Study on Anti-thrombotic Activity, Superoxide Generation in Human Neutrophils and Platelet Aggregation in Human Blood of *Hwao-tang*

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The present paper reports the effects of Hwaotang on atherosclerosis using a spontaneous experimental model, We have also investigated the pharmacological effect of Hwaotang on collagen- and ADP-induced blood platelet aggregation, thrombin-induced conversion of fibrinogen and fibrinolysis in in vitro experiments, and various effects on stimuli-induced superoxide generation in human neutrophils. Hwao-tang 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. Hwao-tang also significantly inhibited fMLP-induced superoxide generation in a concentration-dependent manner, but not that induced by arachidonic acid. Hwao-tang inhibited neutrophil functions, including degranulation, superoxide generation, and leukotriene B4 production, without any effect on 5-lipoxygenase activity. In conclusion, the protection of extracts of Hwao-tang on the ischemic infarction induced artificially might be involved to their inhibition of thrombotic action. The results also indicate that Hwao-tang exerts the effects on superoxide generation related to the inhibition of neutrophil functions.

Key words : Hwao-tang(huàyūtāng, 化瘀湯), thrombosis, activating blood circulation and eliminating blood stasis, aggregation, atherosclerosis, superoxide generation, neutrophils

Introduction

The drugs for invigorating blood circulation and eliminating blood stasis are used for all kinds of syndrome through the blood stasis and thrombosis for hundreds of years in the traditional korean therapeutic system[1,2,3,4].

Hwaotang belongs to the category of the drugs for invigorating blood circulation and eliminating blood stasis in the traditional korean medicine[5]. According to the ancient chinese medicinal literature 《NaShiYakEuiKyng(羅氏會約醫鏡)》, Hwaotang has been known to exhibit activating blood circulation, removing blood stasis and abdominal pain, and has been widely used in Chinese and Korean traditional medicine[5,6]. Hwaotang is consisted of Angelica gigantis Radix, Rehmanniae Radix, Paeoniae Radix, Ciniamomi Cortex, Cnidii Rhizoma, Persicae Semen and Carthami Flos (Scheme 1)[5].

Hwaotang has been reported to have a hypolipidemic effect in patients with hypercholesterolemia[7], and in highcholesterol-induced experimental models.

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Now a days, Hwaotang is mainly used for the treatment of inflammation, hyperlipemia and arteriosclerosis. The pharmacological action of Hwaotang on platelet aggregation has been limitedly studied in regard to ischemic infarction[8,9]. 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 Hwaotang in experimentally induced atherosclerosis in rabbits have been reported[8]. Inhibitory effects of Hwaotang on the atherosclerosis and venous thrombosis have also been reported[10]. However, pharmacological mechanisms of Hwaotang on atherosclerosis formation and anti-thrombosis are poorly understood.

Superoxide generation in human neutrophils is stimulated during phagocytosis and by treatment of cells with various stimuli, such as certain chemoattractants and activators of protein kinase[11,12,13]. However, resting neutrophils in the blood circulation are poorly responsive to agonize. Neutrophil function can be primed by a variety of pro-inflammatory stimuli[14,15,16]. When the cells are exposed to priming agents, such functions as respiratory burst, phagocytosis and degranulation are greatly enhanced. However, the mechanism of such priming has not been clearly defined. Little is known of the biological activity of Hwaotang and previous studies

have focused mainly on their anti-thrombosis[10].

The present paper reports the effects of Hwaotang on atherosclerosis using a spontaneous experimental model, Kurosawa and Kusanagi- hypercholesterolemic (KHC) rabbits[17]. We have also investigated the pharmacological effect of extracts obtained from Hwaotang on collagen- and ADP-induced blood platelet aggregation, thrombin-induced conversion of fibrinogen and fibrinolysis in in vitro experiments, and various effects on stimuli-induced superoxide generation in human neutrophils.

Materials and Methods

1. Drugs

Hwaotang : *Angelica gigantis Radix, Rehmanniae Radix, Paeoniae Radix, Ciniamomi Cortex, Cnidii Rhizoma, Persicae Semen and Carthami Flos* were used as Hwaotang prescription (Scheme 1).

Scheme 1. Composition of Hwaotang (化瘀湯)[5,6,7]

Angelicae gigantis Radix (當歸)	16.0 g
Rehmanniae Radix (熟地黃)	10.0 g
Paeoniae Radix (白芍藥酒炒)	8.0 g
Ciniamomi Cortex (肉桂)	8.0 g
Cnidii Rhizoma (川芎)	4.0 g
Persicae Semen (桃仁)	4.0 g
Carthami Flos (紅花酒炒)	3.2 g
Total amount	53.2 g

Hwaotang is a dried decoctum of a mixture of 7 herbal drugs. A total of 53.2 g of Hwaotang was added to 500 ml of water and boiled for 2 hrs, filtered and then concentrated to 200 ml. 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 Hwaotang 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).

2. Animals

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.

3. Chemicals

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).

