Reevaluation of antithrombotic fruits and vegetables: great variation between varieties

Junichiro Yamamoto1,*, Yoshinobu Ijiri2, Yukinori Tamura3, Masahiro Iwasaki4, Masahiro Murakami5, Yoshio Okada1

1 Kobe Gakuin University, Kobe, Japan; 2 Faculty of Health and Nutrition, Osaka Shoin Women’s University, Osaka, Japan; 3 Faculty of Nutrition, Kobe Gakuin University, Kobe, Japan; 4 Division of Nutrition and Metabolism, Original Nutrition Co., Ltd., Osaka, Japan; 5 Faculty of Pharmacy, Osaka Ohtani University, Osaka, Japan.

1. Introduction

Prevention of arterial thrombotic diseases has higher priority than treatment of existing diseases. Compared to the ineffective Western-style diet, clinical trials provided evidence for reduced risk of arterial thrombosis and death from coronary heart disease in people on Mediterranean, Vegetarian and Japanese-style diets (1-9). As to the mechanism of such an antithrombotic effect, several nutrients and components of foods (omega-3 fatty acids; red wine; onion, garlic, kiwi; chocolate, etc.) were shown to inhibit platelet function in vitro (10).

In finding foods and dietary components with a potential antithrombotic effect, the use of pathologically relevant technique(s) is of crucial importance. Only those test(s) which have already proved to be useful in clinical practice in monitoring the overall thrombotic status and predicting major adverse thrombotic events should be used for screening dietary components and nutrients for the antithrombotic effect. Despite that platelets play a pivotal role in thrombosis, point-of-care platelet function tests failed to materialize clinical expectations. Tailoring antithrombotic medication based on monitoring platelet function by these tests...
did not improve clinical outcome (11-13). At present, prothrombotic status is assessed by measuring platelet aggregation to various soluble agonists (adenosine diphosphate, collagen, arachidonic acid, thrombin), and by extrapolating the results obtained using various biomarkers of coagulation and fibrinolysis. The major shortcoming of all these tests is the use of anticoagulated blood, in which activated platelets do not generate thrombin, the most significant contributor to arterial thrombogenesis. This could be the reason why most platelet function tests which measure platelet aggregation to various soluble agonists failed in guiding cardiac patients antithrombotic medication (14-19).

Evidence has been presented that only those tests, which take the arterial high shear and flow conditions as well as generation of thrombin by activated platelets into account, have relevance for the pathomechanism of occlusive arterial thrombosis in vivo. We compared results obtained from platelet function tests performed with anticoagulated blood and those obtained using shear-induced thrombosis and thrombolysis tests performed from non-anticoagulated blood. Our findings show that the commonly used platelet function tests performed at low shear conditions and from anticoagulated blood do not reflect the overall thrombotic status, while the innovative shear-induced thrombosis tests performed from non-anticoagulated blood do (20,21). We have shown in animal experiments that the combined use of high shear stress-induced thrombosis in vitro tests using non-anticoagulated blood (hemostatometry and global thrombosis test, GTT) in combination with the flow-mediated vasodilation in vivo test (FMV or FMD) provides reliable assessment of the global thrombotic status (22). In addition, GTT has been shown to be clinically useful for monitoring thrombotic status in patients on antithrombotic medication (23,24). It was therefore reasonable to employ these techniques to test fruits and vegetables and herbal drugs for antithrombotic effects.

2. In vitro tests

2.1. Shear-induced platelet-rich thrombus formation in non-anticoagulated blood

2.1.1. Hemostatometry

Details of hemostatometry have been described previously (25,26). Briefly, non-anticoagulated blood was withdrawn from the abdominal aorta of animals and tested with a hemostatometer built for this purpose in Kobe Gakuin University. Blood was forced to flow through a plastic tube by a paraffin oil replacement technique. While blood was flowing in it, the tubing was punched with a fine needle to induce "bleeding" from the holes into the surrounding warm saline. The perfusion pressure was monitored to assess the thrombotic reaction. Punching the tube caused a sharp drop in the perfusion pressure. Eventually "bleeding" stopped due to formation of platelet-rich hemostatic plugs in the holes and with this, the perfusion pressure returned to the pre-punching level. The recorded pressure changes reflect both the hemostatic and coagulation processes. In the recorded pressure curve, areas of 30% (H1) and 90% (H2) pressure recovery reflect the primary and completed hemostasis. Increase or decrease of H1 and H2 reflected inhibition or enhancement of hemostatic plug formation (platelet reactivity), respectively. In some occasions increased pressure was used to induce thrombolysis (Figure 1A).

