A study on anti-thrombotic activity of Hwao-tang

Tae Woo Park, Won Hwan Park*

Departments of Diagnostics, College of Oriental Medicine, Dongguk University

<Abstract>

The thrombosis is the category of blood stasis(瘀血). Blood stasis is a pathologicial state resulting from the reverse or impeded flow of blood in the body or the stagnation of blood flow in local parts as well as abnormal blood outside of the vessles which remains in the body and fails to disperse. Hwao-tang has been reported to have a hypolipidemic effect in patients with hypercholesterolemia, and in highcholesterol-induced experimental models. The present paper reports the effects of HOT on atherosclerosis using a spontaneous experimental model, Kurosawa and Kusanagi-hypercholesterolemic (KHC) rabbits. We have also investigated the pharmacological effect of extracts obtained from HOT on collagen-and ADP-induced blood platelet aggregation, thrombin-induced conversion of fibrinogen and fibrinolysis in in vitro experiments. In conclusion, the protection of extracts of Korean herbs' HOT on the ischemic infarction induced artificially might be involved to their inhibition of thrombotic action.

Key words : Hwao-tang(化瘀湯), thrombosis, blood stasis, atherosclerosis, hypercholesterolia, collagen, ADP.

Introduction

In oriental medicine, the thrombosis is the category of blood stasis(瘀血). Blood stasis is a pathologicial state resulting from the reverse or impeded flow of blood in the body or the stagnation of blood flow in local parts as well as abnormal blood outside of the vessles which remains in the body and fails to disperse[1,2]. As soon as blood stasis is formed, it can further affect the circulation of blood and lead to new pathological changes, causing a variety of diseases and syndromes[1,2]. 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 and thrombosis[3,4,5,6]. The traditinal korean therapeutic system has been used for the treatment of various disease for hundreds of years, including the clinical treatment of atherosclerosis, hypercholesterolimia, diabetes and obesity[7]. Hwao-tang has been reported to have a hypolipidemic effect in patients with hypercholesterolemia[8], and in highcholesterol-induced experimental models. HOT is consisted of *Angelica gigantis Radix, Rehmanniae Radix, Paeoniae Radix, Ciniamomi Cortex, Cnidii Rhizoma, Persicae Semen* and *Carthami Flos*. According to the ancient Chinese medicinal literature 《NaSiHoeYakEuiKyung (羅氏會約醫鏡)》, HOT is activate blood circulation, vital energy and regulate menstruation, and is indicated for irregular menstruation, dysmennorrhea, amenorrhea and metrorrhagia due to blood stasis, and sudden loss of vision caused by retinal hemorrhage[9]. Now a days, HOT is mainly used for the treatment of inflammation, hyperlipemia and arteriosclerosis. The pharmacological action of HOT has been limitedly studied in regard to ischemic infarction[8]. This herbal medicine has been shown to express diverse activities such as immunomodulating, anti-infarction, anti-allergic and anti-inflammatory effects(Kim et al., unpublished results). Antisclerotic effects of HOT in experimentally induced atherosclerosis in rabbits have been reported (Park et al., not shown). Inhibitory effects of HOT on the atherosclerosis and venous thrombosis have also been reported[10]. However, pharmacological mechanisms of HOT on atherosclerosis formation and anti-thrombosis are poorly understood. The present paper reports the effects of HOT on atherosclerosis using a spontaneous experimental model, Kurosawa and Kusanagi-hypercholesterolemic (KHC) rabbits[11]. We have also investigated the pharmacological effect of extracts obtained from HOT on collagen- and ADP-induced blood platelet aggregation, thrombin-induced conversion of fibrinogen and fibrinolysis in vitro experiments.

Materials and Methods

1. Animals

* 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
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Male KHC rabbits weighing 1.5-2.0 kg were purchased from Genetic Resource Center, KRIBB, KIST (Taejon, Korea). They were maintained in the animal facility (room temperature: 23.2℃, relative humidity: 55.10%, all fresh air ventilation: 15-20 times/hrs, 12 hrs light and 12 hrs dark) and subjected to the experiment after a 7 days quarantine period. Male Wistar-King strain rats weighing 150-200 g were used. They were fed on a standard diet for at least 7 days. They were fasted for 24 hrs before the start of the experiments.

