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Review Article

Investigations into the Pathomechanism of thrombotic Disorders with an *Ex Vivo* Global Test Performed from Non-Anticoagulated Blood: From Animal Experiments to Bedside Application

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Abstract

Ex vivo shear-induced and in vivo helium-neon laser-induced thrombosis/fibrinolysis tests were compared in animal models. The results of this paired *ex vivo* global thrombosis test (GTT) and *in vivo* thrombosis test comparison showed that both tests were well-matched. Thrombotic disorders are classified as type I, II, or III. Type I is due to increased thrombotic and decreased fibrinolytic activity, while type II is caused only by decreased fibrinolytic activity. Type III is caused by endothelial dysfunction which is independent of thrombotic and fibrinolytic activities of blood. Based on these animal experiments, specific medications can be considered also for humans. Daily intake of an antithrombotic diet and suitable individual-matched exercise may prevent thrombotic episodes and aid rehabilitation.

Keywords: Endogenous Fibrinolysis; Platelet Aggregation; Shear-Induced Thrombosis; Cancer-Associated Thrombosis; Antithrombotic Drug; Physical Exercise; Antithrombotic Diet

Abbreviations

GTT: Global Thrombosis Test; OT: Occlusion Time; LT: Lysis Time

Introduction

Prevention, early detection, and proper treatment are crucial for thrombotic disorders. We previously used animal models to study the effects of physical exercise and foods with antithrombotic activity on the prevention of thrombotic disorders [1-3]. It was essential to perform a pathologically relevant blood test for these experiments; however, we did not find such a blood test among the platelet function or coagulation tests currently used in clinical practice. Anticoagulation as is commonly used in measuring platelet reactivity, coagulability, and coagulation cascade-related factors is one of the biggest hurdles in this respect. Anticoagulation namely decreases the physiologically important calcium ion concentration. It also inhibits the activity and production of thrombin and other thrombin-generating cascade enzymes. In addition, assessing the impact of individual factors - biomarkers- in a multifactorial thrombotic status is long unsolved.

D-dimer measurement appears to be popular among various clinical blood tests [4-7]. D-dimer is the product of fibrin degradation, and an increase in D-dimer level results from enhanced fibrinolysis. However, most doctors may administer antithrombotic drugs because removing fibrin deposits may increase D-dimer levels. If possible, medication should be determined after confirming the thrombotic and fibrinolytic activities which can be measured at present. We will compare the *in vivo* and *ex vivo* results obtained using animal experiments in our laboratory.

We used the *in vivo* helium-neon laser-induced thrombosis/thrombolysis (fibrinolysis) model in our laboratory to evaluate *in vitro* (*ex vivo*) tests (i.e., Haemostatometry, Thrombotic Status Analyser [TSA], and Global Thrombosis Test [GTT. These tests measure thrombotic and fibrinolytic activities simultaneously. We correlated the results of *in vivo* and *in vitro* tests [8-16].

Arterial thrombi are platelet-rich white thrombi with red cell-rich fibrin tails formed from non-anticoagulated (native) blood. Such thrombi are shear-dependent [17]. GTT is a high shear-induced thrombosis/fibrinolysis test that targets arterial thrombosis. It was first introduced in the literature in 2003 and was used in a clinical study on stroke in 2010 [18]. GTT has also been used to assess venous thrombosis in cancer patients [3,19]. We proposed that the prothrombotic status in these patients was due to suppressed fibrinolysis and was independent of platelet activity, emphasizing the importance of endogenous fibrinolytic activity [20-22]. GTT is a commercially available test that is capable of simultaneously assessing endogenous fibrinolytic and platelet reactivity.

Materials and Methods

Assessment of thrombotic and fibrinolytic activities using *in vivo* thrombosis tests in the vessels of animal models

Thrombi are formed by the interaction between blood vessels and blood under flow (Virchow's triad). This process was examined in animal models. Laboratory animals were anesthetized with pentobarbital. The mesenteric or pial microvessels of rats or the carotid arteries of mice were exposed to facilitate intravenous injection of Evans blue dye. The exposed blood vessels were then irradiated with a helium-neon laser beam. The injected dye specifically absorbs the laser energy and converts it into heat, burning the blood vessel from the inside and inducing thrombus formation. Video monitoring was conducted throughout the process (https://drive.google.com/file/d/1EMaD-Rwt_lDlc_99rwy_q2WeSIWaX-wF/view?usp=sharing;

https://drive.google.com/file/d/1VNMjAjbQFRF18ZgdxEsRSzqpFX0gyh-W/view?usp=sharing).

