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Evaluation of antithrombotic effect: Importance of testing components and methodologies

Junichiro Yamamoto1,* , Yukinori Tamura2, Yoshinobu Ijiri3, Masahiro Iwasaki4, Masahiro Murakami5, Osamu Matsuo6

1 Laboratory of Physiology, Faculty of Nutrition, Kobe Gakuin University, Kobe, Japan; 2 Department of Physiology and Regenerative Medicine, Faculty of Medicine, Kinki University, Osakasayama, Japan; 3 Department of Health and Nutrition, Faculty of Health and Nutrition, Osaka Shoin Women’s University, Osaka, Japan; 4 Division of Metabolism and Clinical Nutrition, Kansai Electric Power Hospital, Fukushima-ku, Osaka, Japan; 5 Laboratory of Pharmaceutics, Faculty of Pharmacy, Osaka Ohtani University, Osaka, Japan; 6 Faculty of Medicine, Kinki University, Osakasayama, Japan.

Summary

The beneficial antithrombotic effect of some dietary components may offer the most promising approach of prevention of cardiovascular diseases and arterial thrombosis. The major stumbling block in finding effective dietary components is the lack of physiologically relevant techniques which can detect potential antithrombotic effect in humans. The presently used platelet function and coagulation tests do not allow the assessment of global thrombotic status and their value in screening dietary components for antithrombotic effect is questionable. Most of these in vitro tests ignore the effect of flow and shear stress, thrombin generation and vascular endothelium, the major contributors to arterial thrombogenesis in humans. As a gold standard, we employed the helium-neon (He-Ne) laser-induced thrombosis test in murine carotid artery and mesenteric microvessels, as the pathomechanism of this test closely reflects arterial thrombogenesis in humans. Results obtained with laser thrombosis test were compared with various shear-induced in vitro platelet function tests which use native blood (Haemostatometry, Thrombotic Status Analyser, Global Thrombosis Test-GTT). Contribution of vascular endothelium to thrombogenesis was assessed by measuring flow-mediated vasodilation (FMV) in vivo. The combination of the two shear-induced ex vivo thrombosis tests (Haemostatometry and GTT) with FMV correlated most closely with the laser-thrombosis test. Our findings suggest that combining the commercially available point-of-care GTT with the FMV test could provide a better assessment of the overall thrombotic status than either of the two tests alone.

Keywords: Global Thrombosis Test, shear-induced thrombosis, flow-mediated vasodilation, helium-neon laser-induced thrombosis, nutrients, traditional kampo medicine

1. Introduction

Prevention and treatment of arterial thrombotic disorders are very important socioeconomic challenges. Compared to the Western-type diet, the Mediterranean-style and the Vegetarian diets reduced the risk of arterial thrombosis and death from coronary heart disease in patients in secondary myocardial infarction prevention trials (1-3). Several nutrients (omega-3 fatty acids; red wine; onion, garlic, kiwi; chocolate, etc.) were shown to inhibit platelet function in vitro (4).

In finding dietary components with potential antithrombotic effect, the use of physiologically relevant techniques is of crucial importance. Only such test(s) which proved to be useful in clinical practice in detecting overall thrombotic status, predicting major adverse thrombotic events should be used for screening diet components and nutrients for antithrombotic effect.
Despite 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 (5). Numerous nutrients inhibited platelet aggregation induced in vitro by various soluble agonists but such effect did not manifest in antithrombotic effect in humans. Therefore, there is still a need to identify tests which accurately reflect the overall thrombotic status in humans. At present, prothrombotic status is assessed by measuring platelet aggregation to various soluble agonists (adenosine diphosphate, collagen, arachidonic acid, thrombin), and by using various biomarkers of coagulation and fibrinolysis and extrapolating the results. All these tests are carried out from anticoagulated blood samples, in which platelet activation do not generate thrombin. This approach has been much less rewarding than it was originally expected. Most of the platelet function tests in clinical use which measure platelet aggregation to various soluble agonists failed in cardiac patients guiding antithrombotic medication (6). It has been shown that only those tests, which take the high shear and flow conditions and the generation of thrombin by platelets into account, have relevance to the pathomechanism of occlusive arterial thrombosis in vivo. Here we compare results obtained by commonly-used platelet function tests performed from anticoagulated blood and those obtained using shear-induced thrombosis and thrombolysis test performed from non-anticoagulated blood. Numerous inconsistencies exist between the commonly used tests and also between these and the shear-induced thrombosis tests. 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, whilst the innovative shear-induced thrombosis tests performed from non-anticoagulated blood do (7,8).

