Protective effects of four traditional Chinese "warming" preparations on endotoxin shock in rats

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Abstract

The present study investigated the effects of four Chinese "warming" preparations (Kanzo-kankyo-to (甘草乾姜湯), Shigyaku-to (四逆湯), Shigyaku-ka-ninjin-to (四逆加人参湯) and Bukuryo-shigyaku-to (茯苓四逆湯)) and one constituent herb, Aconiti tuber, on endotoxin shock in rats. Data showed all preparations and Aconiti tuber alone improved blood pressure, maintained heart rate, attenuated endotoxin-induced hemoconcentration, inhibited the increase of neutrocyte and improved survival rate in various degrees. In addition, the preparations with or without Aconiti tuber showed the obvious differences in the improvements. These results suggest that all preparations have some therapeutic effects on the rats with endotoxin shock. Aconiti tuber plays an important role in protecting the rats from endotoxin shock in our study.

Key words herbs, endotoxin shock, survival rate, blood pressure, heart rate, blood analysis.

Abbreviations AT, Aconiti tuber ; BS, Bukuryo-shigyaku-to (Fuling-sini-tang), 茯苓四逆湯 ; HR, heart rate ; i.p., intraperitoneal ; KK, Kanzo-kankyo-to (Gancao-ganjiang-tang), 甘草乾姜湯 ; MAP, mean arterial pressure ; SG, Shigyaku-to (Sini-tang), 四逆湯 ; SN, Shigyaku-ka-ninjin-to (Sini-jia-renshen-tang), 四逆加人参湯.

Introduction

Endotoxin shock, a highly lethal syndrome, is a profound systemic inflammatory response characterized by hypotension and multiple organ failure. It is initiated by endotoxin (lipopolysaccharide) that is a component of the outer membrane of Gram-negative bacteria. The toxic effects of endotoxin are mostly due to the multiple inflammatory mediators released from monocytes or macrophages, endothelial cells, neutrophils and the others. Based on the pathogenesis, many therapies have been proposed in literature. One common aspect of these treatments was that it prevented the development of a profound systemic inflammation by modulating immune response.[1,2,3]

Many traditional Chinese medicines as modulators of immune response have been examined to treat bacterial infections,[4,5] virus infections,[6] lethal irradiation,[7] autoimmune disease[8,9] and cancer.[10] It was also reported that Sho-saiko-to (小柴胡湯) prevented oxygen toxicity, membrane damage and metabolic disorders during endotoxemia.[11,12] Sho-saiko-to depressed nitric oxide formation in endotoxin activated macrophage cells.[13] But little is known about the effects of traditional Chinese "warming" preparations on endotoxin shock.

The "warming" preparation, Shigyaku-to (SG, Sini-tang, 四逆湯) was prescribed originally for the patients with general weakness, weak pulse and cold extremities,[14]; and these symptoms were observed frequently in endotoxin shock. The other three "warming" preparations were prepared based on SG by adding or removing some constituent herbs. These preparations were Kanzo-kankyo-to (KK, Gancao-ganjiang-tang, 甘草乾姜湯), Shigyaku-ka-ninjin-to

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Table I Composition of the preparations and dosages used in the experimental groups.

Group	n	Treatment: saline	Treatment: ETX	Treatment: preparations	Composition of preparations and dosages used: Glycyrrhizae radix (g)	Zingiberis rhizoma (g)	Aconiti tuber (g)	Ginseng radix (g)	Hoelen (g)	Dosage used (mg/kg/day)
A	6	+	−	distilled water	−	−	−	−	−	−
B	30	−	+	distilled water	−	−	−	−	−	−
C	20	−	+	Kanzo-kankyo-to (KK)	8.0	4.0	−	−	−	530
D	20	−	+	Shigyaku-to (SG)	4.8	3.6	2.4	−	−	830
E	20	−	+	Shigyaku-ka-ninjin-to (SN)	4.8	3.6	2.4	2.4	−	1,020
F	20	−	+	Bukuryo-shigyaku-to (BS)	2.4	1.8	1.2	1.2	4.8	475
G	20	−	+	Aconiti tuber (AT)	−	−	5.0	−	−	1,000

n : number of animals. ETX : endotoxin (9 mg/kg, i.p.).

(SN, Sini-jia-renshen-tang, 四逆加人参湯), and Bukuryo-shigyaku-to (BS, Fuling-sini-tang, 茯苓四逆湯). Each of these three preparations also had an efficacy similar to SG.[15] The constituent herbs of those four preparations are listed in Table I.

