Inhibitory Effects of Hwao-tang on the Atherosclerosis and the Venous Thrombosis

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The inhibitory effects of the traditional herbal medicine Hwao-tang on the progression of the atherosclerotic lesions were studied using the spontaneous familial hypercholesterolemia (FH) model, Kurosawa and Kusanagi-hypercholesterolemic (KHC) rabbits. Hwao-tang is activate blood circulation, vital energy and regulate menstruation, etc. Now a days, Hwao-tang is mainly used for the treatment of inflammation, hyperlipemia and arteriosclerosis. However, pharmacological mechanisms of Hwao-tang on lipid metabolism and atherosclerosis formation are poorly understood. We have investigated the pharmacological effects of Hwao-tang on hypercholesterolemia and atherosclerosis using a spontaneous experimental model. In conclusion, the protection of extracts of HOT and its herbs on the ischemic infarction induced artificially might be related to their inhibitory effects on DIC, platelet coagulation and thrombic action. These suggest that Hwao-tang has inhibitory effects on the development of atheromatous plaque formation in spontaneous FH model rabbits. It is possible that the antioxidative effects of Hwao-tang on LDL led to the beneficial effects observed in this study.

Key words : Hwao-tang(HOT), atherosclerosis, hypercholesterolemia, DIC, platelet aggregation, fibrinogen, fibrin KHC rabbits, total cholesterol, triglyceride, phospholipid, LDL. HDL, HPLC.

Introduction

Hypercholesterolemia is considered to be a major cause of the lesions associated with atherosclerosis[1], and a number of hypercholesterolemic drugs are used to improve the plasma lipid level of patients. The traditinal korean therapeutic system has been used for the treatment of various disease for hundreds of years, including the clinical treatment of hypercholesterolimia, diabetes and obesity[2], Hwao-tang(HOT) has been reported to have a hypolipidemic effect in patients with hypercholesterolemia[3], and in cholesterol-induced experimental models. HOT is consisted of Angelicae 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[4]. 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[3]. 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 Hwao-tang in experimentally induced atherosclerosis in rabbits have also been reported (Park et al., not shown). However, pharmacological mechanisms of Hwao-tang on lipid metabolism and atherosclerosis formation are poorly understood. Although the effectiveness of HOT for ischemic infarction and inflammatory lung diseases has been widely demonstrated by clinical administration, the scientific and acting mechanisms for those are not understood and elucidated. It is generally known that inflammation, hyperlipemia and arteriosclerosis induce disseminated intravascular coagulation (DIC). Therefore, anti-inflammatory activity may be assessed by the effect on DIC.

The present paper reports the effect of extracts obtained from HOT on endotoxin-induced experimental DIC in heperlipemia and normal rats. We have also investigated the pharmacological effects of Hwao-tang on hypercholesterolemia and atherosclerosis using a spontaneous experimental model, Kurosawa and Kusanagi-hypercholesterolemic (KHC) rabbits[5]

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in relation to blood chemistry, pathology and the oxidation of low-density lipoproteins (LDL).

Materials and Methods

1. Animals

Male KHC rabbits weighing 1.5-2.0kg 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: 15-20times/h, 12hrs light and 12hrs dark) and subjected to the experiment after 7days quarantine period. For DIC animal, male Wistar-King strain rats weighing 150-200g were used. They were fed on a standard diet for at least 7 days. They were fasted for 24hrs before the start of the experiments.

2. Drugs

Hwao-tang[4] (化瘀湯, HOT): 當歸(*Angelicae 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 HOT prescription (Table 1).

Table 1. Composition of Hwao-tang

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 53.2g of HOT was added to 500 ml of water and boiled for 2hrs, filtered and then concentrated to 200 ml. 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. Analysis of Hwao-tang by HPLC

Hwao-tang (0.5g) was extracted with 20 ml of methanol under ultrasonication for 30 min followed by centrifugation. The analysis of the supernatant solution by high-performance liquid chromatography (HPLC) equipped with LC-10 AD pumps, an SPD-M10Avp photodiode-array detector, and a CTO-10A column oven (Shimadzu, Kyoto, Japan) was performed using a TSK Gel ODS-80Ts column (250×4.6 mm, Tosoh Co., Tokyo, Japan). The solvents were (A) 0.05 M $AcONH_4$-AcOH buffer (pH 3.6), (B) 100% CH_3CH. A linear gradient of 90% A and 10% B changing over 60 min to 0% A and 100% B was applied (And 0% A and 100% B was continued for 30 min). The flow rate and the column temperature were 1.0 ml/mmin and 40, respectively. The UV data of the effluent from the column ranging from 200 to 450 nm were collected, and the peak analysis and assignment were performed using the system analysis software, CLASS-LC10 (Shimadzu, Kyoto, Japan).

