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Regular Article

Anti-thrombotic effect of proanthocyanidin, a purified ingredient of grape seed

Takashi Sano[a], Etsuko Oda[a], Tsutomu Yamashita[a], Aki Naemura[a], Yoshinobu Ijiri[a], Jun Yamakoshi[b], Junichiro Yamamoto[a,*]

[a]*Laboratory of Physiology, Faculty of Nutrition, and High Technology Research Centre, Kobe Gakuin University, Ikawadani-cho, Nishi-ku, Kobe 651-2180, Japan*
[b]*Research and Development Division, Kikkoman Corporation, Noda, Noda City, Chiba, Japan*

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Abstract

Introduction: Moderate and regular consumption of wine reduces the risk of acute coronary thrombotic events. The mechanism of the anti-thrombotic effect of wine is not clear. Extract or purified ingredients of grapes have not yet been studied for anti-thrombotic effect. *Materials and methods*: Anti-thrombotic effect of proanthocyanidin, a highly purified ingredient of grape seed, was assessed by a shear-induced thrombosis test in vitro and by a laser-induced thrombosis test in the mouse carotid artery, in vivo. *Results and conclusions*. Intravenously (20 mg/kg body weight, BW) or orally (2×200 mg/kg BW) administered proanthocyanidin significantly inhibited the laser-irradiation induced thrombus formation in the carotid artery (both P=0.01). Subsequent to oral administration of proanthocyanidin, in vitro platelet reactivity to shear stress has been inhibited. The latter suggests that the in vivo anti-thrombotic effect of proanthocyanidin may be due to a direct inhibitory effect on platelets.
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Introduction

Prevention of life-style related atherothrombotic diseases such as myocardial infarction and stroke is important and urgent social task in the developed countries. Epidemiological studies provided irrefutable evidence for the causative role

* Corresponding author. Tel.: +81 78 974 1551; fax: +81 78 974 5689.
E-mail address: yamamoto@nutr.kobegakuin.ac.jp (J. Yamamoto).

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of inappropriate diet both in the development and clinical outcome of thrombotic diseases. Regular consumption of diet or nutritional supplements of experimentally proven anti-thrombotic effect may offer effective and economical way of preventing thrombotic events.

Mortality from cardiovascular diseases is significantly lower in French than in people from other countries having similar high fat diet [1]. It has been suggested that the French's habitual and large amount of red wine consumption is responsible for the lower cardiovascular mortality [2]. This so-called "French Paradox" has accelerated laboratory studies for finding anti-thrombotic fruits and vegetables and for identifying the effective ingredients. Grapes and grape products have been studied for platelet inhibitory and anti-thrombotic effects [3–7]. Other studies showed that 3-week intake of proanthocyanidin, a purified substance of grape seeds, protected from myocardial infarction subsequent to reperfusion [8–10]. The aim of the present study was therefore to assess the anti-thrombotic activity of purified proanthocyanidin by experimental models, which reflect arterial thrombogenesis.

The common technique of assessing anti-thrombotic effect of foods and vegetables is to use animal models. By the Folts' model of coronary stenosis, Demrow et al. [3] and Osman et al. [4] demonstrated that red wine (1987 Chateauneuf-du-Pape) and Welch's natural purple grape juice inhibited thrombogenesis in vivo. Freedman et al. [6] and Shanmuganayagam et al. [7] made unsuccessful attempts to identify the active material(s).

In arterial thrombotic diseases such as cardiovascular disease and stroke, platelets play a pivotal role. The conventional in vitro platelet function test is the platelet aggregometry, which measures activation and aggregation response of platelets to different stimuli such as collagen, adenosine diphosphate or epinephrine. However, platelet aggregation induced by high shear forces is more relevant to pathology than the agonists-induced platelet aggregation test [11,12]. Further, because of the effect of all anticoagulants on platelets and coagulation, a test, which is performed from native, non-anticoagulated blood has much more relevance to in vivo than those conventional tests, which use anticoagulated blood [13–20]. In this study, a shear-induced in vitro platelet function test from native blood sample was used for screening followed by the helium–neon laser-induced in vivo thrombogenesis test performed in the carotid artery of atherosclerosis-prone mice.