2. Drugs

當歸(*Angelica gigantis Radix*) 16g, 熟地黃(*Rehmanniae Radix*) 10g, 白芍藥(酒炒)(*Paeoniae Radix*) 8g, 肉桂(*Ciniamomi Cortex*) 8g, 川芎(*Cnidii Rhizoma*) 4g, 桃仁(*Persicae Semen*) 4g and 紅花(酒炒)(*Carthami Flos*) 3.2g were used as Hwao-tang (HOT, 化瘀湯)[12] prescription(Table 1).

Table 1. Composition of Hwao-tang

韓藥名(Pharmacognostic Name)	重量(g)
當歸(*Angelicae gigantis Radix*)	16.0 g
熟地黃(*Rehmanniae Radix*)	10.0 g
白芍藥(酒炒)(*Paeoniae Radix*)	8.0 g
肉桂(*Ciniamomi Cortex*)	8.0 g
川芎(*Cnidii Rhizoma*)	4.0 g
桃仁(*Persicae Semen*)	4.0 g
紅花(酒炒)(*Carthami Flos*)	3.2 g

HOT is a dried decoctum of a mixture of 7 herbal drugs. A total of 28 g of HOT was added to 500 ㎖ of water and boiled for 2 hours, filtered and then concentrated to 200 ㎖. This decoction was spray-dried to give a powdered extract. The yield was 5.2 g., which represents one human dose/day. The aqueous extracts of HOT and its seven composed Korean herbs, which was massproduced as for clinical use, were kindly supplied by the Oriental Medical Hospital of Dongguk University(Kyungju, Korea). Endotoxin (Escherichia coli; 055: B5) was from Difco Lab. (USA). Thrombin, adenosine diphosphate (ADP) disodium salt, plasminogen-containing fibrinogen and urokinase were purchased from Sigma Co. (USA).

3. Pathological evaluation of aorta

The thoracic aortas were opened longitudinally, and the percentages of the areas of atheromatous plaque were calculated according to Kita's method[13]. Some parts of plaque were fixed with 15% buffered formalin. They were embedded in paraffin, and sections were stained with hematoxylan and eosin (HE) or Oil-Red-O.

4. Blood platelet aggregation test

Whole blood samples were collected from heart of pentobarbital-anesthetized rats. Nine ㎖ of the blood and 1 ㎖ of heparin solution (10 U/㎖) were transferred into a plastic tube. And centrifuged at 1,000 rpm for 10 min to give platelet-rich plasma(PRP). PRP was removed with a siliconized pipet, to be stored in a plastic test tube with a screw cap. The remaining red cell precipitate of the blood samples was further centrifuged at 3,000 rpm for 30 min to give platelet-poor plasma (PPP), which was used as a maximal transmittance standard[14]. Platelet aggregation test described by Ekimoto et al[15]. was modified and performed with collagen (500 ㎍/㎖) and ADP (0.05 μM) used as aggregation agents. A 0.2 ㎖ aliquot of PRP was placed in a test tube and the content was stirred at 1,200 rpm, at 37℃, for 1 min to which was added a 10 ㎕ aliquot of a test solution. After 1 min, an aggregation agent was added to the reaction mixture. Changes in the light transmittance of the reaction mixture was continuously recorded with a Husm System platelet aggregometer (Rika Electric Co., Japan) and the transmission at the maximal aggregation after the addition of an aggregating agent was recorded. Then platelet aggregation was expressed as the percent increase in the transmittance taking the transmittance of a control mixture containing no test solutions zero.

5. Thrombin-induced conversion of fibrinogen to fibrin

Fibrinogen (500 ㎎) was dissolved in 100 ㎖ of 150 mM NaCl containing 50 mM Tris-Acetate buffer (pH 7.4). A test solution (0.1 ㎖) was added to 1. 8 ㎖ of the fibrinogen solution with stirring. After 1 min, 0.1 ㎖ of thrombin solution (0.2 U/㎖) was added to the mixture and the whole was gently stirred until a fibrin clot appeared. The time required for clotting was recorded.

