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Antithrombotic Activities of Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang

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Abstract – The possibility of Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang, which have been recommended for stroke patients with constipation in traditional Korean clinics, was evaluated as a novel antithrombotic agent. Chungpesagantang potently inhibited ADP- and collagen-induced rat platelet aggregation *in vitro*, but Sunghyangjunggisan and Yangkyuksanwhatang did not inhibit it. However, all tested herbal formulae inhibited *in vivo* ADP- and collagen-induced rat platelet aggregation and extended the bleeding time of mice tails. All these herbal formulae did not affect certain plasma clotting times such as APTT, PT and TT. Among these herbal formulae, Chungpesagantang showed significant protection against death due to pulmonary thrombosis in mice. Based on these findings, these herbal formulae should prevent thrombosis and cardiovascular diseases such as myocardial infraction stroke, and arteriosclerosis.

Keywords – antithrombosis, Chungpesagantang, Sunghyangjunggisan, Yangkyuksanwhatang, stroke.

Introduction

Platelets play an important role in the pathogenesis of thrombosis. The interactions between the platelets and blood vessel walls are important in the development of thrombosis and cardiovascular diseases, such as myocardial infraction stroke, and arteriosclerosis (Mustard, 1975; Mustard *et al.*, 1990; Dinerman and Mehta, 1990). Once the blood vessels are damaged, platelet aggregation occurs rapidly to form hemostatic plugs or arterial thrombi at the sites of vessel injury or in regions where the blood flow is disturbed. These thrombi are the source of thromboembolic complications of arteriosclerosis, heart attacks, stroke, and peripheral vascular disease (Packham, 1994; Stein and Fuster, 1989; MacMahon and Sharpe, 1991). Therefore, the inhibition of platelet function represents a promising approach for the prevention of thrombosis. A number of anti-platelet herbal medicines and their herbal formulae have been evaluated for their effects in preventing the development of thrombosis or its recurrence. Among herbal formulae, Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang are representative herbal formulae for patients who suffer from stroke in Korea. Therefore, as part of our continuing search for biological active anti-stroke agents from medicinal resources, we have been screened antithrombotic activity of Sunghyangjunggisan, Chungpesagantang and their ingredients. However, the antithrombotic activity of Yangkyuksanwhatang was not studied.

In present study, we compared antithrombotic activities of Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang by determining their inhibitory effect on platelet aggregation induced by various aggregating agents *in vitro* and *ex vivo*, and its antithrombotic effect *in vivo*.

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Experimental

Materials – Adenosine 5'-diphosphate (ADP), epinephrine, collagen, bovine serum albumin, prothrombin, thromboplastin, thrombin and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (USA). The other chemicals were of analytical reagent grade.

Herbal formula materials and extraction – Chungpesagantang is consisted of 16 g Puerariae Radix, 8 g Scutellariae Radix, 8 g Angelicae Tenuissimae Radix, 4 g Raphani Semen, 4 g Platycodi Radix, 4 g Cimicifugae Rhizoma, 4 g Angelicae Dahuricae Radix and 4 g Rhei Rhizoma. Sunghyangjungisan is consisted of 6 g Pogostemi Herba, 4 g Perillae Herba, 4 g Arisaematis Rhizoma, 4 g Saussureae Radix, 2 g Atractylodis Rhizoma Alba, 2 g Aurantii nobilis Pericarpium, 2 g Pinelliae Tuber, 2 g Aurantii Immaturi Pericarpium, 2 g Arecae Pericarpium, 2 g Cinnamomi Cortex, 2 g Zingiberis Rhizoma, 2 g Alpiniae Fructus, 2 g Glycyrrhizae

Radix, 0.5 g Zingiberis Rhizoma and 0.5 g Zizyphi Fructus. Yangkyuksanwhatang is consisted of 8 g Rehmanniae Radix, 8 g Lonicerae Folium, 8 g Forsythiae Fructus, 4 g Gardeniae Fructus, 4 g Menthae Herba, 4 g Anemarrhenae Rhizoma, 4 g Gypsum, 4 g Ledebouriellae Radix and 4 g Nepetae Spica. Materials of herbal formulae were purchased from Kyungdong Crude Drug Market, Seoul, Korea, 1999. They were identified by N-J Kim (East-West Medical Research Institute, Kyung Hee University). Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang were extracted twice with 10-fold boiling water. After evaporation, each extract were used. The yield of each formula was 19.4%, 19.9% and 11.9%, respectively.

Animals – Male Sprague-Dawley rats (180-220 g) and ICR mice (male, 20-24 g) were purchased from Sam Yook Animal Co. (Korea) and acclimatized for one week at a temperature of 22+1°C and a humidity of 55+5% with free access to a commercial pellet diet obtained from Samyang Co. (Korea) and drinking water before the experiments. The animal experiments were carried out in accordance with international guidelines.