Materials and methods

Chemicals

Purified proanthocyanidin preparation (Gravinol-S, GSE) was donated by Kikkoman (Japan); GSE was composed of 89.3% proanthocyanidins, which contained 6.6% dimer, 5.0% trimer, 2.9% tetramer and 74.8% oligomers and polymers bigger than pentamer, 6.6% monomeric flavanols (2.5% (+)-catechin, 2.2% (−)-epicatechin, 1.4% (−)-epigallocatechin and 0.5% (−)-epigallocatechin gallate), 2.24% water, 1.06% protein and 0.8% ash. Sodium pentobarbital was purchased from Abbott. Lab. (USA) and Evans blue dye from Merck (Germany).

Animals

Male C57BL/6 mice were obtained from SLC (Japan). Mice were kept in air-conditioned rooms (22.5±0.5 °C and humidity 50±5%) with 12-h light and dark cycle. Animals had free access to standard solid chow (CE-2, Japan Clea, Tokyo, Japan) and drinking water ad libitum. All procedures were conducted in compliance with the "Guiding Principles for the Care and Use of Animals in the Field of Physiological Science", published by the Physiological Society of Japan.

Laser-induced thrombogenesis test

The helium–neon laser-induced thrombogenesis method has been described in details elsewhere [21–23]. Mice were anaesthetized with Nembutal (60 mg/kg, i.m.). The carotid artery (450–500 μm in diameter) was exposed by incision and a polyethylene tube (PE10, Becton Dickinson and Company, USA) was inserted into the left femoral artery to inject the dye. The mouse was placed on a special microscope stage (Olympus Model BH-2, Olympus, Japan), Evans blue dye (30 mg/kg) was injected and then the center of the exposed carotid artery was irradiated with laser (Model Neo-50 MS, 30 mW power, Nihon Kagaku Engineering, Osaka, Japan). Thrombus formation at the site of irradiation was monitored under epi-illumination and simultaneously recorded on videotape using CCD camera (Model TMC-7, Takenaka System, Kyoto, Japan).

Calculation of thrombus size

Details of this technique have been described [23]. An image-frame of the forming thrombus was

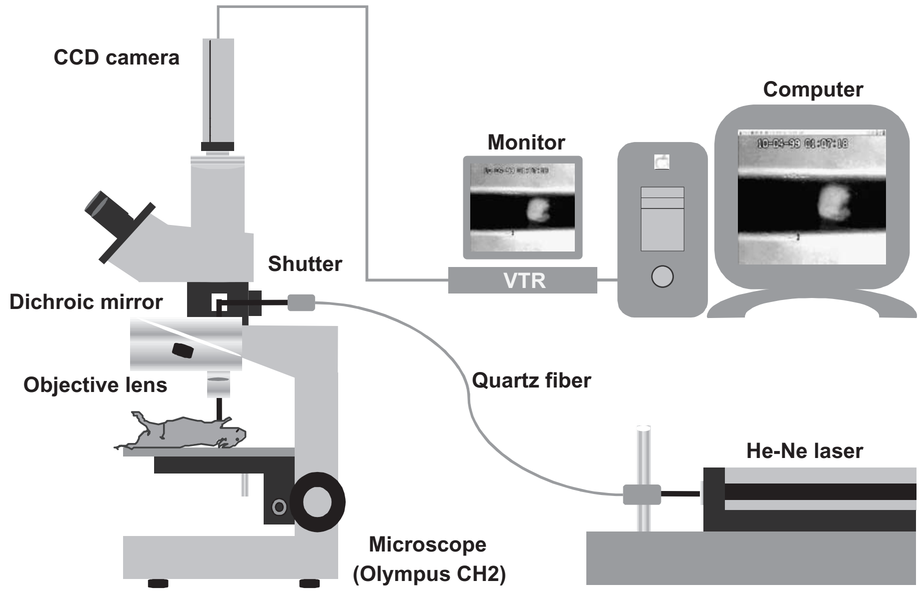

Figure 1 A helium—neon laser-induced thrombogenesis system.

computer-analyzed (Windows) in every 10 s. Thrombus mass was outlined by a defined threshold gray scale and the area of thrombus was calculated. The size of thrombus was calculated by multiplication of the area and gray scale. Image analysis was performed by software, Image-Pro Plus (Media Cybernetics, USA). Thrombotic status was expressed by the total sum of sizes obtained in 10