6. Fibrin plates

Fibrin plates were prepared by the method of Astrup and Mullertz[2]. One % agarose solution in phosphate buffered saline (10 mM phosphate buffer, pH 7.8, in 150 mM NaCl) was kept at 45-50℃ in a water bath. Agarose solution of plasminogen-containing fibrinogen and of plasminogen free-fibrinogen was prepared by dissolving 166 ㎎ of plasminogen-fibrinogen and 200 ㎎ of plasminogen free-fibrinogen in 100 ㎖ of agarose solution at 31℃. A 10 ㎖ aliquot of the mixture and 0.1 ㎖ of thrombin (10 U/㎖) solution were quickly mixed in a test tube, and the contents were immediately poured into a Petri dish. The five wells of diameter 5 mm were made into each fibrin-agar plate.

7. Inhibition of plasminogen

A test solution (0.1 ㎖) and urokinase solution (0.1 ㎖, 100 U/㎖) were mixed, and 200 ㎕ of the mixture was added

to each of the wells in the plasminogen-containing fibrin plate. Twenty ㎕ of a mixture of phosphate buffer (0.1 ㎖) and urokinase solution (0.1 ㎖, 100 U/㎖) was used as a control mixture. The plates were incubated at 31℃ for 20 hr. Then parent rings appeared where the fibrin lysis had occured. Two diameters of such rings were measured and the area was calculated. The inhibitory effect of test samples in this fibrinolytic system was assessed by comparing the lysed area with that of the control. The activity was expressed as an concentration which inhibited the lysis by 50% (IC50: ㎎/㎖)

8. Inhibition of plasmin

Urokinase solution (0.5 ㎖, 100 U/㎖) and plasminogen solution (0.5 ㎖, 0.5 ㎎/㎖) were mixed and incubated at 28℃ for 30 min. To the incubated solution (0.1 ㎖) was added a test solution (0.1 ㎖) of an appropriate concentration. Then 20 ㎕ of the mixture was put into each well in the plasminogen-free fibrin plates. Twenty ㎕ of phosphate buffer was used as control. The plates were incubated at 37℃ for 18 hrs. Two diameters of the lysed area were measured and the area was calculated. The inhibitory effect of samples was assessed by comparison of the lysed area with that of the control. The activity was expressed as the concentration which inhibits plasmin activity by 50% (IC50: ㎎/㎖)

9. Statistical analysis

The statistical significance was established as follows. The ANOVA one-way analysis of variance followed by pairwise comparisons using the Scheff test was used for the multigroup comparisons. The statistical analysis between two groups was evaluated by the F-t test. A probability value of 5% or less was considered indicative of a significant effect. Data are expressed as mean±S.E. The differences were considered significant at $p<0.01$.

Results

1. Effects of HOT on pathological aorta in rabbits

The main areas of atheromatous plaque in the aortic arch at the 4th weeks were 38.21% in normal diet group and 4.0% in descending aorta. in normal diet group. However, HOT groups indicated a decreased aortic arch of 26.54% and increased descending aorta (5.4%) in 4 weeks of administration. In 8 weeks, there were significant differences in the area of atheromatous plaques of the descending aorta or in the histopathological findings of the atherosclerotic lesions between the two groups (Table 2).

Table 2. Percentage of intimal surface area of thoracic aorta involved with atheromatous plaque in KHC rabbits fed with or without HOT

Feeding period	Groups	Aortic arch(%)	Descending aorta(%)
4weeks	Normal	38.21±2.5	4.0±0.5
	HOT	26.54±2.4	5.4±0.6
8weeks	Normal	45.65±3.4	20.3±1.8
	HOT	21.23±2.1*	29.4±3.4

Each tabular value indicates the mean ± S.E. *P<0.05, significantly different from normal diet group.

2. Effects of HOT and its herbs on collagen- or ADP-induced blood platelet aggregation

As shown in Table 3, preincubation of PRP with HOT (0.2, 0.4 or 1.0 ㎎/㎖) produced inhibition of collagen- or ADP-induced blood platelet aggregation to various extent.

