Effect of Silsosangami on Platelet Aggregation, Hemolysis and Inducible Nitric Oxide Synthase

Chang Hwan Kim, Han Geu Kim, Jong Chan Ahan, Soo Kyung Lee,
Tae Wook Chung, June Ki Kim[1], Dall Yeong Choi[1], Cherl Ho Kim[2], Won Hwan Park*

Department of Diagnostics, 1: Department of Pathology, 2: Department of Biochemistry, College of Oriental Medicine, Dongguk University

Silsosangami(SSG) is a formula of treaditional korean medicines as an effective biological response modifier for augmenting host homeostasis of body circulation. Little is known of the biological activity of SSG and previous studies have focused mainly on their anti-thrombosis8). There is a growing interest in the pharmacological potential of the SSG due to the recent finding by our group that SSG and each constituent herbs of SSG were able to inhibit NO and prostaglandin E2 (PGE2) synthesis in murine peritoneal macrophages stimulated with bacterial endotoxin. In this paper, the effects of SSG on platelet aggregation and hemolysis in human blood were studied. SSG provoked remarkable inhibiting effect on platelet aggregation, and APTT were sensitive to the presence of this SSG. Using an in vitro system, APTT was delayed with the increment of the concentrations of these seven compounds. These results suggested that SSG might be used as a novel antithrombotic therapeutic agents in post-myocardial infarction. A SSG reduced NO production in mouse peritoneal macrophages stimulated with lipopolysaccharide, without the influence on the activity of iNOS being observed. SSG significantly reduced mouse paw edema induced by carrageenan. Western blot analysis showed that SSG reduced the expression of iNOS. The results indicate that SSG exerts anti-inflammatory effects related to the inhibition of NO production, which could be due to a decreased expression of iNOS.

Key words : Silsosangami(失笑散加味), platelet aggregation, prostaglandin E2, iNOS

Introduction

Silsosangami(Shī xiào-sǎn jiā wèi, 失笑散加味, SSG) is a formula of oriental medicines as an effective biological response modifier for augmenting host homeostasis of body circulation[1]. SSG is consisted of crude ingredients from 7 medicinal herbs, *Typhae Pollen, Pteropi Faeces, Paeoniae Radicis rubra, Cnidii Rhizoma, Persicae Semen, Carthami Flos,* and *Curcumae Tuber*[1)2)3)]. The pharmacological action of SSG has been limitedly studied in regard to ischemic infarction[1]. This traditional korean medicine has been shown to express diverse activities such as immunomodulating, anti-infarction, anti-allergic and anti-inflammatory effects. The SSG widely used in traditional Korean medicine, has been known to have an anti-diabetic activity and anti-platelet aggregation activity. The study by Shin[4] has reported that SSG and each constituent herbs of SSG inhibited ADP-induced aggregation as well as arachidonic acid-induced aggregation of human platelet, but the role of each herb of SSG on the inhibition of platelet aggregation and hemolytic effect have not yet been investigated in detail. Prostaglandins and nitric oxide (NO) exert numerous vascular and inflammatory effects. Production of prostaglandins or NO by the constitutive isoenzymes, or endothelial NO synthase, is implicated in regulation of vascular tone and homeostatic functions. In contrast, inducible NO synthase (iNOS) is not generally expressed in resting cells, but are induced following appropriate stimulation with pro-inflammatory agents such as cytokines and lipopolysaccharide[5]. The activity of these inducible enzymes results in overproduction of prostaglandins and NO, which play a key role in the pathophysiology of arthritis and other inflammatory conditions[6]. NO is also able to enhance the production of tumour necrosis factor-α and interleukin-1β, which participate in the macrophage-dependent inflammation[7]. Little is known of the biological activity of SSG and previous studies have focused mainly on their anti- thrombosis[8]. There is a growing interest in the pharmacological potential of the SSG due to the recent finding by our group that SSG and each constituent herbs of SSG were able to inhibit NO and prostaglandin E2 (PGE2) synthesis in murine peritoneal macrophages stimulated with bacterial endotoxin. In this

* To whom correspondence should be addressed at : Won Hwan Park, Department of Diagnostics, College of Oriental Medicne, Dongguk University, Sukjang-Dong 707, Kyung-Ju 780-714, Korea.
E-mail: diapwh@mail.dongguk.ac.kr, Tel: 054-770-2373
· Received : 2002/08/19 · Revised : 2002/09/24 · Accepted : 2002/11/25

paper, we used the method of activated partial thromboplastin times (APTT)[9] for the first time to study the inhibition on platelet aggregation activity of each constituent herbs of SSG. The effect of each herb of SSG on hemolysis was also investigated. Three herbs of *Curcumae Tuber, Persicae Semen* and *Paeoniae Radicis rubra* showed the highest hemolysis ability on human blood among the 7 herbs. The present study was undertaken to examine the effects of a new SSG on murine macrophage as well as on several enzymes relevant to the inflammatory process. The results demonstrated the *in vitro* inhibitory effects on cell functions exerted by this SSG, which also exhibited anti-inflammatory activity *in vivo*.

