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Perilla oil improves blood flow through inhibition of platelet aggregation and thrombus formation

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The inhibitory effects of perilla oil on the platelet aggregation *in vitro* and thrombosis *in vivo* were investigated in comparison with aspirin, a well-known blood flow enhancer. Rabbit platelet-rich plasma was incubated with perilla oil and aggregation inducers collagen or thrombin, and the platelet aggregation rate was analyzed. Perilla oil significantly inhibited both the collagen- and thrombin-induced platelet aggregations, in which the thromboxane B_2 formation from collagen-activated platelets were reduced in a concentration-dependent manner. Rats were administered once daily by gavage with perilla oil for 1 week, carotid arterial thrombosis was induced by applying 35% $FeCl_3$-soaked filter paper for 10 min, and the blood flow was monitored with a laser Doppler probe. Perilla oil delayed the $FeCl_3$-induced arterial occlusion in a dose-dependent manner, doubling the occlusion time at 0.5 mL/kg. In addition, a high dose (2 mL/kg) of perilla oil greatly prevented the occlusion, comparable to the effect of aspirin (30 mg/kg). The results indicate that perilla oil inhibit platelet aggregation by blocking thromboxane formation, and thereby delay thrombosis following oxidative arterial wall injury. Therefore, it is proposed that perilla oil could be a good candidate without adverse effects for the improvement of blood flow.

Key words: Platelet aggregation, thromboxane B_2, thrombosis, perilla oil

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Thrombosis due to embolic blood vessel occlusion is one of the major causes of cardiovascular and cerebrovascular diseases including cardiomuscular in farction (angina) and cerebral strokes. For the thrombus formation, platelet aggregation plays a crucial role [1]. Upon endothelial injury, adhesive ligands including collagen and von Willebrand Factors (vWF) and their agonists such as adenosine diphosphate (ADP) and thrombin are up-regulated. Such coagulating factors activate platelets, leading to adhesion to the injured arterial walls and aggregation [2].

Collagen supports the binding of platelets to injured arteries via their surface receptors glucoprotein VI and integrin $\alpha2\beta1$ [3]. Collagen binding activates platelets through tyrosine kinase-mediated signaling pathway, and then the stimulated platelets adhere to the arterial walls, which is dependent on the release of agonists such as ADP and prostaglandin H_2/thromboxane A_2 (TXA_2) from platelet granules [4,5]. TXA_2 is an inducer of vasoconstriction and platelet aggregation, and plays a

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key role in the arterial homeostasis. Thus, TXA_2 is considered as an important etiological mediator in the progress of atherosclerosis and myocardial ischemia [6]. TXA_2 is produced from arachidonic acid during oxidation reaction catalyzed by cyclooxygenase (COX) and thromboxane synthase, and then rapidly oxidized to a stable inactive thromboxane B_2 (TXB_2) [7]. There, the blood concentration of TXB_2 following blood clotting is a specific marker for the assessment of COX-1 activity and platelet aggregation [8].

It is well known that transition metals including Fe^{2+} and Cu^{2+} facilitate oxidative radical formation, inducing cellular and tissue injuries as well as endothelial cell damage leading to thrombosis. So, application of ferric chloride ($FeCl_3$) to arterial outer surface has been used as a model to induce oxidative thrombosis, for the efficacy assessment of anti-thrombotic blood flow enhancers [9].

It is well known that unsaturated fatty acids (UFA) regulate blood lipid profiles, and thereby prevent coronary heart disease [10-13]. Fish oil ω-3 polyunsaturated fatty acids (PUFA) prevented vasoconstriction [14], and inhibited vascular inflammatory response by decreasing production of reactive oxygen species (ROS) [15,16]. Notably, α-linolenic acid (ALA), a well-known ω-3 PUFA rich in perilla oil improved insulin sensitivity and lipemia, and prevented coronary heart disease [17,18]. Especially, in a recent study, we demonstrated that perilla oil possessing a low ω-6/ω-3 ratio not only reduced total cholesterol (TC) and low-density lipoproteins (LDL) causing atherosclerosis, but also delayed and attenuated brain hemorrhage in stroke-prone spontaneously hypertensive rats (SHR-SP), thereby extending their lifespan (unpublished results).