NADPH, ferricytochrome c (cyt. c), superoxide dismutase, N-formyl-methionyl-leucyl-phenylalanine (fMLP), arachidonic acid (AA) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO, USA). Genistein was from Wako Pure Chemicals (Osaka, Japan). The APTT assay kit was purchased from Dade International (Aguade, PR, USA). All other reagents used were of analytical grade and were purchased from Nacalai Tesque (Osaka, Japan) unless otherwise stated.

4. 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[18]. 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.

5. 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[19].

Platelet aggregation test described by Ekimoto et al.[20] 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.

6. 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.

7. Fibrin plates

Fibrin plates were prepared by the method of Astrup and Mullertz[21]. 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.

8. 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% (IC_{50}: ㎎/㎖)

9. 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% (IC_{50}: ㎎/㎖)

10. Isolation of neutrophils

Human peripheral blood polymorphonuclear leukocytes (HPPMNs) were isolated from the peripheral blood of healthy humans by Ficoll-Hypaque (Flow Laboratories) density gradient centrifugation[22] and were washed twice with Krebs-Ringer-phosphate solution, pH 7.4 (KRP)[23]. The cells were counted and resuspended in KRP at a concentration of 1×10^8 cells/ml.

11. Assay of superoxide generation

Superoxide generation was assayed by measuring the reduction of cyt. c at 37℃ using a dual-beam spectrophotometer (Shimadzu UV-3000; Shimadzu, Kyoto, Japan) under constant stirring conditions[24]. The standard assay mixture consisted of 1×10^6 cells/ml, 1 mmol/l $CaCl_2$, 20 mmol/l cyt. c, 10 mmol/l glucose, 0-50 mmol/l steroidal saponin and a stimulus (12.5 nmol/l fMLP, 1 nmol/l PMA or 10 mmol/l AA) in a final volume of 2 ml of KRP. After preincubation for 3 min with a steroidal saponin, the reaction was started by adding a stimulus and the absorbance change at 550-540 nm (A550-540) was monitored for 5 min.

12. Detection of tyrosyl phosphorylation of neutrophil proteins

Neutrophils (1×10^6 cells/ml) were incubated in 1 ml of KRP containing 1 mmol/l $CaCl_2$, 10 mmol/l glucose and 0-100 mmol/l of timosaponins E1 and E2 for 3 min at 37℃, and then 0.5 ml of ice-cold 45% trichloroacetic acid (final concentration, 15%) containing 1 mmol/l sodium vanadate and 2 mmol/l phenylmethylsulfonylfluoride was added to stop the reaction. After incubation for 30 min at 4℃, the mixture was centrifuged at 10,000 g for 15 min at 4℃. The precipitate was washed twice with ice-cold diethyl ether:ethanol (1:1, v/v), dissolved in 50 ml of 62.5 mmol/l Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 0.7 mol/l -mercaptoethanol and 10% glycerol and was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 7.5% gel. The electrophoresed proteins were transferred onto Immobilon-P membranes (Nippon Millipore) using a semidry blotting apparatus (Sartorius) for 60 min at 2 mA/cm2, and the tyrosyl phosphorylated proteins were detected using phosphotyrosine-specific monoclonal antibody (PY-20; ICN Biochemicals), peroxidase-conjugated rabbit anti-mouse immunoglobulin G antibody (E.Y. Laboratories) and the ECL Western Blotting Detection System (Amersham, Japan)[22]. The apparent molecular masses of the proteins were determined using prestained molecular weight standards (14,300-200,000 molecular weight range; Gibco-BRL). To estimate the phosphorylation level, the lanes were scanned using an Epson

GT 8000 (Seiko Epson Co., Japan) and the intensity of the 58 kDa band was analysed using NIH Image software (Nayne Rasband, National Institute of Health, USA).

13. Preparation of human neutrophils

Venous blood was obtained, with informed consent, from healthy volunteers. Leukocytes were obtained and purified as previously described[25]. Viability was more than 95% according to the trypan blue exclusion test. The mitochondrial dependent reduction of 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan26) was used to assess the possible cytotoxic effect of Hwaotang on human neutrophils.

14. Elastase release by human neutrophils

Neutrophils (2.5×10^6 cells/ml) were preincubated with test Hwaotang or vehicle for 5 min and then stimulated with cytochalasin B (10 μM) and N-formyl-L-methionyl-L-eucyl-L-phenylalanine (fMLP, 10 nM) or platelet-activating factor (PAF) (0.5 μM) for 10 min. Elastase activity was estimated in supernatants, using N-tert-butoxy- carbonyl-alanine p-nitrophenyl ester (200 μM) as substrate and p-nitrophenol release was measured. Possible direct inhibitory effects on elastase activity were also assessed[27].

15. Synthesis and release of leukotriene B4 by human neutrophils

A suspension of human neutrophils (5×10^6 cells/ml) was preincubated with test compound or vehicle for 5 min and then stimulated with calcium ionophore A23187 (1 μM) for 10 min at 37℃. Leukotriene B4 levels in supernatants were measured by radioimmunoassay[28]. High-speed (100,000×g) supernatants from sonicated human neutrophils were obtained and incubated under appropriate conditions with 10 μM arachidonic acid to assess 5-lipoxygenase activity[29].