In addition to referring those findings which were obtained by conventional platelet function tests and showed antiplatelet effect of various nutrients, here we focus on the techniques which enabled us to screen large number of fruits, vegetables and nutrients and identified those varieties, which had significant antithrombotic effect. This would provide better understanding of those technologies for assessment of antithrombotic status, leading to efficient discovery and development of an effective antithrombotic medicament.

2. Methodology

2.1. Light transmittance aggregometry

The technique has been described in details elsewhere (9). In citrate-anticoagulated blood sample, platelet activation and aggregation is induced by various soluble agonists, such as adenosine diphosphate, collagen, arachidonic acid, adrenalin, and thrombin. The following nutrients inhibited platelet aggregation to various agonists: berries, cocoa and dark chocolate, coffee, garlic, ginger, kiwi fruit, omega-3 fatty acids, onion, purple grape juice, red vine, white vine, tomato (4).

2.2. PFA-100

PFA-100 is a global test for primary haemostasis, performed from citrate-anticoagulated blood. The principle and the test details are described (9). The measured "closure time" was increased (platelet inhibition) by berries, cocoa and dark chocolate (4).

2.3. Laser-induced thrombosis in the microcirculation and in the carotid artery

Formation of platelet-rich thrombi and their embolization was initiated in the mouse carotid artery or the rat mesenteric microvessels using the He-Ne laser-induced thrombosis technique (10,11). He-Ne laser-induced thrombosis method has been previously described in detail (12). In brief, the mesenteric or pial microvessels of anaesthetized rats or the left femoral artery of anaesthetized mice, was exposed and Evans blue dye was injected through the jugular vein (rat) or tail vein (mice). The centre of the mesenteric or pial microvessel or the carotid artery was irradiated with laser, and the formation of a thrombus at the site of irradiation was monitored and recorded on videotape. Thrombotic status of rats was expressed by the number of thrombosis required to complete occlusion of blood flow and in mice expressed as the cumulative thrombus size. The latter was calculated by continuous observation of the thrombus mass every 10 seconds in the first 10 minutes after irradiation.

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

a) Haemostatometry Details of haemostatometry have been described previously (Figure 1A) (13). Briefly, non-anticoagulated blood was withdrawn from the abdominal aorta of animals and tested with a Haemostatometer purpose-built in Kobe Gakuin University. Blood was forced to flow through a plastic tube by 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 haemostatic plugs in the holes and with this, the perfusion pressure returned to the pre-punching level. The recorded pressure changes reflect both the haemostatic and coagulation processes. In
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 \((d; \text{sec})\) 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, arresting 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

\[\text{Figure 1. Shear-induced thrombosis/thrombolysis and endothelial function tests. (A) Principle of Haemostatometry (a); Haemostatogram (b). H1; H2: indices of platelet reactivity; CT1, CT2: indices of coagulation activity. (B) Principle of Global Thrombosis Test (GTT) (a); real-time recording of occlusion and lysis (b). OT: occlusion time (index of platelet reactivity); LT: lysis time (index of endogenous thrombolytic activity). (C) A typical pattern of flow-mediated endothelium dependent vasodilation (FMV) and endothelium independent vasodilation (NFV). FMV after restoration of blood flow (a); NFV after application of nitroglycerin (b).}\]
or enhancement of platelet reactivity, respectively. Increase or decrease of LT indicates inhibition or enhancement of spontaneous thrombolysis, respectively. The important characteristics of GTT are that GTT can measure platelet reactivity and thrombolytic activity simultaneously for a short time by simple technique.

2.5. Flow-mediated Vasodilation test (FMV)

FMV was used to assess endothelial function (15-17). We have adopted the endothelium-dependent and independent flow-mediated vasodilation technique to mice, as previously described in detail and a typical result is shown in Figure 1C (18). Baseline images of the diameter of the femoral artery of mice were taken before clamping and then at 30 sec intervals over 450 sec after restoration of blood flow. Nitroglycerin-mediated 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 compared.

We used the above techniques to test extract of a variety of carrots (19), rice (20), strawberries (21), tomatoes (22,23) and grapes (24) for antithrombotic effect. From the several varieties tested, we could find some, which had significant experimental antithrombotic effect.

