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Inhibitory effects of total flavones of *Hippophae Rhamnoides L* on thrombosis in mouse femoral artery and in vitro platelet aggregation

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Abstract

Total flavones of *Hippophae Rhamnoides L* (TFH) are extracted from Sea buckthorn, a Chinese herbal medicine. Sea buckthorn has antioxidant, anti-ulcerogenic and hepato-protective actions, and its berry oil is reported to suppress platelet aggregation. Though it is frequently used for patients with thrombosis, the likely mechanism(s) and effects of TFH on thrombogenesis remain unclear. Thus, we have investigated the effect in-vivo of TFH on thrombogenesis and in vitro on platelet aggregation, comparing them to those of aspirin.

We measured thrombotic occlusion time in a mouse femoral artery thrombosis model by the photochemical reaction between intravenously injected rose bengal and green light irradiation. In vitro platelet aggregation in whole blood was measured by single platelet counting. Thrombotic occlusion time was 8.5 ± 0.6 min in the control group. TFH at a dose of 300 μg/kg, intravenously administered 15 min before the rose bengal injection, significantly prolonged it to 11.6 ± 1.0 min ($P < 0.05$), a similar effect on in-vivo thrombogenesis to that of aspirin. TFH at a concentration of 3.0 μg/ml significantly ($P < 0.01$) inhibited in vitro platelet aggregation induced by collagen (2 μg/ml) in a concentration dependent manner, in contrast TFH did not affect aggregation induced by arachidonic acid (80 μM) and ADP (0.3 μM).

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The results of the present study, in which TFH prevented in-vivo thrombogenesis, probably due to inhibition of platelet aggregation, suggest a possible clinical approach for the prevention of thrombosis.

Keywords: Sea buckthorn; Thrombosis; Platelet aggregation; Flavones

Introduction

Platelet activation plays a pivotal role in the pathogenesis of thrombosis, which can lead to fatal diseases such as myocardial or cerebral infarction, and atherosclerosis [7,19,32]. Anti-platelet agents are frequently used for preventing those diseases [3], and many studies have analyzed the influence of various foods on them [2,4]. We demonstrated that geniposide, one of the constituents of Gardenia fruit (*Gardenia jasminoides* ELLIS, Rubiaceae), or genistein, a major isoflavone included in soy, inhibited in vitro platelet aggregation and prevented in-vivo thrombogenesis in an animal model [16,27], and that natto, a traditional fermented vegetable cheese-like soyfood, prevented neointimal formation following endothelial injury in experimental animals [28].

Total flavones of *Hippophae Rhamnoides L* (TFH) are extracted from Sea buckthorn (*Hippophae Rhamnoides L*), a Chinese herbal medicine, which is reported to have antioxidant, anti-ulcerogenic, and hepato-protective effects [5,10,17,26]. Also, its berry oil is reported to suppress platelet aggregation [15]. In China sea buckthorn is frequently used for patients with thrombosis. However, the mechanism(s) that most likely inhibit thrombogenesis remain unclear. Further, there are few reports on the effects of TFH on thrombogenesis. Thus, we have investigated the effects in-vivo of TFH on thrombogenesis and in vitro on platelet aggregation. In this study, the thrombotic occlusion of the mouse femoral artery was induced by photochemical reaction between an intravenous administration of rose bengal, one of the most efficient photosentitizers, and green light irradiation. The photo-excitation of rose bengal by green light produces single molecular oxygen by energy transfer. The singlet oxygen causes endothelial injury followed by platelet adhesion, aggregation and the formation of a platelet rich thrombus at the site of the photochemical reaction. This model is simple, reproducible and practical for evaluating antithrombotic effects of anti-platelet agents [12,23,24].

Method

Materials

Collagen was purchased from Nycomed Pharma GmbH, Munich, Germany. ADP was provided by MC Medical (Tokyo, Japan). Aspirin and arachidonic acid were obtained from Sigma (St. Louis, MO, USA). Rose bengal was from Wako Pure Chemical Co. (Osaka, Japan).

Preparation of total flavones of Hippophae Rhamnoides L

TFH was purchased from Yangguang Saji Co. (Liaoning, China). The extraction procedure was as follows: *Hippophae Rhamnoides* fruit was heated three times with 75% methanol for 90 minutes

each time. After being cooled to room temperature, the suspension was filtered and methanol removed. $NaHCO_3$ was added to neutralize the aqueous solution and the solution then concentrated by evaporation. The extract was dissolved in ethyl acetate and filtered again to remove impurities. After collecting the ethyl acetate, the amount of TFH in a final extract was 13% of the total dry weight. For the study, TFH was dissolved in dimethylsulfoxide (DMSO, 0.1% as a final concentration).

