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# Effect of Two Different Groups of Chinese Medicines on Nitric Oxide Production by Mouse Macrophage-like Cells

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**Abstract.** *Seven Chinese medicines were investigated for their ability to modify nitric oxide (NO) production by unstimulated and lipopolysaccharide (LPS)-stimulated mouse macrophage-like Raw 264.7 cells, in comparison with their radical intensity and scavenging activity. LPS significantly stimulated the NO production by Raw 264.7 cells. Three Chinese medicines, Sho-saiko-to, Hange-shashin-to and Sairei-to (tentatively classified as Group I), significantly reduced the extracellular concentration of NO in the LPS-stimulated cells, slightly below their cytotoxic concentrations. On the other hand, another four Chinese medicines, Byakko-ka-ninjin-to, Hochu-ekki-to, Juzen-taiho-to and Ninjin-yoei-to (tentatively classified as Group II), showed similar effects, but required higher concentrations due to the co-existence of both the inhibitors and stimulators for NO production by activated macrophages. Western blot analysis demonstrated that LPS stimulated the expression of inducible NO synthase (iNOS) at both protein and mRNA levels, and that Sairei-to reduced the LPS-induced iNOS expression more potently than did Juzen-taiho-to. ESR spectroscopy shows that Group I medicines generally produced higher amounts of radicals under alkaline condition, and scavenged superoxide (produced by hypoxanthine-xanthine oxidase reaction) and NO (produced by NOC-7, NO generator) more potently than Group II medicines. These data support the classification of Chinese medicines into two groups: Group I and Group II. The net inhibition of NO production by Group I medicines may be the summation of the radical scavenging activity and the inhibition of iNOS expression due to higher cytotoxicity. Group II medicines showed lower cytotoxicity, lower radical intensity, lower radical scavenging activity, but higher stimulation activity for NO production by macrophages than Group I, suggesting their possible application for immunopotentiation.*

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*Key Words:* Chinese medicine, NO, macrophage, ESR, radical.

Nitric oxide (NO) is produced from L-arginine by NOS (NO synthase) in the presence of NADPH, and displays diverse biological activities such as vasodilation, inhibition of endothelial leukocyte adhesion and regulation of energy metabolism (1). We have previously reported that lignins stimulated the mouse macrophage-like cell line Raw 264.7 to produce not only NO, but also citrulline and asparagine (2), to an extent comparable to that attained by lipopolysaccharide (LPS) (3). This was due to the stimulation of the expression of inducible NOS (iNOS) (unpublished observations) and asparagine synthetase proteins (3) and their mRNAs (unpublished obsevations). On the other hand, phenylpropenoid monomer (precursor of lignins) (4, 5), flavonoids (6) and Himalayan plant extracts (7) did not stimulate, but rather inhibited, LPS-stimulated NO production. This was due to the inhibition of iNOS mRNA and radical scavenging activity (7). These data suggest that these plant materials may modify the functions of macrophages, which play significant both positive and negative roles in immunological reactions.

We have recently found that *Rikko-san* and its major ingredients (*Shoma, Ryutan, Saishin, Bofu*) possess both inhibitors and stimulators for NO production by Raw 264.7 cells (8). The extracellular concentration of NO is determined by the intracellular concentration of iNOS, the enzyme activity of iNOS and the quenching effect of radical scavengers present in the assay system. To further confirm the distribution of stimulators and inhibitors of NO production in various natural products, we investigated here

whether seven Chinese medicines (*Sho-saiko-to, Hange-shashin-to, Byakko-ka-ninjin-to, Hochu-ekki-to, Juzen-taiho-to, Ninjin-yoei-to, Sairei-to*) positively or negatively modify the NO production by LPS-stimulated Raw 264.7 cells. We also investigated, using ESR spectroscopy, whether they produce radicals under neutral to alkaline pH, and scavenge various radical species such as superoxide anion ($O_2^-$) and NO radical, which may modify the effective concentration of NO in the culture medium.