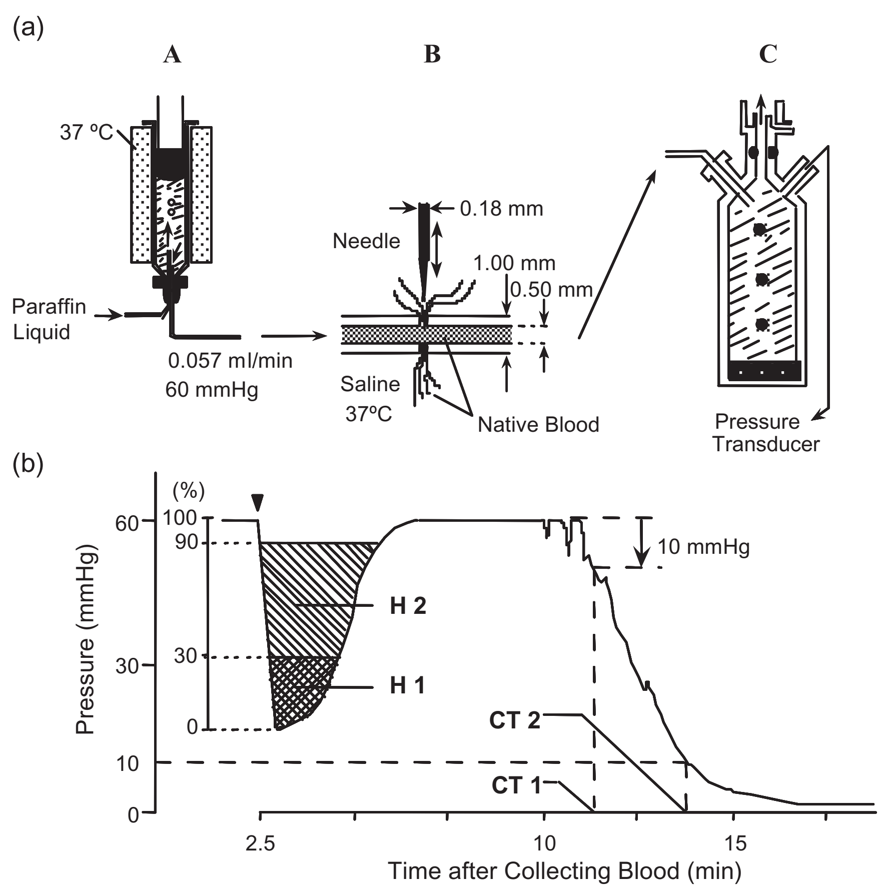

Figure 2 Principle of haemostatometry (a) and a typical haemostatogram (b) H1, 30% pressure recovery; H2, 90% pressure recovery; CT1, start of dynamic coagulation; CT2, completed dynamic coagulation.

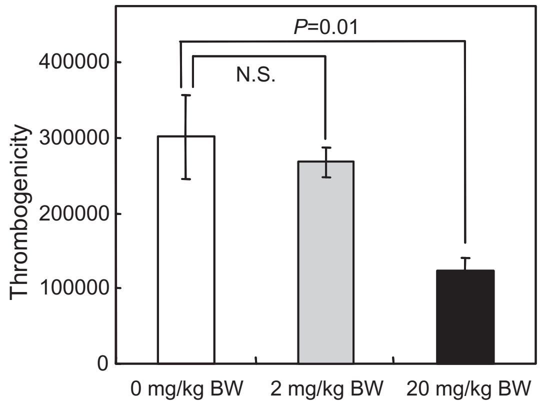

Figure 3 Anti-thrombotic effect of intra-arterially administered proanthocyanidin assessed by a helium—neon laser-induced thrombosis model in the carotid artery of C57/BL6 mice. Proanthocyanidin or the solvent was injected and laser irradiation was started 5 min after injection. Results are expressed in mean±S.E.M. (*n*=4, respectively). N.S.: not significant.

min after irradiation. The helium—neon laser-induced thrombogenesis system is shown in Fig. 1.

Measurement of shear-induced platelet function (haemostatometry)