The severity of the thrombotic reaction was expressed as the number of irradiations required to cause complete stoppage of blood flow. In the mouse carotid artery, video recording was performed for 10 min after irradiation, and the total size of the emboli following irradiation was measured every 10 s [8-9,23-26].

Simultaneous measurement of thrombotic and fibrinolytic activities in non-anticoagulated blood using *ex vivo* shear-induced thrombosis/fibrinolysis tests

In tests using anticoagulated blood, thrombotic status is measured under non-physiological conditions, such as low concentration of calcium ions and inhibition of thrombin and thrombin-generating cascade enzymes. A parallel-plate perfusion chamber, developed by Sakariassen., *et al.* [27], used human non-anticoagulated blood to measure thrombotic status. This chamber has been used in many human clinical studies, including studies with antithrombotic and anti-bleeding therapeutics in healthy humans and in patients with thrombotic risk and with various bleeding disorders [28-42].

Haemostatometry

High shear-induced thrombosis tests using non-anticoagulated blood include Haemostatometry, TSA and GTT [10-16]. We set up a Haemostatometer and purchased TSA and GTT to assess the effects of suitable physical exercise and antithrombotic foods. A syringe was used to deliver blood into a specially manufactured polyethylene tubing (OD, 1.00 mm; ID, 0.50 mm), which was punctured with a 0.18-mm diameter needle. A hemostatic plug was formed by shear forces (375 dyn/cm^2, immediately after puncture). A reservoir detected the pressure changes in the system. The tubing was punctured 2.5 minutes after blood withdrawal, resulting in bleeding, hemostatic plug formation, and coagulation. Plug formation and coagulation were assessed using the changes in pressure, which were recorded and analyzed by a computer. Pressure decreased after the puncture and returned to the initial level (60 mmHg) as the hemostatic plug was formed, followed by a gradual pressure drop caused by subsequent coagulation. The areas with up to 30% (H1, mmHg·s) and 90% (H2) recovery from the maximal pressure drop were used as indices of hemostatic plug formation. The time until a 10-mmHg pressure drop from baseline was used as an index of the onset of coagulation of flowing blood (CT1, min). The time until the pressure could be maintained at 10 mmHg for 1 minute was used as an index of coagulation completion (CT2) [10-12].

Thrombotic status analyser

The principle of TSA is similar to that of Haemostatometry. The parts which cause high shear are ready made in contrast to Haemostatometer [13,14].

Global thrombosis test

The GTT test tube had a conical section in which two ceramic ball bearings were placed. Due to the three flat segments formed on the inner surface of the tube, there were three narrow gaps adjacent to the ball bearings. When blood is added to the tube, it flows through the narrow gaps, and the droplets are collected in a reservoir downstream. Platelets are exposed to high shear stress and become activated while passing through the gaps. Platelet aggregates and thrombin are generated from activated platelets in the space between the two ball bearings. As fibrin-stabilized thrombi reach the lower ball bearing, blood flow gradually decreases and is finally arrested. The instrument detects the time interval (d, s) between two consecutive blood drops falling into the reservoir. At

the start of the test, flow is rapid and hence d is small. Subsequently, the flow rate decreases, and d increases. When the actual default d ≥ 15 s is reached, this time is displayed as the occlusion time (OT, s). Subsequently, the flow is completely arrested. After OT, a preset "thrombi stabilization period" follows, during which the sensors ignore the blood drops. This allows the stabilization of the fully occlusive thrombi. Eventually, flow is partially restored due to endogenous thrombolysis (fibrinolysis), as indicated by the detection of the first blood drop after OT. This was recorded as the lysis time (LT). The GTT-3 test was started by placing a disposable test tube into the heated channel of the instrument, setting the instrument in standby mode, and waiting for the blood sample to start the measurement (https://drive.google.com/file/d/1Up-MgDCFPEBWYN-3hKrmCobMCm47XpTp8/view).