4. Experimental design for plasma total cholesterol, triglyceride and phospholipid levels in KHC rabbits

Rabbits were carefully divided into four groups (two control groups and two experimental groups) of five to eight rabbits each to avoid group differences in plasma total cholesterol, triglyceride and phospholipid levels. The control groups were maintained with a commercial diet (MR-stock, Japan Agriculture Industry Co., Yokohama, Japan). Treated groups were fed MR-stock containing 1g/kg/day of Hwao-tang. After a 4-week period of diet administration, one of the control groups and one of the Hwao-tang-treated groups were euthanized by pentobarbital sodium for the determination of the effects of the drug. After a 8-weeks period of diet administration, the same examinations were performed on the other two groups.

5. Assay of plasma lipids

Blood samples were collected from each rabbits's auricular artery with EDTA-2NA, just before the onset of the diet administration period and every other week after commencement, following an overnight fast. Plasma was separated from whole blood by centrifugation at 3000×g for 15 min at 4℃. Fresh plasma samples were cryopreserved at -20℃ and used for the analysis of plasma lipids. The plasma total cholesterol, triglyceride, phospholipid and HDL-cholesterol levels were measured using an autoanalyzer (Hitachi 7070, Hitachi Co., Tokyo, Japan), and the plasma LDL-cholesterol, VLDL-cholesterol and chylomicron (CM) levels were measured using test kits (Eiken Co., Tokyo, Japan).

6. 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[6]. 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.

7. Evaluation of antioxidative effects on LDL

Plasma LDL was prepared by ultracentrifugation(d 1.006-1.063g/ml), and the oxidation of LDL was investigated by measuring the conjugated dienes formed with 2,2'-azobis (4-methoxy-2, 4-dimethylv aleronitrile; V-70), (Kondo et al., 1994)[7].

8. Anti-endotoxin-induced DIC in high cholesterol diet-treated rats

Endotoxin-induced DIC model was prepared by the method of Kubo[8]. Rats were fed on a high cholesterol diet [salt-free buffer (30%), cholesterol (15%), bile powder (2%) in laboratory chow] for 15 weeks. Extract (200 or 500 mg/kg, daily) was orally administered to those rats for 7 days before the intravenous injection of endotoxin (0.1 mg/kg). The animals were killed by decapitation 4 hrs after the injection of endotoxin, and liver was quickly removed and subjected to microscope examination for hepatic infarct. Results of histological observation were rated as follows: 3, severe hepatic infarct; 2, moderate; 1, slight; 0, non-detectable.

9. Anti-endotoxin-induced DIC effect in normal rats

Experimental DIC was induced by a modification of the method of Kubo[8]. Extracts (400 or 800 mg/kg) was administered orally to healthy rats 1 hr before the injection of endotoxin (0.1 mg/kg) into the tail vein. Four hrs after the injection od endotoxin, the rats were abesthetized with pentobarbital. And blood samples were taken from the heart with a plastic syringe. As an anticoagulant, 10 mM 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.

The number of platelets was countered with an automatic blood counter (Coulter Counter, model S-Plus, Coulter Co., USA). Fibrinogen count was determined according to the method of Nishio[9]. The prothrombin time was measured with a COAG-A-Mate dual-channel device (General Diagnostic, Warner-Lambert Co., USA). Content of fibrin degradation product (FDP) was determined by means of the latex aggregation test (FDPL test U, Teikoku Zoki, Japan).

10. Blood platelet aggregation test

Whole blood samples were collected from heart of pentobarbital-anesthetized rats. Nine ml of the blood and 1 ml of heparin solution (10 U/ml) 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[10].

Platelet aggregation test described by Ekimoto et al[11] was modified and performed with collagen (500 μg/ml) and ADP (0.05 μM) used as aggregation agents. A 0.2 ml 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 $\mu\ell$ 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.