2.1.2. GTT

GTT (Thromboquest Limited, London, UK) has been described in detail (23,24,27,28). Figure 1B shows the embodiment (a) and the principle of the technique (b) and a typical recording (c). There are flat segments along the inner wall of a conical plastic tube and when perfectly round ceramic ball bearings are placed into such a conical tube, the flat segments prevent the ball bearings from occluding the lumen. When non-anticoagulated blood is added to such a tube, it flows through the narrow gaps by the ball bearing and exits in droplets into an adjacent collecting tube. The latter is transilluminated by a light emitter and a sensor opposite the emitter generates a signal whenever a drop of blood interrupts the light path. In essence, the instrument detects the time interval (d; sec) between consecutive blood drops. Blood flows at 37°C by gravity through the narrow gaps formed between the upper ball bearing and the inner wall of the tube, where high shear stress activates and aggregates platelets. Platelet aggregates formed and then captured in the gaps by the lower ball bearing, arrest the blood flow. At the start, blood flow is rapid and hence (d) is small. Subsequently, the flow rate gradually decreases and hence (d) increases. When the actual (d) exceeds 15 seconds (occlusion-d), the instrument displays "Occlusion Time (OT)", which is the time elapsed from the detection of the first drop of blood until OT. Later, the blood flow is completely arrested. Eventually, due to thrombolysis, flow is restored as indicated by the detection of the first drop of blood after complete occlusion (Lysis Time- LT). Compared to controls, increased or decreased OT indicates inhibition or enhancement of platelet reactivity, respectively. Increase or decrease of LT indicates inhibition or enhancement of spontaneous thrombolysis, respectively. GTT can measure platelet reactivity and endogenous thrombolytic activity simultaneously.

3. In vivo tests

3.1. Laser-induced thrombosis in the microcirculation and in the carotid artery of experimental animals
mediated vasodilation (endothelium-independent vasodilation) was induced by placing 70 microliters of 2.2 mM nitroglycerin/saline solution on the artery. A typical pattern of vasodilation after restoration of flow was transferred to a computer and the artery diameter changes were calculated. Changes in vessel diameter after restoration of flow were expressed as percentage of the baseline values (before clamping) and the peak vasodilation was calculated. A typical pattern of these is shown in Figure 3.

4. Screening antithrombotic fruits and vegetables by shear-induced thrombosis/thrombolysis in vitro tests, followed by He-Ne laser-induced in vivo test

Since overall antithrombotic and prothrombotic activities of fruits and vegetables were varied from varieties to varieties and determined by the balance between antithrombotic activity (platelet reactivity) and endogenous thrombolytic activity (fibrinolytic activity), special attention was paid to the sources of fruits and vegetables. Fruits and vegetables were ground using mortar and pestle. Juices obtained were prepared by filtration (test samples). One tenth volume of the test sample was mixed with nine tenths volume of non-anticoagulated rat blood collected from rat abdominal aorta immediately before the tests. Antithrombotic, prothrombotic and thrombolytic activities were
measured by shear-induced thrombosis/thrombolysis *in vitro* test (hemostatometry or GTT) after quick mixing. The intensity of antithrombotic, prothrombotic and thrombolytic activities was expressed as the maximum dilution factor. At first these activities were screened using raw test samples, subsequently assessed using heat-treated (5-10 min) samples. Those samples which showed a significant antithrombotic or thrombolytic effect were administered to mice orally and tested by He-Ne laser-induced thrombosis *in vivo* test.

4.1. *Antithrombotic vegetables*

The overall effect of administered fruit or vegetable extracts on the *in vivo* thrombotic status is determined by the balance between thrombotic activity (effect on the growth of a platelet-rich thrombus) and thrombolytic activity (disintegration or fibrinolysis of the formed thrombus). For this reason, first we used relevant *in vitro* tests for screening and the active varieties were further tested *in vivo* to assess the overall antithrombotic effect.
4.1.1. Tomatoes

Ordinary size tomatoes Twenty-one varieties were measured by hemostatometry in vitro. The antithrombotic activity was dependent on the variety. The varieties were ranked into subgroups according to their activities, i.e. the group inhibiting platelet rich thrombus formation (antithrombotic group), the one enhancing the rate of thrombosis (prothrombotic group) and the group without effect (non-thrombotic group). Ten varieties were antithrombotic, out of them three had a highly significant antithrombotic effect. Two varieties showed a prothrombotic effect while in four varieties the effect was not clear. One variety with the strongest and heat stable antithrombotic effect was selected for further investigation. When tested in vivo, oral administration of this variety showed significant antithrombotic activity. This decreased during maturation (49).