In the present study, we investigated the improvements on endotoxin shock in rats with intraperitoneal endotoxin injection by oral administration of four preparations. To further intensify whether one constituent herb, Aconiti tuber, played an important role on improving endotoxin shock, the efficacy of Aconiti tuber alone was also investigated.

Materials and Methods

Endotoxin : Endotoxin (0111 : B4, *E. coli* lipopolysaccharide ; Boivin method ; Difco Laboratories Co.) dissolved in sterile physiological saline in a concentration of 3 mg/ml was injected intraperitoneally.

Preparations : Five kinds of crude herbs were supplied by Tsumura Co. (Tokyo, Japan). They are Gancao (甘草), Glycyrrhizae radix, *Glycyrrhiza glabra* L. var. *glandulifera* REG. et HERD., a chopped crude herb (Neimenggu, 内蒙古 ; China) ; Ganjiang (乾姜), Zingiberis rhizoma, *Zingiber officinale* ROSC., a chopped crude herb (Sichuan Prov., 四川省 ; China) ; Bushi (附子), Aconiti tuber (AT), *Aconitum carmichaeli* DEBX., powder was purified from a root of Aconiti tuber by autoclaving under high temperature to reduce its toxicity (Iwate Pref. ; Japan) ; Ninjin (人参), Ginseng radix, *Panax ginseng* C.A. MEY., a chopped crude herb (Nagano Pref. ; Japan) and Fuling (茯苓), Hoelen, *Poria cocos* WOLF, a chopped crude herb (Sichuan Prov., 四川省 ; China). The composition of the preparations and the dosages of boiled water extract (KK), the mixtures (SG, SN and BS) of boiled water extract and Acoiti tuber, and Aconiti tuber alone (AT) are listed in Table I. The animal dosage was five folds of human (weight 50 kg) daily dosage. Each preparation was dissolved in distilled water at an appropriate concentration just before it was administered orally to rats.

The composition of four "warming" preparations were as follows :

KK : Glycyrrhizae radix, Zingiberis rhizoma were immersed in 270 ml distilled water at room temperature for 30 min and then heated to 100°C. The heating was stopped when the volume was reduced to 110 ml. The extract solution was dried through vacuum evaporation at the temperature of −60°C, and KK extract was obtained.

SG : AT was added to the extract solution. The other procedures were the same as those described in KK.

SN : Glycyrrhizae radix, Zingiberis rhizoma and Ginseng radix were immersed in distilled water. The other procedures were the same as those in SG.

BS : Glycyrrhizae radix, Zingiberis rhizoma, Ginseng radix and Hoelen were immersed in distilled water. The other procedures were the same as those in SG.

Animals : Male Sprague-Dawley SPF rats (from Charles River Co., Japan), weighing 250-300 g, were housed under standard conditions for one week before the experiment.

Grouping and treatment : There were seven study

groups listed in Table I. The groups were Group A (n=6, saline) : normal control received intraperitoneal saline injection. Group B (n=30, endotoxin) : experimental control received endotoxin intraperitoneally. Experimental groups C (n=20, endotoxin and KK), D (n=20, endotoxin and SG), E (n=20, endotoxin and SN), F (n=20, endotoxin and BS) and G (n=20, endotoxin and AT) were treated separately with the preparations and AT alone under investigation after intraperitoneal endotoxin injection. Rats received saline or endotoxin (9 mg/kg) intraperitoneally after fasting for 24 hours and then were administered orally 2 ml solution of vehicle (distilled water), the preparations or AT alone at 1, 24 and 48 hours after the intraperitoneal endotoxin injection. The study was approved by the Animal Care Committee of Fukui Medical University and conducted according to the protocol of animal use.

Observation of Survival and Measurements of MAP and HR : We observed survival rates between 0 and 72 hours at 8-hour intervals after endotoxin injection. We measured mean arterial pressure (MAP) and heart rate (HR) with BP · MONITOR MK-1000 (Shitsutyokikai Co. Japan). The MAP and HR were obtained by monitoring the pulses from the rat tail artery before the administration of endotoxin and at 24, 48 and 72 hours after the administration of endotoxin, respectively.

Analysis of peripheral blood samples : The survival rats were anesthetized with pentobarbital sodium (30 mg/kg, i.p.) at 72 hours after the administration of endotoxin. Two milliliters of blood were collected from the hearts. The concentration of hemoglobin (Hb), the hematocrit (Hct), the counts of circulating platelet, neutrocyte and lymphocyte were analyzed. In order to compare with the data obtained at 72 hours, we also sampled 2 ml of blood from the other seven groups (n=5 in each group) at 6 hours after endotoxin injection.