3. Comparison of He-Ne laser-induced thrombosis test with tests measuring global thrombotic and thrombolytic status ex vivo.

3.1. Involvement of platelets and fibrin in the platelet-rich thrombi formed in vivo and in vitro.

Platelets and fibrin are the main components of arterial thrombus. The involvement of these two components in the formation and lysis of a thrombus formed from non-anticoagulated (native) blood was measured by the in vivo test of He-Ne laser-induced thrombus and the shear-induced in vitro thrombosis tests performed on native blood sample. In small arterial vessels of the microcirculation, laser-induced thrombi were composed of tightly packed platelets. Electron microscopy and specific antibody staining failed to detect fibrin in the microvessels. However, measurement of lysis or embolization of the formed thrombi suggested the presence of fibrin, as administration of plasminogen activators or inhibitors enhanced or inhibited thrombolysis, respectively (25-29).

High shear-induced haemostatic plug formed by Haemostatometry was composed predominantly of platelets, but minor amount of fibrin was also detected. This was confirmed by electron microscopy. The dominant presence of platelets in the formed haemostatic plug was shown directly by electron microscopy and indirectly by administration of antiplatelet agents such as aspirin, thromboxane A2 receptor antagonist, stable prostacyclin derivative and prostaglandin E1 prior the measured haemostasis (Table 1). When citrated or fully heparinized blood was tested, haemostasis did not occur, indicating the important role of thrombin and fibrin formation in the measured haemostasis (30). In a recent study using the Global Thrombosis Test, we also confirmed the cardinal role of thrombin in the formation of an occlusive thrombus (31).

3.2. Effect of blood flow and shear stress on thrombus formation in microvessels in vivo and haemostatic plug formation in vitro

As the mechanism of platelet-rich thrombus formation at various flow rates has been investigated, we measured the shear rate during thrombus formation in vivo (32) and investigated the mechanism of shear-induced thrombus formation using GTT (33).

Shear rates during thrombus formation in the microcirculation were $641 \pm 40$ s$^{-1}$ in arterioles and $280 \pm 20$ s$^{-1}$ in venules before the onset of thrombus formation. Shear rates increased during platelet-rich thrombus formation and reached a peak of approximately $2,500$ s$^{-1}$ in both arterioles and venules. It was demonstrated that under such conditions

<table>
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<th>Table 1. Effect of various antiplatelet agents on platelet reactivity and coagulation as measured by Haemostatometry</th>
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<td><strong>Items</strong></td>
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<td>-------------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Aspirin 0.3 mM</td>
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<tr>
<td>Aspirin 1 mM</td>
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<tr>
<td>Aspirin 3 mM</td>
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<tr>
<td>TXA2Rant 0.5 μM</td>
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<tr>
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<tr>
<td>sPGI2 0.055 mM</td>
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<td>sPGI2 0.55 mM</td>
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<td>sPGI2 14.1 nM</td>
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<td>sPGI2 141 nM</td>
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TXA2Rant: thromboxane A2 receptor antagonist; sPGI2: stable prostacyclin derivative; sPGE1: stable prostaglandin E1. Final concentrations are shown. Data are expressed as mean ± SEM (n = 6-10 in each group) *p < 0.05; **p < 0.01 vs. control. (Revised; Yamamoto et al. Platelets. 1999; 10:178-187).
von Willebrand factor (vWF) played the major role in forming the initial platelet aggregates and fibrinogen (fibrin) was involved only in venules, but not in arterioles (32). These findings showed similar mechanisms of thrombus formation between the in vivo and in vitro tests used and those experiments which were performed on human blood (33-35).

Further, we demonstrated that thrombus formation in the GTT was similar to thrombus formation at high shear and flow conditions in terms of the activation of adhesive proteins and ligand receptors on platelets (Table 2) (33). The GTT allowed the assessment of the effect of tissue-type plasminogen activator, streptokinase and plasminogen activator inhibitor on the stability of formed thrombi, and therefore assess not only the efficacy of the spontaneous (endogenous) thrombolytic system but also response to activators and inhibitors of thrombolysis.