11. Thrombin-induced conversion of fibrinogen to fibrin

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

12. Fibrin plates

Fibrin plates were prepared by the method of Astrup and Mullertz[12]. 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 mg of plasminogen-fibrinogen and 200 mg of plasminogen free-fibrinogen in 100 ml of agarose solution at 31℃. A 10 ml aliquot of the mixture and 0.1 ml of thrombin (10 U/ml) 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.

13. Inhibition of plasminogen

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

to each of the wells in the plasminogen-containing fibrin plate. Twenty ul of a mixture of phosphate buffer (0.1 ml) and urokinase solution (0.1 ml, 100 U/ml) was used as a control mixture. The plates were incubated at 31℃ for 20 hrs. 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% (IC_{50}: mg/ml)

14. Inhibition of plasmin

Urokinase solution (0.5 ml, 100 U/ml) and plasminogen solution (0.5 ml, 0.5 mg/ml) were mixed and incubated at 28℃ for 30 min. To the incubated solution (0.1 ml) was added a tst solution (0.1 ml) of an appropriate concentration. Then 20 $\mu\ell$ of the mixture was put into each well in the plasminogen-free fibrin plates. 20 $\mu\ell$ 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% (IC_{50}: mg/ml)

15. 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. Also, For plasma total cholesterol, triglyceride and phospholipid levels in KHC rabbits, the statistical analysis of the biochemical and morphometric data was performed using the Mann-Whitney U test. Data are expressed as mean±S.E. The differences were considered significant at $p<0.01$.

Results

1. Plasma lipids of control and Hwao-tang-treated rabbits

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 <Table 1>. The total cholesterol level in the Hwao-tang treated group was similar to that of the control group. However, there were significant differences between the control and Hwao-tang groups in triglyceride, phospholipid or lipoprotein levels ($P<0.05$) <Table 1-5 and Fig. 1-4>.

Table 1. Effect of Hwao-tang on serum total cholesterol, triglyceride and phospholipid concentrations in hypercholesterolemic rabbits

group \ mg/ml	total cholesterol	triglyceride	phospholipid
Normal diet	4059.5±10.7	353.2±43.4	172.3±16.7
Hwao-tang	3585.4±13.5	222.4±18.7*	151.4±11.4

*P(0.05, significantly different from normal diet group. Data represent the mean±S.E.

Table 2. Effect of Hwao-tang on the chylomicron levels as plasma lipoprotein parameters in KHC rabbits

group \ weeks (mg/ml)	0	1	2	3	4	5	6	7	8	9	10	11	12
Normal diet	154	121	89	73	53	50	43	41	38	35	35	30	29
Hwao-tang	153	72*	52*	32*	27*	23*	25	26	28	26	25	22	25

*P(0.05, significantly different from normal diet group.

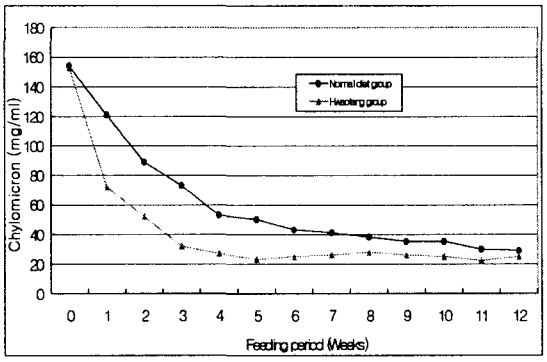

Fig. 1. Effect of Hwao-tang on the chylomicron levels as plasma lipoprotein parameters in KHC rabbits

Table 3. Effect of Hwao-tang on the LDL-cholesterol levels as plasma lipoprotein parameters in KHC rabbits

group \ weeks (mg/ml)	0	1	2	3	4	6	7	8	9	10
Normal diet	2045	4643	4043	3587	3265	3152	3184	3234	3132	3032
Hwao-tang	2041	3465	3576	3043	2834	2764	2554*	2423*	2212*	2221*

*P(0.05, significantly different from normal diet group.