Mini-type tomatoes Antithrombotic activity of four varieties was measured by the in vitro test GTT. Antithrombotic activities showed great variation between varieties, one was antithrombotic while the other three had no such effect. The antithrombotic activity decreased during maturation. Despite earlier suggestion of polyphenolic rich foods are antithrombotic, lycopene content was independent of antithrombotic activity. Thus, lycopene content cannot be used as an index of antithrombotic activity (50).

4.1.2. Onions

Onions from Hokkaido (Northern area in Japan) Antithrombotic activity of ten varieties was measured by the in vitro tests hemostatometry. Three varieties inhibited thrombus formation, one enhanced the rate of thrombus growth while six varieties had no effect on experimental thrombus formation. Five varieties enhanced endogenous thrombolytic activity while five varieties had no effect on it. Considering the balance between thrombotic and thrombolytic activities, one variety with the strongest antithrombotic effect was selected for further investigation. Subsequently, the antithrombotic activity of this variety was tested after oral administration in mice by the He-Ne laser-induced thrombosis in vivo test. This variety was heat stable (51).

Onions from Awaji Island, Hyogo (Middle area in Japan) Antithrombotic activity of five varieties was measured by the in vitro test GTT. One variety, which was antithrombotic by GTT test was further investigated by the laser-induced thrombosis test and the antithrombotic activity was confirmed after oral administration to mice. This activity was heat stable. Another variety inhibited endogenous thrombolytic activity, suggesting an overall prothrombotic activity (22,32).

4.1.3. Strawberry

Antithrombotic activity of seventeen varieties was measured by hemostatometry in vitro. Ten varieties inhibited platelet reactivity (antithrombotic), six varieties had no effect on thrombus formation and one could not be determined by this test. Varieties were ranked into subgroups on the basis of the intensity of their antiplatelet effect. Three varieties with the strongest antiplatelet activity were heat stable. Antithrombotic activity of these three varieties was demonstrated after oral administration in mice by the laser-induced thrombosis test in vivo (53).

4.1.4. Potatoes

Potatoes harvested in the spring Antithrombotic activity of twenty varieties was measured by the in vitro test GTT and ranked into subgroups. Three varieties, all heat-stable, were selected as antithrombotic varieties. Subsequently, antithrombotic activity was measured after oral administration in mice by the laser-induced thrombosis test. All three varieties showed antithrombotic activity in vivo (54).

Potatoes harvested in the autumn Antithrombotic activity of seven varieties was measured by the in vitro test GTT. Six varieties inhibited platelet reactivity and the antithrombotic activities were heat stable. One variety (heated) was further examined by the laser-induced thrombosis test in vivo and demonstrated to be prothrombotic under in vivo conditions (55).

4.1.5. Carrots

Antithrombotic activity of fifteen varieties and heat stability of selected varieties were measured by the in vitro test GTT. Effect on thrombus formation in vivo was measured by the laser-induced thrombosis test. Results of three varieties are shown in Figure 4.

As demonstrated by the laser in vivo test, the variety SAKATA-0421 inhibited platelet reactivity and enhanced endogenous thrombolysis, but after heat treatment, the inhibitory effect on platelets disappeared but the enhancing effect on endogenous thrombolytic activity remained (Figure 4A). SAKATA-0418 did not inhibit platelet reactivity but enhanced endogenous thrombolysis before heat treatment in vitro. After heat treatment, platelet reactivity was enhanced but effect on endogenous thrombolytic activity disappeared, suggesting prothrombotic activity in vivo (Figure 4B). The variety SAKATA-0420 enhanced platelet activity and endogenous thrombolytic activity before heat treatment and had no effect on the in vivo test. After heat treatment, it did not affect platelet reactivity but the effect on endogenous thrombolytic activity remained (Figure 4C). These findings showed that the in vivo effect on thrombosis variables can be predicted by the
Figure 4. Effect of heat treatment of carrot variety (SAKATA-0421) (A), (SAKATA-0418) (B) and (SAKATA-0420) (C) on platelet reactivity (OT) and endogenous thrombolytic activity (LT) measured in vitro by GTT (a) and on thrombosis measured in vivo by He-Ne laser-induced thrombosis test (b). (Revised; Yamamoto et al: Blood Coagul Fibrinolysis 2008; 19:785-792.)
The antithrombotic activity and heat stability of twenty-five herb species were measured by the in vitro test hemostatometry. Herbs were classified into subgroups on the basis of their antithrombotic activity. Thirteen herbs were antithrombotic, five prothrombotic, six non-thrombotic and one undetermined. As to the mechanism of the heat stable antithrombotic effect of some herbs, we found that the antithrombotic effect was due to inhibition of platelet reactivity. Because at that time the GTT technique was not available, the quantitative effect on endogenous thrombolysis could not be measured. The antithrombotic effect was not related to a protection of endothelial function as measured by FMV (57).