Thrombotic occlusion model

In the present study, a photochemical model was used in mouse femoral artery [16,27]. Briefly, 34 male ICR mice (5 weeks old, 24–30 g) were anesthetized with an intraperioneal injection of sodium pentobarbital (80 mg/kg) and fixed in a supine position. A cannula was inserted into the left jugular vein for the administration of the drug and rose bengal. The left femoral artery was carefully exposed and a pulsed Doppler flow probe was placed on it for monitoring blood flow (PDV-20, Crystal Biotech America, Hopkinton, MA, USA). Transillumination of the exposed segment with green light (wavelength = 540 nm) was achieved using a xenon lamp with heat absorbing and green filters (model 4887; Hamamatsu Photonics, Hamamatsu, Japan). The irradiation was led with an optic fiber mounted on a micromanipulator so that the head was approximately 3 mm away from the part of the left femoral artery proximal to the flow probe. After establishing the baseline blood flow, drugs were administered intravenously. Fifteen min after drug administration, irradiation was started and rose bengal (15 mg/kg) was infused for 5 min. The successful formation of occlusive thrombus was indicated by a complete cessation of blood flow for 1 min. The time required to achieve this complete cessation was measured as 'occlusion time'. TFH at doses of 100 and 300 μg/kg, dissolved in 0.1% DMSO, was intravenously administered at a volume of 0.02 ml/10g body at 15 min before the start of rose bengal infusion in 6 different mice. As control for TFH, 0.1% DMSO was administered as the same manner in another 6 mice. In 8 different animals, aspirin at 10 mg/kg, dissolved in 0.1 M $NaHCO_3$, was intravenously administered at a volume of 0.02 ml/10g body at 15 min before the start of rose bengal infusion. As a control for aspirin, 0.1 M $NaHCO_3$ was administered in the same manner to another 8 mice.

In vitro platelet aggregation in whole blood

Platelet aggregation was studied in whole blood by a single platelet counting method, as previously described with slight modification [16,22]. Thirty-one ICR mice were anaesthetized with sodium pentobarbital and blood was drawn from the inferior vena cava into prewarmed (37 °C) syringes containing 1/10 volume of 3.8% sodium citrate. The blood was then diluted with the same volume of prewarmed saline. Blood sampled from one animal was divided into a series of 5 plastic tubes in volumes of 280 μl aliquots. Various doses of drug or vehicle were added to the tubes, followed by incubation at 37 °C for 30 min. And then, 10 μl aliquots of collagen (2 μg/ml), arachidonic acid (80 μM) or ADP (0.3 μM) were added to the tubes which were shaken for 5 min at 140 shakes/min (collagen and arachidonic acid) or for 20 s (ADP) using an automatic shaker (Taiyo Incubator Personal, Taitec, Tokyo, Japan). Formaldehyde (1%) was then added to stop further aggregation or disaggregation. The effects of TFH and aspirin on platelet aggregation induced by collagen and arachidonic acid were tested in 5 and 6 mice, respectively. For evaluation of aggregation induced by ADP, 4 mice treated with TFH and 5 with

aspirin were examined. The number of platelets in each tube was determined using a whole blood cell counter (Celltac, Nihon Kohden, Tokyo, Japan). TFH at concentrations of 0.3 to 3.0 μg/ml dissolved in 0.1% DMSO or aspirin at concentrations of 0.03 to 1.0 mM dissolved in 0.1 M $NaHCO_3$ were added to each tube. As controls for TFH or aspirin, 0.1% DMSO or 0.1 M $NaHCO_3$, respectively were added to the tubes.

Statistical analysis

Results are expressed as mean ± S.E.M. Occlusion time data from animals treated with THF were statistically analyzed by one-way ANOVA (analysis of variance) followed by Dunnett's comparison test. Other data among more than three groups were analyzed by one-way ANOVA by Bonferroni's multiple comparison, and the data between two groups by unpaired student's t–test. A P value of < 0.05 was considered to be significant.

