

Alleviating Effects of Si-Ni-San, a Traditional Chinese Prescription, on Experimental Liver Injury and Its Mechanisms

Jieyun JIANG,^a Chunxiang ZHOU,^a and Qiang XU^{*,a,b}

^aDepartment of Pharmacology for Chinese Materia Medica, China Pharmaceutical University; 24 Tong Jia Xiang, Nanjing 210009, China; and ^bState Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University; 22 Han Kou Road, Nanjing 210093, China. Received November 25, 2002; accepted March 31, 2003

The present study aims at examining the effects of Si-Ni-San, a prescription usually used for treating hepatitis in Traditional Chinese Medicine (TCM), on various experimental liver injury models and its mechanisms. The prescription showed significant hepatoprotection against CCl₄-induced hepatic damage, both *in vivo* and *in vitro*. To the liver injury induced by Bacillus Calmette-Guerin (BCG) with lipopolysaccharide (LPS), Si-Ni-San also provided significant alleviation through enhancing nitric oxide (NO) release by macrophages. Against the liver injury induced by a delayed-type hypersensitivity reaction to picryl chloride (PCI-DTH), Si-Ni-San alleviated it remarkably when administered during either the induction or effector phase. A significant reduction of *in-vitro* hepatotoxicity, as measured by the inhibition of serum transaminase evaluation, was observed in non-parenchymal cells from liver-injured mice treated with Si-Ni-San. Si-Ni-San facilitated apoptosis in non-parenchymal cells from liver-injured mice, as well as in spleen cells activated by PCI *in vivo* or by Con A *in vitro*. These results suggest that Si-Ni-San provides alleviating effects against liver injury through multiple mechanisms, including protection of the hepatocyte membrane, enhancement of NO release, and dysfunction of liver-infiltrating cells mainly through causing their apoptosis.

Key words Si-Ni-San; experimental liver injury; alleviating effect; hepatocyte membrane; nitric oxide; apoptosis

Si-Ni-San, a prescription from the Treatise on Febrile Diseases—one of the most influential medical classics in China, is made from four Traditional Chinese Drugs: Radix bupleuri, Radix Paeoniae Alba, Fructus Aurantii Immaturus and Radix Glycyrrhizae. In Traditional Chinese Medicine (TCM), Si-Ni-San has been believed to be effective for improving disorders of digestive system, alleviating mental depression, etc. and is widely used as a medication recipe to clinically treat hepatitis and neuralgia. However, as seen in most other traditional prescriptions, Si-Ni-San also lacks scientific evidence of how it was formulated and how it takes effect.

Recently, it has been reported that Si-Ni-San has remarkable curative effects on many types of hepatitis, such as acute hepatitis with jaundice, chronic hepatitis, etc.^{1–3)} The prescription shows both hepatoprotective activity and a virus-clearing effect in patients with hepatitis.⁴⁾ These findings have driven us to explore its effectiveness from an experimental aspect and to find evidence that the prescription cures liver diseases. In the present study, therefore, we examined the effects of Si-Ni-San on several experimental liver injury models and investigated its mechanisms.

MATERIALS AND METHODS

Animals Four- to six-week-old Kunming strain of mice were obtained from the Experimental Animal Center of China Pharmaceutical University (Nanjing, China), and BALB/c mice were from Sino-British SIPPR/BK Lab Animals Ltd. (Shanghai, China). All the animals were maintained with free access to pellet food and water in plastic cages at 20±2 °C, and kept on a 12 h light/dark cycle. This study complied with the current ethical regulations for animal research of this institute, and all mice used in the experiment received humane care.

Drugs Radix Bupleuri Chinensis, Radix Paeoniae Alba,

Fructus Citri Aurantii and Radix Glycyrrhizae Uralensi were purchased from Nanjing Medicinal Material Co. (Nanjing, China) and identified by Dr. Boyang Yu (Department of Chinese Medicinal Prescription, China Pharmaceutical University) as *Bupleurum chinense* DC, *Citrus aurantium* L., *Paeonia albiflora* PALL. and *Glycyrrhiza uralensis* FISCH, respectively. The ethanol extract from Si-Ni-San was made by a common method. Briefly, equal amounts of each material were mixed and extracted twice with 8 times the volume (v/w) of 70% (v/v) ethanol in distilled water under reflux for 1 h. After filtrating and reclaiming the ethanol, the extract was lyophilized to obtain a powder in 18.0% yield. This powder was used for the dosage of Si-Ni-San in this study.