Materials and Methods

1. Materials and extraction of SSG Extracts

SSG[1)2)] is consisted of herbs such as *Typhae Pollen*(蒲黃) 4g, *Pteropi Faeces*(五靈脂) 4g, *Paeoniae Radicis rubra*(赤芍藥) 4g, *Cnidii Rhizoma*(川芎) 4g, *Persicae Semen*(桃仁) 4g, *Carthami Flos* (紅花) 4g and *Curcumae Tuber*(鬱金) 4g. A total of 28g of SSG was added to 500ml of water and boiled for 2hrs, filtered and then concentrated to 200ml. This decoction was spray-dried to give a powdered extract. The yield was 5.2g., which represents one human dose/day. The aqueous extracts of SSG and its seven composed Korean herbs, which were massproduced for clinical use, were kindly supplied by the Oriental Medical Hospital of Dongguk University, Kyongju, Korea. The APTT assay kit with a composition of actin and calcium was purchased from Dade International Inc. (manufactured by Dade Diagnostics of P.R. Inc., Aguade, PR00602-0865, USA). L[9,10-3H]oleic acid and L-3- phosphatidylcholine 1-palmitoyl-2-arachidonyl [arachidonyl- 1-14C] were purchased from Du Pont (CA, USA).

iNOS specific poly -clonal antisera, N- (2-cyclohexyloxy-4-nitrophenyl) methane-sulfonamide (NS398) and N-(3-(aminomethyl) benzyl)acetamidine di- hydrochloride (1400W) were purchased from Cayman Chem.(MI, USA). The rest of reagents were from Sigma Chem.(MO, USA).

2. Determination of platelet aggregation

Nine volumes of healthy venous blood were added into one volume of 3.8% sodium citrate in a plastic tube. After centrifuged at 3000r.p.m. for 10min, the plasma was pipetted and then adjusted to 1.0ml aliquots containing various concentration of water extracts from SSG. In the control experiment, the equivalent volume of 0.9% NaCl was added to reaction mixture in place of the extracted compounds. Each 0.1ml aliquot of the reaction mixture was incubated at 37℃ for 1min, then 0.1ml of actin was added and the mixture was incubated for 2min. After the addition of 0.1ml of 0.02M $CaCl_2$, The activated partial thromboplastin times(APTT) was recorded[10)]. The APTT was performed using an activated commercial reagent (Dade International Inc.).

3. Hemolysis measurement

Fresh blood from healthy human were collected (9 parts of blood: 1 part of 3.8% sodium citrate) in the plastic tubes, and red blood cells were separated by centrifugation at 2500r.p.m. for 10min. The precipitates (red blood cells) were washed with two volume of physiological saline solution, then centrifuged at 3000r.p.m. for 10min. The red blood cells were then suspended in 0.9% saline solution (10% erythrocyte suspension). 0.25ml of 10% cells suspension was mixed with SSG and each herb of SSG. The mixture was incubated at 37℃ for 5min with shaking and then centrifuged at 1100r.p.m. for 5min. 0.2ml of supernatant was added into 3.3ml distilled water and the absorbance of the supernatant was measured at 550nm (Asample). The percent hemolysis (H%) of each sample was calculated using the following equation: H%= Asample/A100 100% where A100 is the absorbance of 100% hemolysis cells, i.e. 0.25ml of cells suspension was incubated in 8.5ml of distilled water[10)].

4. Isolation and culture of mouse peritoneal macrophages

Female Swiss mice weighing 25-30g were used to obtain highly purified peritoneal macrophages. Cells were harvested by peritoneal lavage 4days after i.p. injection of 1ml of 10% thioglycolate broth. Cells were resuspended in culture medium (120mM NaCl, 4.7mM KCl, 1.2mM $CaCl_2 \cdot 7H_2O$, 1.2mM KH_2PO_4, 25mM $NaHCO_3$, 10mM HEPES, 1 mM arginine, and 10mM glucose) supplemented with 10% foetal bovine serum, 2mM glutamine, 100U/ml penicillin, 100g/ml streptomycin, and incubated at 37℃ for 2hrs. Thrse adhrserent cells were used to perform thrse experiments described below. Cytotoxicity was assessed by thrse reduction of MTT[11)].