4. Correlation between He-Ne laser-induced platelet reactivity measurement in vivo and high shear-induced platelet reactivity in vitro test using non-anticoagulated blood

In a study measuring thrombotic status of diabetic rats, the in vivo He-Ne laser-induced thrombosis and the in vitro high shear-induced platelet reactivity test were compared. The thrombotic status of two kinds of spontaneously diabetic, insulin independent rats (Goto-Kakizaki [GK] rats and Otsuka Long-Evans Tokushima Fatty [OLETF] rats) was measured (Figure 2A, 2B) (36,37). GK rats were in a prothrombotic status as detected by the in vivo laser test and this corresponded with enhanced platelet reactivity measured by the in vitro Haemostatometry. Also the inhibited thrombotic status of OLETF rats as measured with the laser test corresponded with suppressed platelet reactivity, measured with Haemostatometry. These results indicate that thrombotic status is predominantly governed by platelet reactivity which is detected similarly by the in vivo laser-thrombosis and in vitro shear-induced platelet reactivity tests.

5. Thrombotic status is determined by the balance between platelet reactivity and endothelial function

In contrast to diabetic rats, in SHRSP rats there was no correlation between the laser-thrombosis test and the shear-induced platelet reactivity test. While the laser-thrombosis test measured a prothrombotic state in SHRSP rats, the ex-vivo shear-induced platelet function test showed suppressed platelet reactivity (Figure 2C) (38-40). To try and identify the reason for

<table>
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<th>Table 2. GTT assessment of the effect of specific platelet inhibitors on human platelet reactivity</th>
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<td>Aurin tricarboxylic acid (μmol/L)</td>
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<tr>
<td>OT (s)</td>
</tr>
<tr>
<td>6B4 (μg/L)</td>
</tr>
<tr>
<td>OT (s)</td>
</tr>
<tr>
<td>12E4 (μg/L)</td>
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<td>OT (s)</td>
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<td>MA16N7C2 (μg/L)</td>
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<tr>
<td>TAK-029 (nmol/L)</td>
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<td>Anti-vWF (μg/L)</td>
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<td>Anti-fibrinogen (μg/L)</td>
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<td>OT (s)</td>
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<td>Argatroban (μmol/L)</td>
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<td>OT (s)</td>
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Aurin tricarboxylic acid: an inhibitor of the association of von Willebrand factor and platelet glycoprotein Ib (GPIb); 6B4 and 12E4: monoclonal antibodies against GPIb; MA16N7C2 and Abciximab: monoclonal antibodies against platelet glycoprotein Ib/IIa (GPIb/ IIa); TAK-029: an inhibitors of platelet glycoprotein Ib/IIa; Anti-vWF: polyclonal antibody against von Willebrand Factor; Anti-fibrinogen: polyclonal antibody against fibrinogen; Argatroban: a thrombin inhibitor. Zero (0) shows vehicle (saline) controls. *p < 0.05; **p < 0.01; ***p < 0.001 vs. control; n = 4-6 human subjects in each group. (Revised; Yamamoto et al. Blood Coagulation & Fibrinolysis 2003; 14:31-39).

Figure 2. Thrombotic status and platelet reactivity of spontaneously diabetic GK rats (A), OLETF rats (B) and stroke-prone spontaneously hypertensive rats (SHRSP) (C). Results are expressed as means ± SEM. A: n = 6-14 in each group; B: n = 6-8 in each group; C: n = 6-8 in each group; *p < 0.05; **p < 0.01 vs. controls. (A: Revised; Taka et al. Platelets 2002; 13: 313-316; B: Taka et al. Diabetes Frontier 1999; 10: 582-583; C: Revised; Noguchi et al. Haemostasis 1997; 27:237-245, Yamashita et al. Thrombosis Research 2002; 105:507-511).
this inconsistency, endothelial function testing (FMV) was performed. FMV in vivo performed in SHRSP rats detected severely dysfunctional endothelium whilst the endothelium-independent vasodilation was normal (Figure 3A) (39). Similar inconsistencies were found between the in vivo and in vitro thrombosis tests in congenital apoE and LDLR deficient mice (Figure 3B) (41,42).

The laser-induced thrombosis test demonstrated a prothrombotic state in mice fed on high-triglyceride but not in mice on high-diglyceride diet. This was inconsistent with that detected with the shear-induced in vitro test, suggesting that the prothrombotic state may be due to endothelial dysfunction caused by the high-triglyceride diet.

These results provided evidence that the overall thrombotic status is determined by the balance between platelet reactivity, the determinant of the thrombotic status of blood, and the function of the vascular endothelium. For this reason, the sensitive in vivo FMV functional test is recommended as a test additional to platelet reactivity assessment, for the assessment of overall thrombotic status (Figure 4) (43).