Reagents Reagents employed in this study were as follows: Picryl chloride (PCI, Tokyo Kasei Industry Co. Ltd., Tokyo, Japan), Cyclophosphamide (Cy, Shanghai 12th Pharmaceutical Factory, Shanghai, China) and biphenyl dimethyl dicarboxylate (BDD, Beijing Xiehe Pharmaceutical Co., Ltd., Beijing, China). Lipopolysaccharide (LPS), N ω -nitro-L-arginine (L-NNA) and Concanavalin A (Con A) were from Sigma. Diphenylamine and sodium dodecyl sulfate (SDS) (Shanghai Chemical Reagents Company, Shanghai, China); trichloroacetic acid (Yuanhang Chemical Factory, Shanghai, China); alanine transaminase (ALT) measurement kit, aspartate transaminase (AST) measurement kit and nitrate reductase NO measurement kit (Nanjing Jiangcheng Bioengineering Institute, Nanjing, China) were all used.

Liver Injury Induced by Local PCI-DTH Liver injury was induced according to our previous reports.^{5,6)} Namely, mice were sensitized twice by painting 0.1 ml of 1% PCI in ethanol on the skin of their abdomens at an interval of 5 d. Five days after the second sensitization, they were injected with 10 μ l of 0.5% PCI in olive oil into the liver, followed 18 h later by bleeding and isolating the serum. The serum ALT and AST were used as the parameters for indicating hepatic damage.⁷⁾ The activities of ALT and AST were deter-

* To whom correspondence should be addressed. e-mail: molpharm@163.com

mined by using commercial kits according to the guidelines indicated.

Liver Injury Induced by BCG+LPS in Mice Mice were injected *via* the tail vein with 1 mg of BCG in 0.2 ml saline (approximately 5×10^7 viable units per mouse) or with saline alone; 10 d later, the mice were injected intravenously with 10 μ g of LPS in 0.2 ml saline or with saline alone. Ten hours after the LPS injection, the mice were bled and the blood was collected for serum ALT and nitric oxide (NO) assay. The levels of serum ALT and NO were determined using commercial kits according to the guidelines indicated.

Liver Cell Preparation Parenchymal and nonparenchymal cells were isolated from naive or liver injured mice 12 h after the challenge with PCI by the modified two-step perfusion method. In brief, mice received pentobarbital intraperitoneally at a dose of 40 mg/kg. The liver was first perfused *in situ* *via* the portal vein with Ca^{2+} - and Mg^{2+} -free Hank's balanced salt solution (HBSS) supplemented with 0.5 mM EGTA (ethylene glycol-bis-(α -amino ethyl)- N,N' -tetraacetic acid) (Dojindo Chemical Inst., Ltd., Kumamoto, Japan) and 25 mM HEPES (N -2-hydroxyethylpiperazine- N -2-ethane sulfonic acid) (pH 7.4) at 37 °C until the blood in the liver was completely removed. Then, the solution was exchanged with 0.1% collagenase (182 units/mg, Wako Pure Chemical Industries, Ltd., Osaka, Japan) (Wako) in HBSS containing 4 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.8 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. After a few minutes of perfusion, the liver was excised rapidly from the body cavity and dispersed into cold HBSS. The cell suspension generated was filtered through a 100-gauze mesh, and parenchymal hepatocytes were separated from nonparenchymal cells by differential centrifugation at 50 g for 2 min. The pellet containing hepatocytes was washed twice to remove dead cells and debris, and then suspended in William's Medium E with 2 mM L-glutamine (GIBCO BRL) containing 10% (v/v) newborn-calf serum, 100 U/ml of penicillin and 100 U/ml of streptomycin (WE medium). The supernatant obtained after the first centrifugation of the slurry generated by perfusion of the liver with collagenase was centrifuged at 300 g for 10 min to obtain nonparenchymal cells. The nonparenchymal cell pellets were washed twice with RPMI 1640 (Gibco BRL) containing 100 U/ml of penicillin, 100 μ g/ml of streptomycin and 10% new born bovine serum (RPMI 1640 medium). The hepatocytes and nonparenchymal cells were found to be about 90% viable, as estimated by trypan blue exclusion, and were generally used immediately for culture or for apoptosis measurement.

Cell Culture and Transaminase-Releasing Assay Hepatocytes were suspended in WE medium at a density of 1×10^5 cells/ml. Portions (0.2 ml) were seeded onto 96-well microplates (Nunc) and cultured in a humidified incubator at 37 °C with 5% (v/v) CO_2 in air. After 5 h, the hepatocyte monolayers were washed twice with WE medium, and 8×10^4 nonparenchymal cells were added to the wells. After 3 h of further culture, the supernatant was collected and used for the assay of ALT activity.