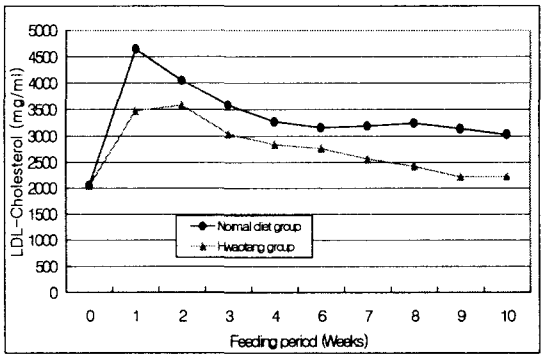

Fig. 2. Effect of Hwao-tang on the LDL-cholesterol levels as plasma lipoprotein parameters in KHC rabbits

Table 4. Effect of Hwao-tang on the VLDL-cholesterol levels as a plasma lipoprotein parameter in KHC rabbits

group \ weeks (mg/ml)	0	1	2	3	4	6	7	8	9	10
Normal diet	984	898	778	723	703	698	676	676	665	604
Hwao-tang	986	743	665	601	556*	543*	535*	530*	467*	463*

*P(0.05, significantly different from normal diet group.

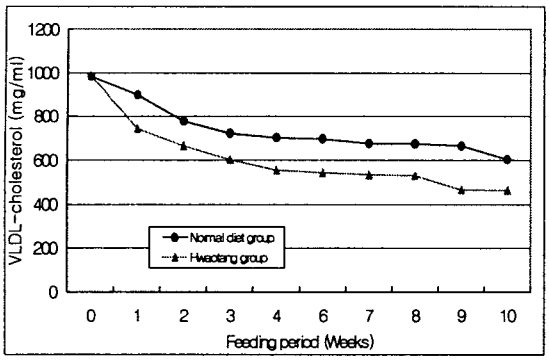

Fig. 3. Effect of Hwao-tang on the VLDL-cholesterol levels as a plasma lipoprotein parameter in KHC rabbits

Table 5. Effect of Hwao-tang on the HDL-cholesterol levels as a plasma lipoprotein parameter in KHC rabbits

group (mg/ml) \ weeks	0	1	2	3	4	6	7	8	9	10
Normal diet	7.5	5.9	4.9	3.9	3.4	3.5	4.2	4.3	3.9	4.4
Hwao-tang	7.7	7.4	7.1*	6.7*	6.5*	6.0*	5.9	5.5	4.7	4.8

*P<0.05, significantly different from normal diet group.

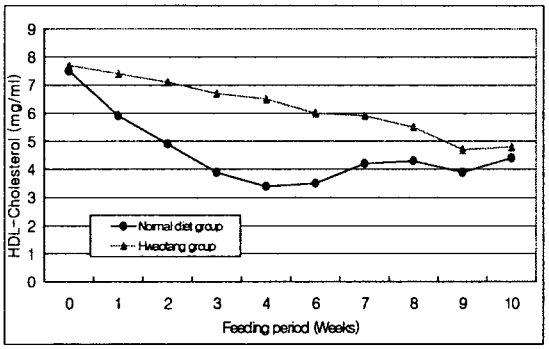

Fig. 4. Effect of Hwao-tang on the HDL-cholesterol levels as a plasma lipoprotein parameter in KHC rabbits

2. Pathological finding of aorta

The main areas of atheromatous plaque in the aortic arch at the 4th week were 38.21% in normal diet group and 4.0% in descending aorta in normal diet group. However, Hwao-tang 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 ($P<0.05$) in the area of atheromatous plaques of the descending aorta or in the histopathological findings of the atherosclerotic lesions between the two groups <Table 6>.

Table 6. Percentage of intimal surface area of thoracic aorta involved with atheromatous plaque in KHC rabbits fed with or without Hwao-tang

Feeding period	Group	Aortic arch (%)	Descending aorta (%)
4 weeeks	Normal diet	38.21±2.5	4.0±0.5
	Hwao-tang	26.54±2.4	5.4±0.6
8 weeks	Normal diet	45.65±4.3	20.3±1.8
	Hwao-tang	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.

3. Antioxidative effects of Hwao-tang on LDL

A significant prolongation of the lag time was found in the 8th and 10th week of the experiment, indicating that Hwao-tang had antioxidative effects on KHC rabbits <Table 7, Fig. 5>.