## Materials and Methods

*Materials.* The following chemicals and reagents were obtained from the indicated companies: *Sho-saiko-to* (catalogue No. 9) [40 g contained the dried extract mixture of Bupleuri Radix (7.0 g), Pinelliae Tuber (5.0 g), Scutellariae Radix (3.0 g), Zizyphi Fructus (3.0g), Ginseng Radix (3.0g), Glycyrrhizae Radix (2.0 g) and Zingiberis Rhizoma (1.0 g)], *Hange-shashin-to* (No. 14) [30.8 g contained the dried extract mixture of Pinelliae Tuber (5.0 g), Scutellariae Radix (2.5 g), Zizyphi Fructus (2.5 g), Ginseng Radix (2.5 g), Glycyrrhizae Radix (2.5 g), Coptidis Rhizoma (1.0 g) and Zingiberis Siccatum Rhizoma 2.5 g]], *Byakko-ka-ninjin-to* (No. 34) [56.7 g contained the dried extract mixture of Ginseng Radix (1.5 g), Glycyrrhizae Radix (2.0 g), Oryzae Semen (8.0 g), Gypsum Fibrosum (15.0 g) and Anemarrhenae Rhizoma (5.0 g)], *Hochu-ekki-to* (No. 41) [36 g contained the dried extract mixture of Bupleuri Radix (2.0 g), Zizyphi Fructus (2.0 g), Ginseng Radix (4.0 g), Glycyrrhizae Radix (1.5 g), Zingiberis Rhizoma (0.5 g), Astragali Radix (4.0 g), Atractylodis Lanceae Rhizoma (4.0 g), Angelicae Radix (3.0 g), Aurantii Nobilis Pericarpium (2.0 g) and Cimicifugae Rhizoma(1.0 g)], *Juzen-taiho-to* (No. 48) [42.8 g contained the dried extract mixture of Ginseng Radix (3.0 g), Glycyrrhizae Radix (1.5 g), Astragali Radix (3.0 g), Cinnamomi Cortex (3.0 g), Rehmanniae Radix (3.0 g), Atractylodis Lanceae Rhizoma (3.0 g), Hoelen, Angelicae Radix (3.0 g), Paeoniae Radix (3.0 g) and Cnidii Rhizoma (3.0 g)], *Ninjin-yoei-to* (No. 108) [46.5 g contained the dried extract mixture of Ginseng Radix (3.0 g), Glycyrrhizae Radix (1.0 g), Astragali Radix (1.5 g), Cinnamomi Cortex (2.5 g), Rehmanniae Radix (4.0 g), Hoelen (4.0 g), Angelicae Radix (4.0 g), Paeoniae Radix (2.0 g), Aurantii Nobilis Pericarpium (2.0 g), Polygalae Radix (2.0 g), Atractylodis Rhizoma (4.0 g) and Schisandrae Fructus (1.0 g)], *Sairei-to* (No. 114) [60 g contained the dried extract mixture of Bupleuri Radix (7.0 g), Pinelliae Tuber (5.0 g), Scutellariae Radix (3.0 g), Zizyphi Fructus (3.0 g), Ginseng Radix (3.0 g), Glycyrrhizae Radix (2.0 g), Zingiberis Rhizoma (1.0 g), Cinnamomi Cortex (3.0 g), Atractylodis Lanceae Rhizoma (2.0 g), Hoelen (3.0 g), Polyporus (3.0 g) and Alismatis Rhizoma (5.0 g)] (Tsumura Corp., Tokyo, Japan) (Major components of each ingredient in seven Chinese medicines are listed in Table I); DMEM, phenol red-free DMEM (Gibco BRL, NY, USA); fetal bovine serum (FBS), LPS from *Escherichia coli.* (serotype 0111:B4), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), phenylmethylsulfonyl fluoride (PMSF), hypoxanthine (HX), xanthine oxidase (XOD) (Sigma Chem Co., St. Louis, MO, USA); diethylenetriamine-pentaacetic acid (DETAPAC) (Wako Pure Chem Ind., Osaka, Japan); 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), superoxide dismutase (SOD) from bovine erythrocytes, 1-hydroxyl-2-oxo-3-*N*-3-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO)(Dojin, Kumamoto, Japan).

*Cell culture.* Raw 264.7 cells (9) were cultured in DMEM supplemented with 10% heat-inactivated FBS, under a humidified 5% $CO_2$ atmosphere.

*Determination of cytotoxic activity.* Near confluent Raw 264.7 cells were treated for 24 hours with various concentrations of each test sample. Cells were washed once and incubated for 4 hours at 37 ˚C with 0.1 mL of fresh medium containing 0.2 mg/mL MTT. After removal of the medium, the cells were lysed with 0.1 mL of DMSO and the absorbance at 540 nm ($A_{540}$) of the cell lysate, which reflects the relative viable cell number, was then determined. The 50% cytotoxic concentration ($CC_{50}$) was determined from the dose-response curve (5-7).

*Assay for NO production.* Near confluent Raw 264.7 cells were incubated for 24 hours with various concentrations of each test sample in phenol red-free DMEM supplemented with 10 % FBS. The extracellular concentration of NO was quantified with Griess reagent, using the standard curve of $NO_2^-$ (5-7). It is possible that the brownish color of the sample might interfere with the precise calculation of NO production. To avoid this, the net NO production was calculated by subtracting the value measured without cells from that measured with the cells. The concentration of each extract to inhibit the LPS-induced NO production by 50% ($IC_{50}$) was determined from the dose-response curve. The inhibitory effect of NO production was evaluated by the selectivity index (SI), which was calculated by the following equation:

$$SI = CC_{50}/IC_{50}$$

*Western blotting.* The cells were lysed with 100 μL of lysis buffer (10 mM Tris-HCl, pH 7.6, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA and 2 mM PMSF) for 10 minutes in ice water, and then incubated for 50 minutes at 4˚C with RT-5 ROTATOR (Titec, Saitama, Japan). Cell lysates were centrifuged at 16,000 xg for 20 minutes at 4˚C to remove insoluble materials and the supernatant was collected. The protein concentration of the supernatant was determined by Protein Assay Kit (Bio Rad, Hercules, CA, USA). Cell lysates (containing 10 μg protein) were mixed with 2 x sodium dodecyl sulfate (SDS)-sample buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromphenol blue, 1.2% 2-mercapto-ethanol), boiled for 10 minutes and applied to SDS-8% polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in phosphate-buffered saline plus 0.05% Tween 20 overnight at 4˚C and incubated with anti-iNOS antibody (1:1,000) (Santa Cruz Biotechnology, Delaware, CA, USA) for 90 minutes at room temperature, before incubation with horseradish peroxidase-conjugated anti-rabbit IgG for 1 hour at room temperature (8). Immunoblots were developed by Western Lightning™ Chemiluminescence Reagent *Plus* (Perkin Elmer Life Sciences, Boston, MA, USA), and analyzed on a Macintosh (Power Macintosh 7600/120) computer, using the public domain NIH Image program (developed at the U.S. National Institute of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, USA, part number PB95-500195GEI).

Table I. *Major components of ingredients in Chinese medicines.*

| Ingredients | Major components |
|---|---|
| Bupleuri Radix | saikosaponin a-f, α-spinasterol, *l*-anomalin |
| Pinelliae Tuber | homogentisic acid, 3,4-dihydroxybenzaldehyde, *l*-ephedrine |
| Scutellariae Radix | baicalein, baicalin, wogonin, campesterol |
| Zizyphi Fructus | oleanolic acid, betulinic acid, zyzyphus saponin |
| Ginseng Radix | ginsenodise-Ro, Ra-Rn, 20-glucoginsenoside-Rf, panaxynol |
| Glycyrrhizae Radix | glycyrrhizin, liquiritin, glabric acid, licoricidin |
| Zingiberis Rhizoma | zingiberen, zingerone, gingerol, shogaol, limonen |
| Astragali Radix | astragaloside I, nicotianamin, formononetin |
| Cinnamomi cortex | cinnamic aldehyde, *O*-methoxycinnamic aldehyde |
| Rehmanniae Radix | capalpol, aucubin, β-sitosterol |
| Atractylodis Lanceae Rhizoma | hinesol, β-eudemol, atractylodin |
| Hoelen | eburicoic acid |
| Angelicae radix | ligustilide n-butylidenephathalide, falcarinol, falcarindol |
| Paeonic Radix | paneoniflorin, oxypaeoniflorin, benzoylpaeoniflorin |
| Aurantii Nobilis Pericarpium | *d*-limonene, *l*-synephrine, nobiletin, hesperidine |
| Coptidis Rhizoma | berberine, coptisine, worenine, palmatine, jateorrhizine |
| Polyporus | ergosterol, α-hydroxytetracosaic acid |
| Oryzae Semen | dextrin, vitamin $B_1$, oryzabran A-D |
| Cinidii Rhizoma | butylphthalide, butylidenephthalide, ligustilide |
| Cimicifugae Rhizoma | cimicifugoside, cimigenol, acerinol |
| Polygalae radix | onjisaponin A-G |
| Zingiberis Siccatum Rhizoma | zingiberene, zingerone |
| Alismatis Rhizoma | alisol A, B, lechitin |
| Glypsum Fibrosum | $CaSO_4.2H_2O$ |
| Atractylodis Rhizoma | atractylon, atractylenolide I, II, III |
| Anemarrhenae Rhizoma | sarsasaponin, trimosaponin, neogitogenin |
| Schisandrae Fructus | Schizandrin, gomisin A, sesquicarene |

*Assay for mRNA expression.* Total RNA was isolated by PURESCRIPT RNA Isolation kit (Gentra systems, Minneapolis, MN, USA) protocol. Raw 264.7 cells were lysed in 300 μL cell lysis solution, then 100 μL Protein-DNA precipitation solution was added. The cell lysates were centrifuged at 15,000 x g for 3 minutes. To the supernatant, 300 μL isopropanol was added. After centrifugation at 15,000 x g for 3 minutes, the pellet was washed in 300 μL 75% ethanol. After centrifugation at 15,000 x g for 1 minute, the pellet was air dried for 15 minutes and dissolved in DEPC-treated $H_2O$. A reverse transcriptase reaction (RT) was performed with 1.0 μg of total RNA, using the Rever Tra Ace (Toyobo Co., Ltd., Osaka, Japan), using oligo $(dT)_{20}$ primer. Single-strand cDNA obtained by RT reaction was amplified, using the KOD plus (Toyobo), using iNOS specific primer of (5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' and 5'-GGCTG TCAGAGCCTCGTGGCTTTGG-3'), human β-actin F specific primers (5'-GAGGCCCAGAGCAAGAGAGG-3' and 5'-TACA TGGCTGGGGTGTTGAA-3'), according to the manufacturer's instruction. RT-PCR products were applied to 2% agarose gel and the ethidium bromide-stained gel was then photographed under UV light.