Table 3. Effects of the HOT and aspirin on collagen- or ADP-induced blood platelet aggregation

Dose(㎎/㎖)		Inhibitory rate(%)	
		Collagen	ADP
HOT	0.2	10.2±1.3	12.4±1.2
	0.4	22.5±3.2	18.7±1.7
	1.0	28.7±4.3	31.3±3.4
Heparin	2	43.4±4.6	18.4±1.7
	5	48.9±6.5	21.6±2.4
	10	72.4±8.6	24.5±3.5

Each value represents the mean ± S.E. of 5 experiments

On the other hand, preincubation of PRP with 1.0 ㎎/㎖ of each herb inhibited collagen- or ADP-induced blood platelet aggregation as shown in Table 4. Treatment of each 1.0 ㎎/㎖ of *Angelica gigantis Radix, Rehmanniae Radix, Paeoniae Radix, Persicae Semen* and *Ciniamomi Cortex* inhibited collagen- or ADP-induced blood platelet aggregation to various extent.

Table 4. Effects of the HOT's herbs on collagen- or ADP-induced blood platelet aggregation

Drug or Herbs	Dose(㎎/㎖)	Inhibitory rate(%)	
		Collagen	ADP
Aspirin	10	76.6±8.9	24.7±2.3
Angelica gigantis Radix	1	32.2±6.5	29.4±3.2
Rehmanniae Radix	1	30.3±5.3	32.7±2.3
Paeoniae Radix	1	24.4±3.2	26.3±2.5
Persicae Semen	1	23.7±2.1	25.5±2.5
Ciniamomi Cortex	1	27.6±2.7	27.5±5.4
Carthami Flos	1	11.5±2.3	20.3±3.5
Cnidii Rhizoma	1	10.4±1.7	17.8±5.3

Each value represents the mean ± S.E. of 5 experiments

3. Effects of HOT and its herbs on conversion of fibrinogen to fibrin induced by thrombin

As shown in Table 5, the clotting time of the control containing no test solution was 252.6±17.3 sec. The clotting time was prolonged significantly by incubation with 0.5 or 1.0 ㎎/㎖ of HOT.

Table 5. Effects of the HOT and heparin on conversion of fibrinogen to fibrin induced by thrombin

Dose(㎎/㎖)		Clotting time of fibrinogen solution (sec)
Control		252.6±17.3
HOT	0.2	248.9±26.6
	0.5	276.6±32.5**
	1.0	280.6±25.6**
Heparin	(10U/㎖)	312.5±36.8**

** : Significantly different from control, p(0.01, Each value represents the mean ± S.E. of 5 experiments.

On the other hand, the clotting time of the test solutions was significantly increased by incubation of herb as shown in Table 6. Treatment of each 1.0 ㎎/㎖ of *Angelica gigantis Radix, Persicae Semen, Rehmanniae Radix* and *Paeoniae Radix* elongated the clotting time for conversion of fibrinogen to fibrin when induced by thrombin.

Table 6. Effects of the HOT'herbs and heparin on conversion of fibrinogen to fibrin induced by thrombin

Herbs	Dose (㎎/㎖)	Clotting time of fibrinogen solution (sec)
Control		243.6±31.2
Heparin (10 U/㎖)		313.5±14.3**
Angelica gigantis Radix	1	295.6±17.9**
Persicae Semen	1	287.9±17.6**
Rehmanniae Radix	1	286.2±14.5**
Paeoniae Radix	1	280.7±8.7**
Ciniamomi Cortex	1	248.9±21.6
Carthami Flos	1	254.4±15.7
Cnidii Rhizoma	1	258.0±14.7

** : Significantly different from control, p(0.01, Each value represents the mean ± S.E. of 5 experiments.

4. Effects of HOT extracts on inhibition of plasminogen or plasmin

As shown in Table 7, the 50% inhibitory concentrations (IC_{50} ㎎/㎖) of HOT on plasminogen and plasmin were 0.96 and 3.25 ㎎/㎖ respectively.

Table 7. Effects of the HOT and Trasylol on fibrinolytic system in fibrin plate

Dose(㎎/㎖)	IC_{50}(㎎/㎖) for plasminogen	IC_{50}(㎎/㎖) for plasmin
HOT	0.960	3.250
Trasylol	0.014	0.013

Each value represents the mean ± S.E. of 5 experiments.