5. Nitrite production in mouse peritoneal macrophages

Peritoneal macrophages (4×10^5/well) were incubated with Escherichia coli [serotype 0111:B4] lipopolysaccharide (10 μg/ml) at 37℃ for 24hrs in the presence of test compounds or vehicle. Nitrite levels were determined in culture supernatants by a fluorometric method[12)] and by radioimmunoassay[13)], respectively. In another set of experiments, lipopolysaccharide-stimulated cells were collected to determine iNOS expression by Western blot analysis as described below.

6. Inducible NOS activity in intact cell

Macrophages stimulated with lipopolysaccharide for 24hrs(4×10^5/well) were washed and fresh medium supplemented with L-arginine (0.5mM) and arachidonic acid (10 μM) was added for a further 2hrs incubation with test compounds to assess the effects of compounds on induced enzyme activity. Supernatants were collected for the measurement of nitrite accumulation for the last 2hrs. Nitrite concentration, as reflection of NO released, was assayed fluorometrically.

7. Inducible NOS activity in broken cell preparations

High-speed (100,000×g) supernatants from peritoneal macrophages stimulated with E. coli lipopolysaccharide were obtained as described above. Aliquots of supernatants were used to determine NO synthase activity by monitoring the conversion of L-[3H]arginine to L-[3H]citrulline[14]. Briefly, supernatants (100g protein/tube) were incubated at room temperature for 60min with NADPH (1mM) and a mixture of unlabeled and L-[3H]arginine (10M, 1 μCi/ml). Incubations were terminated by adding 20mM HEPES (1 ml, pH 5.5) containing 1mM EGTA and 1mM EDTA. L-[3H]citrulline was separated from arginine by adding 1.5ml of a 1:1 suspension of DOWEX (50W) in water. Radioactivity was measured in supernatants by liquid scintillation counting.

8. Western blot analysis

Inducible NOS protein expression were studied in the cytosolic and microsomal fractions, respectively, from lipopolysaccharide-stimulated peritoneal macrophages and cell pellets obtained by centrifugation of air pouch exudates. Equal amounts of protein were loaded on 12.5% polyacrylamide gel electrophoresis- sodium dodecyl sulphate (PAGE-SDS) and transferred onto polyvinyl-idene difluoride membranes for 90min at 125mA. Membranes were blocked in phosphate buffer saline (0.02M, pH7.0)-Tween-20 (0.1%) containing 3% w/v unfatted milk. For iNOS, membranes were incubated with specific anti-iNOS polyclonal antiserum (1/1000). Membrane was incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (1/20,000) and peroxidase-conjugated rabbit anti-goat/sheep IgG (1/20,000), respectively. The immunoreactive bands were visualized using an enhanced chemi-luminescence system (ECL, Amersham Korea, Korea).

9. Carrageenan paw edema

The anti-inflammatory activity of SSG was assessed by the carrageenan paw oedema test in mice according to the method of Sugishita et al.[15]. SSG (10, 50, 100, 300 mg/100g by oral administration), indomethacin (5mg/kg), or vehicle (tween 80/saline 1:99, v/v) was administered intraperitoneally 1h before injection of carrageenan (0.05ml; 3% w/v in saline) into the subplantar area of the right hind paw. The volumes of injected and contralateral paws were measured 1, 3 and 5hrs after induction of edema by using a plethysmometer. The volume of edema was expressed in each animal as the difference between the carrageenan-injected and contralateral paws. After the last determination of paw edema (5hrs), the animals were killed by cervical dislocation and the right hind paws were homogenized in 2ml of saline. Aliquots of supernatants were used to determine elastase activity as above.

10. Statistical analysis

The results are presented as means±standard error. Inhibitory concentration 50% (IC_{50}) or inhibitory dose 50% (ID_{50}) values were calculated from at least four concentrations (n=6 ; n represents the number of experiments). The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's t-test for multiple comparisons. The significance of the difference between the each herbs of SSG and controls with respect to their effects on hemolysis (Fig. 1, 2, 3, 4 and Fig. 5) and APTT (Table 1) were determined with the Wilconxon's test. P-values less than 0.05 were considered to represent a significant difference.

Results

1. Effects of seven herbs on hemolysis of human erythrocytes

The effects of seven natural herbs extracted from of SSG on hemolysis of erythrocytes were shown in Table 1. *Curcumae Tuber*, *Persicae Semen* and *Paeoniae Radicis rubra* showed the highest hemolysis ability on human blood, and *Typhae Pollen* and *Pteropi Faeces* had a slight effect on hemolysis among the seven herbs, other two herbs of *Cnidii Rhizoma* and *Carthami Flos* had no effect on hemolysis. The degree of hemolysis in these three herbs was *Curcumae Tuber* > *Persicae Semen* > *Paeoniae Radicis rubra*.