Blood was drawn from the antecubital vein, and the tourniquet constriction was limited to a short time, only during needle insertion. A two-syringe blood collection technique was used to avoid platelet activation and coagulation during the blood draw. The first draw of 3-4 mL was used for laboratory tests, and only 4 mL of blood in the second syringe was used for GTT measurement. The non-anticoagulated blood sample was transferred from the syringe into the GTT test tube within 15 s of withdrawal. The test automatically started once the blood was transferred into the test tube. Measurements for OT/thrombus stability and LT/rate of thrombolysis were taken, displayed, and simultaneously recorded on an SD memory card to allow for the display of the measurement in a graph form later using a specialized algorithm, GTT-Draw.

Results and Discussion

Thrombi formed in animal microvessel *in vivo* (A) and shear rate dependent thrombi *ex vivo* (B-1)
These are shown in figure 1.

Figure 1

Correlation between the in vivo and the *ex vivo* tests were confirmed by animal experiments

Comparison between two types of congenitally spontaneous diabetic rats, the Goto-Kakizaki (GK) strain and the Otsuka Long-Evans Tokushima Fatty (OLETF) strain

The thrombotic statuses of two types of congenitally spontaneous diabetic rats, GK rats and OLETF rats, were assessed by *in vivo* helium-neon (He-Ne) laser-induced thrombosis test and *ex vivo* shear-induced thrombosis test using non-anticoagulated blood. Results are shown in Figure 2 (GK in A and OLETF in B) [43-44]. In GK rats, the numbers of irradiation laser pulses required to occlude the blood flow in arterioles and venules were significantly fewer than those in control rats. Haemostatometry, an *ex vivo* shear-induced thrombosis test, revealed that the indices of thrombotic activity (i.e., H1 and H2) were significantly lower than those of Wistar rats, indicating that GK rats have higher thrombotic activity. *In vivo* tests were congruent and supported the finding of prothrombotic activity in GK rats. In contrast, both *ex vivo* and *in vivo* tests revealed that OLETF rats had antithrombotic activity compared to the control rats (LETO). These results showed that the *in vivo* thrombosis test and the *ex vivo* shear-induced thrombosis test were significantly and positively correlated in non-anticoagulated blood.

Comparison of the effect of two grape varieties on thrombotic status

The effect of juice from two grape varieties, Cabernet Sauvignon and Neo Muscat B, on thrombotic status was assessed by *ex vivo* GTT. The juice was added to non-anticoagulated rat blood, mixed, and added to a disposable GTT tube, and its effect on thrombotic