*Assay for radical intensity.* The radical intensity of the test sample was determined at 25˚C in 0.1 M Tris-HCl buffer (pH 7.4), 0.1 M $NaHCO_3/Na_2CO_3$ buffer (pH 9, 10) or in 0.1 M KOH (pH 12.5), using ESR spectroscopy (JEOL JES RE1X, X-band, 100 kHz modulation frequency). Instrument settings: center field, 336.0±5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT; gain, 630; time constant, 0.03 seconds; scanning time, 2 minutes. The radical intensity was defined as the ratio of the peak height of these radicals to that of MnO (10).

To determine $O_2^-$, produced by HX-XOD reaction (total volume: 200 μL) [2 mM HX in 0.1 M phosphate buffer, pH 7.4 (PB) 50 μL, 0.5 mM DETAPAC 20 μL, 8 % DMPO 30 μL, PB 20μL, sample (in $H_2O$) 50 μL, XOD (0.5 U/mL in PB) 30 μL], the gain, time constant and scanning time were changed to 500, 0.1 seconds and 1 minute, respectively. The radical intensity was determined 1 minute after mixing. The $O_2^-$ scavenging activity was expressed as SOD unit/mg, by calibration with the standard curve of SOD (10).

For the determination of NO radical, the sample was added to the reaction mixture of 20 μM carboxy-PTIO and 50 μM NOC-7 in 0.06 M phosphate buffer, pH 7.4. The gain and scanning time were changed to 250 and 2 minutes, respectively. The NO radical intensity was defined as the ratio of the peak height of the 1st peak of carboxy-PTI (indicated by arrows in Figure 4C), which was produced by the reaction of NO (derived from NOC-7) and carboxy-PTIO (11), to that of MnO. To determine the general reduction activity, the sample was added to the carboxy-PTI solution (which was produced by preincubation for 10 minutes with carboxy-PTIO and NOC-7) and 3 minutes later the radical intensity was determined.