Table 1. Effect of seven herbs on hemolysis of human erythrocytes

Herbs		Hemolysis of erythrocytes(% of control)	
		0.2 mg/10 ml	0.5 mg/10 ml
H_2O		100	100
NaCl		3.5±0.4	2.8±0.6
Persicae Semen	(PS)	37.7±12.6*	44.2±5.9*
Paeoniae Radicis rubra	(PR)	35.7±3.8*	39.2±1.8*
Typhae Pollen	(TP)	11.3±2.1	15.4±1.8
Curcumae Tuber	(CT)	43.3±12.6*	49.7±6.8*
Pteropi Faeces	(PF)	14.7±1.9	18.5±1.3
Carthami Flos	(CF)	1.8±0.5	2.4±0.4
Cnidii Rhizoma	(CR)	2.4±0.4	2.2±0.2
SSG		28.4±2.6*	31.6±3.1*

The assays were carried out as described under materials and methods. The final concentrations of these seven herbs added were 0.2 mg and 0.5 mg, respectively. In the control experiment, the same volume of 0.9% NaCl was added. Hemolysis by water was shown as 100%. The results of five independent experiments were averaged and summarized in table (mean±standard error, * : P-value(0.05).

2. Concentration-dependent effect of *Curcumae Tuber, Persicae Semen, Paeoniae Radicis rubra* and SSG on hemolysis of human erythrocytes

Typical curves of hemolysis induced by *Curcumae Tuber, Persicae Semen, Paeoniae Radicis rubra* and SSG are shown in Fig. 1, Table 2. The effects of herbs such as *Curcumae Tuber, Persicae Semen, Paeoniae Radicis rubra* and SSG on hemolysis depend on the increment of concentrations of those herbs. As a similar result, it was reported that the saponin such as anemarrhenasaponin from Anemarrhena asphodeloides Bunge (Liliaceae) had stronger effect on hemolysis than the other compounds as reported by Niwa et al[12]. The effects of hemolysis observed with *Curcumae Tuber, Persicae Semen, Paeoniae Radicis rubra* and SSG were almost same as that of anemarrhenasaponin.

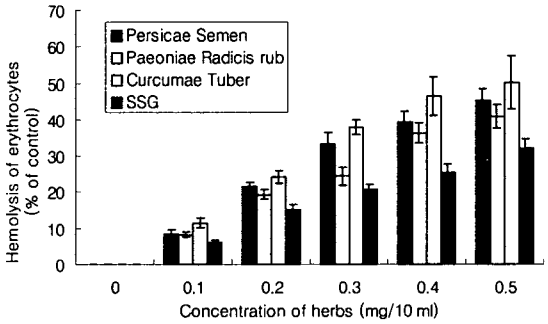

Fig. 1. Effect of various concentration of *Persicae Semen, Paeoniae Radicis rubra, Curcumae Tuber* and SSG on hemolysis of human erythrocytes. The assays were carried out as described under materials and methods. Hemolysis by water was shown as 100%. The results of five independent experiments were averaged and summarized in a bar figure (mean ±standard error, P<0.05).

Table 2. Effect of various concentration of *Persicae Semen, Paeoniae Radicis rubra, Curcumae Tuber* and SSG on hemolysis of human erythrocytes

Concentration (mg/10 ml)	Hemolysis of erythrocytes (% of control)			
	PS	PR	CT	SSG
0.0	0	0	0	0
0.1	8.4±1.1	8.3±0.8	11.5±1.3	6.1±0.6
0.2	21.5±1.2	19.3±1.3	24.3±1.7	15.3±1.3
0.3	33.3±3.1*	24.4±2.4	37.9±2.1*	20.6±1.7
0.4	39.5±2.7*	36.3±2.7*	46.4±5.2*	25.5±2.1
0.5	45.2±3.2*	40.7±3.2*	50.2±7.3*	32.1±2.7*

The assays were carried out as described under materials and methods. Hemolysis by water was shown as 100%. The results of five independent experiments were averaged and summarized in table (mean±standard error, * ; P-value<0.05).