Statistical analysis : All data are expressed as mean±S.E. Differences between groups were analyzed by ANOVA and survival data were evaluated by χ^2 test. The condition of $p<0.05$ was considered to be statistically significant.

Results

Survival rate

All treated groups showed various improvements on survival rate (20 %-50 %) over experimental control group (17 %, Fig. 1). The survival rates in all groups with endotoxin decreased over time. But the survival rates in groups D and F (50 %) after 16 hour were significantly different from group B (17 %) ($p<0.05$). The difference of the survival rate between group B (17 %) and group D (50 %) did not change after 24 hours ($p<0.05$). The survival rate of group D was the highest (35 %) after 72 hours. Groups E, F and G showed a rate of 30 % and group C showed the lowest rate among the treated groups (20 %). The

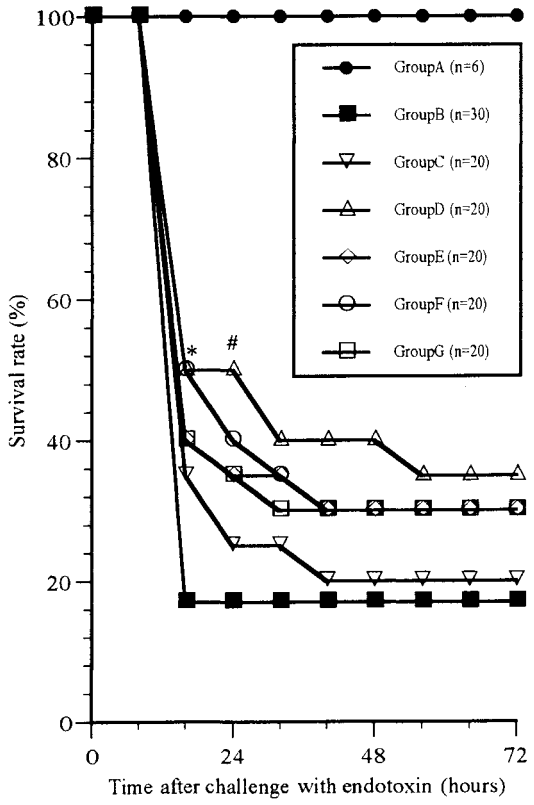

Fig 1 Effects of the preparations on survival rate. Group A : saline, Group B : endotoxin (ETX), Group C : ETX+Kanzo-kankyo-to, Group D : ETX+Shigyaku-to, Group E : ETX+Shigayku-ka-ninjin-to, Group F : ETX+Bukuryo-shigyaku-to, Group G : ETX+Aconiti tuber, * : $p<0.05$, groups D and F compared with group B. # : $p<0.05$, group D compared with group B.

survival rate of group A maintained 100 % until 72 hours.

Changes in MAP and HR

The initial values of MAP and HR did not show significant difference between the groups. All treated groups demonstrated an improvement in MAP during later time points and displayed protection in maintaining HR (Tale II).

The MAP of group B fell significantly during the first 24 hours. The MAP of group B at 48 hours further decreased a level lower than that at 24 hours ($p<0.05$). However, the MAPs of the treated groups increased gradually during the next 48 hours in comparison with the values at 24 hours and the MAPs of

Table II Effect of the preparations on mean arterial pressure and heart rate.

		MAP (mmHg) / HR (beats/min)							
		Hours after endotoxin challenge							
Group	n	0		24		48		72	
A (saline)	6	101±4	/393±15	98±6	/372±20	104±8	/384±10	104±3	/371±10
B (ETX)	5	108±4	/377±9	62±5[a]†	/271±15[a]†	50±2[a]†‡	/291±15†	51±4[a]†	/301±21†
C (ETX+KK)	4	108±5	/377±27	40±5†	/295±25	48±7†	/304±22	56±9†	/316±19
D (ETX+SG)	7	102±11	/374±10	55±10†	/336±21	59±8†	/328±16	77±6[b]	/356±12
E (ETX+SN)	6	96±2	/356±21	44±5†	/303±26	51±4†	/317±21	63±6†‡	/333±24
F (ETX+BS)	6	98±7	/368±6	42±4†	/366±29	56±4†	/338±29	59±5†‡	/355±31
G (ETX+AT)	6	103±5	/405±25	59±11†	/370±32	62±12†	/341±30	64±8†	/360±28

Data from rats surviving until 72 hours after endotoxin injection. MAP : mean arterial pressure. HR : heart rate. n : number of animals. ETX : endotoxin (9 mg/kg, i.p). KK : Kanzo-kankyo-to, SG : Shigyaku-to, SN : Shigayku-ka-ninjin-to, BS : Bukuryo-shigyaku-to. AT : Aconiti tuber. a : $p<0.05$ compared with group A, b : $p<0.05$ compared with group B. † : $p<0.05$ compared with 0 hour, ‡ : $p<0.05$ compared with 24 hours. Data are mean±S.E.