16. Wetern blot analysis

iNOS or COX-2 protein expression was studied in the cytosolic or 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 polyvinylidene difluoride membranes for 90 min at 125 mA. Membranes were blocked in phosphate buffer saline (0.02 M, 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); for COX-2, membranes were incubated with specific anti-COX-2 polyclonal antiserum (1/1000). Both membranes were 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 chemiluminescence system (ECL, Amersham Korea, Korea).

17. 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 Hwaotang 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, Hwaotang 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 1).

Table 1. Percentage of intimal surface area of thoracic aorta involved with atheromatous plaque in KHC rabbits fed with or without Hwaotang

Feeding period	Groups	Aortic arch(%)	Descending aorta(%)
4weeks	Normal	38.21±2.5	4.0±0.5
	Hwaotang	26.54±2.4	5.4±0.6
8weeks	Normal	45.65±3.4	20.3±1.8
	Hwaotang	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 Hwaotang and its herbs on collagen- or ADP-induced blood platelet aggregation

As shown in Table 2, preincubation of PRP with Hwaotang (0.2, 0.4 or 1.0 ㎎/㎖) produced inhibition of collagen- or ADP-induced blood platelet aggregation to various extent. 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 3. 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 2. Effects of the Hwaotang and aspirin on collagen- or ADP-induced blood platelet aggregation

Dose(mg/ml)		Inhibitory rate(%)	
		Collagen	ADP
Hwaotang	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

Table 3. Effects of the Hwaotang's herbs on collagen- or ADP-induced blood platelet aggregation

Drug or Herbs	Dose(mg/ml)	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	14.5±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 Hwaotang and its herbs on conversion of fibrinogen to fibrin induced by thrombin

As shown in Table 4, 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 mg/ml of Hwaotang.

Table 4. Effects of the Hwaotang and heparin on conversion of fibrinogen to fibrin induced by thrombin

Dose(mg/ml)		Clotting time of fibrinogen solution (sec)
Control		252.6±17.3
Hwaotang	0.2	248.9±26.6
	0.5	276.6±32.5**
	1.0	280.6±25.6**
Heparin (10U/ml)		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 5. Treatment of each 1.0 mg/ml 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.

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

As shown in Table 6, the 50% inhibitory concentrations (IC50 mg/ml) of Hwaotang on plasminogen and plasmin were 0.96 and 3.25 mg/ml respectively.

Table 5. Effects of the Hwaotang's herbs and heparin on conversion of fibrinogen to fibrin induced by thrombin

Herbs	Dose(mg/ml)	Clotting time of fibrinogen solution (sec)
Control		243.6±31.2
Heparin (10 U/ml)		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.

Table 6. Effects of the Hwaotang and Trasylol on fibrinolytic system in fibrin plate

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

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

5. Effect of Hwaotang on fMLP- (A) and PMA- (B) induced superoxide generation in human neutrophils

The effect of Hwaotang on fMLP- and PMA- induced superoxide generation in human neutrophils was investigated by measuring superoxide generation by HPPMNs in order to examine the pharmacological action of Hwaotang. fMLP-induced superoxide generation was inhibited by Hwaotang in a concentration-dependent manner (Fig. 1).

On the other hand, PMA-induced superoxide generation was enhanced by Hwaotang in a concentration-dependent manner (Fig. 2). The effect of Hwaotang on AA-induced superoxide generation could not be observed (data not shown).

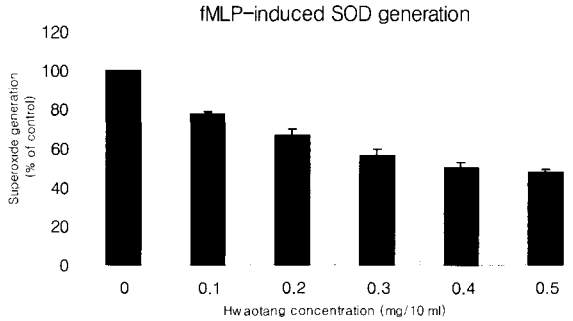

Fig. 1. Effect of Hwaotang on fMLP-induced superoxide generation in human neutrophils

The cells were preincubated with Hwaotang (0-0.5 mg/10 ml) for 3 min prior to the addition of 12.5 nmol/l fMLP. The experimental conditions are as described in Material and

Methods. Results are expressed as the mean±S.E. from three independent experiments.

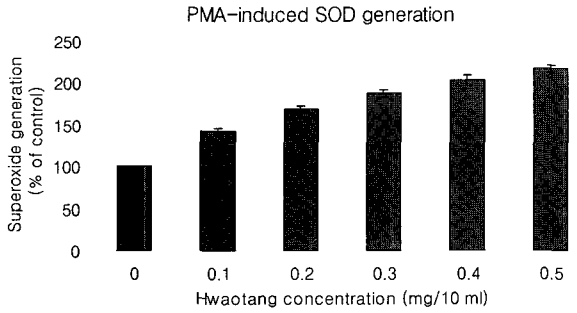

Fig. 2. Effect of Hwaotang on PMA-induced superoxide generation in human neutrophils

The cells were preincubated with Hwaotang (0-0.5 mg/10 ml) for 3 min prior to the addition of 1 nmol/l PMA. The experimental conditions are as described in Material and Methods. Results are expressed as the mean±S.E. from three independent experiments.