3. Effects of seven herbs on platelet aggregation

The APTT is a rapid and the most widely used screening test to detect hereditary and acquired coagulation defects. APTT is also used to adjust heparin sodium dosage[10]. Therefore, the effects of seven herbs extracted from SSG on the APTT were also investigated. The inhibition effects of the seven herbs extracted from SSG were shown in Table 3. All of these extracted herbs showed remarkable inhibiting effects on platelet aggregation, and APTT were sensitive to the presence of these seven herbs. The plasma incubated with one of the seven herbs showed significant longer APTT than that of control. Using an *in vitro* system, APTT was delayed with the increment of concentrations of these seven herbs. The effectiveness of these herbs on antithrombin activity is *Curcumae Tuber* > *Persicae Semen* > *Paeoniae Radicis rubra* > SSG > *Pteropi Faeces* > *Typhae Pollen* > *Carthami Flos* > *Cnidii Rhizoma*

Table 3. Effects of seven herbs on platelet aggregation

Herbs (0.5 mg/10 ml)		APTT (sec)	Delaya (sec)
0.9% NaCl		25.3±1.9	0.0
Persicae Semen	(PS)	29.5±1.8	2.34
Paeoniae Radicis rubra	(PR)	29.1±2.1	2.21
Typhae Pollen	(TP)	28.2±1.9	1.83
Curcumae Tuber	(CT)	30.3±2.7	2.61
Pteropi Faeces	(PF)	28.1±2.4	1.67
Carthami Flos	(CF)	27.8±2.7	1.52
Cnidii Rhizoma	(CR)	27.3±3.2	1.34
SSG		28.5±3.1	1.94

a : The time compared with the APTT induced by the same volume of 0.9% NaCl. Data are presented as mean±standard error(n=5), all APTT values are significantly different from control.

4. MTT assay of SSG

SSG did not cause different levels of cellular toxicity with the increment of concentrations in this experiment as determined by reduction of MTT (data not shown).

5. Production of nitrite in stimulated mouse peritoneal macrophages

Incubation of 24 hrs lipopolysaccharide-stimulated mouse peritoneal macrophages with SSG caused a concentration-dependent inhibition of nitrite (as index of NO generation) production. Table 4 shows the IC_{50} values of test SSG for nitrite, respectively. As expected, 1400W (selective inhibitor of iNOS activity) reduced nitrite levels and dexamethasone inhibited metabolites at 32.5nM concentrations. None of these compounds affected cellular viability, as assessed by mitochondrial reduction of MTT after 24 hrs (data not shown) indicating that they were not cytotoxic.

Table 4. IC_{50} values for inhibition of nitrite accumulation in stimulated macrophages

Treatments	IC_{50}[a] nitrite
SSG	6.9 ug/ml
1400W	2.1 uM
NS398	N.D.
Dexamethasone	32.5 nM

Peritoneal macrophages stimulated with lipopolysaccharide for 24hrs produced 976.6ng nitrite/ml, compared with 84.4ng nitrite/ml in untreated cells. a : Fifty % Inhibitory concentration of each drug in ptroducing nitrite

6. Effect of SSG on iNOS activity in mouse peritoneal macrophages

The following experiments were designed to determine if the inhibition of nitrite production in macrophages was due either to interference with enzyme induction or to direct inhibition of enzyme activities. Cells treated with lipopolysaccharide for 24 hrs were washed and 12 ug/ml of SSG was added and 10 uM of each test products were also added, followed by 2 hrs incubation in fresh culture medium supplemented with L-arginine and arachidonic acid. No significant reduction of nitrite levels was observed for SSG after this 2 hrs period (Table 5). Nevertheless, 1400W caused a very significant reduction of nitrite(66%) production respectively.

Table 5. Effects of SSG and enzyme inhibitors on iNOS activities in intact peritoneal macrophages after 24 hrs of lipopolysaccharide stimulation

Treatments	iNOS (ng/ nitrite/ml)
Basal	1.6±0.2**
Control	42.5±1.7
SSG	38.4±2.71
1400W	15.3±1.3**
NS398	N.D.

All compounds except for SSG (12 ug/ml) were incubated at 10 M for 2 hrs after the s timulation period. Data shown, means±standard error (n=4-6). Basal : cells were not st imulated with lipopolysaccharide. N.D. : not determined. ** : P-value<0.01.

7. iNOS activity in broken cells preparations

To confirm the results obtained with intact cells, we examined the effects of this SSG on iNOS activity in broken cell preparations (Table 6). SSG at 10 uM was inactive in all the enzymatic activities assayed. In contrast, 1400W reduced significantly the production of citrulline (86% inhibition) respectively, in these subcellular preparations.

Table 6. Effect of SSG and enzyme inhibitors on iNOS activities in high speed supernatants or microsomes of 24 hrs lipopolysaccharide-stimulated macrophages, respectively

Treatments	iNOS (pmol citrulline/mg protein, min)
Control	15.2±1.2
SSG	12.3±1.1
1400W	1.9±0.3**
NS398	N.D.
Indomethacin	N.D.

Data shown, means±standard error, (n=4-5). N.D. : not determined. SSG was assayed at 24 ug/ml and at 10 uM. ** : P-value<0.01.