Since PUFA affects both platelets and endothelial cells that play a crucial role in the regulation of thrombosis and haemostasis [19], we investigated the blood flow-improving activity of perilla oil in a $FeCl_3$-induced carotid artery thrombosis model, in addition to the effects on the TXB_2 formation and platelet aggregation as action mechanisms.

Materials and Methods

Materials

Perilla oil was obtained from Misuba RTech Co. (Asan, Korea). Perilla oil was extracted under a cold-pressed method at 30-48ºC, and analyzed with Varian 3800 gas chromatograph (Varian Inc., Walnut Creek, CA, USA) equipped with a Supelcowax 10 fused-silica capillary column (Supelco, Bellefonte, PA, USA). From the fatty acid analysis, it was found that perilla oil contains 72.12% PUFA, 19.1% monounsaturated fatty acids (MUFA), and 8.49% saturated fatty acids (SFA). Especially, among PUFA, 57.47% was ω-3 ALA [18:2(n-3)].

Animals

Six-month-old male New Zealand white rabbits (body weight 2.0 kg) and 7-week-old male Sprague-Dawley rats (body weight 200-220 g) were procured from Daehan-Biolink (Eumseong, Korea), and subjected to the experiment after 1-week acclimation to the laboratory environment. The animals were housed in each cage with free access to feed and water under constant environmental conditions (22±2ºC temperature; 40-70% relative humidity; 12-hour light-dark cycle; 150-300 lux brightness). All the animal experiments were conducted according to the Standard Operation Procedures, and approved by the Institutional Animal Care and Use Committee of Chungbuk National University, Korea (CBNUA-514-13-01).

Measurement of platelet aggregation

Blood sample was collected from the ear artery of rabbits directly into anti-coagulant citrate dextrose solution containing 0.8% citric acid, 2.2% trisodium citrate, and 2% dextrose. Platelet-rich plasma (PRP) was obtained by centrifugation at 230×*g* for 10 min. Platelets were sedimented by centrifugation of the PRP at 800×*g* for 15 min and washed with a HEPES buffer (pH 6.5) [9,20]. The washed platelets were resuspended (3×10^8 cells/mL) in the HEPES buffer (pH 7.4).

Platelet aggregation was measured with an aggregometer (Chrono-Log Co., Harbertown, CA, USA) according to the turbidimetric method of Born [21] as previous described [20]. In brief, the washed platelet suspension was preincubated with perilla oil (100-800 μg/mL) or aspirin (100-200 μg/mL) as a reference control at 37ºC in the aggregometer under stirring at 1,000 rpm. After 3-min preincubation, platelet aggregation was induced by adding collagen (2.5 μg/mL) or thrombin (0.1 U/mL). The extent of aggregation was expressed as a percentage of the vehicle control value stimulated with collagen or thrombin alone.

Analysis of thromboxane formation

TXB_2 released from platelets was assessed using a kit according to the manufacturer's instructions. In brief, washed rabbit platelets (4×10^8 cells/mL) were preincubated with perilla oil (100-800 μg/mL) or aspirin (100 μM) as a reference control at 37°C for 3 min in an aggregometer, and aggregation was induced by adding collagen (2.5 μg/mL) [9,20]. The reaction was stopped by adding 5 mM indomethacin and 2 mM EGTA, centrifuged at 1,200 rpm for 2 min, and analyzed for the concentration of TXB_2 by enzyme-linked immunosorbent assay (ELISA).