Table 7. Antioxidant status of LDL of KHC rabbits fed with or without Hwao-tang

group (mg/ml) \ weeks	4	6	8	10	12	14
Normal diet	28.2±4.2	24.3±2.3	23.2±4.2	20.5±2.4	17.4±2.1	13.6±1.0
Hwao-tang	23.4±4.3	26.4±2.4	36.3±5.3**	35.2±6.2**	28.2±3.4*	28.1±3.2*

Antioxidant status of LDL was expressed as lag phase(min) for LDL oxidation. Each tabular value indicates the mean ± S.E.. *P<0.05, **P<0.01, significantly different from normal diet group.

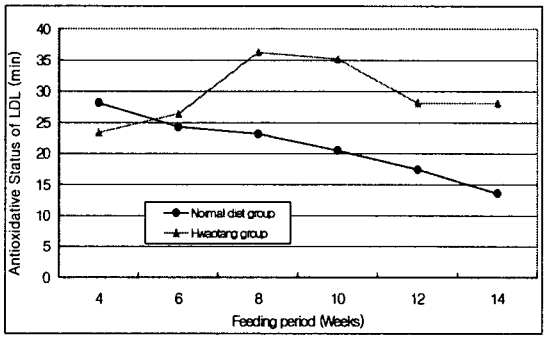

Fig. 5. Antioxidant status of LDL of KHC rabbits fed with or without Hwao-tang

4. Effects of HOT and its 7 herbs on endotoxin-induced DIC in high cholesterol-treated rats (in vivo)

In high cholesterol-fed rats, intravenous injection of endotoxin induced hepatic infarct with partial or straggling thrombus and hemorrhage. <Table 8> showed that oral administration of the HOT (100, 200, 400 mg/100 g) significantly inhibited the formation of hepatic infarct in high cholesterol diet and endotoxin-treated rats.

Table 8. Effects of HOT on the Endotoxin-induced DIC in high cholesterol diet-treated rats

Dose (mg/100g)	Number of animals	Hepatic infarct scorea)				
		4	3	2	1	0
Control (0)	6[b]	3	2	1	0	0
100	6	2	1	2	1	0
200	6	1	0	1	2	1
400	6	0	1	1	2	2

a) Hepatic infarct score : 4, severe hepatic infarct; 3, moderate hepatic infarct; 2, low hepatic infarct; 1, slight hepatic infarct; 0, non-detectable. b) Number of rats

Protective effects of each herb extract against endotoxin-induced DIC in high cholesterol diet-treated rats are also shown in <Table 9>. 400mg/100g(body weight) of each medicine reduced hepatic infarct score. Treatments of *Angelicae gigantis Radix, Rehmanniae Radix, Paeoniae Radix* and *Persicae Semen* showed significant protection of DIC in five different applications. Ciniamomi Cortex showed a moderate protection of DIC. However, *Carthami Flos* and *Cnidii Rhizoma* were not effective for the protection on DIC <Table 9>.

Table 9. Effects of each herb extract on the endotoxin-induced DIC in high cholesterol diet-treated rats

Dose (400 mg/100g)	Number of animals	Hepatic infarct scorea)				
		4	3	2	1	0
Control (0)	5[b)]	3	1	1	0	0
Angelicae gigantis Radix	6	1	0	1	2	2
Rehmanniae Radix	6	0	1	2	2	1
Paeoniae Radix	6	1	1	1	1	2
Ciniamomi Cortex	7	1	2	3	1	0
Persicae Semen	6	1	0	1	2	2
Carthami Flos	6	2	1	2	1	0
Cnidii Rhizoma	7	2	2	2	1	0

a) Hepatic infarct score: 4, severe hepatic infarct; 3, moderate hepatic infarct; 2, low hepatic infarct; 1, slight hepatic infarct; 0, non-detectable. b) Number of rats

5. Effects of HOT on endotoxin-induced DIC in normal rats (in vivo)

The injection of endotoxin (0.1 mg/500g) into the tail vein may induce DIC accompanied by a decrease in the number of blood platelets and fibrinogen, prologation of prothrombin time and an increase in FDP. Oral administration of 200 or 400 mg/100g of HOT or 20 mg/100g of aspirin before the injection of endotoxin prevented endotoxin-induced DIC as shown in Table 10-13. The platelet count was 73.4±9.6 x 10^4/mm3 in normal rats only with saline and 19.3±2.6 x 10^4/mm3 in rats injected with 0.1 mg/500 g of endotoxin control rats. Oral administration of 200 or 400 mg/100 g of HOT counteracts the endotoxin-induced decrease in the platelet count significantly <Table 10>.