Preparation of Splenocytes Splenocytes were obtained from PCI-twice sensitized or naive mice. Briefly, the spleen was removed in a sterile manner and cells were dissociated in 5 ml of RPMI 1640 medium. The cell suspension was centrifuged at 200 g for 5 min. After removing the supernatant, the solution containing 0.17 M Tris (hydroxymethylamino-

methane) and 0.75% NH_4Cl was used to remove erythrocytes. After being washed twice, the cells were re-suspended in RPMI 1640 medium.

Culture of Macrophage and Measurement of NO J774A.1 cells (Shanghai Institute of Cell Biology) were cultured with RPMI 1640 medium (without phenol red) in 24-well plates (2×10^6 macrophages per well in a volume of 1 ml) (Nunc) at 37 °C in 5% CO_2 /95% air for 2 h adherence. Then they were incubated in the absence or presence of LPS (final concentration 10 μ g/ml) for 48 h for NO assay. Each assay was carried out in triplicate.

The serum NO level was determined as nitrite and nitrate, the stable metabolites of NO, by a commercial NO measurement kit. As the kit instructions indicated, the nitrate in serum was reduced into nitrite by nitrate reductase with a conversion rate of more than 90%. On the other hand, the NO release from J774A.1 cells in culture supernatant was determined as nitrite by Griess reagent.⁸⁾ Briefly, 100 μ l of culture supernatant from 2×10^5 J774A.1 cells were incubated at room temperature for 10 min with 100 μ l of Griess reagent (0.5% sulfanilamide, 0.05% N -(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4). The OD values of the samples were then read at 540 nm. A standard curve using NaNO_2 was then used to calculate NO_2^- concentrations.

Apoptosis Measurement DNA fragmentation was quantified by the diphenylamine method with minor modification.^{9,10)} Briefly, cells were lysed with 0.4 ml of lysis buffer (10 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0, 0.5% Triton X-100) at room temperature for 30 min. The lysate was centrifuged at 13000 g at 4 °C for 10 min to separate the intact from fragmented chromatin. The supernatant, containing fragmented DNA, was transferred into a separate tube. Both sediment and supernatant were brought to 12.5% trichloroacetic acid and left overnight at 4 °C. After being centrifuged at 20000 g at 4 °C, the DNA in the precipitates was hydrolyzed by heating to 90 °C for 10 min in 80 μ l of 5% trichloroacetic acid, and then 160 μ l of diphenylamine reagent (0.15 g diphenylamine, 0.15 ml sulfuric acid, 2.5 μ l of 40% acetaldehyde/10 ml glacial acetic acid) were added into each tube. After color development overnight at room temperature, the absorbance was read at 595 nm. The ratio of DNA fragmentation was recorded as the percentage of fragmented DNA to total DNA.

DNA electrophoresis was measured as described by Yamada.¹¹⁾ Briefly, portions of 1×10^6 cells with or without drug treatment were washed and spun down. Pellets were re-suspended in 0.6 ml of lysis buffer and incubated for 10 min on ice. After centrifugation at 20000 g , supernatants were mixed gently for 2—3 min with an equal volume of TE-saturated phenol (Wako), followed by centrifuging and mixing supernatants with chloroform: isoamylalcohol (24:1). Then, the supernatants containing DNA after centrifugation at 20000 g were precipitated overnight at -20 °C in 0.3 M NaCl and 70% ethanol. The loading buffer (Wako) containing 0.02% bromophenol blue, 0.02% xylene cyanol FF, 50% glycerol and 0.1% sodium dodecyl sulfate was then added to the samples at 1:10 (v/v) ratio. Electrophoresis was carried out in 2% agarose for 90 min at 50 V, and DNA was visualized with ethidium bromide.

Statistics Data were expressed as mean \pm S.D. Statistical analysis was evaluated by one-way analysis of variance

(ANOVA), followed by the Student-Newman-Kanuls test for multiple comparisons which was used to evaluate the difference between two groups. $p < 0.05$ was considered significant.

RESULTS

Hepatoprotection Activity of Si-Ni-San from CCl₄ in Vivo and in Vitro Si-Ni-San (100, 200 mg/kg) and BDD (150 mg/kg) were administered *p.o.* for 7 d. One hour after the final administration, mice were injected *i.p.* with 0.15% of CCl₄ in olive oil (0.2 ml/20 g body weight). The mice were sacrificed 22 h later for assaying the serum ALT and AST activities. As shown in Table 1, treatment with CCl₄ caused a remarkable enzymatic elevation (CCl₄ group) compared with normal mice. Both 100 and 200 mg/kg of Si-Ni-San, as well as the positive control, BDD, showed significant inhibition of this CCl₄-induced ALT and AST elevation.