Blood flow monitoring in $FeCl_3$-induced thrombosis model

Rats (n=10/group) were orally administered with perilla oil (0.5, 1 or 2 mL/kg) or aspirin (30 mg/kg) for 1 week. Forty min after the final administration, the animals were anesthesized by intramuscular injection of Zoletil® (1 mL/kg). Under constant maintenance of body temperature (36-37°C) using a heating pad, the right carotid artery of rats were exposed, and dissected away from the vagus nerve and surrounding tissues. Aortic blood flow rate was monitored with a laser Doppler flowmeter (AD Instruments, Colorado Springs, CO, USA). At the time point of 1 hour after the final administration, arterial thrombosis was induced by wrapping the artery with a Whatman No. 1 filter paper (3 mm in diameter) saturated with 35% $FeCl_3$ solution near (5 mm anterior to) the flowmeter probe for 10 min [9,20]. The blood flow was monitored for 90 min. A part of the animals (n=3/group) were sacrificed at the time point of 50 min from the application of $FeCl_3$, and the arteries were cut to observe the thrombus in the artery.

Statistical analysis

The results are presented as means±standard deviation. The significance of differences of all results was analyzed by one-way analysis of variance followed by the Dunnett's multiple-range test correction, using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was set a priority at $P<0.05$.

Results

Perilla oil significantly inhibited platelet aggregation induced by collagen (2.5 μg/mL) in a concentration-dependent manner, inhibiting by approximately 24, 35, and 54% at the concentrations of 250, 500, and 800 μg/mL respectively (Figure 1), although the effect of perilla oil was inferior to that of aspirin. Aspirin significantly inhibited by approximately 78 and 85% at the concentrations of 100 and 200 μg/mL.

Perilla oil markedly suppressed the platelet aggregation induced by collagen (2.5 μg/mL) as well as thrombin (0.1 U/mL), in which 500 μg/mL perilla oil inhibited the collagen- and thrombin-induced aggregation by 40% and 52%, respectively (Figure 2). Aspirin was stronger than perilla oil, inhibiting the collagen- and thrombin-

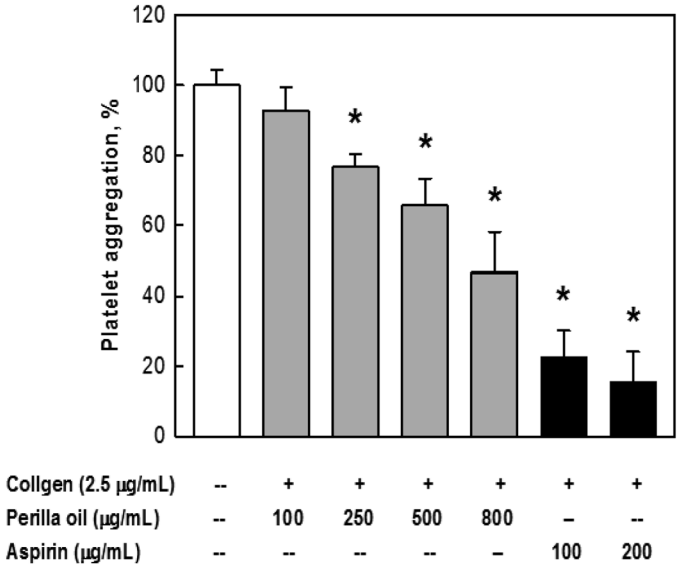

Figure 1. Inhibition by perilla oil (100-800 μg/mL) or aspirin (100-200 μg/mL) of platelet aggregation induced by collagen (2.5 μg/mL). *Significantly different from vehicle control (collagen alone)($P<0.05$).