6. Thrombotic status of blood is determined by the balance between prothrombotic and thrombolytic (fibrinolytic) activities

The overall thrombotic status of blood is determined by the balance between prothrombotic and thrombolytic factors. While platelet reactivity is measured by platelet function tests and thrombelastography measures the overall coagulation activities, conventional thrombosis tests cannot measure the (spontaneous) thrombolytic/fibrinolytic activity of blood. Using the GTT, we could detect both thrombotic and thrombolytic activities in sequence from one blood sample. Though, the influence of diet and nutrients on platelet function using conventional tests has been published (4), with this technique for many years we have been screening fruits and vegetables for antithrombotic effects. Importantly we demonstrated that such activity can be specific for a particular variety while other varieties do not show antithrombotic effects (19-24). Effects of specific varieties of red and white grapes on in vitro and in vivo thrombogenesis and thrombolysis are shown in Figure 5 (24). The use of GTT and in vivo laser thrombosis tests showed that a specific variety of red grapes inhibited platelet reactivity and enhanced thrombolysis, suggesting a potential antithrombotic effect. On the other hand, one variety of white grapes enhanced platelet reactivity and inhibited thrombolysis, suggesting a potential prothrombotic effect.

Platelet reactivity and thrombolytic activity of carrot varieties before and after heat treatment are shown in Figure 6. One variety SAKATA-0418 had no effect on platelet reactivity but enhanced thrombolysis before heat treatment. After heat treatment, as measured both with in vitro (GTT) and in vivo (laser thrombosis) techniques, this carrot variety enhanced platelet reactivity and lost the effect on thrombolysis. This finding suggests that heat treatment profoundly alters the pro- or antithrombotic activities of some fruit and vegetables.

Besides searching effective foods for preventing thrombosis, we have applied those methodologies to evaluation of antithrombotic effects of several traditional herbal drugs or Kampo medicines (44) and demonstrated the balance between prothrombotic and thrombolytic factors. While platelet reactivity is measured by platelet function tests and thrombelastography measures the overall coagulation activities, conventional thrombosis tests cannot measure the (spontaneous) thrombolytic/fibrinolytic activity of blood. Using the GTT, we could detect both thrombotic and thrombolytic activities in sequence from one blood sample. Though, the influence of diet and nutrients on platelet function using conventional tests has been published (4), with this technique for many years we have been screening fruits and vegetables for antithrombotic effects. Importantly we demonstrated that such activity can be specific for a particular variety while other varieties do not show antithrombotic effects (19-24). Effects of specific varieties of red and white grapes on in vitro and in vivo thrombogenesis and thrombolysis are shown in Figure 5 (24). The use of GTT and in vivo laser thrombosis tests showed that a specific variety of red grapes inhibited platelet reactivity and enhanced thrombolysis, suggesting a potential antithrombotic effect. On the other hand, one variety of white grapes enhanced platelet reactivity and inhibited thrombolysis, suggesting a potential prothrombotic effect.

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Besides searching effective foods for preventing thrombosis, we have applied those methodologies to evaluation of antithrombotic effects of several traditional herbal drugs or Kampo medicines (44) and demonstrated
that some of the medications for blood stagnation have significant antithrombotic effect as well. On the other hand, antithrombotic fractions from *Centella asiatica* have been also investigated (45). Thus, combination of shear-induced thrombosis test, Haemostatometry or GTT, and FMV should be useful in searching and developing antithrombotic drugs.

7. Measurement of thrombotic status of healthy volunteers and patients with the Haemostatometer and GTT

We assessed the thrombotic status of a small number of healthy volunteers and patients with thrombotic disorders using Haemostatometry and GTT. These tests proved to sensitively detect changes in the thrombotic status after physical exercise-load and gender differences, age and habitual smokers, and race differences between healthy Japanese and British volunteers (46-54).

8. Conclusions

The value of shear-induced *in vitro* Global Thrombosis Test and Haemostatometry in testing various components of nutrient and herbal drugs for antithrombotic effect was assessed. Results obtained with these *in vitro* tests were compared with the results obtained using the He-Ne laser-induced carotid artery and mesenteric microvessel thrombosis model *in vivo*. Our findings suggest that the use of Global Thrombosis Test together with the Flow-mediated Vasodilation *in vivo* test provides better assessment of the global thrombotic status than using the platelet function test alone.

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