Hepatocytes isolated from naive mice were pre-treated with Si-Ni-San for 3 h. After being washed twice with the medium, 0.1 ml of 0.2% of CCl₄ was added for another 1 h incubation, followed by determining the ALT activity in the supernatant. Si-Ni-San inhibited the CCl₄-induced ALT release in a dose-dependent manner (Fig. 1).

Alleviation of BCG+LPS-Induced Liver Injury in Mice by Si-Ni-San Si-Ni-San (100, 200 mg/kg) and BDD

(150 mg/kg) were administered *p.o.* for 10 d from the day of BCG injection. L-NNA was injected *i.p.* twice at 150 mg/kg at 2 h before and 0 h after LPS injection. As shown in Table 2, a remarkable elevation in serum ALT activity was observed in BCG+LPS treated mice (BCG+LPS group) as compared with the normal level. Compared to the BCG+LPS group, both doses of Si-Ni-San caused a significant decrease in serum ALT level, with an increase in NO level, while the co-administration of L-NNA almost completely blocked the inhibitory activity of Si-Ni-San on the elevation in ALT, with a significant reduction in serum NO level. However, BDD significantly reduced the ALT release without affecting the NO production.

Enhancement of LPS-Induced NO Production in J774A.1 Cells by Si-Ni-San J774A.1 cells were cultured in RPMI 1640 medium in 24-well plates (2×10^6 cells per well in a volume of 1 ml) (Nunc) at 37 °C in 5% CO₂/95% air for 2 h adherence. Then they were incubated in the absence or presence of LPS (final concentration 10 µg/ml) and/or Si-Ni-San. As shown in Table 3, Si-Ni-San significantly enhanced the LPS-induced NO production in a dose-dependent manner.

Improvement of the Liver Injury Induced by PCI-DTH in Mice by Si-Ni-San with Reduced Hepatotoxicity of Nonparenchymal Cells Si-Ni-San (100, 200 mg/kg) was

Table 1. Protective Activity of Si-Ni-San against CCl₄-Induced Hepatic Damage in Mice

Groups	Dose (mg/kg)	No. of mice	ALT (Karmen unit)	AST (Karmen unit)
Normal	—	10	48 ± 8	125 ± 34
CCl ₄	—	10	1123 ± 538**	589 ± 336**
CCl ₄ + Si-Ni-San	100	10	224 ± 125 ^{##}	292 ± 233 [#]
	200	10	338 ± 231 [#]	186 ± 86 [#]
CCl ₄ + BDD	150	10	125 ± 87 ^{##}	245 ± 196 [#]

Si-Ni-San (100, 200 mg/kg) and biphenyl dimethyl dicarboxylate (BDD, 150 mg/kg) were administered *p.o.* for 7 d. One hour after the final administration, mice were injected *i.p.* with 0.15% of CCl₄ in olive oil (0.2 ml/20 g body weight). They were sacrificed 22 h later for assaying the serum ALT and AST activities. Each value indicates the mean ± S.D. One-way ANOVA revealed a significant effect at $p < 0.01$. ** $p < 0.01$ vs. normal; [#] $p < 0.05$, ^{##} $p < 0.01$ vs. CCl₄ group (Student-Newman-Kanuls test).

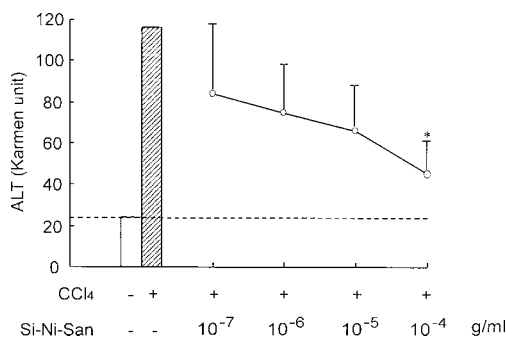


Fig. 1. Effect of Si-Ni-San on CCl₄-Induced Hepatocyte Damage *in Vitro*

Hepatocytes (2×10^4 cells in 0.2 ml WE medium) were pre-cultured in a 96-well microplate for 5 h. After being washed twice, they were treated with the various concentrations of Si-Ni-San for 3 h. Then, the hepatocytes were exposed to 0.2% CCl₄ for 1 h, followed by collection of the supernatant to assay alanine transaminase. Each datum represents the mean ± S.D. of 3 experiments, and each experiment included triplicate sets. One-way ANOVA revealed a significant effect at $p < 0.01$. * $p < 0.05$, ** $p < 0.01$ vs. CCl₄ group (Student-Newman-Kanuls test).