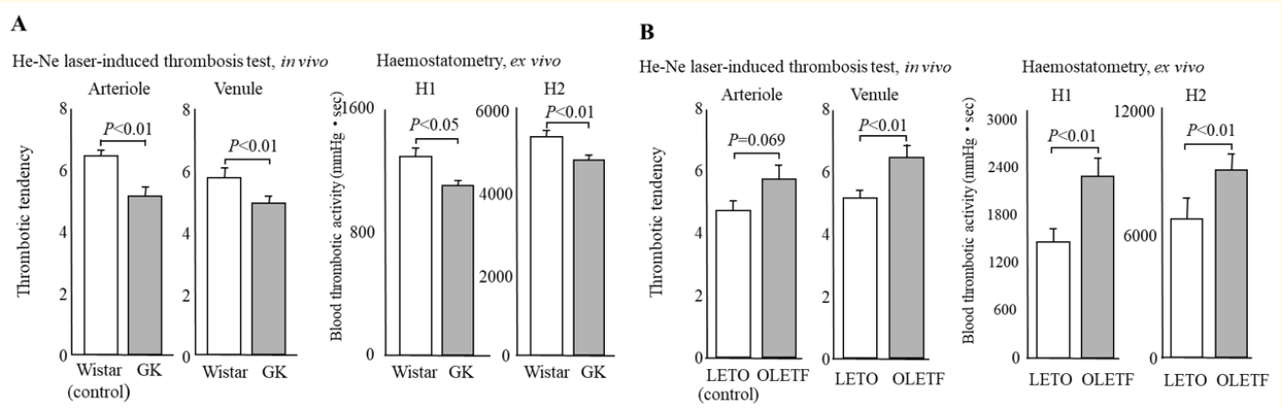

Figure 2

Figure 3

status was then assessed. The results are shown in Figure A. Cabernet Sauvignon increased OT and decreased LT (A A-1) (Cabernet sauvignon), suggesting that Cabernet Sauvignon inhibits thrombosis *in vivo*. Subsequently, the juice was orally administered to mice, and the effect of the juice was assessed using an *in vivo* He-Ne laser-induced thrombosis test. Antithrombotic effect was confirmed *in vivo* (B A-1) (Cabernet sauvignon). In contrast, Neo Muscat B decreased OT and increased LT in the *ex vivo* GTT (A A-2) (Neo muscat (A-2), suggesting that Neo Muscat B may enhance thrombosis, which was confirmed *in vivo* (B A-2) [45]. A similar relationship between the *in vivo* and *ex vivo* tests has also been confirmed using carrot varieties [46]. These results also demonstrated a positive correlation between the *in vivo* thrombosis test and the *ex vivo* shear-induced thrombosis test in non-anticoagulated blood.

Inconsistencies between the results obtained by the *in vivo* test and the *ex vivo* test in stroke-prone spontaneously hypertensive rat (SHRSP)

The correlation between the *in vivo* and the *ex vivo* tests was examined in SHRSP rats (Figure 4). The *in vivo* He-Ne laser-induced thrombosis test caused occlusion with small irradiation number, showing that SHRSP rats had increased thrombotic activity in the arterioles and venules [A] [47]. However, the *ex vivo* Haemostatometer demonstrated an antithrombotic status (B) [48]. Endothelial function was assessed in SHRSP rats and compared to its control, a normotensive rat WKY, by flow-mediated vasodilation (FMV or FMD) to resolve these contradictory findings (C). In the control rats, the femoral artery was dilated after the reflow of blood, showing that endothelial function was normal. In contrast, the femoral artery of the SHRSP did not dilate, indicating that endothelial func-

Figure 4

tion was damaged [48]. These findings suggest that thrombotic status may be determined by endothelial function in SHRSP.

In case that the *ex vivo* blood test cannot detect the thrombotic status

The above results indicate that it is essential to clarify the contribution of endothelial function to overall thrombotic status. Therefore, FMV was used to assess endothelial function in the femoral arteries of mice [49]. Vasodilation after the release of blood flow stasis is shown as a percentage of the diameter before stasis. Peak vasodilation percentage was used to assess endothelial function.

Mice were fed four types of diets for 8 weeks: a Japanese-style low-fat diet (LF), a Western-style high-fat diet (HF), an experimental high triacylglycerol diet (TAG), and an experimental high diacylglycerol diet (DAG). The *in vivo* He-Ne laser-induced thrombosis test and *in vivo* endothelial function test (FMV) were subsequently performed. The results are shown in Figure 5 [50,51]. The *in vivo* He-Ne laser-induced thrombosis test showed that TAG and HF resulted in higher prothrombotic activity compared to LF and DAG diets. However, this difference was not observed in the *ex vivo* shear-induced thrombosis test. The HF and TAG diets significantly lowered vasodilation compared to the LF and DAG diets, indicating that the HF and TAG diets damaged endothelial function. These findings show that thrombotic status cannot be detected only by the *ex vivo* blood test in cases where endothelial function is dominant in determining thrombotic status. Based on these results, thrombotic status is recommended for assessment, as shown in figure 6.

Types of thrombotic disorders classified by the *ex vivo* GTT in humans.

Type I: Thrombotic disorders caused by increased thrombotic and decreased fibrinolytic activity.

Taomoto., *et al*. compared the thrombotic status of stroke patients acutely after the onset of the event with that of healthy volunteers and those of stroke patients before and after 14 days medication [18]. The occlusion time (OT) of the patients was significantly lower than that of the healthy volunteers, suggesting that thrombotic activity was enhanced. Conversely, the lysis time (LT) of the patients was significantly prolonged, indicating that the fibrinolytic activity was suppressed. Strokes may have been caused by either of these findings.

The thrombotic status of the patients before and 2 weeks after the antithrombotic therapy was assessed. OT was significantly increased, and LT was significantly decreased after 14 days of the medication, shifting towards the level of fibrinolytic and thrombotic activities seen in healthy patients and indicating recovery.