8. iNOS protein expression in mouse peritoneal macrophages

Western blot assays of 24hrs lipopolysaccharide-stimulated cells were performed to assess possible effects on iNOS protein expression. Fig. 2 shows a representative experiment where co-incubation of SSG at 12ug/ml with lipopolysaccharide (10 ug/ml) for 24hrs, caused reduction in the expression of both isoenzymes.

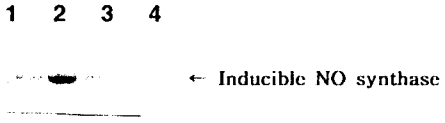

Fig. 2. Effect of SSG and dexamethasone (DX) (10 uM) on iNOS expression on 24 hrs lipopolysaccharide (LPS)-stimulated murine peritoneal macrophages. The figure is representative of three similar experiments.

9. Carrageenan paw edema

After i.p. administration, SSG caused a dose-dependent reduction in carrageenan-induced edema 3 and 5 hrs after induction of inflammation (Table 7). The greatest effect was observed 3 hrs later, with the inhibition percent of 18.7%, 44.7% and 63% at the doses of 60, 120 and 360 mg/100g, respectively. Indomethacin (5 mg/kg i.p.) was assayed as reference compound, showing a significant reduction in swelling 3 (57.7%) and 5 hrs (44.4%) after the administration of carrageenan.

Table 7. Effect of SSG and indomethacin on carrageenan mouse paw edema, 1, 3 and 5 hrs after the induction of inflammation (μℓ)

Time(hr)	Control	Indomethacin (5mg/kg)	SSG(mg/100g) 60	120	360
1 hr	9.5±0.7	8.3±0.4	7.9±0.4	7.3±0.7	4.9±0.3*
3 hrs	20.8±0.4	8.8±0.3**	16.9±0.5*	11.5±1.1*	7.7±0.6**
5 hrs	16.0±0.3	8.9±0.4**	10.0±1.4**	7.3±1.0**	5.3±1.0**

Control : vehicle (tween 80/saline 1:99, v/v) was administered intraperitoneally 1 h before injection of carrageenan (0.05 ml; 3% w/v in saline) into the subplantar area of the right hind paw. * : P-value<0.05, ** : P-value<0.01 (n=6-7 animals). SSG at the doses of 60, 120 and 360 mg/100g was administrated orally. Others were i.p. administrated.

The last evaluation of edema (5 hrs) was followed by killing of the animals and the paws injected with carrageenan were homogenized to determine the levels of elastase (Table 8). The results indicate that elastase activity was significantly and dose dependently decreased by the three doses of SSG assayed. Indomethacin also significantly reduced elastase activity in homogenates of inflamed paws. SSG reduced the levels of this prostanoid at the doses of 120 mg/100g and 360 mg/100g (Table 8).

Table 8. Inhibition by SSG and indomethacin of elastase activity (control value 153.6±6.5 nmol p-nitrophenol released/ml) in homogenates of inflamed paws(control values 92.2±5.4 and 16.4±1.4 ng/ml, respectively)

Dose	Elastase activity(% Inhibition)
SSG (60 mg/100g) oral	28.4±2.2*
SSG (120 mg/100g) oral	44.4±4.2*
SSG (360 mg/100g) oral	53.2±4.3*
Indomethacin (5.0mg/kg) i.p	40.3±2.4*

Data represent means±standard error, (n=5-6 animals). * : P-value<0.05.

Discussion

The traditinal korean therapeutic system has been used for the treatment of various blood circulation disease, including the clinical treatment of thrombosis[16]. Nowadays, the occurrence rate of the blood circulation system disease has been increased. And the thrombosis importantly came to the front as the risk factor of these circulation system's disease[17][18]. Thrombosis plays a major role in ischemic heart disease. Patients who have sustained an acute myocardial infraction are at increased risks of developing vascular events such as sudden death, recurrence of infraction and thromboembolic stroke. In traditional korean medicine, the thrombosis in the category of blood stasis and this blood stasis present the generalize or local blood circulation disturbance that generated by all kinds of pathological fact or blood stream retention accompanying with a series of syndrome. As the syndrome, stabbing pain fixed at certain region, squamous and dry skin, fullness and pain of the chest and hypochondrium, firmness and fullness of the lower abdomen and so forth have been created. And it becomes the pathopoiesis cause that the symtom complex with a mass or swelling in the abdomen, apoplexy etc[19]. Moreover, the drugs for invigorating blood circulation and eliminating blood stasis or drugs for removing blood stasis are used for all kinds of syndrome through the blood stasis[20][21]. On this study, SSG was used for investigating reaction of enzymes acted on mechanism of thrombosis formation. SSG is consisted of *Typhae Pollen, Pteropi Faeces, Paeoniae Radicis rubra, Cnidii Rhizoma, Persicae Semen, Carthami Flos* and *Curcumae Tuber*[1][2][3]. SSG has been reported to have a hypolipidemic effect in patients with hypercholesterolemia and in cholesterol-induced experimental models[1]. Also SSG has been reported to have an inhibitory effects on the atherosclerosis in hypercholesterolemic rabbits[4]. The results of recent clinical studies indicate that antithrombotic therapy significantly reduces the number of vascular events in these patients.