Table 2. Effect of Si-Ni-San on BCG+LPS-Induced Liver Injury in Mice

Group	Dose (mg/kg)	No. of mice	ALT (Karmen unit)	NO ₂ ⁻ level (µmol/l)
Normal	—	10	47.3 ± 19.8	42.7 ± 7.6
BCG+LPS	—	10	429.1 ± 153.4**	91.5 ± 17.0**
BCG+LPS	100	10	208.6 ± 186.0 ^{##}	134.9 ± 27.5 ^{##}
+ Si-Ni-San	200	10	204.4 ± 120.0 ^{##}	140.5 ± 28.4 ^{##}
BCG+LPS	200	10	409.1 ± 151.1 ^{a)}	56.5 ± 20.6 ^{a)}
+ Si-Ni-San + L-NNA				
BDD	150	10	240.8 ± 164.6 [#]	105.4 ± 19.1

Mice were injected *via* the tail vein with 1 mg of BCG in 0.2 ml saline or with saline alone; 10 d later, the mice were injected intravenously with 10 µg of LPS in 0.2 ml saline or with saline alone. L-NNA was injected *i.p.* twice at a dose of 150 mg/kg 2 h before and 0 h after LPS injection. Ten hours after the LPS injection, the mice were bled and the blood was collected for serum ALT and NO assay. Si-Ni-San (100, 200 mg/kg) and biphenyl dimethyl dicarboxylate (BDD, 150 mg/kg) were administered *p.o.* for 10 d from the BCG injection day. Each value indicates the mean ± S.D. One-way ANOVA revealed a significant effect at $p < 0.01$. ** $p < 0.01$ vs. normal; [#] $p < 0.05$, ^{##} $p < 0.01$ vs. BCG+LPS group; ^{a)} $p < 0.01$ vs. BCG+LPS+Si-Ni-San 200 group (Student-Newman-Kanuls test).

Table 3. Effect of Si-Ni-San on LPS-Induced NO Production by J774A.1 Cells *in Vitro*

Group	Dose (g/ml)	NO ₂ ⁻ level (nmol/l)
Spontaneous	—	20.7 ± 3.9
LPS	—	71.3 ± 5.4
LPS + Si-Ni-San	10 ⁻⁷	70.9 ± 5.1
	10 ⁻⁶	82.8 ± 4.7*
	10 ⁻⁵	86.8 ± 4.3*
	10 ⁻⁴	96.7 ± 8.1**

J774A.1 cells were cultured with RPMI 1640 medium in 24-well plates (2×10^6 macrophages/ml/well) (Nunc) at 37 °C in 5% CO₂/95% air for 2 h adherence. Then, they were incubated in the absence or presence of LPS (final concentration 10 µg/ml) and/or Si-Ni-San for 48 h for NO assay. Each datum represents the mean ± S.D. of 3 experiments, and each experiment included triplicate sets. One-way ANOVA revealed a significant effect at $p < 0.01$. * $p < 0.05$, ** $p < 0.01$ vs. LPS group (Student-Newman-Kanuls test).

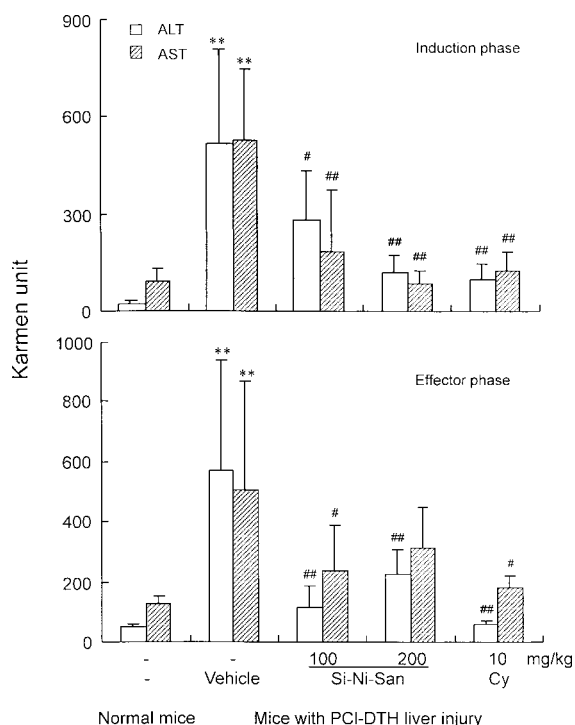


Fig. 2. Effect of Si-Ni-San and Cyclophosphamide (Cy) on the Elevation of Serum Alanine Transaminase (ALT) and Aspartate Transaminase (AST) Activities in Mice with Liver Injury Induced by Delayed-Type Hypersensitivity to Picryl Chloride