All patients with cerebral infarctions received intravenous antiplatelet or anticoagulant drugs in addition to oral aspirin, cilostazol, or both. Lacunar infarctions were treated with intravenous ozagrel hydrochloride or ozagrel plus edaravone. Patients with atherothrombotic infarctions were managed with intravenous argatroban plus edaravone or heparin, while those with cardioembolic infarctions were administered oral warfarin and antiplatelet or anticoagulant drugs. Medication was continued for 14 days. Antiplatelet drugs succeeded in restoring thrombotic activity to nor-

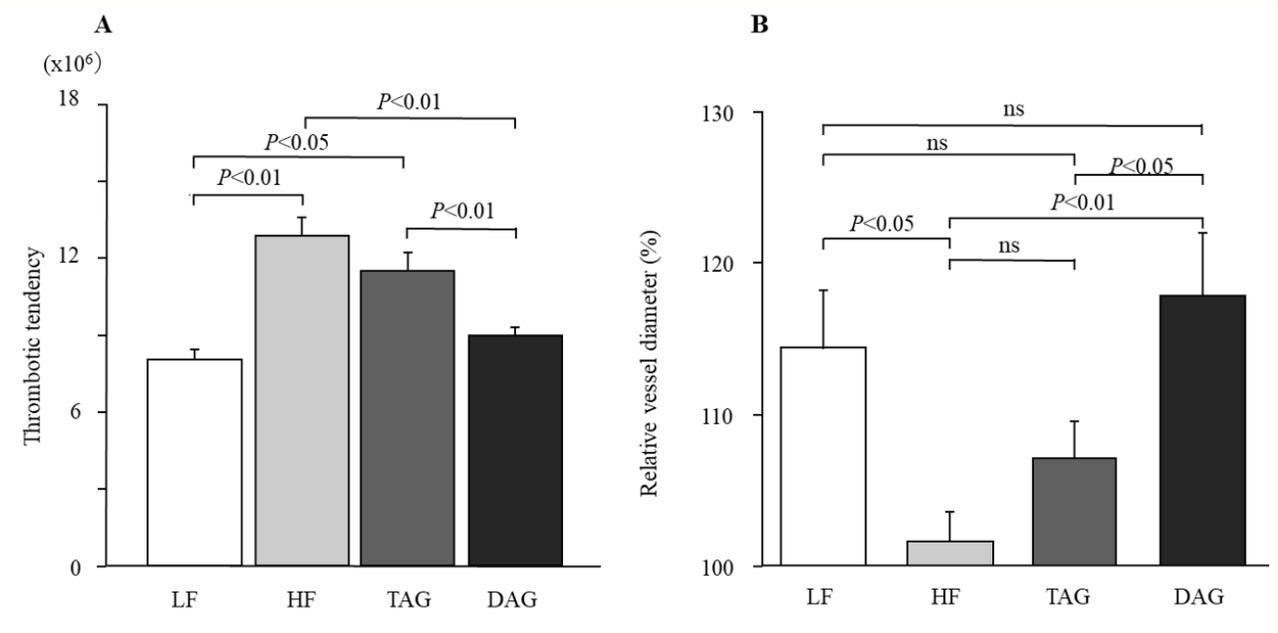

Figure 5

Figure 6

mal levels; however, the question of how fibrinolytic activity, represented by LT, improves remains unclear. It is unclear whether the normalization of fibrinolytic activity is secondary to the recovery of thrombotic activity.

In this study, the antiplatelet drug, cilostazol, was frequently used. Cilostazol acts not only on platelets but also on the endothelium. Cilostazol enhances endothelial function in flow-mediated vasodilation (FMV) tests [52-55]. Drugs targeting platelets may be

useful in this type of thrombosis. However, direct oral anticoagulants (DOAC), including inhibitors of thrombin, factor Xa, and factor XIa, can be particularly beneficial because thrombin is a platelet agonist. However, attention must be paid to bleeding because antiplatelets and anticoagulants might cause bleeding as aspirin does [56]. OT and LT measurements may be beneficial to avoid this side effect.

Type II: Thrombotic disorders caused by decreased fibrinolytic activity.

We compared the thrombotic status of patients with and without cancer [3,19]. The OTs of the patients with cancer were lower than those of patients without cancer, but the difference was not statistically significant. In contrast, the LTs of patients with cancer were significantly higher than those of patients without cancer ($p < 0.001$), indicating that the prothrombotic status of cancer patients is due to suppressed fibrinolytic activity. Thus, novel drugs that enhance fibrinolytic activity should be considered in these patients, including plasminogen activator inhibitor-1（PAI-1） inhibitors and activated thrombin activatable fibrinolysis inhibitor (thrombin activatable fibrinolysis inhibitor a (TAFIa)) inhibitors [57-59]. Statins may also be beneficial because they decrease PAI-1 levels [60,61]. Such drugs may alleviate venous thrombosis in cancer patients. Drugs that act on endothelial cells and increase fibrinolytic activity should also be considered. However, as some anti-cancer drugs cause thrombosis [62,63], the thrombotic and fibrinolytic activities of individuals should be assessed with GTT after chemotherapy.

