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Effects of Soyasaponin on Experimental Disseminated Intravascular Coagulation. I

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The antithrombic activity of soyasaponins obtained from soybean was evaluated in the experimental model of disseminated intravascular coagulation (DIC) induced by infusion of endotoxin or thrombin in rats, as well as *in vitro* models (blood aggregation, conversion of fibrinogen to fibrin and activation of the fibrinolytic system).

Total soyasaponin (TS) prevented the decrease of blood platelets and fibrinogen, and the increase of fibrin degradation products (FDP) in the endotoxin-induced DIC. TS also inhibited the formation of fibrin thrombi in the renal glomeruli in the thrombin-induced DIC.

In vitro experiments, TS and soyasaponins, I, II, III, A_1 and A_2 inhibited the conversion of fibrinogen to fibrin. TS and soyasaponins I and II promoted activation of the fibrinolytic system in a plasminogen-containing fibrin plate.

Keywords——soyasaponin; disseminated intravascular coagulation; endotoxin; thrombin; blood platelet; fibrinogen; plasminogen; fibrinolytic system

Soybean (*Glycine max* MERR., seeds) is a very important cereal which is used on a large scale for the manufacture of edible oil and processed foodstuffs, and also as a medicine (it is described in many ancient herbal books as an anti-inflammatory drug or anti-maturative).

The possibility has been suggested recently that continuous intake of soybeans may be effective for the prevention of adult diseases, such as arteriosclerosis, hyperlipemia and obesity. Five glucuronide-saponins, soyasaponins I, II, III, A_1 and A_2 have been isolated from soybeans[1] and the effects of these saponins on lipid metabolism in isolated fatty cells from rats have also been reported.[2] In addition, total soyasaponin (TS) has been shown to be clinically effective for hyperlipemia and obesity.

It is well known that the syndrome of disseminated intravascular coagulation (DIC) (closely related to arteriosclerosis and hyperlipemia) is an acquired hemorrhagic disorder characterized by the apparent simultaneous activation of blood coagulation, fibrinolysis and kinin generation, combined with the pathologic consequences of fibrin deposition in the microcirculation. The pathophysiological aspects of DIC have been reported in animal models based on infusion of endotoxin or thrombin.[3] It is also well known that the infusion of endotoxin in animals results in the activation of Hageman factor, injury to the endothelium, blood platelet aggregation and activation of complement.

The purpose of the present investigation was to study the preventive effect of soyasaponins on experimental DIC induced by endotoxin or thrombin administration to rats, as well as on blood platelet aggregation, the conversion of fibrinogen to fibrin and the fibrinolytic system (*in vitro* models).

Materials and Methods

Materials——TS and soyasaponins I, II, III, A_1 and A_2 were obtained from the saponin fraction of defatted soybean powder as described previously.[1] The sources of materials were as follows: endotoxin (*Escherichia coli* 055:B5, Difco Lab., U.S.A.), thrombin (Mochida Ltd., Japan), urokinase (Midorijuji Ltd., Japan), collagen, adenosine diphosphate (ADP) disodium salt and arachidonic acid (Sigma Chemical Co., U.S.A.) and plasminogen-containing fibrinogen (Nakarai Chemical Ltd., Japan).

Animals——Male Wistar–King strain rats weighing between 150—200 g were used for the experiments involving endotoxin- and thrombin-induced DIC, and blood platelet aggregation. They were fed a standard diet (Nihon Clea, Japan) for a minimum period of 7 d and then fasted for 24 h before the start of the experiments.

Endotoxin-Induced DIC——Experimental DIC was induced by a modification of the method of Schoendorf *et al.*[4] TS (10, 50, 100 or 200 mg/kg) was administered orally to the rats 1 h before the injection of endotoxin (0.1 mg/kg) into the tail vein. Blood samples were withdrawn from the heart into plastic syringes at 4 h after the injection of endotoxin, while the rats were anesthetized with pentobarbital. As an anticoagulant, 0.01 M sodium ethylenediaminetetraacetic acid (EDTA) was used for platelet counts and a 1:9 volume of 3.8% sodium citrate for prothrombin time and fibrinogen determination.

Platelets were counted with an automatic blood cell counter (Coulter counter, model S-Plus, Coulter Co., U.S.A.). Fibrinogen was determined according to the method of Quick.[5] The prothrombin time was measured with a COAG-A-Mate dual-channel device (General Diagnostic, Warner–Lambert Co., U.S.A.). Fibrin degradation product (FDP) was determined by means of the latex aggregation test (FDPL test U, Teikoku Zoki, Japan).