Mice were sensitized twice by painting 0.1 ml of 1% picryl chloride in ethanol on the skin of their abdomens at an interval of 5 d. Five days after the second sensitization, they were injected with 10 μ l of 0.5% picryl chloride in olive oil into the liver, followed 18 h later by bleeding. Si-Ni-San (*p.o.*), and Cy (*i.p.*) were given for 10 d from the first sensitization (induction phase), or for 3 times at 0, 5 and 10 h after the picryl chloride challenge in the liver (effector phase). Each column represents the mean \pm S.D. ($n=10$). One-way ANOVA revealed a significant effect at $p<0.01$. ** $p<0.01$ vs. normal; # $p<0.05$, ## $p<0.01$ vs. liver injury treated with vehicle (Student-Newman-Kanuls test).

administered *p.o.*, and cyclophosphamide (10 mg/kg) was given *i.p.* for 10 d from the 1st PCI sensitization (induction phase), or for 3 times, at 0, 5 and 10 h after the PCI challenge (effector phase), respectively. As shown in Fig. 2, the elevation in serum ALT and AST activities was significantly inhibited by Si-Ni-San and cyclophosphamide when given in either the induction or effector phase. In histopathological examinations, the main changes in liver injury were inflammatory infiltration and hepatocellular coagulation necrosis, as reported previously.^{5,6,12-14} These changes were remarkably reduced in most of the mice by Si-Ni-San and cyclophosphamide (data not shown).

In the case in which Si-Ni-San (200 mg/kg) was given 3 times (0, 5, 10 h) after picryl chloride challenge, the nonparenchymal cells and hepatocytes were also isolated 2 h after the last administration. Compared with the liver injury group, nonparenchymal cells obtained from mice administered *p.o.* with Si-Ni-San showed significantly lowered hepatotoxicity, consistent with the significant inhibition of serum ALT elevation (Fig. 3).

Facilitated Apoptosis by Si-Ni-San of Nonparenchymal Cells and Splenocytes from Mice with PCI-DTH Liver Injury as Well as in Splenocytes Activated by Con A *in Vitro* Nonparenchymal cells and spleen cells were isolated from normal mice and mice with PCI-DTH liver injury 12 h after the PCI challenge. In another case, splenocytes from

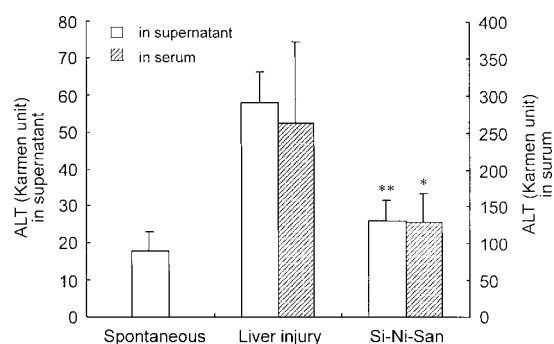


Fig. 3. Effects of Oral Administration of Si-Ni-San on Serum Alanine Transaminase (ALT) Activity and the Potential of Isolated Nonparenchymal Cells to Cause ALT Release from Hepatocytes *in Vitro*

Liver injury was induced by PCI-DTH. Si-Ni-San was given three times (0, 5, 10 h) after the picryl chloride challenge of liver. Two hours after the last administration, serum was collected and liver nonparenchymal cells and hepatocytes were isolated. Hepatocytes (2×10^4) in WE medium (0.2 ml) were precultured in a 96-microplate for 5 h. After being washed twice, they were co-cultured with 8×10^4 nonparenchymal cells at 37°C for a further 3 h. The supernatant was then collected and assayed for ALT activity. Each column represents the mean \pm S.D. of 3-4 mice. One-way ANOVA revealed a significant effect at $p<0.01$. * $p<0.05$, ** $p<0.01$, vs. liver injury group (Student-Newman-Kanuls test).