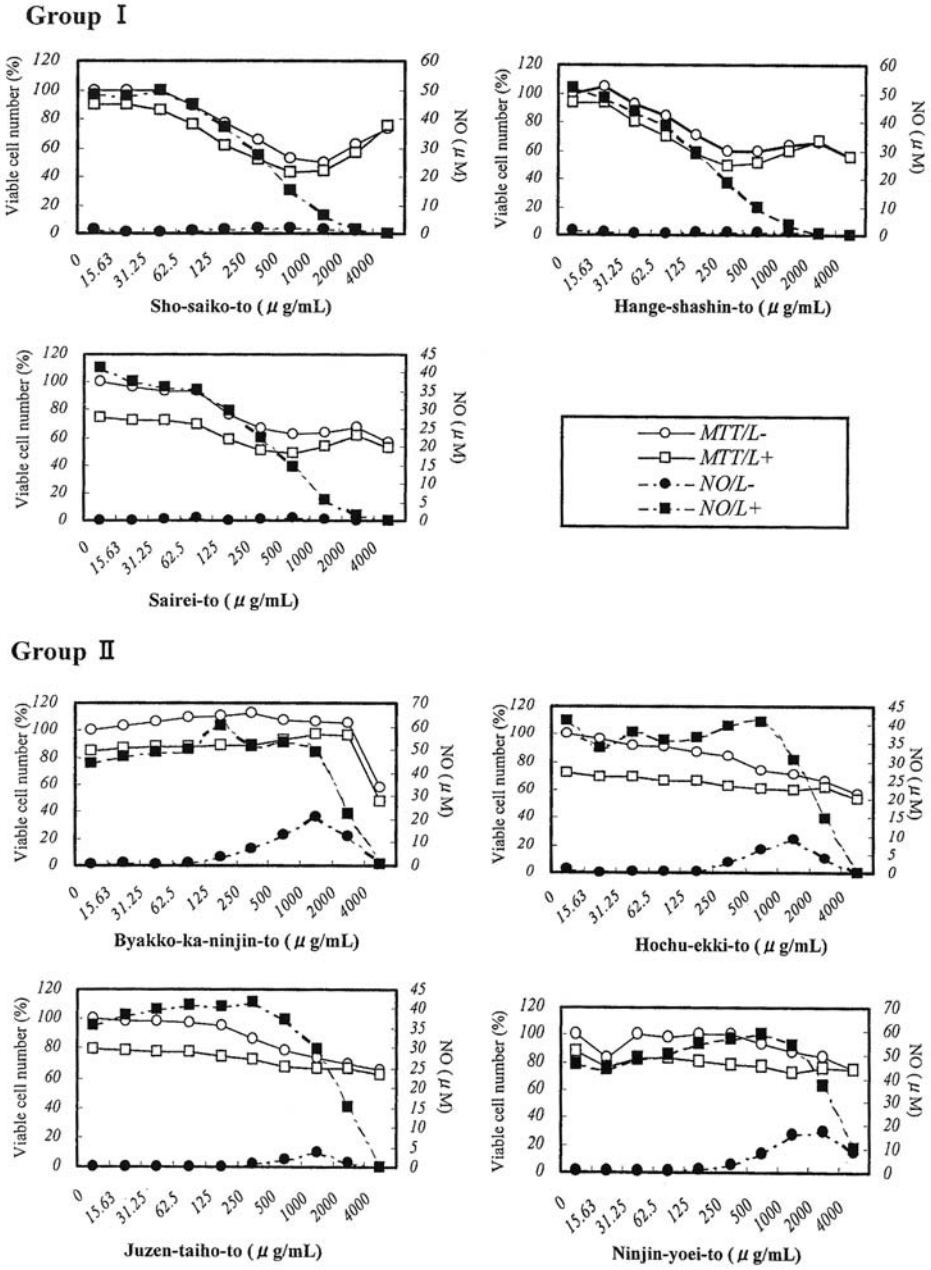


Figure 1. *Effect of seven Chinese medicines on the viable cell number and NO production by unstimulated or LPS-stimulated Raw 264.7 cells. Near confluent 264.7 cells were incubated for 24 hours with 0.1 mL of fresh phenol red-free-DMEM supplemented with 10% FBS containing the indicated concentrations of each sample in the presence (□,■) or absence (○,●) of 100 ng/mL LPS. The viable cell number (% of control) (○,□) and extracellular NO concentration (●,■) were then determined. Each symbol represents the mean of 4 determinations.*

## Results

*NO production.* LPS (100 ng/mL) significantly stimulated the NO production by Raw 264.7 cells, from the background level to approximately 50 μM (Figure 1). Among seven Chinese medicines, *Sho-saiko-to* (No. 9), *Hange-shashin-to* (No. 14) and *Sairei-to* (No. 114) (tentatively classified as Group I) showed varying extents of cytotoxic activity ($CC_{50}$=950, 525 and >4000 μg/mL, respectively), which was further enhanced in the presence of LPS ($CC_{50}$=291, 243 and 375 μg/mL, respectively) (Table II). Group I medicines alone did not significantly induce the NO production by unstimulated Raw 264.7 cells, but effectively inhibited the

Table II. *Inhibition by Chinese medicines of LPS-induced NO production in Raw 264.7 cells.*

| Chinese medicine | $CC_{50}$ (μg/mL) | | $IC_{50}$ (μg/mL) | SI= $CC_{50}$/ $IC_{50}$ |
|---|---|---|---|---|
| | LPS (-) | LPS (+) | LPS(+) | |
| Group I: | | | | |
| *Sho-saiko-to* (No. 9) | 950 | 291 | 318 | 0.91 |
| *Hange-shashin-to* (No. 14) | 525 | 243 | 160 | 1.51 |
| *Sairei-to* (No. 114) | >4000 | 375 | 303 | 1.24 |
| Group II: | | | | |
| *Byakko-ka-ninjin-to* (No. 34) | >4000 | 3900 | >4000 | <0.97 |
| *Hochu-ekki-to* (No. 41) | >4000 | 3750 | 1641 | 2.29 |
| *Juzen-taiho-to* (No. 48) | >4000 | >4000 | 1825 | >2.19 |
| *Ninjin-yoei-to* (No. 108) | >4000 | >4000 | 3048 | >1.12 |

These values were calculated from Figure 1.

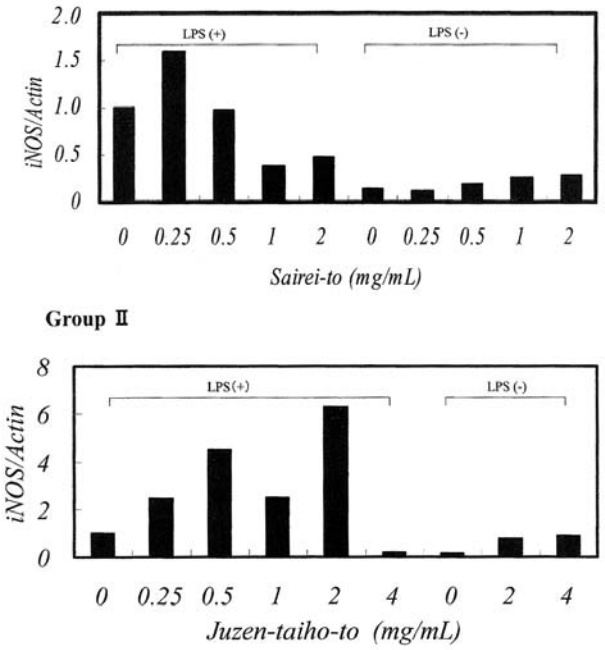


Figure 2. *Effect of Sairei-to (No. 114) and Juzen-taiho-to (No. 48) on iNOS protein expression in Raw 264.7 cells. Raw 264.7 cells were incubated for 24 hours with the indicated concentrations of Sairei-to (No. 114) or Juzen-taiho-to (No. 48) in the absence or presence of 100 ng/mL LPS, and iNOS protein was quantified by Western blot analysis.*