Results

Effect of TFH on thrombotic occlusion in the femoral artery

In animals injected with 0.1% DMSO as vehicle, the blood flow was thrombotically occluded by 8.5 ± 0.6 min after the start of rose bengal infusion under green light irradiation. Treatment with TFH at a dose of 300 μg/kg significantly ($P < 0.05$) prolonged occlusion time to 11.6 ± 1.0 min, while 100 μg/kg TFH did not affect it (Fig. 1A). TFH at 300 μg/kg had a similar effect to that of aspirin at the 10 mg/kg

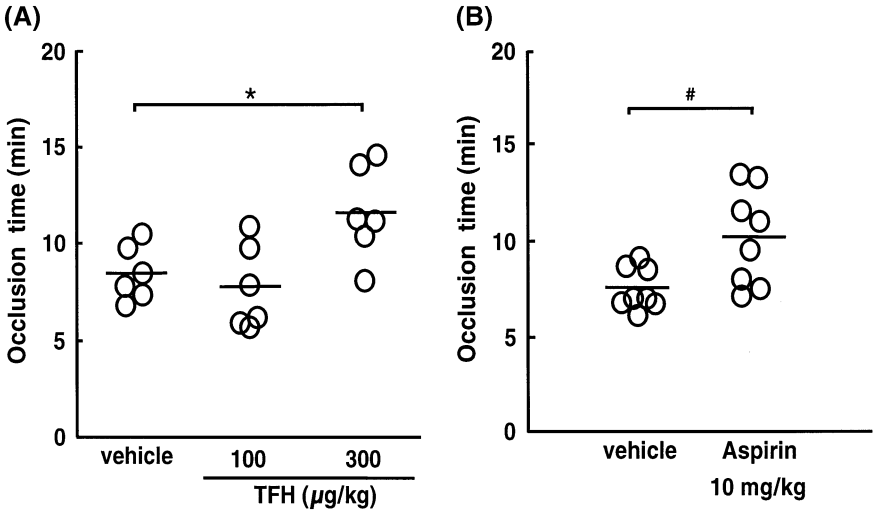

Fig. 1. Effects of (A) TFH and (B) aspirin on thrombotic occlusion time in mouse femoral artery induced by photochemical reaction. Each circle indicates individual data and bars represent mean values. *: $P < 0.05$ vs. control by Dunnett's multiple comparison test. #: $P < 0.05$ vs. control by unpaired student's t–test.

dose, which prolonged the occlusion time from 7.6 ± 0.4 min to 10.3 ± 0.9 ($P < 0.05$, Fig. 1B). There was no significant difference in the occlusion times between animals treated with 0.1% DMSO or 0.1 M $NaHCO_3$ as vehicles.

Effect of TFH on in vitro platelet aggregation in whole blood

The platelet counts with or without stimulants for platelet aggregation were not different between animals treated with 0.1% DMSO and 0.1 M $NaHCO_3$ as vehicles.

The stimulation with collagen (2 μg/ml) reduced platelet counts, which indicated the induction of platelet aggregation. TFH inhibited platelet aggregation induced by collagen in a concentration-dependent manner and the inhibitory effect of TFH at a concentration of 3.0 μg/ml reached statistical significance ($P < 0.01$, Fig. 2A). The inhibitory effect of aspirin on platelet aggregation induced by collagen is shown in Fig. 2B. Aspirin at a concentration of 1.0 mM significantly suppressed platelet aggregation ($P < 0.01$). TFH had a similar inhibitory effect to that of aspirin on platelet aggregation induced by collagen.

On the other hand, TFH did not inhibit arachidonic acid-induced platelet aggregation (Fig. 3A), in contrast to aspirin, which at concentrations of 0.1 and 0.3 mM significantly inhibited aggregation induced by arachidonic acid ($P < 0.01$, Fig. 3B). The inhibitory effect of aspirin on platelet aggregation induced by arachidonic acid was similar to that by collagen. Neither TFH nor aspirin affected platelet aggregation induced by ADP (0.3 μM, Fig. 4).