Table 10. Effects of the HOT and asprin on the blood platelets of endotoxin-induced DIC in normal rats

Dose (mg/100g)	Number of rats	Blood platelet counts (×10^4/㎣)
Normal	5	73.4±9.6
Control (0)	7	19.3±2.6
100	6	30.2±3.2
200	6	36.6±3.5a)
400	8	53.5±6.4b)
Aspirin 5	6	51.3±3.0b)
10	6	54.3±8.0b)
20	6	56.5±4.0b)

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

The level of fibrinogen was 225±23.3 mg/ml in normal rats treated only with saline and 91.2±9.8 mg/ml in DIC rats control. Oral administration of 200 mg/ml and 400 mg/100 g of HOT countereacted the endotoxin-induced decrease in fibrin level significantly <Table 11>.

Table 11. Effects of the HOT and aspirin on the fibrinogen of endotoxin-induced DIC in normal rats

Dose (mg/100g)	Number of rats	Fibrinogen(mg/ml)
Normal	5	225.0±23.3
Control (0)	7	91.2±9.8
100	7	115.0±15.3
200	6	142.0±15.7[a)]
400	7	163.7±20.4[b)]
Aspirin 5	6	174.0±15.6[b)]
10	6	182.0±14.6[b)]
20	7	185.0±15.6[b)]

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

The prothrombin time was 11.2±0.55 sec in normal rats and 17.2±2.12 sec in DIC rats. In rats treated with oral administration of 200 mg/100 g or 400 mg/100 g of HOT, the prothrombin time was found to be shorter than that of control group <Table 12>.

Table 12. Effects of the HOT and aspirin on the prothrombin of endotoxin-induced DIC in normal rat

Dose (mg/100g)	Number of rats	Prothrombin time (sec)
Normal	5	11.2±0.55
Control (0)	6	17.2±2.12
100	6	16.4±1.43
200	6	15.2±1.22[a)]
400	7	13.8±1.68[b)]
Aspirin 5	5	14.6±1.72[a)]
10	5	14.7±1.74[a)]
20	5	15.5±1.63[a)]

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

The FDP level was 0.3±0.02 μg/ml in normal rats and 5.4±0.4 μ g/ml in DIC rats control. Administration to 200 mg/100 g and 400 mg/100 g of HOT for 1 hr before the injection of endotoxin counteracted the endotoxin-induced elevation of FDP level significantly. A marked preventive effect of aspirin, which was used as a standard drug, was observed on endotoxin-induced blood platelets decrease and fibrinogen decrease, however, little effect on endotoxin-induced prothrombin time increase or FDP increase <Table 13>.

Table 13. Effects of the HOT and aspirin on the FDP of endotoxin-induced DIC in normal rats

Dose (mg/100g)	Number of rats	FDP (㎍/ml)
Normal	5	0.3±0.02
Control (0)	6	5.4±0.4
100	7	2.3±0.2[a)]
200	6	3.5±0.4[a)]
400	8	3.4±0.2[a)]
Aspirin 5	5	3.2±0.2[a)]
10	6	4.4±0.3[b)]
20	7	4.8±0.4[b)]

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

6. Effects of the HOT's 7 herbs on the blood platelets of drug-induced DIC in normal rats

Oral administration of 400 mg/100 g of each herb before the injection of endotoxin prevented endotoxin-induced DIC as shown in Table 14-15. Oral administrations of 400 mg/100 g of *Angelicae gigantis Radix, Rehmanniae Radix, Paeoniae Radix,* and *Persicae Semen* counteracted the endotoxin-induced decrease in the platelet count significantly <Table 14>.

Oral administrations of 400 mg/100 g of *Angelicae gigantis Radix, Rehmanniae Radix, Paeoniae Radix,* and *Persicae Semen* countereacted the endotoxin-induced decrease in fibrin level significantly. *Ciniamomi Cortex* showed a moderate activity. However, *Carthami Flos* and *Cnidii Rhizoma* were not effective <Table 15>.

