homogenate obtained was deproteinized with ethanol. After extraction with n-hexane, α-tocopherol was estimated fluorometrically by the method of Thompson et al.17 with modification.18

Nonprotein Sulphydryl Nonprotein sulphydryl (NpSH) was estimated by the method of Jeffries.20

Estimation of Serum Lactate Dehydrogenase (LDH) Isozyme Electrophoresis Serum LDH isozyme was assayed by the method in which NADH was formed in the presence of phentazene methosulfate and NBT was reduced to a strongly colored formazan. Agar gel electrophoresis was performed using the LDH Isozyme-Test Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Acid Phosphatase Activity of serum acid phosphatase (Acp) was estimated by the Acid Phospha K-Test Wako (Wako Pure Chemical Industries, Ltd.).

Isolation of Plasma Membrane from Liver The isolation of plasma membranes from liver was performed by a modification of the method of Emmet et al.21 The livers from 10 mice were pooled and perfused with 0.25 M sucrose solution containing 10 mM Tris buffer, pH 7.6. The washed liver tissues were homogenized in 15 volumes of a solution containing 1 mM

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CaCl₂ buffered with 1 mm NaHCO₃, pH 7.6. The homogenate was centrifuged at 1500 × g for 10 min and the pellet was resuspended in the homogenizing medium and was washed twice by centrifugation at 1220 × g for 10 min. The suspension of pellet was subjected to sucrose density gradient centrifugation (d: 1.22, 1.20, 1.18 and 1.16) at 105000 × g for 60 min. The fraction isolated at a density between d: 1.18 and 1.16 was again subjected to sucrose density gradient centrifugation (d: 1.16, 1.17, 1.18, and 1.22). The fraction obtained (d: 1.17) was washed with 0.25 M sucrose solution containing 1 mM EDTA buffered with 1 mM NaHCO₃ pH 7.4.

**SDS-Disk Gel Electrophoresis Pattern of Solubilized Liver Plasma Membrane** Each sample was suspended to give a final protein concentration of 10-20 μg/ml in a 2% SDS solution containing 10 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, 10% sucrose and 1% β-mercaptoethanol, and then heated at 100°C for 3 min. Samples were then subjected to electrophoresis in 5.6% polyacrylamide disk gel (0.8 × 13 cm) containing sodium dodecyl sulfate (SDS) according to the method of Fairbanks et al. After electrophoresis the gels were stained for protein by 0.1% Coomassie blue. Protein bands of the membrane were numbered in order from the top of the gel, and the molecular weight of each protein band was estimated by comparison with those of the standard proteins.

**Reagents** All reagents for measurements were of analytical grade: NADPH, xanthine, thiobarbituric acid, and NBT (Sigma Chemical Co., St. Louis, Mo., U.S.A.). Nonfluorescent Dinitrophenols (n-hexane, ethanol, and distilled water) of Doin Kogyo Co., Kumamoto, Japan, was used for the assay of α-tocopherol.

## Results

**Effects of TJ-9 Administration on Lipid Peroxide Level and Xanthine Oxidase Activity in Endotoxin-Poisoned Mice**

As shown in Table I, the MDA value showed a marked increase to about 6 times control value at 18 h after endotoxin administration, then tended to decrease at 24 h, thereafter returning to about 1.65 times after 2 d. These results coincided with those described in a previous report. The following experiments were therefore carried out 18 h after endotoxin administration. As shown in Fig. 1, the levels of lipid peroxide and XOD activity in endotoxin-injected mice livers were greater at 18 h postintoxication than those in control, and the levels of endotoxin–TJ-9-treated mice were markedly lower than those in endotoxin-treated mice. In contrast, the SOD activity was markedly decreased in endotoxin-treated mice, while it showed a

<p>| Table I. Time Course of Lipid Peroxide Levels in Livers of Endotoxin-Poisoned Mice |
|-------------------------------|-------------------------------|</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver lipid peroxide MDA³⁰ nmol/g wet liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 h</td>
</tr>
<tr>
<td>Control</td>
<td>212 ± 53²⁰</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>567 ± 89²⁰</td>
</tr>
<tr>
<td></td>
<td>18 h</td>
</tr>
<tr>
<td>Control</td>
<td>361 ± 81²⁰</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>2196 ± 357²⁰</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Control</td>
<td>308 ± 49²⁰</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>1508 ± 283²⁰</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
</tr>
<tr>
<td>Control</td>
<td>382 ± 71²⁰</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>627 ± 108²⁰</td>
</tr>
</tbody>
</table>

a) Mice were injected intraperitoneally with 6 mg/kg of endotoxin, and control mice were injected with saline. b) MDA, malondialdehyde. c) Mean value ± S.E. of 8 mice. d) p<0.05.