Details of haemostatometry have been described [13—15,17]. A three-channel Haemostatometer was used, identical with the original Haemostatometer [13]. The instrument was purpose-built in the Laboratory of Physiology, Faculty of Nutrition, Kobe Gakuin University and it was significantly different from the once marketed instrument called Clot Signature Analyzer (CSA). Blood was withdrawn from the abdominal aorta of Nembutal-anaesthetized mice (60 mg/kg, i.m.). The principle of haemostatometry is shown in Fig. 2. Briefly, liquid paraffin was pumped into the blood sample thereby displacing blood from the syringe through a plastic tube into a reservoir. The blood flow in the tubing was 0.057 ml/min, which resulted in a steady 60 mm Hg perfusion pressure. The tubing was pierced through with a fine needle (diameter: 0.18 mm) at (B) 150 s after blood withdrawal, causing "bleeding" into the surrounding warmed saline. The initial shear stress was 375 dyn/mm^2. Eventually, platelet-rich haemostatic plugs occluded the holes, so the "bleeding" has stopped. Subsequent to such haemostasis, coagulation occurs, which arrests the flow in the main tubing (dynamic clotting time). Pressure changes, which accompanied the above events, were monitored and analyzed by computer. Areas of the pressure recordings showing 30% (H1) and 90% (H2) pressure recovery after the initial pressure drop due to piercing were used as indices of platelet reactivity. The time from blood withdrawal until the first drop of perfusion pressure ≥10 mm Hg (CT1) and to a level of ≤10 mm Hg (CT2) reflect coagulation. H1 and H2 were used as indices of platelet reactivity (platelet adhesion plus platelet aggregation) and CT1 and CT2 as dynamic coagulation. Increase of H1 and H2 indicated inhibited platelet reactivity, while decrease of H1/H2 showed enhanced platelet reactivity. Prolongation of CT1 and CT2 indicated inhibition of dynamic coagulation while shortening of CT1/CT2 showed hypercoagulation.

Administration of proanthocyanidin

Proanthocyanidin was dissolved in saline—dimethylsulfoxide mixture (4:1; 2 or 20 mg/ml).

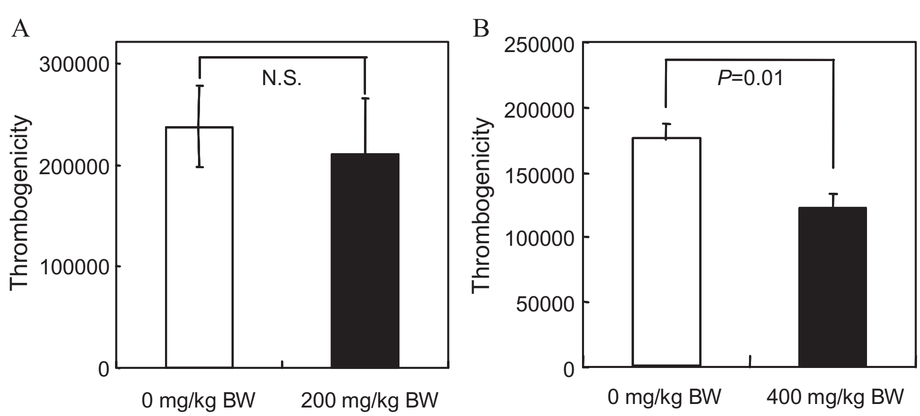

Figure 4 Anti-thrombotic effect of orally administered proanthocyanidin assessed by helium—neon laser-induced thrombosis model in the carotid artery of C57/BL6 mice. Proanthocyanidin or the solvent was orally administered once in A (200 mg/kg BW, *n*=4 in each group) and twice with 30 min interval in B (200 mg/kg BW×2, *n*=7 in each group). Laser irradiation was started 60 min after the last administration. Results are expressed as mean±S.E.M. N.S.: not significant.

Proanthocyanidin was injected into the left femoral artery (2 or 20 mg/kg body weight, BW). Laser-irradiation started 5 min after intra-arterial injection of proanthocyanidin. In case of oral administration, laser irradiation started or blood was withdrawn from abdominal aorta 60 min after a single dose or a second dose of proanthocyanidin (20 mg/ml; 200 mg/kg BW). In case of twice two doses, there was a 30-min interval between the two administrations.

Statistical analysis

Results were expressed as means±S.E.M. Calculating significance, H1 and H2 were converted to logarithmic values. Statistical evaluation was performed by ANOVA, followed by Fisher's post hoc test using commercially available statistical package Stat View (ver. 5.0, SAS Institute, NC, USA). $P<0.05$ was considered to be statistically significant.

Results

Effect of intra-arterial proanthocyanidin

Results are shown in Fig. 3. A 2 mg/kg BW had no significant anti-thrombotic effect (300541±55922 vs. 267081±20602; proanthocyanidin vs. controls), but 20 mg/kg BW significantly inhibited thrombus formation (300541±55922 vs. 136051±16998; proanthocyanidin vs. controls).

Effect of oral proanthocyanidin

Results are shown in Fig 4. A single administration of 200 mg/kg BW did not inhibit thrombus formation (262229±44447 vs. 223014±32149; proanthocyanidin vs. controls) but repeated (×2) 200 mg/kg BW dose (400 mg/kg BW) significantly inhibited laser-induced thrombogenesis (174638±13502 vs. 121094±13000; proanthocyanidin vs. controls).