Type III: Thrombotic disorders caused by endothelial dysfunction.

Thrombotic disorders can result from endothelial dysfunction and occur independently of thrombotic and fibrinolytic activities. Thus, they cannot be assessed through GTT. Instead, an endothelial function test, such as an FMV test, may be useful. If endothelial dysfunction is detected, drugs that improve endothelial function may be useful. In addition, endothelial cell-effective antiplatelet drugs such as cilostazol might be beneficial [52-55]. We have previously reported that antithrombotic apple varieties prevent thrombosis by causing tissue plasminogen activator (tPA) release from endothelial cells [64]. Thus, daily intake of antithrombotic fruit and vegetable varieties might be helpful. As trans-fatty acids promote thrombus formation in mice by aggravating anti-thrombogenic endothelial functions via toll-like receptors, avoidance of trans-fatty acids is recommended [65].

Prevention and rehabilitation of thrombotic disorders in daily life.

We assessed the effects of aging and habitual smoking on thrombotic and fibrinolytic activities. Smoking did not affect thrombotic activity (OT) but lowered fibrinolytic activity (LT), indicating that habitual smoking worsened thrombotic status. A similar effect was observed with aging [66], and these findings are similar to those of type II thrombotic disorders. Smoking cessation is recommended and daily intake of antithrombotic fruit and vegetable varieties, which are especially effective in increasing fibrinolytic activity, may be beneficial.

We selected fruit and vegetable varieties with antithrombotic activity using *in vivo* He-Ne laser-induced thrombosis test and *ex vivo* shear-induced thrombosis/fibrinolysis tests using non-anticoagulated blood. Fruit and vegetable varieties were classified as with or without antithrombotic activity [1-3,46,64,67]. Results obtained from animal experiments have been confirmed in humans [68,69].

Exercise is widely believed to contribute to thrombosis prevention. However, sudden death during exercise or "*exercise paradox*" is also acknowledged. A breakthrough in the exercise paradox has been long-awaited. We attempted to solve this problem using Haemostatometry and GTT [70-73]. Strenuous, short-duration exercise increased thrombotic activity but did not affect fibrinolytic activity. In contrast, mild, long-term exercise decreased thrombotic activity but not fibrinolytic activity. These results suggest that long-term individually matched mild exercise after assessing individual thrombotic status using GTT is recommended to avoid sudden death. This trial may be beneficial for the prevention and treatment of thrombotic disorders or rehabilitation.

Individual and racial differences in thrombotic and fibrinolytic activities

Differences in thrombotic and fibrinolytic activities among individuals and races using GTT have been reported [74,75]. To the best of our knowledge, GTT is the sole commercially available point-of-care blood test for assessing thrombotic and fibrinolytic activities. GTT enables worldwide medication for thrombotic disorders.

Conclusion and Future Perspective

Thrombotic disorders can be classified as type I, II, or III by the point-of-care *ex vivo* GTT, which enables simultaneous measurement of thrombotic and endogenous fibrinolytic activities. Adjunct endothelial function tests, such as FMV, may be beneficial for deciding the suitable medication. Antiplatelet drugs or DOACs should be considered and investigated for type I disease, which is characterized by increased thrombotic activity and decreased fibrinolytic activity. For type II disorders, which result from decreased fibrinolytic activity, fibrinolytic drugs such as PAI-1 inhibitors and TAFIa inhibitors should be studied and examined. FMV could be beneficial in assessing type III disorders because they show no change

in thrombotic and fibrinolytic activity. Drugs that affect endothelial cells and induce the release of factors should also be considered and investigated for type III disease. Daily intake of antithrombotic fruit and vegetable varieties, coupled with regular and mild physical exercise, may also be beneficial in the prevention and treatment of thrombotic disorder, including rehabilitation. GTT enables universal treatment and development of antithrombotic drugs for thrombotic disorders.