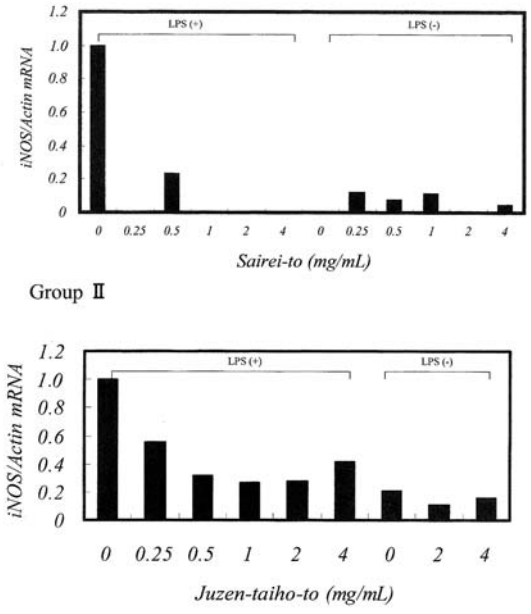


Figure 3. *Effect of Sairei-tou (No. 114) and Juzen-taiho-to (No. 48) on iNOS mRNA expression. Raw 264.7 cells were incubated for 24 hours with the indicated concentrations of Sairei-to (No. 114) or Juzen-taiho-to (No. 48) in the absence or presence of 100 ng/mL LPS. RNA was then isolated, and the RT-PCR product was applied to agarose gel electrophoresis. Expression of iNOS mRNA was plotted as a ratio to β-actin mRNA expression.*

NO production by LPS-stimulated Raw 264.7 cells ($IC_{50}$=318, 160 and 303 μg/mL, respectively), giving rise to the selectivity index (SI) value of 0.91, 1.51 and 1.24, respectively (Figure 1, Table II). The lower value of SI may be due to the enhanced cytotoxicity in the presence of LPS.

On the other hand, *Byakko-ka-ninjin-to* (No. 34), *Hochu-ekki-to* (No. 41), *Juzen-taiho-to* (No. 48) and *Ninjin-yoei-to* (No. 108) (tentatively classified as Group II) showed much lower cytotoxic activity, regardless of the presence ($CC_{50}$>3750 μg/mL) or absence ($CC_{50}$>4000 μg/mL) of LPS (Figure 1). These samples also inhibited the NO production by LPS-stimulated Raw 264.7 cells, at markedly higher concentrations ($IC_{50}$=>4000, 1641, 1825 and 3048 μg/mL, respectively) (SI=<0.97, 2.29, >2.19 and >1.12, respectively) (Table II). Group II medicines stimulated the NO production by unstimulated Raw 264.7 cells (Figure 1).

*iNOS expression.* Western blot analysis demonstrated that LPS stimulated the expression of iNOS protein in Raw 264.7 cells (Figure 2). *Sairei-to* (No. 114), which belongs to Group I, dose-dependently inhibited the LPS-stimulated iNOS expression. The inhibitory effect of *Sairei-to* appeared above 1 mg/mL. On the other hand, *Juzen-taiho-to* (No. 48), which belongs to Group II, stimulated the LPS-stimulated iNOS protein expression at lower concentrations (0.25-2 mg/mL), but inhibited it at higher concentration (4 mg/mL), further confirming the coexistence of both stimulators and inhibitors of NO production (Figure 2). It should be noted that *Juzen-taiho-to* alone also produced iNOS protein, more potently than *Sairei-to*.

These two Chinese medicines inhibited the LPS-stimulated expression of iNOS mRNA (Figure 3). Their