Preparation of platelets – Blood was collected from the rats by cardiac puncture into a flastic flask containing 2.2% sodium citrate (1:9 v/v). The platelet rich plasma (PRP) was prepared by centrifuging the blood at 120×g for 15 min, and then again at 850×g for 10 min to prepare the platelet poor plasma (PPP) (Teng and Ko, 1988). The supernatants were pooled and centrifuged at 600×g for 15 min at room temperature. The platelet pellets were washed with modified Tyrode-HEPES buffer (129 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 2 mM EGTA, 5.6 mM glucose, 10 mM HEPESs, and 0.35% BSA, pH 7.4) and centrifuged at 600×g for 15 min. Then, platelet pellets were gently resuspended in Tyrode-HEPES buffer.

***In vitro* antiplatelet aggregation** – The platelet aggregation was measured by turbidometry using a dual channel Whole Lumini-Ionized Calcium Aggregometer (Chrono-Log Co., Ltd, Havertown, PA, USA) according to the method of Born and Cross (1963). Briefly, rat PRP (300 μl) was incubated at 37°C for 2 min in the aggregometer with stirring at 1200 rpm, and then stimulated with ADP and collagen. The herbal medicines or aspirin (as a reference agent) were incubated with PRP for 3 min, followed by addition of the aggregation agents. Any changes in light transmission were recorded for 10 min after stimulation with these agents. Each inhibition rate was obtained from the maximal aggregation induced by the respective agonist at the concentration derived from Equation 1, and then the values of IC_{50} were calculated from the data using a probit method.

Equation 1:

$$\text{Inhibition rate (\%)} = \frac{\text{1-maximal aggregation rate of sample treated PRP}}{\text{maximal aggregation rate of vehicle treated PRP}} \times 100$$

***Ex vivo* antiplatelet aggregation** – Male SD rats were used after overnight fasting. 1 g/kg of herbal medicine extract (or 50 mg/kg of aspirin) was administered orally to the rats as a vehicle for three days. The blood was collected 3 h after the final sample treatment, and the PRP was previously described. Platelet aggregation was induced by 80 μg/ml of collagen or 8 μM of ADP. The antiplatelet activities of the sample were investigated according to the method of Kimmura *et al.* (1985).

***In vivo* anti-thrombotic activity** – The anti-thrombotic effects of herbal medicines were investigated by the mouse thromboembolism test according to the method of Di Minno and Silver (1983). Male ICR mice were used after overnight fasting. The herbal medicines (1 g/kg), or aspirin (50 mg/kg) as a positive control, and 0.5% CMC solution were administered orally. A mixture solution of collagen (110 μg) and epinephrine (13 μg) was injected into the mouse tail vein 3 h after the sample treatment. The number of dead or paralyzed mice was recorded for up to 15 min, and the percentage of protection was calculated as follows: [1-(dead+paralyzed)/ total]×100

Tail bleeding time in conscious mice – The bleeding time was measured as described by Hornstra *et al.* (1981). The bleeding time is designed to determine the blood's ability to form a hemostatic plug, in which the platelet, plasma factor, and blood vessel wall are involved. In short, the herbal medicine (1 g/kg) or aspirin (50 mg/kg) and the 0.5% CMC solution were administered once a day for three days. 3 h after the oral administration of the samples, the tail of the male ICR mouse was transected 2 mm from the tip, and 1.5 cm of the distal portion was vertically immersed in saline at 37°C.

***In vitro* coagulation parameters** – The plasma clotting times, activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) were measured by a modification of Hara's method (1994). The PPP was incubated with the samples for 7 min at 37°C, and the coagulation was started with by adding 100 μl of 20 mM $CaCl_2$, 100 μl of thromboplastin, and 100 μl of bovine thrombin into the 100 μl of incubated plasma for the APTT, PT and TT assays, respectively

Results

***In vitro* antiplatelet aggregation effect** – The *in vitro* inhibitory activity of Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang on platelet aggregation was

Table 1. Effect of Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang on *in vitro* antiplatelet aggregation (aggregation inducer, ADP or collagen)

Herbal formula	IC_{50} (mg/ml)[a]	
	ADP	Collagen
Aspirin	0.04	0.17
Chungpesagan-tang	1.5	1.5
Sunghyangjunggi-san	>15	>15
Yangkyuksanwha-tang	>15	>15

[a]50% inhibitory concentration (IC_{50}) was calculated as follows: (control aggregation (%)-herbal medicine-treated aggregation (%))/ control aggregation (%)×100 = inhibition (%).

compared (Table 1). Chungpesagantang potently inhibited ADP- and collagen-induced rat platelet aggregations *in vitro* in a dose-dependent manner, with an IC_{50} value of 1.5 mg/ml. However, the other tested herbal formulae did not inhibit them.

***In vitro* anticoagulation effect** – The effect of herbal medicines on plasma clotting time was evaluated by APPT, PT and TT assays using human platelet poor plasma (Table 2). The tested herbal formulae did not affect the tested plasma clotting times.