Table 14. Effects of the HOT's 7 herbs on the blood platelets of Endotoxin-induced DIC in normal rats

Dose (400 mg/100g)	Number of rats	Blood platelet counts ($\times 10^4$/㎖)
Normal	5	70.3±7.4
Control (0)	7	17.6±2.6
Angelicae gigantis Radix	6	47.7±6.5[b)]
Rehmanniae Radix	6	49.6±7.1[b)]
Paeoniae Radix	6	47.2±6.3[b)]
Ciniamomi Cortex	7	36.7±3.7[a)]
Persicae Semen	6	45.7±5.3[b)]
Carthami Flos	6	24.3±3.6
Cnidii Rhizoma	7	28.7±3.5

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

Table 15. Effects of the HOT's 7 herbs on the fibrinogen of Endotoxin-induced DIC in normal rats

Dose (400 mg/100g)	Number of rats	Fibrinogen (mg/ml)
Normal	5	226.0±25.3
Control (0)	7	90.1±9.7
Angelicae gigantis Radix	6	163.3±12.6[b)]
Rehmanniae Radix	6	157.3±9.5[b)]
Paeoniae Radix	6	160.7±16.4[b)]
Ciniamomi Cortex	7	134.3±12.3[a)]
Persicae Semen	6	112.4±13.3
Carthami Flos	6	126.1±13.5
Cnidii Rhizoma	7	113.2±12.2

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

Discussion

The cause that the increase of animality fat intakes, under exercise, fatness, adding the stress, advanced age etc., the occurrence rate of the circulation system disease has been increased[13,14]. And the thrombosis and atherosclerosis importantly came to the front as the risk factor of these circulation system's disease. Also the thrombosis came from the platelet aggregation[15]. Platelet do not attache to the normal hemangioendothelial cell. But when it stimulated by endothelium peronia and so on, it attache to the injury endothelium or rise aggregation between the platelet. And the activation of abnormal platelet occur the platelet grume and thrombogenesis. So it bring up the occlusive angiosis, so to speak, cardiovascular disease, cerebrovascular disease, arterial sclerosis[16,17,18]. In oriental 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, black stool, dark purple tongue or with ecchymoses and petechiae etc. have been created. And it becomes the pathopoiesis cause that the convulsion and palpitation, severe palpitatiion, tympanites, the symtom complex with a mass or swelling in the abdomen, insanity, stricken by wind etc. 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 atherosclerosis[19,20,21]. 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[4]. HOT is additional prescripton 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)》. HOT is consisted of *Angelicae gigantis Radix, Rehmanniae Radix, Paeoniae Radix, Ciniamomi Cortex, Cnidii Rhizoma, Persicae Semen* and *Carthami Flos*. Now a days, The HOT is applied as an effective biological response modifier for augmenting host homeostasis of body circulation[3]. The pharmacological action of HOT has been limitedly studied in regard to ischemic infarction[3]. 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 Hwao-tang in experimentally induced atherosclerosis in rabbits have also been reported (Park et al., not shown). However, pharmacological mechanisms of Hwao-tang on lipid metabolism and atherosclerosis formation are poorly understood. Hence, the result of this studies is as follow, Hwao-tang showed inhibitory effects on the progression of atherosclerosis lesions, without beneficial effects on the total cholesterol and with significant beneficial effects on the triglyceride, phospholipid or other chemical parameters of KHC rabbits. We also investigated hepatic lipids (total cholesterol, triglyceride and phospholipid) (data not shown). The administration dose of Hwao-tang 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[5] and other physiological characters or pharmacological response to fibrate in KHC rabbits have been revealed[22]. Our results show that Hwao-tang does improve hypercholesterolemia in KHC rabbits, a severe spontaneous FH model. Although Hwao-tang inhibited the progression of atherosclerotic lesions macroscopically, there were no significant difference in the histopathological findings of the lesion between the control and Hwao-tang groups. Further study and examination are desirable for a better understanding