6. Effects of protein kinase inhibitors on PMA-induced superoxide generation by Hwaotang-treated HPPMNs

To determine if protein kinase C and protein tyrosine kinase participate in the mechanism for priming of HPPMNs by Hwaotang, the effects of protein kinase inhibitors on PMA-induced superoxide generation were examined. PMA-induced superoxide generation by Hwaotang was inhibited by staurosporine, an inhibitor of protein kinase C, in a concentration-dependent manner. On the other hand, the effect of genistein, an inhibitor of protein tyrosine kinase, on superoxide generation was negligible (Fig. 3, Fig. 4).

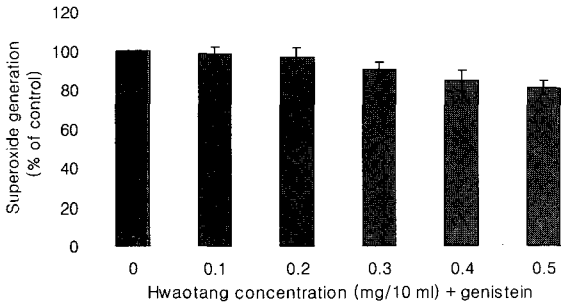

Fig. 3. Effect of protein kinase inhibitor, genistein, on PMA-induced superoxide generation by Hwaotang-treated HPPMNs.

The cells were preincubated with 0.4 mg/10 ml Hwaotang in the presence or absence of a protein kinase inhibitor for 3 min at 37℃ prior to the addition of PMA. The data were obtained at 4 min after the addition of PMA. Other conditions were the same as in Fig. 1. Results are expressed as the mean±S.E. from three independent experiments. Effect of genistein on Hwaotang-induced superoxide generation.

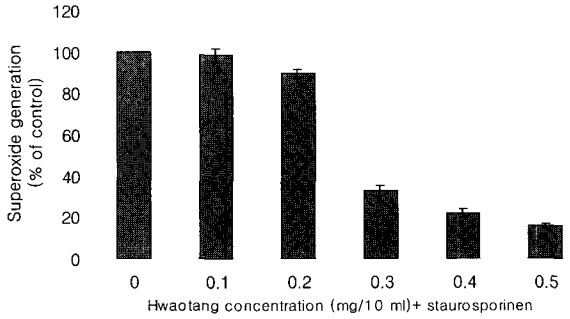

Fig. 4. Effect of protein kinase inhibitor, staurosporine on PMA-induced superoxide generation by Hwaotang-treated HPPMNs

The cells were preincubated with 0.4 mg/10 ml Hwaotang in the presence or absence of a protein kinase inhibitor for 3 min at 37℃ prior to the addition of PMA. The data were obtained at 4 min after the addition of PMA. Other conditions were the same as in Fig. 1. Results are expressed as the mean±S.E. from three independent experiments. Effect of staurosporinen on Hwaotang-induced superoxide generation.

7. Effect of Hwaotang on fMLP-induced tyrosyl phosphorylation of HPPMN proteins

The effect of Hwaotang on the phosphorylation of tyrosine residues of HPPMN protein in fMLP-induced neutrophils was examined. When the fMLP-induced neutrophils were incubated with Hwaotang, Hwaotang inhibited the phosphorylation of tyrosine residues of the 58 kDa protein in fMLP-induced neutrophils, in parallel with a decrease in superoxide generation(Fig. 5, Table 7).

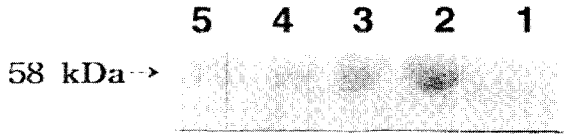

Fig. 5. Western analysis of Hwaotang on fMLP-induced tyrosyl phosphorylation of HPPMN proteins

Proteins with phosphorylated tyrosine residues were detected by immunoblotting with anti-phosphotyrosine antibody. The results are expressed as the mean from three independent experiments.

8. Effect of Hwaotang on fMLP-induced p47 phox synthesis in human neutrophils

The effect of Hwaotang on 47 kDa protein in neutrophils by using human monoclonal antibody to human p47 phox was examined. When the fMLP-induced neutrophils were incubated with Hwaotang, the amount of p47 phox protein decreased in a concentration-dependent manner (Fig. 6, Table 8). Hwaotang inhibited the synthesis of p47 phox protein in fMLP-induced neutrophils.