4.1.7. Sesame

Whole grains of six accessions (varieties) were roasted at 110°C for 10 min and crushed. Diet containing whole grain flour was given to mice for 12 weeks and antithrombotic activity was measured by the laser-induced in vivo thrombosis test. Two accessions were antithrombotic and one variety showed prothrombotic effect (58).

4.1.8. Rice

The antithrombotic activity of five varieties (non-glutinous white rice) was measured. Diet containing non-glutinous white rice was given to mice for 3 months and antithrombotic activity was measured by the laser-induced in vivo thrombosis test. Four varieties had no effect on the overall thrombotic status while one variety had a prothrombotic effect (59).

4.2. Antithrombotic fruits

4.2.1. Apples (Aomori Prefecture, northern area in Japan)

Antithrombotic activity of sixteen varieties and heat stability of selected varieties were measured by the in vitro test GTT. Subsequently, antithrombotic activity in vivo was measured after oral administration to mice by the laser-induced in vivo thrombosis test. Sixteen apple varieties were classified into subgroups: antithrombotic, prothrombotic, and varieties having no effect on experimental thrombosis. It was demonstrated that antithrombotic activity in apple varieties was determined by the enhanced endogenous thrombolytic activity and not the effect on platelet reactivity. In apples the endogenous thrombolytic activity was caused by heat stable factors which increased the release of tissue plasminogen activator (t-PA), from endothelial cells and/or blood cells (60).

4.2.2. Mulberries

According to the GTT test results, eleven varieties were classified into three subgroups: antithrombotic, prothrombotic and without significant effect on experimental thrombosis. Subsequently, antithrombotic or prothrombotic effect was determined after oral administration to mice by the laser-induced in vivo thrombosis test. Combination of the effects on platelet reactivity and endogenous thrombolysis, as measured by GTT in vitro predicted the overall effects on thrombosis in vivo (61).

In contrast to vegetable varieties, much attention has to be paid to the area where these fruits were harvested. The antithrombotic activity of some fruits harvested in one area was different from those harvested in a different area (unpublished). Vegetable varieties but not fruit varieties are grown from the respective seeds and this may be the reason for the dependence of the measured antithrombotic effect on the harvest area.

4.2.3. Grapes

Antithrombotic activity of forty-six grape varieties (27 red grapes; 19 white grapes) donated from three institutes and heat stability of the selected varieties were measured by the in vitro test GTT. Effects of these varieties (raw) on platelet reactivity and endogenous thrombolytic activity are shown in Table 1. Three red varieties (Cabernet Sauvignon, Concord, Berry A) and one white variety (Honey Venus) were classified into an antithrombotic subgroup because of their effect on platelet reactivity and/or endogenous thrombolytic activity. The effect of Cabernet Sauvignon donated from two institutes (Cabernet Sauvignon A, Cabernet Sauvignon B) was different. Cabernet Sauvignon A inhibited platelet reactivity and enhanced endogenous thrombolytic activity, suggesting that Cabernet Sauvignon A could be considered as antithrombotic. Cabernet Sauvignon B enhanced platelet reactivity and inhibited endogenous thrombolytic activity, suggesting that Cabernet Sauvignon B was prothrombotic (Figure 5) (62). The results of mulberry and grape varieties showed that classification of fruit varieties according to their effect on experimental thrombosis should be re-defined together and the harvest areas should be considered.

The so-called French Paradox have prompted many epidemiological and laboratory studies on investigating antithrombotic grapes and wines (63,64).
Epidemiological studies have provided evidence that intake of fresh fruits and vegetables could help to prevent cardiovascular disease and stroke (65-69), while some studies have cast doubt on the red wine hypothesis (70,71).