Fig. 1. Effects of TJ-9 Preadministration on Lipid Peroxide Level (a), Xanthine Oxidase (b) and SOD Activities (c) in Mouse Liver 18 h after Endotoxin Challenge

A. saline, B. endotoxin (6 mg/kg, i.p.), C. endotoxin and TJ-9 (500 mg/kg/d, p.o.), D. TJ-9 (500 mg/kg/d, p.o.). Each bar represents the mean ± S.E. of 10 mice. a) Significant difference from the value of endotoxin-poisoned mice at p<0.05.

Fig. 2. Changes in Glutathione Peroxidase (GSH-Px) Activity (a) and α-Tocopherol (b) and Nonprotein SH(NpSH) (c) Levels in Liver of TJ-9-Pretreated Mice 18 h after Endotoxin Administration

A. saline, B. endotoxin (6 mg/kg, i.p.), C. endotoxin (6 mg/kg, i.p.) and TJ-9 (500 mg/kg/d, p.o.), D. TJ-9 (500 mg/kg/d, p.o.). Each bar represents the mean ± S.E. of 10 mice. a) Significant difference from the value of endotoxin-poisoned mice at p<0.05.
significant increase in the liver of endotoxin-TJ-9-treated mice.

Effects of TJ-9 Challenge on Free Radical Scavengers in Liver of Mice Given Endotoxin We examined the effect of TJ-9 on GSH-Px activity and on NpSH and α-tocopherol levels in livers (Fig. 2). The scavenger levels (GSH-Px, NpSH, α-tocopherol) in liver of endotoxin-treated mice were lower than those in control at 18 h postintoxication, while they showed a more significant increase in the liver of endotoxin-TJ-9-treated mice than in mice treated with endotoxin. These results indicate the protective effect of TJ-9 from endotoxin-induced free radical formation.

![Fig. 3. Effects of TJ-9 Preadministration of LDH Electrophoretic Patterns and on Activities of LDH Activity (a) and Acid Phosphatase (b) in Mouse Sera 18 h after Endotoxin Challenge](image)

A, saline; B, endotoxin (6 mg/kg, i.p.); C, endotoxin (6 mg/kg, i.p.) and TJ-9 (500 mg/kg/d, p.o.); D, TJ-9 (500 mg/kg/d, p.o.). Dose in each group is shown in Figs. 1 and 2. Each bar represents the mean ± S.E. of 10 mice. a) Significant difference from the value of endotoxin-poisoned mice at p<0.05.

![Fig. 4. Scanograms of SDS–Polyacrylamide Gel Electropherograms of Liver Plasma Membrane Protein](image)

(a), control; (b), endotoxin; (c), endotoxin + TJ-9; (d), TJ-9. Endotoxin (6 mg/kg) was administered i.p. into TJ-9 (500 mg/kg/d, p.o.)-pretreated mice. After treatment, the mice were sacrificed at 18 h postintoxication. Each sample (10–20 μg of protein) was subjected to SDS-gel electrophoresis as described in Materials and Methods. Gels were stained for protein with 0.1% Coomassie blue and decolored. Each plasma membrane preparation was from 10 mice. ——, endotoxin-induced membrane protein damage.
Effects of TJ-9 Administration on Serum LDH Electrophoretic Patterns and Acid Phosphatase (Acp) Activity in Mice Given Endotoxin

Figure 3a shows serum LDH isozyme electrophoretic scanograms at 18 h after endotoxin administration. In the electrophoretic patterns of the isozyme, the leakage of serum LDH-5 was markedly less in mice treated with endotoxin-TJ-9 as compared with that in the endotoxemic mice. Figure 3b shows Acp leakage in the mouse serum. In endotoxin-TJ-9-treated mice, serum Acp activity was significantly less than that in mice given endotoxin. There was no significant difference in this activity between normal and TJ-9-treated mice.

SDS-Polyacrylamide Gel Electrophoretic Scannograms of Plasma Membrane Protein in Mouse Liver

As can be seen in Fig. 3, LDH isozyme and Acp activity showed lower leakage in serum from liver of mice given TJ-9, revealing the protective effect of TJ-9 administration on plasma membrane injury in liver following endotoxin injection. The distribution of the plasma membrane protein is shown in Fig. 4. The differences in electrophoretic profiles of the membrane proteins between endotoxin-poisoned and control mice were largely in the molecular weight regions near 60000—150000, while the administration of TJ-9 was found to prevent most of the membrane protein damage caused by endotoxin challenge.