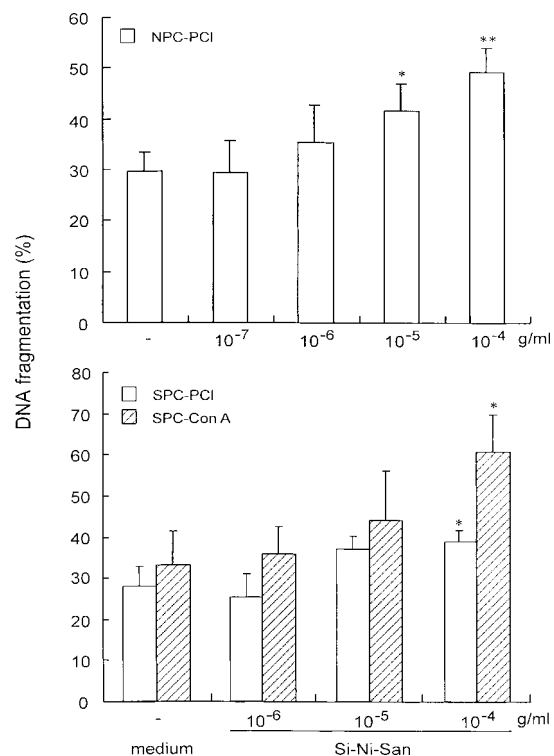


Fig. 4. Effect of Si-Ni-San on DNA Fragmentation in Nonparenchymal Cells and Splenocytes Isolated from Mice with PCI-DTH Liver Injury and in Splenocytes Activated by Con A *in Vitro*

Nonparenchymal cells and splenocytes were isolated 12 h after the PCI challenge. In another case, splenocytes from naive mice were activated by 5 μ g/ml of Con A at 37°C in 5% CO₂ humidified air for 48 h. After being co-cultured with Si-Ni-San for 6 h, DNA fragmentation in the cells was quantified by the diphenylamine method, as described in Materials and Methods. Each column represents the mean \pm S.D. of 3-4 mice. One-way ANOVA revealed a significant effect at $p<0.01$. * $p<0.05$, ** $p<0.01$ vs. medium control (Student-Newman-Kanuls test).

naive mice were activated by 5 μ g/ml of Con A at 37°C in 5% CO₂ humidified air for 48 h. After co-culture with Si-Ni-San for 6 h, DNA fragmentation in nonparenchymal cells and splenocytes from mice with PCI-DTH liver injury was induced in a Si-Ni-San-dose-dependent manner, as well as in

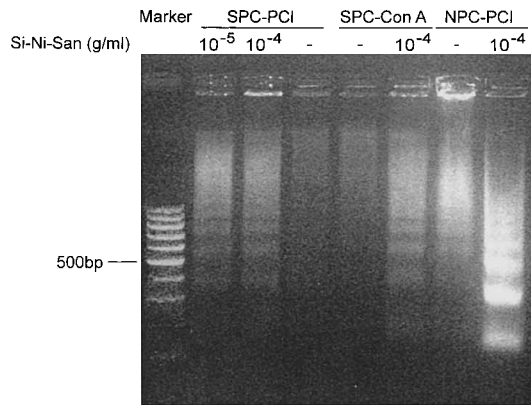


Fig. 5. Gel Agarose Electrophoresis of DNA from Nonparenchymal Cells and Splenocytes, and the Effect of Si-Ni-San on It

Nonparenchymal cells and splenocytes were isolated 12 h after the PCI challenge. In another case, splenocytes from naive mice were activated by 5 μ g/ml of Con A at 37 °C in 5% CO₂ humidified air for 48 h. After being co-cultured with Si-Ni-San for 6 h, DNA electrophoresis was determined as described in Materials and Methods. The results are representative of two separate experiments. SPC-PCI: splenocytes from mice with PCI-DTH liver injury; SPC-Con A: splenocytes from normal mice and activated by Con A *in vitro*; NPC-PCI: nonparenchymal cells from mice with PCI-DTH liver injury.

splenocytes activated by Con A *in vitro* (Figs. 4, 5). No effect of Si-Ni-San on nonparenchymal cells or splenocytes isolated from normal mice was observed (data not shown).

DISCUSSION

The present study first examined the effects of Si-Ni-San on CCl₄-induced liver injury in mice. A 7-d preventive administration of the prescription significantly inhibited the elevation of ALT and AST levels as did BDD, a hepatoprotective agent (Table 1). Similarly, the prescription demonstrated significant hepatoprotection against CCl₄-induced hepatic damage *in vitro* in a dose-dependent manner (Fig. 1). These results suggest that Si-Ni-San may protect the hepatocyte membrane.

The pathogenesis of LPS-elicited liver damage in BCG-pretreated mice includes the infiltration of monocytes or macrophages into the liver and the release of soluble factors toxic to hepatocytes. To this liver injury, Si-Ni-San also showed significant inhibition of the ALT elevation with an increased serum NO level (Table 2). This improving effect of Si-Ni-San against BCG+LPS-induced liver injury was markedly blocked by L-NNA, an inhibitor of NO production. Additionally, Si-Ni-San significantly enhanced LPS-induced NO production in J774.1 cells, the cell line of macrophages (Table 3). Since the protective role of NO on liver injury has been well reported,^{15,16} the above results suggest that the improvement of Si-Ni-San against liver injury may be related to the enhancement of NO production.