As explanation in introduction, neutrophils are essential for host defense and their contribution to the propagation and maintenance of acute and chronic inflammation includes several mechanisms.

The APTT is a rapid and the most widely used screening test to detect hereditary and acquired coagulation defects. APTT is also used to adjust heparin sodium dosage[12].

All of these extracted herbs showed remarkable inhibiting effect on platelet aggregation, and APTT were sensitive to the presence of these seven herbs. The plasma incubated with one of the seven herbs showed significant longer APTT than that of control. Using an *in vitro* system, APTT was delayed with the increment of concentrations of these herbs ($P<0.01$). The effectiveness of these herbs on antithrombin activity is *Curcumae Tuber* > *Persicae Semen* > *Paeoniae Radicis rubra* > SSG > *Pteropi Faeces* > *Typhae Pollen* > *Carthami Flos* > *Cnidii Rhizoma*. These results suggest that three herbs are important for antithrombin activity and that further studies of these herbs on pharmaceutical functions may bring a significant development to clinical uses.

In the present work, SSG reduced the degranulation induced by cytochalasin B+fMLP or cytochalasin B+PAF, as well as the leukotriene B4 synthesis induced by ionophore A23187, thus exerting inhibitory effects on neutrophil functions triggered by structurally divergent agonists. DTD seems to affect cell activation at a site common to different signaling pathways as it inhibit responses induced by fMLP, PAF, TPA or ionophore A23187.

The induction of inducible NO synthase greatly increase the synthesis of NO. iNOS inhibition resulted in modulation of the inflammatory response and delayed paw swelling induced by carrageenan in mice[22]. Furthermore, NO has been shown, in *in vitro* and *in vivo* studies, to increase the production of pro-inflammatory prostaglandins[23]. SSG inhibited the production of NO in murine peritoneal macrophages stimulated by lipopolysaccharide. The inhibition was dose-dependent without any triggering cytotoxic effect. Western blot analysis of mouse peritoneal macrophages lysates showed that iNOS protein expression were reduced by the presence of SSG during lipopolysaccharide treatment, indicating that SSG inhibits the induction rather than the activity of enzymes. In a model of inflammation, the mouse paw edema induced by carrageenan, SSG exerted potent inhibitory effects. Interestingly, SSG reduced the elastase content in the inflamed paw, an index of migration.

In sum, the present study demonstrated that SSG can control NO overproduction by selective inhibition of the enhanced expression of enzymes. SSG inhibited the oxidative burst in human neutrophils and murine peritoneal macrophages. SSG exerts acute anti-inflammatory effects by reducting of leukocyte activation and by inhibiting of iNOS expression.

Conclusion

The effects of SSG on platelet aggregation and hemolysis in human blood were studied. SSG provoked remarkable inhibiting effect on platelet aggregation, and APTT are sensitive to the presence of this SSG. Using an *in vitro* system, APTT was delayed with the increment of the concentrations of

these seven compounds. In the seven herbs, only *Curcumae Tuber* and *Persicae Semen* and *Paeoniae Radicis rubra* had the strongest effects on hemolysis, and *Typhae Pollen, Pteropi Faeces* had a slight effects on hemolysis and *Cnidii Rhizoma* and *Carthami Flos* had no effect on hemolysis. These results suggested that SSG might be used as a novel antithrombotic therapeutic agents in post-myocardial infarction.

A SSG reduced nitric oxide(NO) production in mouse peritoneal macrophages stimulated with lipopolysaccharide, without the influence on the activity of iNOS being observed. SSG significantly reduced mouse paw edema induced by carrageenan. Western blot analysis showed that SSG reduced the expression of iNOS. The results indicate that SSG exerts anti-inflammatory effects related to the inhibition of NO production, which could be due to a decreased expression of iNOS.