Discussion

Because of the increase of animality fat intakes, lack of exercise, fatness, the stress, advanced age etc., the occurrence rate of the circulation system disease has been increased[16,17]. And the thrombosis and atherosclerosis importantly came to the front as the risk factor of these circulation system's disease. According to the ancient Chinese medicinal literature 《NaSiHoeYakEuiKyung(羅氏會約醫鏡)》, HOT activate blood circulation, vital energy and regulate menstruation, and is applied for irregular menstruation, dysmennorrhea, amenorrhea and metrorrhagia due to blood stasis, and sudden loss of vision caused by retinal hemorrhage[9]. HOT is additional prescription of 'Decoction Containing Four Drugs with Persicae and Carthami(桃紅四物湯)' from 《Golden Mirror of Medicine(醫宗金鑑)》, and 'Decoction Containing Four Drugs with Persicae and Carthami(桃紅四物湯)' is an alias of Decoction Containing Four Drugs with Addition(加味四物湯)' from 《OkGiMiEui(玉機微義)》[18]. HOT is consisted of *Angelica gigantis Radix, Rehmanniae Radix, Paeoniae Radix, Ciniamomi Cortex, Cnidii Rhizoma, Persicae Semen* and *Carthami Flos*[18]. Now a days, The HOT is applied as an effective biological response modifier for augmenting host homeostasis of body circulation[8]. The pharmacological action of HOT has been limitedly studied in regard to ischemic infarction[8]. This herbal medicine has been shown to express diverse activities such as immunomodulating, anti-infarction, anti-allergic and anti-inflammatory effects (Kim et al., unpublished results). Antisclerotic effects of HOT in experimentally induced atherosclerosis in rabbits have also been reported[19]. However, pharmacological mechanisms of HOT on anti-thrombosis and atherosclerosis formation are poorly understood.

In the previous paper[19], the plasma total cholesterol levels increased up to 2 weeks after the onset of the diet period and reached 1 plateau in the control group. The total cholesterol level in the HOT treated group was similar to that of the control group. However, there were significant differences between the control and HOT groups in triglyceride, phospholipid or lipoprotein levels. Hence, the result of this study is as follow, HOT showed inhibitory effects on the progression of atherosclerosis lesions, without beneficial effects on other chemical parameters of KHC rabbits. The administration dose of HOT was determined as 1g/kg/day according to a preliminary dose-setting study. The mechanism of onset of hypercholesterolemia in KHC rabbits, similar to that of FH rabbits, is attributed to a deficiency of the LDL-receptor[11] and other physiological characters or pharmacological response to fibrate in KHC rabbits have been revealed[4]. The results show that HOT inhibited the progression of atherosclerotic lesions macroscopically, there were no significant differenced in the histopathological findings of the lesion between the control and HOT groups. Further study and examination are desirable for a better understanding of the histopathological effects of HOT. The extract derived from HOT and its herbs were then used for its effect on blood platelet aggregation, thrombin action and fibrinolytic action in vitro. The results show that the extracts of HOT and its herbs of *Angelica gigantis Radix, Persicae Semen,*

Rehmanniae Radix and *Paeoniae Radix* effectively inhibited the platelet aggregation induced by various aggregating agents, such as ADP and collagen and that the extract inhibited conversion of fibrinogen to fibrin. In fibrinolytic system, this extract had inhibitory effect of plasminogen and plasmin action. Further work is in progress on the effect of main components, flavonoids of HOT, on blood coagulative and fibrinolytic system.

Conclusion

The inhibitory effects of the traditional herbal medicine HOT on the progression of the atherosclerotic lesions were studied. The anti-thrombotic properties of HOT were also investigated. In in vitro experiments, the extract was shown to have inhibitory effect on collagen- and ADP-induced blood platelet aggregation, on thrombin-induced conversion of fibrinogen to fibrin and on the activity of plasminogen or plasmin. In conclusion, the protection of extracts of Korean herbs' HOT on the ischemic infarction induced artificially might be involved to their inhibition of thrombotic action.

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