Discussion

Most administered endotoxin locates in cells of the RES in animals, particularly in Kupffer cells and splenic macrophages, and also in the macrophages, when stimulated with endotoxin, release lysosomal enzymes of cytokines. One of the cytokines, tumor necrosis factor (TNF) has frequently been reported to cause a shock syndrome similar to endotoxin shock, and has been suggested to possibly the major mediator of this shock. We recently reported that Sho-saiko-to protects the TNF-induced lethality in galactosamine-hypersensitized mice, and also that Sho-saiko-to-treated mice are protected against the falling rectal temperature after TNF administration. We therefore suggested that Sho-saiko-to may protect mice from the severe shock syndrome induced by TNF. Kampo medicines are a system of drug therapy developed from clinical experience accumulated over some thousands of years in China. Sho-saiko-to, one such kampo prescription, is applied clinically as a therapy for chronic hepatitis. Our own studies suggested that lipid peroxidation by free radicals occurs in the ischemic state, probably induced by disseminated intravascular coagulation, in endotoxin-poisoned mice. Therefore, we investigated whether or not TJ-9 can defend mice from damage by free radicals generated in the ischemic state of tissues during endotoxemia.

Despite marked high levels of lipid peroxide in the liver of endotoxemic mice, it was noted here that lipid peroxide formation was markedly lower in liver of endotoxemic mice given TJ-9. As shown in Figs. 1 and 2, however, XOD activity notably increased in liver of these mice, while SOD and GSH-Px activities showed a significant recovery in endotoxin-TJ-9-treated mice. SOD dismutates from \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) formation, and the ensuing molecular interaction generates a more toxic hydroxyl radical and perhaps singlet oxygen, while GSH-Px is the enzyme responsible for the destruction of \( \text{H}_2\text{O}_2 \) and organic hydroperoxide compounds inducing cellular membrane damage. It follows, therefore, that the upkeep of these enzymatic activities by TJ-9 pretreatment may play a key role in providing a defense against endotoxin-induced \( \text{O}_2^- \) toxicity by catalytically scavenging \( \text{O}_2^- \). Moreover, as shown in Fig. 2, the NpSH and z-tocopherol levels showed a significant increase in the liver of endotoxic mice given TJ-9 than in endotoxemic mice. Previously, we observed that z-tocopherol can be helpful in preventing membrane instability in endotoxin-poisoned mice. Furthermore, GSH plays an important role in tissues, especially in the oxidation-reduction system which is responsible for the inner tissue metabolism, and also protects SH-enzymes and membrane SH from free radical attack. Miyahara and Tsutsumi suggested that Sho-saiko-to markedly inhibited iron-induced lipid peroxidation in microsome and mitochondria in rat liver, and also identified active components as baicalin and ginsenoside Rf. We suggest from this study that the administration of TJ-9 protects against toxicity of free radical during endotoxemia.

We observed on the SDS-disk electrophoresis pattern that TJ-9 had almost repaired damage to the liver plasma membrane 18 h after endotoxin injection (Fig. 4). Thus, it may be inferred that the administration of TJ-9 prevented the peroxidation of membrane lipid by superoxide-free radicals generated in endotoxicosis as described above. The role of lysosome as an intracellular target for endotoxin has been proposed on the basis that endotoxin induces lysosomal instability and causes the release of lysosomal enzymes into tissues. In own experiment, endotoxin-TJ-9-treated mice exhibited less leakage of LDH and Acp in the serum, suggesting that the liver tissue membrane was stabilized by the administration of TJ-9. Judging from the protective effect of LDH isozyme leakage in serum of endotoxin-poisoned mice after TJ-9 administration, we conjectured that TJ-9 might be primarily associated with an increased stability of hepatic lysosomal particles.

We also assumed that Sho-saiko-to enhanced the host defense mechanism by changing the RES functions. For example, the lipid peroxide formation after endotoxin injection was not observed in the livers of C3H/HeJ mice (nonresponder strain). Wahl et al. found that livers of C3H/HeN (responder strain) mice produce a significant amount of prostaglandin E, one of the lipid peroxides, when exposed to endotoxin, whereas macrophages from C3H/HeJ mice are unresponsive. It is, therefore, conjectured that pretreatment of TJ-9 may give mice the ability to endure an attack of endotoxin by suppressing the endotoxin-induced release of lysosome enzymes from cells and/or of cytokines (TNF etc.) from macrophages. Further investigation must be done on this point.

References