Effect of oral proanthocyanidin on platelet reactivity ex vivo

Subsequent to two oral proanthocyanidin doses (30 min apart), blood was withdrawn from mice and the effect on shear-induced platelet reaction was assessed by haemostatometry. Results are shown in Fig. 5. Compared to controls, proanthocyanidin increased H1 and H2 thus inhibiting platelet reactivity (H1: 452.3±52.9 vs. 588.1±58.4, $P=0.08$; H2: 2289±209.2 vs. 3669±403.5, $P<0.05$). This treatment did not affect clotting times CT1 and CT2 (data not shown).

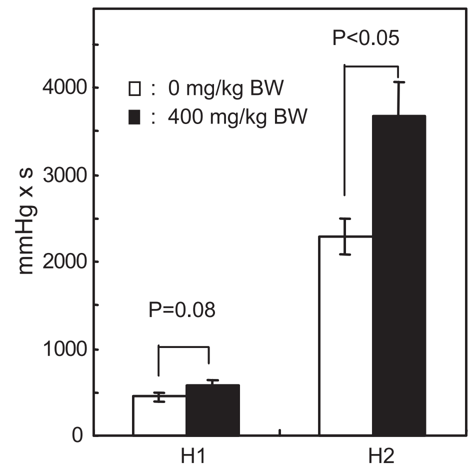

Figure 5 Inhibitory effect of orally administered proanthocyanidin on shear-induced platelet reactivity (ex vivo). Blood was withdrawn 60 min after the second administration of proanthocyanidin or the solvent (control). Proanthocyanidin was administered twice as in Fig. 4B (200 mg/kg BW×2). The effect on platelet function was measured by haemostatometry ($n=11$ in each group). Results are expressed mean±S.E.M.

Discussion

Grapes, their ingredients and grape products were shown to inhibit platelet aggregation in vitro and thrombus formation in vivo [3–7]. Earlier studies in rodents have shown that intake of proanthocyanidin, a purified ingredient of grape seeds protects from myocardial infarction after reperfusion injury [8–10]. However, the chemical characteristic and the mechanism of action of the effective ingredient(s) of grapes have not yet been clarified. There are numerous epidemiological and experimental studies on grapes and their products, but the findings are inconsistent [3,4,24,25]. This may partly be due to the different sources of grapes and grape products. In our earlier study on tomatoes, we emphasized the importance of sources in assessing the anti-thrombotic effect of fruits and vegetables [26].

Previous studies showed that grape juice inhibited ADP-induced platelet aggregation and arterial thrombus formation [3,6]. Antioxidant activity of grapes was suggested as a possible mechanism for the anti-thrombotic effect [3,6]. Partially purified materials from seeds and skins of grapes further increased the anti-platelet activity of grape juice [7]. Other studies demonstrated a cardioprotective effect of proanthocyanidin, and suggested that the reactive oxygen species scavenging activity of proanthocyanidin could be a mechanism of the cardioprotective effect [8–10]. In addition to such protective effect against vascular damage, our present study demonstrates a significant anti-platelet and anti-thrombotic effect of proanthocyanidin. The mechanism of the latter may also involve the antioxidant activity, which may protect both platelets and coronary vessels. It has been shown that highly polymerized proanthocyanidins have increased antioxidant activity [27]. The current proanthocyanidin preparation contains 74.8% pentamer or bigger polymers out of 89.3% content of proanthocyanidin, suggesting that phenol groups of highly polymerized proanthocyanidin may play important role in antioxidant and anti-thrombotic activities.

Although in the present study the effective dose of proanthocyanidin (400 mg/kg, p.o.) was higher than that reported earlier (50–100 mg/kg, p.o.), the latter was observed after daily intake over 3 weeks [8–10]. This suggests that long-term regular intake may decrease the effective dose considerably. Taken into account the resistance of rodents to antiplatelet drugs, so that the effective dose of aspirin in rats is ×30 higher [28], and the effective dose of ticlopidine in rats is ×80 higher [29] than in humans, we think that less than 1 g of proanthocyanidin can exert an anti-thrombotic effect in human.

In conclusion, physiologically relevant animal models of platelet function and arterial thrombus formation demonstrated a significant anti-thrombotic effect of proanthocyanidin. The mechanism may involve direct protective effect on platelets and blood vessels. Potentially, proanthocyanidin could be beneficial in atherothrombotic diseases in humans.

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