References

1. Jiang, C.H., Huo Xue Huo Yu yanjiu xinbian, pp.521-522, Shanghai Science Technical Press, Sanghai, 1990.
2. Lee, S.I., Herbal Theory, pp.54-56, SuSeodang, Seoul, 1975.
3. Xie Zhufan, Huang Xiaokai, Dictionary of Traditional Chinese Medicine, pp.192-198, The Commercial Press, Hong Kong, 1984.
4. Shin, S.S. etc., An experimental study on inhibitory effects of Silsosangami on the atherosclerosis in hypercholesterolemic rabbits, Korean J. Oriental Physiology & Pathology 15(5), 818-823, 2001.
5. MacMicking, J., Xie, Q.W. and Nathan, C., Nitric oxide and macrophage function, Annu. Rev. Immunol. 15, 323-350, 1997.
6. Kang, R.Y., Freire Moar, J., Sigal, E. and Chu, C.Q., Expression of cyclooxygenase-2 in human and an animal model of rheumatoid arthritis. Br. J. Rheumatol. 35, 711-718, 1996.
7. Marcinkiewicz, J., Grabowska, A. and Chain, B., Nitric oxide up-regulates the release of inflammatory mediators by mouse macrophages. Eur. J. Immunol. 25, 947-951, 1995.
8. Hong, M.Y., Choi, D.Y., Kim, C.H., Kim, B.J., Kim, H.G., Chung, W.T. and Park, W.H., Inhibitory effects of Hwaotang(HOT) on the atherosclerosis and the venous thrombosis. Korean J. Oriental Physiology & Pathology 16(2), 380-388, 2002.
9. Murray, D., Pennell, B. and Olson J., Variability of prothrombin time and activated partial thromboplastin time in the diagnosis of increased surgical bleeding. Transfusion 39, 56-62, 1999.
10. Niwal, A., Takeda, O., Ishimaru, M., Nakamoto, Y., Yamasaki, K., Kohda, H., Nishino, H., Segawa, T., Fujimura, K. and Kurimoto, A., Screening test for platelet aggregation inhibitor in natural products. The active principle of Anemarrhenae Rhizoma. Yakugaku Zasshi 108, 555-561, 1988.
11. Gross, S.S. and Levi, R., Tetrahydrobiopterin synthesis. An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. J. Biol. Chem. 267, 25722-25729, 1992.
12. Misko, T.P., Schilling, R.J., Salvemini, D., Moore, W.M. and Currie, M.G., A fluorometric assay for the measurement of nitrite in biological samples. Anal. Biochem. 214, 11-16, 1993.
13. Moroney, M.A., Alcaraz, M.J., Forder, R.A., Carey, F. and Hoult, J.R.S., Selectivity of neutrophil 5-lipoxygenase and COX inhibition by an anti-inflammatory flavonoid glycoside and related aglycone flavonoids. J. Pharm. Pharmacol. 40, 787-792, 1988.
14. Mitchell, J.A., Sheng, H., Forstermann, U. and Murad, F., Characterization of nitric oxide synthases in non-adrenergic non-cholinergic nerve containing tissue from the rat anococcygeus muscle. Br. J. Pharmacol. 104, 289-291, 1991.
15. Sugishita, E., Amagaya, S. and Ogihara, Y., Anti-inflammatory testing methods: comparative evaluation of mice and rats. J. Pharmacobio-Dyn. 4, 565-575, 1981.
16. Zhang Enqin, Clinic of Traditional Chinese Medicine (I) in A Practical English-Chinese Library of TCM, pp. 102-107, House of Shanghai College of Traditional Chinese Medicine, Shanghai, 1990.
17. Kim, J.G., clinic lipid, pp.241-270, Medical Science Press, Seoul, 1995.
18. Seo, S.G., Adult & Aged disease, p.27, 38-53, Korea medical science, Seoul, 1992.
19. Park, W.H., etc., A study of the influence of both of Shunqidaotantang and Huayutang on thrombosis, contusion-hyperemia, and hyperlipidemia, The J.of DGOM V.2, No.1, 19-54, 1993.
20. Shin, M.G., Clinic Herbs Science, p.143, 453, NamSanDang, Seoul, 1986.
21. 久保道徳, 松田秀秋, 松田玲子, 抗血栓形成作用について, 牧丹皮の研究, 第8報, pp.38, 307-312, 1994
22. Ianaro, A., O'Donnell, C.A., Di Rosa, M. and Liew, F.Y., A nitric oxide synthase inhibitor reduces inflammation, down-regulates inflammatory cytokines and enhances interleukin-10 production in carrageenin-induced oedema in mice. Immunology 82, 370-375, 1994.
23. Salvemini, D., Wang, Z.Q., Bourdon, D.M., Stern, M.K., Currie, M.G. and Manning, P.T., Evidence of peroxynitrite involvement in the carrageenan-induced rat paw ooedema. Eur. J. Pharmacol. 303, 217-220, 1996.