Table 7. Effect of Hwaotang on fMLP-induced tyrosyl phosphorylation of HPPMN proteins

Treatment	Intensity	% of control
Control (without Hwaotang)	104.0	100
25 nmol/l fMLP	232.0	224.6
25 nmol/l fMLP + 0.2 mg Hwaotang/10 ml	213.0	212.6
25 nmol/l fMLP + 0.5 mg Hwaotang/10 ml	186.0	174.7
25 nmol/l fMLP + 1.0 mg Hwaotang/10 ml	173.0	167.5

The extent of tyrosyl phosphorylation was estimated by densitometry. The results are expressed as the mean from three independent experiments.

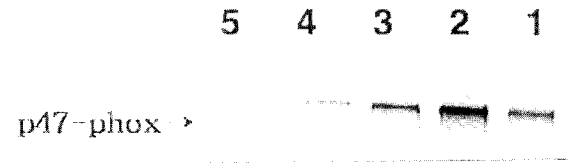

Fig. 6. Western blot of Hwaotang on fMLP-induced p47 phox synthesis in human neutrophils

p47 phox proteins were detected by immunoblotting with human monoclonal antibody. The results are expressed as the mean from three independent experiments.

Table 8. Effect of Hwaotang on fMLP-induced p47 phox synthesis in human neutrophils

Treatment	Intensity	% of control
Control (without Hwaotang)	385.0	100
25 nmol/l fMLP	396.0	105.6
25 nmol/l fMLP + 0.2 mg Hwaotang/10 ml	387.0	211.5
25 nmol/l fMLP + 0.5 mg Hwaotang/10 ml	246.0	68.4
25 nmol/l fMLP + 1.0 mg Hwaotang/10 ml	156.0	44.2

The extent of p47 phox proteins was estimated by densitometry. The results are expressed as the mean from three independent experiments.

9. Elastase release by human neutrophils

We assayed Hwaotang in the degranulation process of human neutrophils activated by two different stimuli. Preincubation of isolated human neutrophils with the test compound elicited a concentration-dependent inhibition of cytochalasin B+fMLP and cytochalasin B+PAF-induced degranulation measured as elastase release. The IC_{50} was 4.6 ug/ml (Fig. 7). Direct inhibitory effects on elastase activity were not observed (data not shown).

10. Synthesis and release of leukotriene B4 by human neutrophils

Hwaotang at 10 ug/ml completely abolished leukotriene B4 release by human neutrophils stimulated with ionophore A23187. The concentration-dependent study showed an IC_{50} value of 5.6 ug/ml. Nevertheless Hwaotang failed to modify leukotriene B4 synthesis by high-speed supernatants from human neutrophils at concentrations up to 1 ug/ml (Fig. 8). Thus, it appears that the reduction of leukotriene B4 release by Hwaotang in intact neutrophils is not due to direct inhibition of 5-lipoxygenase activity.

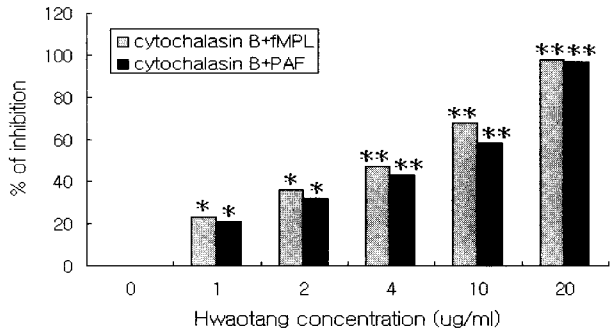

Fig. 7. Inhibition by Hwaotang of neutrophil activation Elastase release induced by cytochalasin B+fMPL or cytochalasin B+PAF. Data represent means±S.E., n=4-5., * : P<0.05, ** : P<0.01.

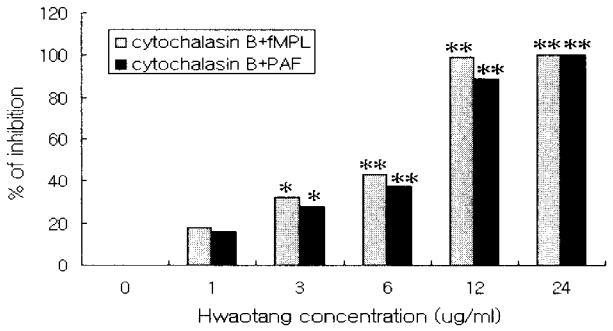

Fig. 8. Inhibition by Hwaotang of release of leukotriene B4 by human neutrophils Leukotriene B4 release induced by cytochalasin B+fMPL or cytochalasin B+PAF. Data represent means±S.E., n=3-5.
* : P<0.05, ** : P<0.01.

Discussion

Nowadays, the occurrence rate of the blood circulation system disease has been increased because that the increase of under exercise, fatness, adding the stress, advanced age etc. And the thrombosis and atherosclerosis importantly came to the front as the risk factor of these circulation system's disease[30,31].

Thrombosis is the formation of a blood clot(thrombus) in the heart or a blood vessel. Contributing factors include injury to a blood vessel's lining from inflammation(thrombophlebitis) or atherosclerosis, blood flow that is turbulent (e.g., from an

aneurysm) or sluggish (e.g., from prolonged bed rest), or coagulation abnormalities (e.g., from high numbers of platelets or excessive fats in the blood). A thrombus can block blood flow at the point of clot formation or break free to block it elsewhere[32].