Thrombin-Induced DIC——Experimental DIC was induced by the method of Margarreten.[6] TS (50, 100 or 200 mg/kg) was administered orally to the rats 1 h before the intravenous infusion of thrombin ($4\ U \cdot min^{-1} \cdot kg^{-1}$, for 30 min) dissolved in saline. Both kidneys were dissected out immediately after the final infusion of thrombin while the rats were anesthetized with pentobarbital, and cryostat sections were prepared. The sections were stained with phosphotungstic acid-hematoxylin for histological examination. A hundred glomeruli were counted and the percentage of glomeruli having fibrin thrombi was determined.

Blood Platelet Aggregation——Whole blood samples were collected into the plastic syringes from the heart of rats anesthetized with pentobarbital. Next, 9 ml of blood and 1 ml of heparin solution (10 U/ml) were transferred into plastic tubes. Platelet-rich plasma (PRP) was obtained by centrifugation of the mixture at 1000 rpm for 10 min. PRP was removed with a siliconized pipet, and samples were stored in capped plastic test tubes. The samples were gently stirred at 5—10 °C for 30 min prior to use. The red cell portion of the samples was centrifuged a second time at 3000 rpm for 30 min to produce platelet-poor plasma (PPP) that was used as the maximal transmittance standard.

The experiments on platelet aggregation were performed by the method of Born *et al.*[7] Aggregating agents used were collagen (500 μg/ml), ADP (0.05 μM), arachidonic acid (50 mM) and endotoxin (500 μg/ml). A 0.2 ml aliquot of PRP was placed in a tube and stirred at 1200 rpm, 37 °C, for 1 min followed by addition of a 10 μl aliquot of the test solution (500 μg/ml). After 1 min, an aggregating agent was added to the reaction mixture. Platelet aggregation was monitored by continuous recording of light transmittance in a Husm System platelet aggregometer (Rika Electric Co., Japan). The extent of aggregation was estimated from the percent increase in the transmission at the maximal aggregation after addition of the aggregating agent.

Convertion of Fibrinogen to Fibrin Induced by Thrombin——Fibrinogen (500 mg) was dissolved in 100 ml of 0.05 M tris acetate buffer (pH 7.4) in 0.15 M NaCl. A test solution (0.1 ml) was added to 1.8 ml of a fibrinogen solution with stirring. After 1 min, 0.1 ml of thrombin solution (0.2 U/ml) was added and the whole was gently stirred until a fibrin clot appeared. The time required for clotting was recorded.

Activation of Fibrinolytic System——Fibrin plates were prepared by the method of Noren *et al.*[8] Agarose (1 g) was dissolved in 100 ml of 0.01 M phosphate buffer (pH 7.8) in 0.15 M NaCl in a boiling water bath and then allowed to cool in a water bath at 45—50 °C. Plasminogen-containing fibrinogen (166 mg) was dissolved in 100 ml of agarose at 31 °C. A 10 ml aliquot of the mixture was placed in each tube and 0.1 ml of thrombin (100 U/ml) was added. The tube was quickly inverted against a piece of plastic film for mixing, and the contents were immediately poured into a Petri dish. The test solution (0.1 ml) prepared at the required concentration and urokinase solution (0.1 ml, 10 U/ml) were mixed, then 20 μl of the mixture was added to each hole in a plate. The plates were incubated at 31 °C for 20 h.

Two diameters of the lysed area were measured and the area was calculated. The effects of samples on the activation of this fibrinolytic system were assessed by comparing the lysed area with that of the control. The control was 20 μl of the mixture of phosphate buffer (0.1 ml) and urokinase solution (0.1 ml, 10 U/ml). The activity was expressed as a percentage.

Results

Endotoxin-Induced DIC

It was shown that DIC could be induced by injection of endotoxin (0.1 mg/kg) into the

tail vein, resulting in a decrease of blood platelets and fibrinogen, prolongation of the prothrombin time and an increase of FDP. Before the injection of endotoxin, 10, 50, 100 or 200 mg/kg of TS, or 50, 200 or 500 mg/kg of aspirin was administered orally, and the preventive effect against the endotoxin-induced DIC was examined (Table I).

The platelet count was $88 \pm 4 \times 10^4/mm^3$ in normal rats injected with saline only. It was reduced to $34 \pm 4 \times 10^4/mm^3$ in rats injected with 0.1 mg/kg of endotoxin. When rats were orally given 200 mg/kg of TS, the reduction of the platelet count was significantly smaller.

The level of fibrinogen was 179 ± 22 mg/dl in normal rats given saline only. The level decreased to 50 ± 9 mg/dl in DIC rats. The decrease of fibrinogen level was significantly less in rats administered orally with 100 or 200 mg/kg of TS.

Prothrombin time was 19.4 ± 2.4 s in the normal rats. It was prolonged to 32.4 ± 3.9 s in the DIC rats. Some shortening of prothrombin time was observed in rats orally given 100 or 200 mg/kg of TS, as compared with the control.