***Ex vivo* antiplatelet aggregation effect** – The *ex vivo* inhibitory activities of Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang on platelet aggregation were measured, after they were orally administered once a day for three days into SD rats (Table 3). These herbal formulae significantly inhibited ADP- or collagen-induced platelet aggregations from that of the control group. These herbal formulae inhibited ADP-induced platelet aggregation similarly to aspirin 50 mg/kg. The difference between these herbal formulae was not significant. However, Sunghyangjunggisan and Yangkyuksawhatang potently inbibited collagen-induced platelet aggregation, although Chungpesagantang weakly inhibited it.

Effect on the tail bleeding time of mice – The effect of herbal medicines on bleeding time was studied using the mouse tail bleeding system. As shown in Table 4, the bleeding time of the control mice was 68±20.5 s. However, Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang markedly prolonged the mouse tail bleeding time compared with the control. Sunghyangjunggisan showed the most potent activity and was more potent than a 50 mg/kg dose of aspirin.

***In vivo* antithrombotic effect** – The *in vivo* antithrombotic activities of Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang were measured (Table 5). All tested herbal formulae exhibited the antithrombitic activity. Chungpesagantang showed siginificant protection from death due to pulmonary thrombosis in mice. However, these herbal formulae did not show more potent antithrombotic activity than aspirin at a dose of 50 mg/kg.

Table 3. Effect of Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang on *ex vivo* antiplatelet aggregation

Herbal formula	*Ex vivo* antiplatelet aggregation (%)	
	ADP	Collagen
Control	60.5 ± 0.7	56.0 ± 1.4
Aspirin	42.5 ± 10.6*	39.5 ± 7.8*
Chungpesagan-tang	44.7 ± 13.9*	46.0 ± 8.0*
Sunghyangjunggi-san	43.8 ± 11.6*	26.0 ± 28.4*
Yangkyuksanwha-tang	43.9 ± 13.8*	31.6 ± 25.2*

The samples were orally administered once a day for three days before the test.
The results were expressed as mean ± SD (n=5).
Platelet aggregation was induced by 80 μg/ml of collagen or 8μM of ADP.
*Significantly different to control ($p<0.05$).

Table 4. Effect of Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang on bleeding time of mice tails

Herbal formula	Mouse tail bleeding time (sec)
Control	68.0±20.5
Aspirin (50 mg/kg)	258.0± 8.5*
Chungpesagan-tang	108.0±19.6*
Sunghyangjunggi-san	>600*
Yangkyuksanwha-tang	172.0±52.0*

The results were expressed as mean ± SD (n=5).
*Significantly different to control ($p<0.05$).

Table 2. Effect of Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang on human plasma coagulation time

Herbal formula	Concentration (mg/ml)	Coagulation time (s)		
		APTT	PT	TT
Control		36.1 ± 1.2	40.8 ± 2.6	34.1 ± 2.4
Chungpesagantang	3	34.0 ± 2.3	34.6 ± 2.5	31.6 ± 1.1
Sunghyangjunggisan	3	36.4 ± 0.1	31.2 ± 3.1	34.3 ± 0.7
Yangkyuksanwhatang	3	36.3 ± 4.8	29.3 ± 0.1	37.1 ± 0.4
Heparin	0.003	174.8 ± 22.4*	57.0 ± 7.7*	>500*

The results were expressed as mean ± SD (n=3).
*Significantly different to control ($p<0.05$).

Table 5. Antithrombotic activity of Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang

Herbal formula	Protection (%)
Control	20
Aspirin (50 mg/kg)	80
Chungpesagan-tang	60
Sunghyangjunggi-san	40
Yangkyuksanwha-tang	40

The samples were orally administered 3 h before tail vein injection of epinephrine and collagen.

Discussion

Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang have been used frequently in Korean Oriental clinics for patients who suffer from stroke, but their anti-stroke activities have not been evaluated. Therefore, we investigated Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang as part of our continuing search for anti-stroke agents from medicinal resources. The *ex vivo* antiplatelet aggregation activities of these herbal formulae were significantly effective, but their *in vitro* anti-platelet aggregation activities except Chungpesagantang was not.

These results suggest that the components of herbal medicines could be transformed to the active or inactive components for anti-platelet aggregation by human intestinal bacteria. For example, puerarin and daidzin, main components of Puerariae Radix, could be transformed to daidzein by human intestinal bacteria (Kim *et al.*, 1998). The metabolite daidzein showed more potent antiplatelet aggregation activity than puerarin and daidzin (Choo *et al.*, 2002). These herbal formulae did not showed urokinase-like activity, although they activated weakly urokinase (Data not shown).

When the prolongation activity of Chungpesagantang, Sunghyangjunggisan and Yangkyuksanwhatang on bleeding time of mouse tails was investigated, these herbal formulae were all effective. Sunghyangjunggisan exhibited the most potent thrombotic activity, followed by Yangkyuksanwhtang and Chungpesagan-tang. These results support that the antithrombotic activity of these herbal formulae could be caused by the activities of urokinase and antiplatelet aggragation. These herbal formulae also exhibited the antithrombotic activity on thromboembolic mice induced by collagen and epinephrine.

These inhibitory activities are important in the prevention of thrombosis and cardiovascular diseases, such as myocardial infraction stroke, and arteriosclerosis. These formulae could prevent the development of thrombosis or its recurrence.

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