In oriental medicine, the thrombosis belong to the category of blood stasis. Blood stasis is a term in oriental medicine. It refers to blood stasis in the body resulting from unsmooth circulation or stagnation of blood and retention of blood in channels or zang-fu organs[6,7]. 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. And it becomes the pathopoiesis cause that the symtom complex with a mass or swelling in the abdomen, apoplexy etc[9]. 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[3,4].

According to the ancient Chinese medicinal literature NaSiHoeYakEuiKyung(羅氏會約醫鏡)》, Hwaotang 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[7].

Hwaotang 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)》[5]. The present time, too, Hwaotang has been used for the treatment of various blood circulation disease, including the clinical treatment of thrombosis[6]. Hwaotang has been reported to have an inhibitory effets of Hwaotang on the atherosclerosis and the venous thrombosis[10]. Hwaotang is also applied as an effective biological response modifier for augmenting host homeostasis of body circulation[9]. The pharmacological action of Hwaotang has been limitedly studied in regard to ischemic infarction[9]. 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 Hwaotang in experimentally induced atherosclerosis in rabbits have also been reported[10]. However, pharmacological mechanisms of Hwaotang on anti-thrombosis and atherosclerosis formation are poorly understood.

This paper reports the effects of Hwaotang on anti-thrombotic activity. The result of this study is as follow, Hwaotang showed inhibitory effects on the progression of atherosclerosis lesions, without beneficial effects on other chemical parameters of KHC rabbits. The administration dose of Hwaotang 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[17] and other physiological characters or pharmacological response to fibrate in KHC rabbits have been revealed[2]. The results show that Hwaotang inhibited the progression of atherosclerotic lesions macroscopically, there were no significant differenced in the histopathological findings of the lesion between the control and Hwaotang groups. Further study and examination are desirable for a better understanding of the histopathological effects of Hwaotang.

The extract derived from Hwaotang 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 Hwaotang 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.

On this study, Hwaotang was also used for investigating the reaction of enzymes acted on mechanism of thrombosis formation and the effects on superoxide generation in human neutrophils. When the cells were preincubated with Hwaotang for 3 min, inhibition of fMLP-induced superoxide generation was observed. According to the time course experiment, 3 min was sufficient for the preincubation time (data not shown). fMLP-induced superoxide generation was inhibited by Hwaotang in a concentration-dependent manner.

On the other hand, PMA-induced superoxide generation was enhanced by Hwaotang in a concentration-dependent manner (Fig. 2). The results indicate that Hwaotang are typical priming factors for the agonist-mediated respiration burst of neutrophils. The rates of the priming effects of Hwaotang on fMLP- and PMA-induced superoxide generation were different from each other. The reverse was true of the rates of enhancement of PMA-induced superoxide generation. PMA-induced superoxide generation by Hwaotang was inhibited by staurosporine, an inhibitor of protein kinase C. However, genistein, an inhibitor of protein tyrosine kinase did not inhibited the superoxide generation(Fig.3 and Table 7), suggesting that Hwaotang enhance superoxide generation in

neutrophils via activation of protein kinase C.

It was previously reported that the phosphorylation process of tyrosine residues of HPPMN proteins was inhibited by genistein and herbimycine A, inhibitors of tyrosine protein kinase[16,33]. Therefore, the effect of Hwaotang on the phosphorylation of tyrosine residues of HPPMN protein in fMLP-induced neutrophils was examined. Hwaotang inhibited the phosphorylation of tyrosine residues of the 58 kDa protein in fMLP-induced neutrophils (Fig. 3). These results indicate that Hwaotang decrease superoxide generation in neutrophils via inhibition of tyrosine protein kinase.

It was reported detecting several priming factors for the phosphorylation of tyrosine residues in a 45 kDa protein of human neutrophils, and that the phosphorylation was inhibited by the tyrosine kinase inhibitors, genistein and herbimycin A, but not by the protein kinase C inhibitors, staurosporine and H-7. The priming factors enhanced superoxide generation induced by PMA and AA. The function of Hwaotang in agonist-mediated superoxide generation in human neutrophils was different from that of other priming factors investigated in previous papers. At present, the mechanism for inhibition of fMLP-induced superoxide generation, for inhibition of protein tyrosine kinase and for activation of PMA-induced superoxide generation by Hwaotang is unknown. Whether Hwaotang alter the properties of fMLP- and PMA-receptors by direct binding or indirectly by changing the membrane environment is also unknown. It has been reported that a 47 kDa protein, one of the cytosolic proteins for activation of NADPH oxidase in human neutrophils, was phosphorylated in human neutrophils after stimulation with fMLP or PMA, based on an analysis using SDS-PAGE34). The mechanism for inhibition of protein synthesis p47 phox by Hwaotang is not clear

In the present work, the respiratory burst elicited in human neutrophils by TPA was potently inhibited by Hwaotang, showing a minor scavenging action in the cell-free system. Hwaotang reduced the degranulation induced by cytochalasinB+fMLP or cytochalasinB+PAF, as well as the leukotriene B4 synthesis induced by ionophore A23187, thus exerting inhibitory effects on neutrophil functions triggered by structurally divergent agonists. Hwaotang may either prevent or slow the progression of neutrophil-mediated tissue injury. Hwaotang seems to affect cell activation at a site common to different signaling pathways as it inhibited responses induced by fMLP, PAF, TPA or ionophore A23187.