Table III Effect of the preparations on peripheral blood changes.

Time (hours)	Group	n	Hb (g/dl)	Hct (%)	Platelets ($\times10^4$/mm^3)	Neutrocytes ($\times10^3$/mm^3)	Lymphocytes ($\times10^3$/mm^3)
6	A (saline)	5	14.2±0.4	44.8±1.2	99.1±5.6	0.45±0.09	5.11±0.38
	B (ETX)	5	16.5±0.3[a]	52.6±0.9[a]	15.6±3.2[aa]	0.29±0.14	2.51±0.95
	C (ETX+KK)	5	15.5±0.2[b]	49.1±0.7[b]	14.9±1.3	0.12±0.04	2.54±0.22
	D (ETX+SG)	5	15.2±0.5[b]	47.1±1.1[b]	20.1±2.2	0.22±0.07	3.67±0.54
	E (ETX+SN)	5	15.0±0.3[b]	47.7±0.3[b]	18.0±1.8	0.33±0.11	3.39±0.49
	F (ETX+BS)	5	13.9±0.1[b]	43.3±0.8[b]	25.1±6.4	0.48±0.24	4.27±1.28
	G (ETX+AT)	5	15.3±0.9[b]	47.4±0.6[b]	18.1±2.4	0.28±0.06	3.61±0.55
72	A (saline)	6	14.5±0.2	45.2±0.5	101.5±4.0	0.69±0.18	4.27±0.34
	B (ETX)	5	15.9±0.3	49.5±0.9	3.8±2.1[αα]	4.09±0.57[α]	4.39±0.68
	C (ETX+KK)	4	15.4±0.4	48.2±1.7	3.1±0.4	2.98±0.23	5.58±0.45
	D (ETX+SG)	6	14.9±0.7	46.0±2.0	17.3±15.5	2.19±0.43[β]	5.75±0.77
	E (ETX+SN)	5	15.5±0.2	46.7±1.0	17.8±6.7	2.29±0.44[β]	5.79±0.35
	F (ETX+BS)	5	13.5±1.1[β]	43.8±1.7[β]	5.8±3.3	2.08±0.67[β]	5.82±0.42
	G (ETX+AT)	5	14.7±1.2	46.4±2.2	2.7±0.2	2.78±0.50	5.49±0.76

a : $p<0.05$ compared with group A after 6 hours, aa : $p<0.0001$ compared with group A after 6 hours, b : $p<0.05$ compared with group B after 6 hours. α : $p<0.05$ compared with group A after 72 hours, $\alpha\alpha$: $p<0.0001$ compared with group A after 72 hours, β : $p<0.05$ compared with group B after 72 hours. Other abbreviations are the same as shown in Table II. Data are mean±S.E.

groups E and F at 72 hours increased significantly compared with the values at 24 hours ($p<0.05$). Furthermore, the MAP of group D at 72 hours was significantly higher than that of group B ($p<0.05$).

The value of HR in group B decreased significantly during 72 hours. In contrast, the HR in all treated groups did not change significantly (Table II).

Analysis of peripheral blood samples

The effects of four "warming" preparations and AT alone on the endotoxin-induced hematologic alteration were summarized in Table III. (a) : All treated groups inhibited the increases of Hb and Hct at 6 hours after endotoxin administration ($p<0.05$). Treated group F also inhibited the increases of Hb and Hct at 72 hours after endotoxin administration ($p<0.05$). (b) : The decreases in the platelet counts due to endotoxin were not prevented in all treated groups. (c) : Neutrocytes in group B increased significantly compared with group A after 72 hours ($p<0.05$). However, the treated groups tended to decrease neutrocytes, especially the neutrocytes in groups D, E and F decreased significantly compared with group B ($p<0.05$). There was no significant change in lymphocyte between all groups.

Discussion

The shock syndrome is considered as a *Yang* depletion in the view of traditional Chinese medicine. The therapeutic rules for the *Yang* depletion are warming of the weakened organs and recuperating the depleted *Yang* to save the body from collapse. In the present study, four representative preparations, SG, KK, SN and BS, which could be found in the *"Recipes for Recuperating Depleted Yang"*,[15] were used on rats with endotoxin shock. All preparations showed some protective effects on endotoxin shock rats.