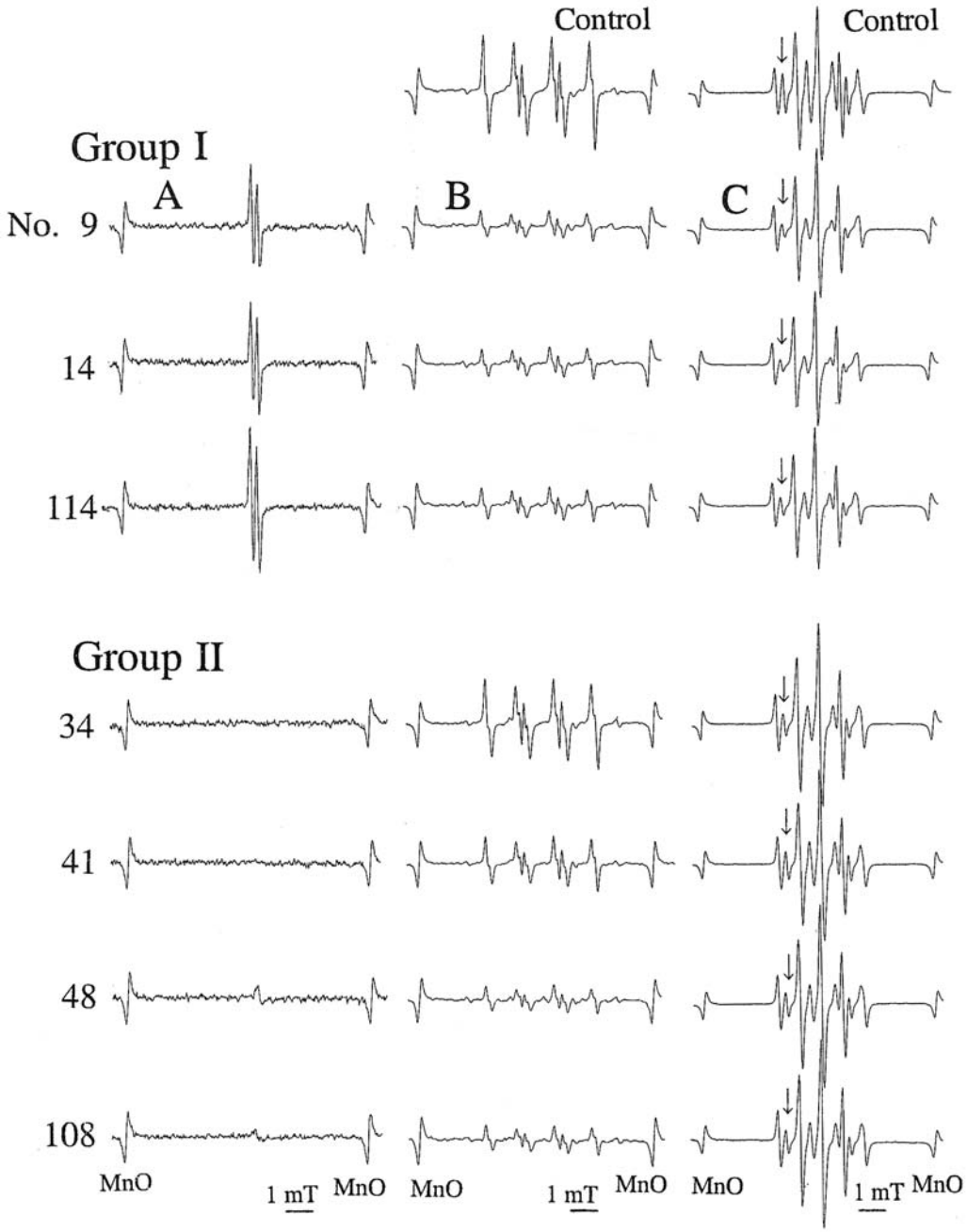


Figure 4. *Radical intensity (A), $O_2^-$ scavenging activity (B) and NO radical scavenging activity (C) of Group I [Sho-saiko-to (No. 9), Hange-shashin-to (No. 14), Sairei-to (No. 114)] and Group II [Byakko-ka-ninjin-to (No. 34), Hochu-ekki-to (No. 41), Juzen-taiho-to (No. 48), Ninjin-yoei-to (No. 108)]. (A) Sample was added at 1 mg/mL in 0.1 M $Na_2CO_3$/$NaCO_3$ buffer (pH 9.0). (B) Sample was added at 1 mg/mL. (C) Sample was added at 1 mg/mL.*

inhibitory effects were observed above 0.25 mg/mL. *Juzen-taiho-to*, but not *Sairei-to*, slightly enhanced the LPS-stimulated iNOS mRNA expression (Figure 3).

*Radical generation.* ESR spectroscopy shows that Group I medicines (No. 9, 14, 114) produced radicals under alkaline condition (Figure 4A). Their radical intensity was below the detection limit at pH 7.4, became detectable at pH 8.0, reached a maximum level at pH 9.0, and declined at higher pH (data not shown). The radical intensity of Group I medicines (No. 9, 14, 114) measured at pH 9.0 (1.97, 1.87 and 2.48, respectively) was larger than Group II medicines