Further work is in progress on the effect of main components, flavonoids of Hwaotang, on blood coagulative and fibrinolytic system. Besides studies are also required to find if the inhibition of enzyme expression by Hwaotang is related to an effect on the generation of reactive oxygen species and/or on the regulation of transcription factors such as NF-κB.

Conclusion

We have reported that Hwaotang had the anti-thrombotic activity and various effects on stimulus-induced superoxide generation in human neutrophils. 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. The effects of these Hwaotang on superoxide generation in human neutrophils were also investigated. Hwaotang significantly inhibited fMLP-induced superoxide generation in a concentration-dependent manner, but not that induced by arachidonic acid (AA). On the other hand, both Hwaotang enhanced superoxide generation induced by phorbol 12-myristate 13-acetate (PMA) in a concentration-dependent manner. The superoxide generation induced by PMA with Hwaotang was suppressed by staurosporine, an inhibitor of protein kinase C, but was not suppressed by genistein, an inhibitor of protein tyrosine kinase. Tyrosyl phosphorylation of a 58 kDa protein, which was increased by fMLP, was inhibited by Hwaotang. Hwaotang also inhibited the generation of a 47 kDa protein and platelet aggregation in human blood. The results suggest that protein tyrosine kinase participates in fMLP-mediated superoxide generation by Hwaotang-treated human neutrophils.

A Hwaotang inhibited neutrophil functions, including degranulation, superoxide generation, and leukotriene B4 production, without any effect on 5-lipoxygenase activity. In conclusion, the protection of extracts of Korean herbs' Hwaotang on the ischemic infarction induced artificially might be involved to their inhibition of thrombotic action. The results also indicate that Hwaotang exerts the effects on superoxide generation related to the inhibition of neutrophil functions.

Acknowledgement

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References

1. Kubo, M. and Matsuda, H., Studies on Scutellaria Radix (VIII) Anti-thrombic actions of 70% methanolic extract and various fractions from Scutellariae Radix. Shoyakugaku Zasshi 38, 302-306, 1984.