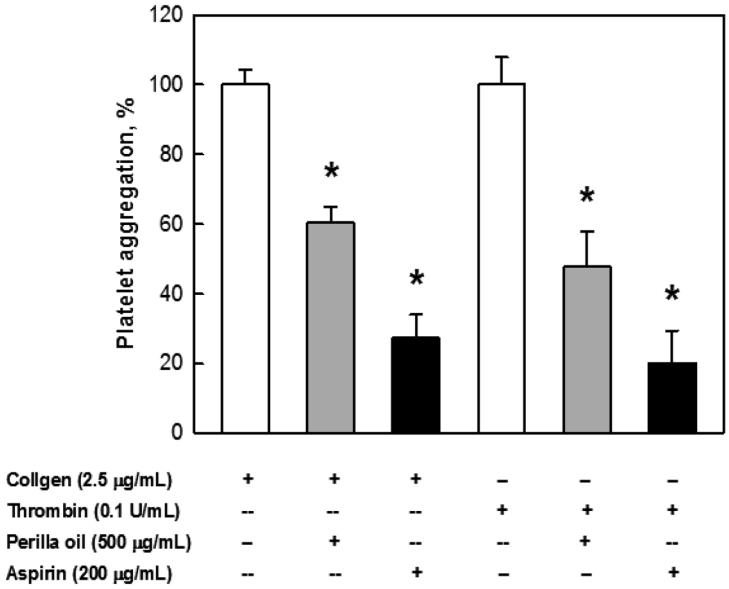

Figure 2. Inhibition by perilla oil (500 μg/mL) or aspirin (200 μg/mL) of platelet aggregation induced by collagen (2.5 μg/mL) or thrombin (0.1 U/mL). *Significantly different from vehicle controls (collagen or thrombin alone) (P<0.05).

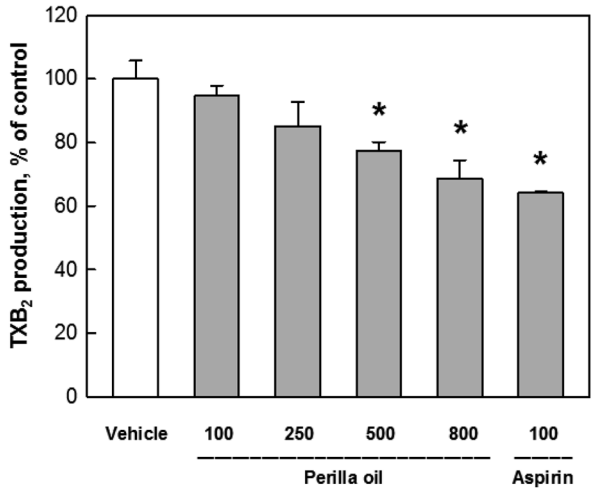

Figure 3. Inhibition by perilla oil (100-800 μg/mL) or aspirin (100 μM) of thromboxane B_2 production from rabbit platelets induced by collagen (2.5 μg/mL). *Significantly different from vehicle control (collagen alone) (P<0.05).

induced aggregation by 73 and 80%, respectively, at 200 μg/mL.

During collagen-induced platelet aggregation, TXB_2 formation was inhibited by perilla oil in a concentration-dependent manner, showing decrease by 23 and 31% at 500 and 800 μg/mL, respectively (Figure 3). Notably, the effect of a high dose (800 μg/mL) of perilla oil was comparable to that (36%) of aspirin (100 μM).

Application of 35% $FeCl_3$ to the external surface of carotid artery for 10 min induced rapid decrease in the blood flow that practically ceased in 30 min (Figure 4). However, 1-week feeding perilla oil delayed the blood flow blockade. Especially, the effect of a high dose (2 mL/kg) of perilla oil was similar to that of aspirin (30 mg/kg).

The mean occlusion time in the vehicle control group was calculated to be 28.4 min, based on the time point when the blood flow dropped to 10% (practical cessation) of initial flow rate (Figure 5). In comparison, treatment with 0.5, 1, and 2 mL/kg of perilla oil extended the occlusion time to 52.8, 55.6, and 82.1 min, respectively. Notably, the blood flow-elongation effect of a high dose (2 mL/kg) of perilla oil was comparable to that (97.0 min) of aspirin (30 mg/kg) (Figures 4 and 5).