Folts and his co-workers have investigated the mechanism of the French Paradox using the Folts animal model. This is the measurement of cyclic flow reductions (CFRs) in coronary blood flow after mechanical stenosis of the coronary artery and some damage to the vascular wall. The effect of grapes on platelet reactivity was measured by collagen-induced platelet aggregation in anticoagulated whole blood. The red wine (1987 Chateauneuf-du-Pape) and Welch’s 100% natural purple grape juice inhibited thrombosis in vivo but the white wine (1990 Chateau Villotte Bordeaux) did not. The antithrombotic activity of the red wine was demonstrated in vivo. Whether the collagen-induced platelet aggregation test was suitable or not to screen and predict antithrombotic effect was not discussed at that time. Epidemiological verification of the antithrombotic effect of red wine consumption stimulated studies aimed to analyze red wine for certain chemical components like quercetin, rutin, resveratrol and antioxidants, which could be responsible for the antithrombotic effect (72-76).

Platelets play a pivotal role in arterial thrombotic diseases. Platelet function in vitro/ex vivo is widely assessed with platelet aggregometry using anticoagulated blood or platelet rich plasma (PRP), which measures platelet aggregation induced by various chemical agonists (10). Despite the recognition of the cardinal role of thrombin in thrombogenesis (28,77-79), a thrombin-induced platelet aggregation test could not be performed from (citrate) anticoagulated whole blood or PRP. Recently, high shear-induced thrombosis/thrombolysis tests using non-anticoagulated blood have become available for the measurement of thrombotic status or thrombotic and thrombolytic activities ex vivo and in vitro. In these tests, generation of thrombin from (shear) activated platelets plays the decisive role (12,20,21,23-28). Recent animal experiments show that the shear-induced thrombosis/thrombolysis in vitro/ex vivo tests using non-anticoagulated blood are useful for screening foods, dietary components and nutrients for antithrombotic effect (22).

5. Correlation between biologically active components

<table>
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<th>Items</th>
<th>No. of red grape varieties</th>
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<td>Inhibition of endogenous thrombolytic activity</td>
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<td>6</td>
</tr>
<tr>
<td>No effect</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>Enhancement of endogenous thrombolytic activity</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Not determined</td>
<td>2</td>
<td>1</td>
</tr>
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</table>


Figure 5. Effect of a red grape variety donated from two institutes (Cabernet Sauvignon A and B) on platelet reactivity (OT) and endogenous thrombolytic activity (LT) in vitro measured by GTT. (Revised; M. Iwasaki: Master's thesis of Kobe Gakuin University, 2006.)
of fruits and vegetables and the antithrombotic/thrombolytic activities

Polyphenolics and antioxidant rich diets have been investigated for prevention of thrombotic diseases (80-82). We did not find a correlation between polyphenolics/antioxidant contents of various fruits and vegetables and their experimental antithrombotic effect (Table 2). Although purified polyphenolics or antioxidants were shown to have antithrombotic activity (83,84), our results indicate that polyphenolics and/or antioxidants content of fruits and vegetables are not markers of the antithrombotic effect and cannot be used for screening such an effect.

6. Effect of different cultivating fields and harvest times on antithrombotic activity

The antithrombotic strawberry variety, KYSt-4, was planted in the same field and harvested in December, January, February, March and April and antithrombotic activity was measured by GTT. In addition, KYSt-4 was planted in four different fields far from each other at the same time in Gifu Prefecture, Japan and harvested in April. We found that the antithrombotic activity of strawberry varieties grown in different environments (soil, fertilizer, temperature) were similar, thus this effect is probably governed by genes and it is resistant to environmental changes (85).

7. Effect of intake of strawberry varieties with and without antithrombotic activity in humans

Whole juice prepared from experimentally antithrombotic strawberry variety (KYSt-4) was given to healthy volunteers and the thrombotic status was measured by GTT two hours after intake. KYSt-4 juice significantly inhibited the shear-induced thrombosis test (GTT) ex vivo but whole juice from non-thrombotic variety (KYSt-10; Control 1) and water (Control 2) did not (85). This suggests that juices from experimentally antithrombotic fruit and vegetable varieties could prevent arterial thrombosis.

8. Conclusions

Vegetable and fruit varieties were screened for experimental antithrombotic effect by using shear-induced in vitro thrombosis tests (hemoatometry; GTT), followed by a laser-induced thrombosis in vivo test. The in vivo test of FMV was also used to detect any possible effect of the active varieties on endothelial function. Antithrombotic activities of fruits and vegetables were different from variety to variety even in the same species. Measurement of biologically active components in fruits and vegetables, which were suggested earlier to be responsible for the antithrombotic effect did not provide additional benefits in our screening. Further clinical studies are needed to prove the effectiveness of dietary components with experimental antithrombotic effect in humans and that daily intake of an antithrombotic diet is beneficial to prevent thrombotic disorders in humans.

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