of the histopathological effects of Hwao-tang. Antioxidative effects of Hwao-tang were found only at 4 and 8 weeks of the diet administration period. The reasons for this discontinuous significance are unclear. However, Persicae Semen and Carthami Flos, which are one of constitutional herbal drugs of Hwao-tang , was reported to have inhibitory effects on lipid peroxide production. Although the antioxidative effects of Hwao-tang do not fully explain our results, we suspect that these effects have important implications for the inhibition of the progression of atherosclerotic lesions. It was reported a relationship between the oxidative modification of LDL and foam cells[23], and since then, it has been widely accepted that oxidized LDL plays a key role in the formation of monocyte-macrophage foam cells, a major cell present in fatty streaks and atherosclerosis fibrous plaques[24]. Several epidemiological studies on oxidized LDL and atherosclerosis in humans have been reported[25], and the clinical application of antioxidants is thought to inhibit the progression of atherosclerosis[26]. In our results, antioxidative effects were observed. Antiatherogenic agents with antioxidative effects might not be able to lower plasma LDL levels in severe FH model animals. However, these results also present the possibility that combination theraphy with other drugs or methods that are known to have marked lowering effects on plasma cholesterol levels might be more effective in the pevention of atherosclerosis formation. The present pharmacological effects of Hwao-tang on lipid metabolism and atherosclerosis formation include an inhibitory effect on the absorption of dietary cholesterol, a lowering effect on the lipid biosynthesis in animals(not shown). Also, our results revealed that Hwao-tang had inhibitory effects on the development of atheromatous plaque formation in the aortic arch of FH model rabbits and raised the possibility that antioxidative effects on LDL participated in the beneficial effect. HOT has been considered as an effective agent for the treatment of inflammation, hyperlipemia and atherosclerosis. A syndrome referred to as DIC (closely related to atherosclerosis and hyperlipemia) is an hemorrhagic disorder apparently caused by simultaneous activation of blood coagulation, fibrinolysis and kinin generation, accompanied by consequent fibrin deposition in the microcirculation. The pathophysiological aspects of DIC produced by infusion of endotoxin in animals have been fully described[27]. In the present studies, extracts of HOT and its 7 herbs was tested for its effect on experimental DIC, which is considered to be closely related to thrombosis. The extract of HOT and its herbs of *Angelicae gigantis Radix, Persicae Semen, Rehmanniae Radix* and *Paeoniae Radix* prevented the endotoxin-induced hepatic venous thrombosis in high cholesterol diet-treated rats. A significant preventive effect on experimental DIC was noted in three parameters (excepting for prothrombin time) in rats when they were treated orally with 100 mg- 500 mg /100 g of the extract. Thus, HOT may be useful for the prevention of DIC in man. However, there is no evidence as to whether HOT is effective or not when used after the onset of DIC.

Conclusion

The inhibitory effects of the traditional herbal medicine Hwao-tang on the progression of the atherosclerotic lesions were studied using the spontaneous familial hypercholesterolemia (FH) model, Kurosawa and Kusanagi-hypercholesterolemic (KHC) rabbits. Changes in blood chemistry, pathology and low-density lipoprotein (LDL) oxidation were measured in a control group and a Hwao-tang group. In the control group, the area of atheromatous plaques of the aorta progressed between 4 (36.65%) and 8 (46.22%). This progressin of atherosclerotic lesions did not happen in the Hwao-tang-treated group between 4 (24.24%) and 8 (22.34%). Antioxidative effects on LDL were seen in the Hwao-tang in weeks 12 and 14. Hwao-tang improved the hypercholestrolemia in the KHC rabbits. These results suggest that Hwao-tang has inhibitory effects on the development of atheromatous plaque formation in spontaneous FH model rabbits. It is possible that the antioxidative effects of Hwao-tang on LDL led to the beneficial effects observed in this study. On the other hand, the anti-thrombic properties of HOT were investigated. The extracts of HOT and its herbs, except *Cnidii Rhizoma* and *Carthami Flos*, inhibited the endotoxin-induced hepatic venous thrombosis in high cholesterol diet-treated rats. However, *Ciniamomi Cortex* showed a very weak inhibitory effect on the endotoxin-induced hepatic venous thrombosis. Also the extract inhibited the endotoxin-induced decrease in blood platelets and fibrinogen, and endotoxin-induced increase in fibrin degradation products (FDP) on disseminated intravascular coagulation in normal rats. In conclusion, the protection of extracts of HOT and its herbs on the ischemic infarction induced artificially might be related to their inhibitory effects on DIC, platelet coagulation and thrombic action.

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