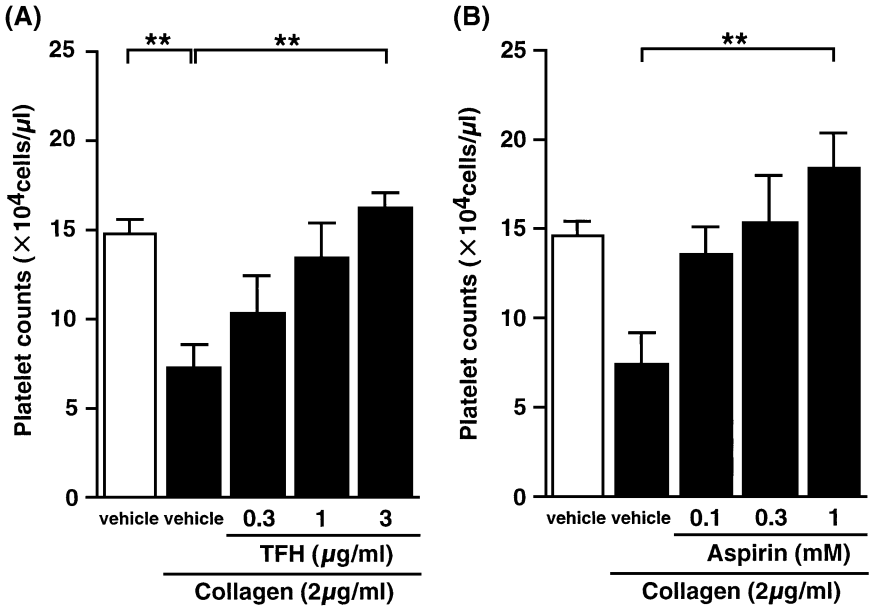

Fig. 2. Effects of (A) TFH and (B) aspirin on platelet aggregation induced by collagen (2 μg/ml) in whole blood in 5 mice. An open column indicates platelet counts in the tubes with added vehicle without collagen. Data are presented as means ± S.E.M. **; $P < 0.01$ by Bonferroni's multiple comparison test.

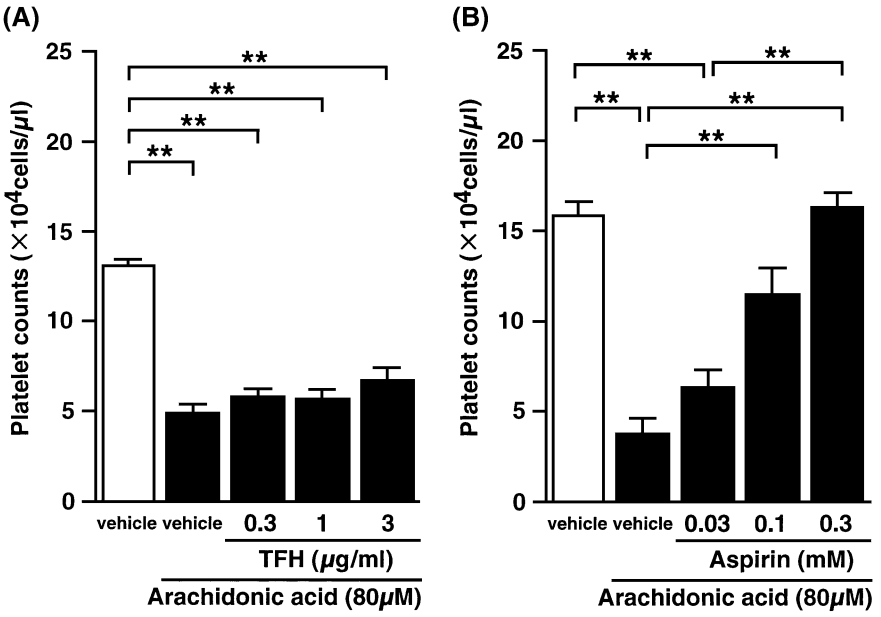

Fig. 3. Effects of (A) TFH and (B) aspirin on platelet aggregation induced by arachidonic acid (80 μM) in whole blood in 6 mice. An open column indicates platelet counts in the tubes with added vehicle without arachidonic acid. Data are presented as means ± S.E.M. **; $P < 0.01$ by Bonferroni's multiple comparison test.