The present study revealed that all preparations improved the MAP during later time points. This can be explained mainly as follows.

One reason is probably due to the cardiotonic effects of Zingiberis Rhizoma and AT. In the previous studies, Yamada *et al.* showed that Zingiberis Rhizoma increased cardiac contractility and cardio-autonomic movement.[16] Yakazu also demonstrated that the extraction of Aconite root had obviously cardiotonic effects on isolated guinea pig hearts.[17] In humans, Liu *et al.* demonstrated that higenamine (i.e., an active component of AT) showed cardiotonic effects in patients with heart failure,[18] and Chen *et al.* reported that AT alone had positive inotropic and chronotropic effects when used in patients with left ventricular failure.[19] In our experiments, all preparations (except KK including Zingiberis Rhizoma only) included the constituent herbs of Zingiberis Rhizoma and AT. Therefore, the cardiotonic effects of Zingiberis Rhizoma and AT contribute to improve MAP. The positive chronotropic effects of Zingiberis Rhizoma and AT are also a reason to explain that all preparations revealed protection in maintaining HR.

Another reason is probably due to the inhibition of increased microvascular permeability. The microvascular permeability increases in endotoxin shock. As a result of the increased microvascular permeability, plasma extravasation enhances and the blood volume reduces. Eventually, the blood pressure drops. It is also well known that the increased microvascular permeability results to a high Hct. In our experiments, the result (Table III) indicates that the preparations inhibit the increases of microvascular permeability. The inhibition of increased microvascular permeability contributes to the improvement of MAP.

The treatment with BS maintained the lowest level of Hb and Hct from early phase to 72 hours after the endotoxin administration (Table III). This may be a result from the effect of Hoelen.

The present study also revealed that treated groups tended to inhibit the increases of neutrocyte at 72 hours after endotoxin injection. Especially treatment with SG, SN and BS inhibited the severe increases of neutrocyte compared with vehicle treated group (Table III). The result suggests that the preparations probably have a function to inhibit the overinflammatory response in endotoxin shock state.

Although all preparations showed the protective effects on endotoxin shock, there were still some differences in the improvements. The treatment with SG (containing AT) improved the survival rate significantly after 24 hours, increased the MAP and inhibited the neutrocyte increase significantly after 72

hours, but no significant effect was observed in the case of KK (containing no AT). Furthermore, SN and BS (containing AT) displayed the similar effects as SG. In addition, AT alone showed some protective effects on the endotoxin shock. The data suggest that AT is an important constituent herb on improving the endotoxin shock in our study. It was also reported in the paper of Zhou *et at.* that the water-soluble fraction of Radix Aconiti showed therapeutic effects in cats with endotoxin shock.[20]

However, none of the preparations prevented the endotoxin-induced severe and sustained thrombocytopenia significanty (Table III). The data suggest that the preparations do not provide an effective means for modulating the decrease in platelet during endotoxin shock.

Endotoxin shock is deeply related to the increased inflammatory mediators, such as interleukins IL-1, IL-6, IL-8 and tumor necrosis factor (TNF), which lead to hypotension and multiple organ failure. To make clear the mechanism of the protective effects of the preparations, it is essential to do further investigation.

Conclusions

The present study showed that the four "warming" preparations improved survival rate by improving MAP, maintaining HR, attenuating the endotoxin-induced hemoconcentration and inhibiting the increase of neutrocyte. The preparations with or without AT showed the obvious differences in the improvements. In addition, AT alone displayed some protective effects on the endotoxin shock. The results suggest that four "warming" preparations have some protective effects on endotoxin shock and AT plays an important role in protecting the rats from the endotoxin shock in our experimental model.

和文抄録

4種類の熱剤（甘草乾姜湯，四逆湯，四逆加人参湯，茯苓四逆湯）と附子単独のエンドトキシンショック・ラットに対する予防効果を検討した。4種類の熱剤及び附子単独の投与により血圧は上昇し，心拍数は維持され，血液濃縮は軽減され，好中球数の上昇を抑制し，生存率を改善した。また，附子を含む熱剤と附子を含まない熱剤の間での予防効果に大きな違いをみた。以上の結果は4種類の熱剤がエンドトキシンショックに対する予防効果を示唆している。また，構成生薬の1つである附子は本実験で効果発現に重要な生薬であることを示唆している。

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