Considering the role of T cell-mediated cellular immunity in clinic hepatitis^{17–20} and the effect of Si-Ni-San on T lymphocytes,²¹ the Si-Ni-San was applied to the third model of liver injury induced by a delayed-type hypersensitivity (DTH) reaction to picryl chloride that was established by us, and compared with cyclophosphamide, an immunosuppressor. The key role of the CD4⁺ T lymphocytes that mainly constitute liver-infiltrating leukocytes in this model has been reported in our previous papers.^{5,6,12} Against this model, Si-

Ni-San showed remarkable alleviation when administered during either the induction or effector phase, as did cyclophosphamide (Fig. 2). These findings suggest that the prescription alleviates liver injury not only by protecting the hepatocyte membrane, as BDD does, but also by affecting the immunity, similarly to cyclophosphamide. To elucidate its mechanism, we further investigated the effect of Si-Ni-San on the cytotoxic activity of liver-infiltrating cells against hepatocytes by using a nonparenchymal cell-hepatocyte co-culture assay. This assay has been described previously,⁶ where we showed that nonparenchymal cells from liver injured mice induced the release of ALT and AST from hepatocytes *in vitro*, and the peak potential for ALT release was observed in the nonparenchymal cells isolated 12 h after the induction of liver injury. In this study, consistent with the inhibition of serum ALT by Si-Ni-San, the isolated nonparenchymal cells from Si-Ni-San-administered mice with liver injury lost their hepatotoxic potential almost completely (Fig. 3). These results indicate that the inhibition of serum transaminase activity by Si-Ni-San may be due to the dysfunction of liver nonparenchymal cells, and the *in vitro* co-culture assay could well reflect the *in vivo* mechanism. Furthermore, Si-Ni-San remarkably inhibited the proliferation of spleen cells induced by Con A.²² These findings suggest that Si-Ni-San may alleviate immunological liver injury not only through inhibiting the activation of liver-infiltrating T lymphocytes, but also by inducing the dysfunction of activated lymphocytes.

We⁶ have previously analyzed the cell populations contained in nonparenchymal cells, *i.e.* the population expressing LFA-1 molecule increased from 32–36% at 0 h to 72.6–78% at 12 h of liver injury. This change was due to the infiltration of lymphocytes to the liver, and the nonparenchymal population at 12 h included 47.9% CD4⁺ T cells and 23.2% CD8⁺ T cells. Treatment of nonparenchymal cells with anti-CD4 or anti-CD8 monoclonal antibody plus complement after 12 h, when hepatotoxicity was at its peak, abolished the release of ALT from parenchymal hepatocytes completely or slightly, respectively. Since the nonparenchymal cells at 0 h were not hepatotoxic, these findings suggested that the liver injury was caused by liver-infiltrating lymphocytes in the nonparenchymal cells rather than by resident liver cells. This was supported by the result that Kupffer cell-enriched cells in nonparenchymal cells had no hepatotoxic potential. Based on these findings, the next experiment was performed to examine how Si-Ni-San affects the function of liver-infiltrating cells. Several studies have shown the importance of apoptosis in regulating the development and maturation of the immune response.^{23,24} Based on these findings, we next examined the apoptosis of nonparenchymal cells. Both the diphenylamine method and DNA electrophoresis revealed that the prescription significantly induced apoptosis in nonparenchymal cells and splenocytes from mice with PCI-DTH liver injury, as well as in splenocytes activated by Con A *in vitro* (Figs. 4, 5), but not in normal nonparenchymal cells or normal splenocytes (data not shown). These findings imply that the apoptosis in nonparenchymal cells, mainly the liver-infiltrating cells, induced by Si-Ni-San might be an effective means of causing their dysfunction.

Overall, the present study together with our previous results²² provided an evidence for the alleviating effects of Si-

Ni-San against liver injury through multiple mechanisms. Its mechanisms include protection of the hepatocyte membrane, production of NO in macrophages as a protective factor, inhibition of lymphocyte activation, and the dysfunction of liver-infiltrating cells through causing their apoptosis. Although the anti-hepatotoxic effect of Glycyrrhizae Radix, a constituent medicinal plant of Si-Ni-San, has previously been reported, we have found that the effect of Si-Ni-San is stronger than that of Glycyrrhizae Radix. Detailed studies are in progress to determine which components of Si-Ni-San promote NO production, protect the hepatocyte membrane, inhibit lymphocyte activation, and cause liver-infiltrating cell apoptosis.

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