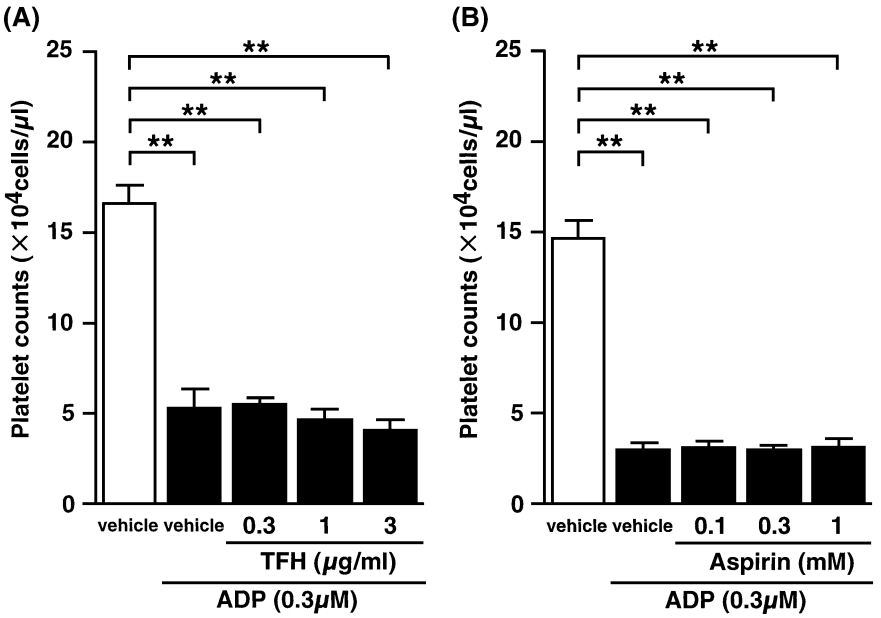

Fig. 4. Effects of (A) TFH and (B) aspirin on platelet aggregation induced by ADP (0.3 μM) in whole blood in 4 and 5 mice, respectively. An open column indicates platelet counts in the tubes with added vehicle without ADP. Data are presented as means ± S.E.M. **; $P < 0.01$ by Bonferroni's multiple comparison test.

Discussion

We have investigated in-vivo the effects of TFH on thrombogenesis, and in vitro on platelet aggregation, and compared them with those of aspirin. TFH and aspirin had similar effects on in-vivo thrombogenesis. TFH inhibited in vitro platelet aggregation induced by collagen, but not by arachinonic acid and ADP. Aspirin is known to suppress thromboxane A_2 production by inhibiting cyclooxygenase. In the present study, aspirin suppressed platelet aggregation induced by both collagen and arachidonic acid, while TFH inhibited just that induced by collagen. These findings suggest that TFH mainly inhibits platelet aggregation due to suppression of arachidonic acid synthesis by stimulation of the collagen receptor.

It has been reported that collagen receptor stimulation leads to tyrosine phosphorylation of Syk or Src, followed by phospholipase C-gamma 2 activation [13,21]. Tyrosine kinase activation increases intracellular calcium and activates phospholipase A_2 (PLA_2), followed by synthesis of arachidonic acid from phospholipids in plasma membrane [14,18]. When the flavones in *Hippophae Rhamnoides L* were analyzed using multidimensional counter-current chromatograms, the contents were mainly isorhamnetin, kaempferol and quercetin [33]. It has been reported that quercetin inhibited tyrosine kinase activity in rat lung [25], but there was no effect on intracellular PLA_2 from human platelets [9]. Further, quercetin inhibited the rise of intracellular calcium concentration in human platelets [31]. Kaempeferol has been reported to inhibit platelet aggregation by suppression of tyrosine kinase activity [1,6]. In contrast, isorhamnetin was reported to be ineffective on platelet aggregation [30]. These findings suggest that TFH may suppress platelet aggregation induced by collagen, probably due to the inhibition of tyrosine kinase activity.

However, quercetin has been reported to inhibit rabbit platelet aggregation induced by arachidonic acid and ADP [29]. In the present study, TFH did not inhibit mice platelet aggregation induced by arachidonic acid and ADP. The discrepancy between our results and evidence reported by Tzeng et al. might be explained by species difference and/or different preparations of platelets. Further, TFH mainly consists of flavones, but also includes a lot of unknown lipophilic impurities, which may contribute to any inhibition of platelet aggregation [33]. It is also possible that other substances in TFH enhanced the effect of flavones. For instance, the combination of quercetin and catechin is reported to synergistically suppress collagen-induced platelet aggregation [20]. Thus, a combination(s) of some flavones in TFH may have strongly suppressed collagen-induced platelet aggregation.

In the present study, TFH had an effect similar to that of aspirin on in-vivo thrombogenesis; and it has been reported that aspirin prevents secondary events in cardiac and cerebral diseases [8]. Based on these findings, a daily taking of TFH is also expected to prevent such events. Further, TFH protects the oxidation of low-density cholesterol and inhibits inflammation by immunomodulation [10,11]; effects that might contribute to the prevention of the progression of atherosclerosis.

In conclusion, TFH prevented in-vivo thrombogenesis, probably due to the inhibition of platelet aggregation. The results of the present study suggest a potential clinical approach for the prevention of cardiac and cerebral thrombosis.

Acknowledgements

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The experimental protocol was reviewed and approved by the Animal Experiments Committee of Hamamatsu University School of Medicine.

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