The FDP level was 3 ± 1 μg/ml in normal rats injected with saline only. The level increased to 45 ± 8 μg/ml in the DIC rats. When 100 or 200 mg/kg of TS was administered to rats 1 h before the injection of endotoxin, the FDP level was reduced significantly.

A clear preventive effect of aspirin (used as a standard drug) was recognized on blood platelets and fibrinogen, but not on prothrombin time or FDP.

Thrombin-Induced DIC

Histological examination of the kidneys collected from rats given intravenous infusion of thrombin (4 U/ml, for 30 min) was carried out. A hundred glomeruli were checked, and the number containing fibrin thrombi is given as a percentage in Table II.

In the kidneys of the normal rats infused with saline only for 30 min, thrombi-containing glomeruli were not detected at all. In the control rats pretreated with thrombin (4 U/min, for 30 min), as many as $69.3 \pm 6.0\%$ of the glomeruli contained thrombi. However, when rats were orally given 50, 100 or 200 mg/kg of TS, the formation of fibrin thrombi in glomeruli was prevented significantly compared with that in the control rats.

Collagen-, ADP-, Arachidonic Acid- or Endotoxin-Induced Blood Platelet Aggregation

The incubation of TS, or soyasaponin I, II, III, A_1 or A_2 (each 500 or 1000 μg/ml) with PRP did not produce any inhibitory effect on aggregation induced by collagen, ADP,

TABLE I. Effects of TS and Aspirin on Endotoxin-Induced Experimental DIC in Rats Injected with 0.1 mg/kg of Endotoxin

Treatment	Dose (mg/kg)	No. of rats	Blood platelets ($\times 10^4/mm^3$)	Fibrinogen (mg/dl)	Prothrombin time (s)	FDP[a] (μg/ml)
Normal		8	88 ± 4	179 ± 22	19.4 ± 2.4	3 ± 1
Control		8	34 ± 4	50 ± 9	32.4 ± 3.9	45 ± 8
TS	10	8	33 ± 5	48 ± 8	33.5 ± 6.5	48 ± 8
TS	50	8	45 ± 10	58 ± 15	31.8 ± 4.8	41 ± 11
TS	100	8	48 ± 10	83 ± 5[b]	22.8 ± 5.0	30 ± 10
TS	200	8	54 ± 9[b]	90 ± 20[b]	19.8 ± 1.5	13 ± 3[c]
Normal		8	82 ± 12	280 ± 17	12.6 ± 0.3	2 ± 1
Control		8	32 ± 5	77 ± 12	26.7 ± 3.2	60 ± 17
Aspirin	50	8	44 ± 4[b]	141 ± 17[c]	24.4 ± 1.7	43 ± 10
Aspirin	200	8	50 ± 4[b]	131 ± 26[b]	25.3 ± 3.3	46 ± 23
Aspirin	500	8	48 ± 2[b]	159 ± 15[c]	23.7 ± 3.2	24 ± 10

a) Each value represents the mean ± S.E.
b) Significantly different from control, $p < 0.05$.
c) Significantly different from control, $p < 0.01$.

TABLE II. Effects of TS and Heparin on Thrombin-Induced Experimental DIC in Rats Infused with 4 U/min of Thrombin for 30 min

Treatment	Dose	No. of rats	Percentage of fibrin deposits in glomeruli[a]
Normal		10	0.0±0.0
Control		10	69.3±6.0
TS	50 mg/kg	10	44.9±8.5[b]
TS	100 mg/kg	10	41.9±8.4[b]
TS	200 mg/kg	10	29.1±6.2[c]
Heparin	$3\,U\cdot min^{-1}\cdot kg^{-1}$	10	3.8±3.4[c]

a) Each value represents the mean±S.E.
b) Significantly different from control, $p<0.05$.
c) Significantly different from control, $p<0.01$.

TABLE III. Effects of TS, Soyasaponins I, II, III, A_1 and A_2 and Heparin on the Conversion of Fibrinogen to Fibrin Induced by Thrombin

Treatment	Clotting time of fibrinogen solution (s)[a]			
	0	50	100	250 (μg/ml)
Control	206±3			
TS		251±9[c]	304±6[c]	350±6[c]
Soyasaponin I			218±8	249±16[c]
Soyasaponin II		225±7[b]	211±3	269±7[c]
Soyasaponin III			196±3	249±10[c]
Soyasaponin A_1			203±4	239±10[c]
Soyasaponin A_2		214±6	254±6[c]	330±3[c]
Heparin (10 U/ml)	266±12[c]			

a) Each value represents the mean±S.E. of 5 experiments.
b) Significantly different from control, $p<0.05$.
c) Significantly different from control, $p<0.01$.