2. Kanamasa, K., Ishida, N., Kato, H., Sakamoto, S., Nakabayashi, T., Otani, N., Ishikawa, K., Katori, R., Recombinant tissue plasminogen activator prevents intimal hyperplasia after balloon angioplasty in hypercholesterolemic rabbits. Jpn. Circulation Journal 60, 889-894, 1996.
3. M.G.,Shin, Clinic Herbs Science, Seoul; NamSanDang, p. 143, 453, 1986.
4. 久保道德, 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.
5. Peng Pu In, Great dictionary of prescription in traditional chinise medicine, beijing; Inmin Hygiene Press, P. 1868, 3464, 8439, 1994.
6. Zhang Enqin, Clinic of Traditional Chinese Medicine (I) in A Practical English-Chinese Library of TCM, Shanghai; House of Shanghai College of Traditional Chinese Medicine, pp. 102-107, 1990.
7. Ming, O. Chinese-English manual of common-used prescriptions in TCM, Hong kong; Guangdong Science & Technology Publishing House, pp.206-208, 1989.
8. Park WH, Park SY, Kim HM, Kim CH, Effects of a Korean traditional formulation, Hwaotang, on superoxide generation in human neutrophiles, platelet aggregation in human blood, and nitric oxide, prostaglandin E2 production and paw oedema induced by carrageenan in mice, Immunopharmacol Immunotoxicol 26(1): 53-73, 2004.
9. W. H., Park 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.
10. M.Y. Hong, Inhibitory effets of Hwaotang on the atherosclerosis and the venous thrombosis. Thesis for the degree of Doctor, Dongguk University, Kyungju,Korea. 2001.
11. Rossi, F., The O2- forming NADPH oxidase of the phagocytes: nature, mechanisms of activation and function. Biochim Biophys Acta 853, 65-89, 1986.
12. Takahashi, R. Edashige, K., Sato, E.F., Inoue, M., Matsuno, T. and Utsumi, K., Luminol chemi-luminescence and active oxygen generation by activated neutrophils. Arch Biochem Biophys 285, 325-330, 1991.
13. McCall, C.E., Bass, D.A., Dechatelt, L.R., Link, A.S. and Mann, M., In vitro responses of human neutrophils to N-formyl-methionyl-leucyl-phenylalanine: correlation with effects of acute bacterial infection. J Infect Dis 140, 277-286, 1979.
14. Zhang, J., Sugahara, K., Sagara, Y., Fontana, M., Dupre, S., and Kodama, H., Effect of cystathionine ketimine on the stimulus coupled responses of neutrophils and their modulation by various protein kinase inhibitors. Biochem Biophys Res Commun 218, 371-376, 1996.
15. Shimazaki, Y., Zhang, J., Wakiguchi, H., et al., Different effect of diastereoisomers of cystathionine sulfoxide on the stimulus coupled responses of human neutrophils. Biochem Biophys Res Commun 247, 387-391, 1998.
16. Zhang, J., Sagara, Y., Fontana, M., Dupre, S., Cavallini, D. and Kodama, H., Effects of cystathionine and cystathionine metabolites on the phosphorylation of tyrosine residues in human neutrophils. Biochem Biophys Res Commun 224, 849-854, 1996.
17. Kurosawa, T., Kisanagi, M., Yamasaki, Y., Senga, Y., Yamamoto, T., New mutant rabbit strain with hypercholesterolemia and atherosclerotic lesions produced by serial inbreeding. Laboratory Animal Science 45, 385-392, 1995
18. Kita, T., Nagano, Y., Yokode, M., Ishii, K., Kume, N., Ooshima, A., Yoshida, H., Kawai, C., Probucol prevents the progression of atheroclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesteromoa. Proc. Natl. Acad. Sci. USA 84, 5928-5931, 1987
19. Manabe, S., Wada, O., Matsui, H., Takeda, M., Kobayashi, N. and Maekawa, T., Triphenyltin fluoide in vitro inhibition of rabbit platelet collagen-induced aggregation and ATP secretion and blockage of arachidonic acid mobilization from membrane phospholipids. Biochem. Pharmacology 32, 1627-1634, 1983
20. Ekimoto, H., Irie, Y., Araki, Y., Han, G.Q., Kadota, S. and Kikuchi, T., Platelet aggregation inhibitors from the seeds of Swietenia mahagoni: inhibition of in vitro and in vivo platelet-activating factor-induced effects of tetranortriperpenoids related to swietenine and swietenolide. Planta Med. 57, 1609-1611, 1991
21. Astrup, T. and Mullertz, S. The fibrin plate method for estimating fibrinolytic activity. Arch. Biochem. Biophys. 40, 346-251, 1952
22. Weiss, S.J., Klein, R., Slivka A. and Wei, M., Chlorination of taurine by human neutrophils. Evidence for hypochlorous acid generation. J Clin Invest 70, 598-607, 1982.
23. Henderson, W.R., The role of leukotrienes in inflammation. Ann. Intern. Med. 121, 684-697, 1994.
24. Yamamoto, M., Saeki, K. and Utsumi, K., Isolation of human salivary polymorphonuclear leukocytes and their stimulation-coupled responses. Arch Biochem Biophys 289, 76-82, 1992.
25. Bustos, G., Ferrandiz, M.L., Sanz, M.J., Paya, M. and

Alcaraz, M.J., A study of the novel anti-inflammatory agent florifenine. Topical anti-inflammatory activity and influence on arachidonic acid metabolism and neutrophil functions. Naunyn-Schmiedeberg's Arch. Pharmacol. 351, 298-304, 1995.

26. 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.
27. Escrig, V., Ubeda, A., Ferrandiz, M.L., Darias, J., Sanchez, J.M., Alcaraz, M.J. and Paya, M., Variabilin: a dual inhibitor of human secretory and cytosolic phospholipase A2 with anti-inflammatory activity. J. Pharmacol. Exp. Ther. 282, 123-131, 1997.
28. Moroney, M.A., Alcaraz, M.J., Forder, R.A., Carey, F. and Hoult, J.R.S., Selectivity of neutrophil 5-lipoxygenase and cyclo-oxygenase inhibition by an anti-inflammatory flavonoid glycoside and related aglycone flavonoids. J. Pharm. Pharmacol. 40, 787-792, 1988.
29. Tateson, J.E., Randall, R.W., Reynolds, C.H., Jackson, W.P., Bhattacherjee, P., Salmon, J.A. and Garland, L.G., Selective inhibition of arachidonate 5-lipoxygenase by novel acetohydroxamic acids: biochemical assessment in vitro and ex vivo. Br. J. Pharmacol. 94, 528-539, 1988.
30. J.G.,Kim, clinic lipid, Seoul ; medical science press, pp.241-270, 1995
31. S.G.,Seo, Adult & Aged disease, Seoul; Korea medical science, p.27, 38-53, 1992
32. A.D.T.Govan, P.S. Macfarlane, R.Callander, (trans; J. D., Lee), Pathology, Seoul ; Korea medical science, pp. 127-134, 573, 1991
33. Ito, O., Zhang, J. and Zhang, M., Priming effect of N-acetyl-S-(3-oxo-3-carboxy-n-propyl) cysteine in human neutrophils and tyrosyl phosphorylation of 45 kDa protein. Clin Chim Acta 266, 93-104, 1997.
34. Schneider, C., Zanetti, M. and Romeo, D., Surface-reactive stimuli selectively increase protein phosphorylation in human neutrophils. FEBS Lett 127, 4-8, 1981.