As dissected 50 min after the application of $FeCl_3$, the arteries were found to be entirely plugged with thrombi in vehicle control rats (Figure 6). However, in animals treated with 0.5 or 1 mL/kg of perilla oil, the thrombi were small and loose, without fully obstructing the arterial lumens. Notably, only minimal thrombi were observed in animals treated with a high dose (2 mL/kg) of perilla oil or aspirin (30 mg/kg).

Discussion

Perilla oil substantially inhibited both the collagen- and thrombin-induced platelet aggregations. Such results indicate that perilla oil not only inhibits blood clotting

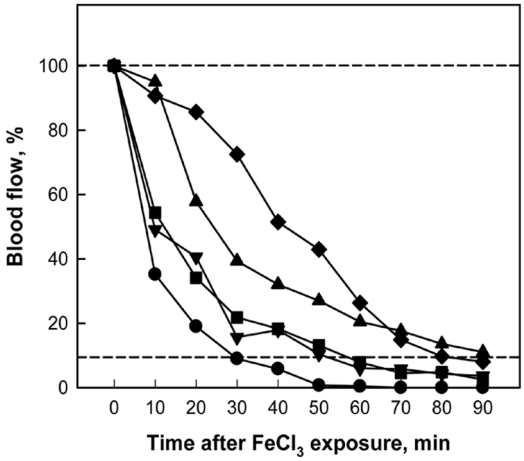

Figure 4. Time-course of carotid arterial blood flow following $FeCl_3$ application outside the arterial wall. Perilla oil and aspirin were orally administered for 1 week prior to $FeCl_3$ exposure. Lower dot line indicates a practical cessation of blood flow.●, vehicle; ▼, 0.5 mL/kg perilla oil; ■, 1 mL/kg perilla oil; ◆, 2 mL/kg perilla oil; ▲, 30 mg/kg aspirin.

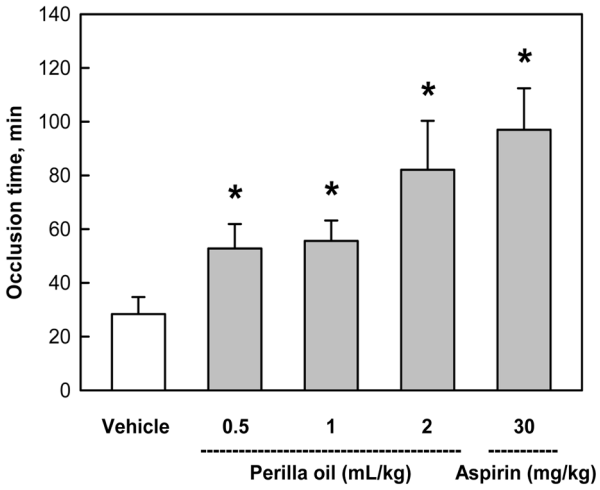

Figure 5. Time to occlusion of carotid arteries after application of $FeCl_3$ outside the arterial wall. Perilla oil (0.5-2 mL/kg) and aspirin (30 mg/kg) were orally administered for 1 week prior to $FeCl_3$ exposure. *Significantly different from vehicle control (P<0.05).

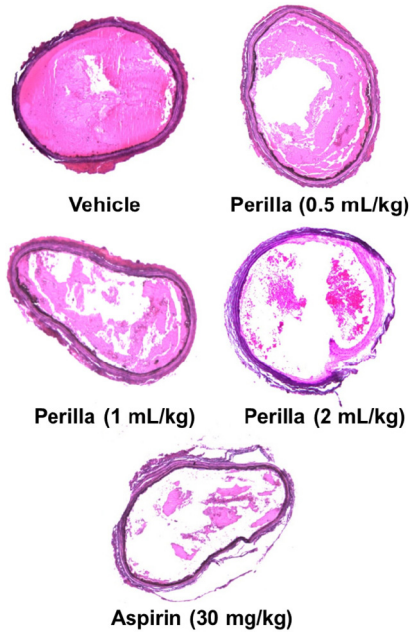

Figure 6. Representative findings of arterial thrombi produced by $FeCl_3$ application outside the arterial wall. Perilla oil (0.5-2 mL/kg) and aspirin (30 mg/kg) were orally administered for 1 week prior to $FeCl_3$ exposure (H&E, magnification ×40).