TABLE IV. Effects of TS, Soyasaponins I, II, III, A_1 and A_2 and Dextran Sulfate on the Fibrinolytic System in a Fibrin Plate

Treatment	Activation percentage[a]			
	10	50	100	250 (μg/ml)
TS	24.5±1.8	30.0±3.0	33.8±2.6	40.0±2.4
Soyasaponin I	5.5±1.8	20.0±3.0	25.0±2.1	30.0±3.1
Soyasaponin II	0.5±0.3	2.0±1.1	22.0±2.5	35.0±3.2
Soyasaponin III	—	—	—	—
Soyasaponin A_1	—	—	—	—
Soyasaponin A_2	—	—	—	—
Dextran sulfate	2.8±1.0	28.6±3.0	31.3±2.0	43.1±2.2

a) Each value represents the mean±S.E. of 5 experiments as compared with the control.

arachidonic acid or endotoxin, whereas aspirin inhibited these aggregations at a concentration of 500 μg/ml.

Conversion of Fibrinogen to Fibrin Induced by Thrombin

As shown in Table III, the clotting time of the control without addition of any test solution was 206 ± 3 s. The clotting time was prolonged significantly by incubation with 250 μg/ml of TS, or soyasaponin, I, II, III, A_1 or A_2 before addition of thrombin.

Activation of Fibrinolytic System

Plasminogen-containing fibrinogen was extensively lysed by addition of a mixture of the test solution and urokinase solution as compared with the control. The activity was expressed as the percentage of the area lysed by a test solution as compared with the control. As shown in Table IV, when TS, or soyasaponin I or II was incubated at a concentration of 10, 50, 100 or 250 μg/ml, relatively strong, dose-dependent effects on the activation of the fibrinolytic system were recognized. A similar result was obtained by incubation with dextran sulfate.

Discussion

Soybeans are quite widely used in foodstuffs and also as a health food which may aid in the prevention of arteriosclerosis, hyperlipemia and obesity. The efficacy of soyasaponins against hyperlipemia and obesity has been substantiated by clinical and biological investigations.[2)]

Accordingly, studies were conducted on the effect of TS and five glucuronide-saponins against experimental DIC, which is closely related to thrombosis. As compared with the control, a significant preventive effect against experimental DIC was noted in three parameters (but not prothrombin time) in rats orally given 100 or 200 mg/kg of TS. Thus, TS may be useful for prevention of DIC in man. However, there is no evidence that TS is effective after the onset of DIC.

It is considered that the effect of TS is associated with inhibition of the conversion of fibrinogen to fibrin by soyasaponins I, II, III, A_1 and A_2, and promotion of activation of the fibrinolytic system by soyasaponins I and II. TS had no effect on the *in vitro* platelet aggregation induced by various aggregating agents, such as ADP, collagen, arachidonic acid and endotoxin, but significantly inhibited *in vivo* platelet aggregation in the endotoxin-induced DIC in rats. The inhibitory effect observed in the *in vivo* experiment might be ascribable to metabolites of TS or degradation products which may be formed from TS by gastric juice in the stomach.

Further work is in progress on the effects of soyasaponins on experimental DIC in rats.

References

1) *a*) I. Kitagawa, M. Yoshikawa and I. Yoshioka, *Chem. Pharm. Bull.*, **24**, 121 (1976); *b*) I. Kitagawa, M. Saito and M. Yoshikawa, Abstracts of Papers, 26th Annual Meeting of the Japanese Society of Pharmacognosy, Tokyo, 1979, p. 21. *c*) I. Kitagawa, M. Yoshikawa, H. K. Wang, M. Saito, V. Tosirisuk, T. Fujiwara and K. Tomita, *Chem. Pharm. Bull.*, **30**, 2294 (1982).
2) H. Ohminami, T. Okuda, M. Yoshikawa and I. Kitagawa, *Proc. Symp. WAKAN-YAKU*, **14**, 157 (1981).
3) R. Oyama and M. Takiguchi, *Igaku No Ayumi*, **109**, 754 (1979).
4) T. H. Schoendorf, M. Rosenberg and F. K. Beller, *Am. J. Path.*, **65**, 51 (1971).
5) A. J. Quick, "Hemorrhagic Diseases," 1st ed., Lea and Febiger, Philadelphia, 1957, p. 279.
6) W. Margarreten, I. Csavossy and D. G. McKay, *J. Lab. Invest.*, **13**, 552 (1964).
7) G. V. R Born and M. J. Cross, *J. Physiol.* (London), **168**, 178 (1963).
8) I. Noren, G. Ramstrom and P. Wallen, *Haemostasis*, **4**, 110 (1975).