*Table III. Radical intensity and scavenging activity of Chinese medicines*

| | Radical intensity at 9.0 | $O_2^-$ scavenging activity $IC_{50}$ μg/mL | SOD unit/mg | NO scavenging activity $IC_{50}$ μg/mL |
|---|---|---|---|---|
| Group I: | | | | |
| *Sho-saiko-to* (No. 9) | 1.97 | 41.5 | 21.7 | 104.7 (53%)[a)] |
| *Hange-shashin-to* (No. 14) | 1.87 | 20.1 | 47.3 | 81.6 (63%) |
| *Sairei-to* (No. 114) | 2.48 | 40.9 | 22.0 | 159.4 (73%) |
| Group II: | | | | |
| *Byakko-ka-ninjin-to* (No. 34) | 0.03 | 191.8 | 4.8 | 261.5 (5%) |
| *Hochu-ekki-to* (No. 41) | 0.03 | 87.9 | 10.2 | >400.0 (20%) |
| *Juzen-taiho-to* (No. 48) | 0.35 | 17.0 | 56.5 | 318.7 (42%) |
| *Ninjn-yoei-to* (No. 108) | 0.22 | 33.0 | 27.6 | 243.1 (25%) |

These values were calculated from the experiments included in Figure 4.
[a)]Number in the parenthesis represents % of reducing activity in NO scavenging activity. To determine the general reduction activity, the sample was added to the carboxy-PTI solution and 3 minutes later the radical intensity was determined.

(No. 34, 41, 48, 108) (0.03, 0.03, 0.35 and 0.22, respectively) (Figure 4A, Table III).

Group I medicines showed slightly higher $O_2^-$ scavenging activity (SOD activity=21.7, 47.3 and 22.0 SOD unit/mg, respectively) than Group II medicines (SOD activity=4.8, 10.2, 56.5 and 27.6 SOD unit/mg, respectively) (Figure 4B, Table III).

Group I medicines showed higher NO scavenging activity ($IC_{50}$=104.7, 81.6 and 159.4 μg/mL, respectively) than Group II medicines ($IC_{50}$=265.1, >400, 318.7 and 243.1 μg/mL, respectively) (Figure 4C, Table III). About 53, 63 and 73% of the NO scavenging activity of Group I medicines was due to their direct reducing activity, whereas only 5, 20, 42 and 25% of NO scavenging activity of Group II medicines was derived from their reducing activity (Table III). Therefore, the NO radical scavenging activity of Group I and II medicines seems not merely to be due to their reducing activity. Their NO radical scavenging activity might reduce the effective concentration of NO, released from the activated macrophages.

Table IV. *Comparison between Group I and Group II Chinese medicines of various biological activities.*

| | Group I | Group II |
|---|---|---|
| | No. 9, 14, 114 | 34, 41, 48, 108 |
| Cytotoxicity | High | Low |
| Radical generation | High | Low |
| Radical scavenging activity | | |
| $O_2^-$ | High and low | High and low |
| NO | High | Low |
| Spontaneous NO production | Background level | Observed |

## Discussion

The present study demonstrates, for the first time, that Chinese medicines can be classified into two groups, depending upon the absence or presence of stimulators of NO production for macrophages: Group I (No. 9, 14, 114), which only contains the inhibitors and Group II (No. 34, 41, 48, 108), which contains both the inhibitors and stimulators (Table IV).

Group I medicines showed higher cytotoxicity, higher radical intensity, higher radical scavenging activity and much lower spontaneous NO production, suggesting the possible link between the cytotoxicity and radical generation/scavenging activity (Table IV). The net inhibition of NO production by Group I medicines may be the summation of the radical scavenging activity and the inhibition of iNOS expression due to higher cytotoxicity.

Group II medicines showed lower cytotoxicity, lower radical intensity, lower radical scavenging activity, but higher spontaneous NO production than Group I medicines (Table IV). These properties of Group II medicines suggest their possible immunopotentiation capability.

Most previous works with plant materials have shown the presence of inhibitors of NO production by macrophages (9), but not that of stimulators. We found that most of the Chinese medicines (4 out of 7) contain not only the inhibitors, but also the stimulators, of macropahges. We found that *Rikko-san*, which also contains both the inhibitors and stimulators, and that some ingredients of *Rikko-san* (*Shoma, Ryutan, Saishin, Bofu*) stimulated NO production by Raw 264.7 cells to a comparable extent as that attained by LPS (8). This suggests that the relative amount of the inhibitor and stimulator for NO production may determine the extent of inhibition of NO production by macrophages. Further study is required to identify these inhibitors and stimulators.

The regulation mechanism of NO production by macrophage through Chinese medicines has not yet been elucidated. The study with the NOS inhibitor suggests that *Sairei-to* induces the diuretic response *via* the stimulation of NO production (12). A higher molecular weight fraction of *Sairei-to*, *Juzen-taiho-to* and another 16 Chinese medicines stimulated the NO production by Raw 264.7 cells, however, the stimulators have not been analyzed in detail yet (13). It has recently been reported that *Juzen-taiho-to* alone did not induce NO production, but enhanced LPS-stimulated NO production by Raw 264.7 cells (14). These findings, including ours about the NO production by *Juzen-taiho-to*, contradict each other. Further study to identify the stimulators in *Juzen-taiho-to* is urgent to elucidate the modulation mechanism of NO production by Chinese medicines.

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