triggered by thrombin, but also blocks TXA_2-mediated adhesion of platelets to the injured vessel walls as confirmed in the collagen-induced TXB_2 formation [3-5]. It is inferred from the results that the effects of perilla oil are similar to those of aspirin, a well-known blood flow enhancer exerting its effect via both mechanisms.

$FeCl_3$ triggers oxidative vascular endothelial damage, causing exposure of subendothelial extra cellular matrix. Then platelets interact with collagen and vWF in the matrix via their respective platelet surface receptors, leading to platelet adhesion. Activated platelets undergo calcium mobilization and the release of ADP and TXA_2 to further accelerate recruitment and aggregation of platelets for thrombus formation [22]. According to the *in vitro* results, *in vivo* anti-thrombotic efficacy of perilla oil has been anticipated. Indeed, oral administration of perilla oil delayed the occlusion time in a $FeCl_3$-induced artery thrombosis model. Notably, the effects of crude perilla oil at a high dose (2 mL/kg) was comparable to those of aspirin (30 mg/kg), a purified drug. Notably, perilla oil doubled the occlusion time at 0.5 mL/kg.

It was reported that ω-3 PUFA has antioxidative and anti-inflammatory activities; it inhibited C-reactive protein in an atherosclerosis model [16], increased mucosal blood flow by inhibiting leukotriene production in an inflammatory bowel disease model [23], and improved cardiovascular diseases [13]. Also, in the present study, perilla oil containing a high concentration (72.12%) of PUFA markedly suppressed the thrombus formation in the $FeCl_3$-induced endothelial injury model. Notably, in our gas chromatographic analysis of perilla oil, 57.47% was ALA out of 72.12% PUFA. Supportively, it was recently demonstrated that ALA inhibited platelet activation and arterial thrombus formation [24]. Activated platelets attach to vascular endothelial walls injured during

oxidative reaction mediated by oxidized LDL, aggregate there, and form thrombus and atherosclerosis. Therefore, perilla oil has attracted investigators' attention, because a diet rich in PUFA may be helpful in preventing heart diseases [18,25,26] and blood coagulation [13]. More importantly, it was demonstrated that most of the plant oils with high ω-6/ω-3 fatty acid ratios including canola oil, safflower oil, olive oil, corn oil, and soybean oil, increased hemorrhagic stroke in SHR-SP and shortened lifespan, except only perilla oil with a low ω-6/ω-3 fatty acid ratio [11,12,27,28].

Besides perilla oil, perilla seed extracts also have anti-allergic and anti-tumor activities [29,30]. In addition, perilla leaf extracts ameliorates obesity and dyslipidemia induced by a high-fat diet [31], and exerts anti-tumor [32], antioxidant, and neuroprotective effects [33].

It is well known that non-steroidal anti-inflammatory drugs including aspirin can induce gastric ulcers and bleeding at high doses [34]. Accordingly, there is a need for an effective improvement of blood flow without risk of adverse effects, and natural products should fulfill this requirement. In the present study, the perilla oil displayed excellent anti-platelet aggregation and anti-thrombotic activities *in vitro* and *in vivo*. Although additional exact action mechanisms remain to be clarified, it is suggested that perilla oil could be the first choice for the improvement of blood flow, especially in the hypertensive patients with a high risk of hemorrhagic stroke.

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