Limitation

In most of the published clinical studies on GTT referred to here, GTT-2 model had been used. There are no or very few publications on the clinical usefulness of the new model GTT-3. The cause might be converting the standard internal medicine wards and Intensive Care Units (ICUs) into isolation units during Coronavirus disease 2019 (COVID-19) pandemic years, thus preventing the use of techniques like GTT which should be performed near patients.

Bibliography

1. Yamamoto J., *et al*. "Prevention of thrombotic disorders by antithrombotic diet and exercise: evidence by using global thrombosis tests". *Future Science OA.* 4.4 (2018): FSO285.

2. Yamamoto J., *et al*. "Reevaluation of antithrombotic fruits and vegetables: great variation between varieties". *Drug Discoveries and Therapeutics* 10.3 (2016): 129-140.

3. Murakami M., *et al.* "Global thrombosis test for assessing thrombotic status and efficacy of antithrombotic diet and other conditions". *Future Science OA* 8.3 (2022): FSO788.

4. Paniccia R., *et al.* "Platelet function tests: a comparative review". *Vascular Health and Risk Management* 11 (2015): 133-148.

5. Anghel L., *et al.* "From classical laboratory parameters to novel biomarkers for the diagnosis of venous thrombosis". *International Journal of Molecular Sciences* 21.6 (2020): 1920.

6. Kim AS., *et al.* "Mechanisms and biomarkers of cancer-associated thrombosis". *Translational Research* 225 (2020): 33-53.

7. Gorog DA., *et al*. "Current and novel biomarkers of thrombotic risk in COVID-19: a consensus statement from the international COVID-19 thrombosis biomarkers colloquium". *Nature Reviews Cardiology* 19.7 (2022): 475-495.

8. Kovacs IB., *et al.* "Evans blue: an ideal energy-absorbing material to produce intravascular microinjury by HE-NE gas laser". *Microvascular Research* 10.1 (1975): 107-124.

9. Yamamoto J., *et al.* "Effect of physical training on thrombotic tendency in rats: decrease in thrombotic tendency measured by the He-Ne laser-induced thrombus formation method". *Haemostasis* 19.5 (1989): 260-265.

10. Ratnatunga CP., *et al.* "High-dose aspirin inhibits shear-induced platelet reaction involving thrombin generation". *Circulation* 85.3 (1992): 1077-1082.

11. Yamamoto J., *et al.* "Effect of leucocyte products on platelet thrombus formation, coagulation and spontaneous thrombolysis, as measured from native human blood, *in vitro*". *Thrombosis Research* 71.4 (1993): 281-287.

12. Ikarugi H., *et al.* "Norepinephrine, but not epinephrine, enhances platelet reactivity and coagulation after exercise in humans". *Journal of Applied Physiology* 86.1 (1999): 133-138.

13. Gorog D., *et al.* "Thrombotic status analyser. Measurement of platelet-rich thrombus formation and lysis in native blood". *Thrombosis and Haemostasis* 73.3 (1995): 514-520.

14. Nakajima S., *et al.* "A global platelet test of thrombosis and thrombolysis detects a prothrombotic state in some patients with non-insulin dependent diabetes and in some patients with stroke". *Platelet.* 11.8 (2000): 459-466.

15. Yamamoto J., *et al.* "Gorog Thrombosis Test: a global in-vitro test of platelet function and thrombolysis". *Blood Coagulation and Fibrinolysis* 14.1 (2003): 31-39.v

16. Yamamoto J., *et al.* "Global thrombosis test (GTT) can detect major determinants of haemostasis including platelet reactivity, endogenous fibrinolytic and thrombin generating potential". *Thrombosis Research* 133.5 (2014): 919-926.

17. Sakariassen KS., *et al.* "Recollections of the development of flow devices for studying mechanisms of hemostasis and thrombosis in flowing whole blood". *Journal of Thrombosis and Haemostasis* 2.10 (2004): 1681-1690.

18. Taomoto K., *et al.* "Platelet function and spontaneous thrombolytic activity of patients with cerebral infarction assessed by the global thrombosis test". *Pathophysiology of Haemostasis and Thrombosis* 37.1 (2010): 43-48.

19. Shioyama W., *et al.* "Effect of cancer chemotherapy on platelet reactivity and thrombolytic activity". Presented at the 25th Kinki Thrombosis Research Society Meeting. Osaka, Japan (2020).

20. Kovacs IB., *et al.* "Spontaneous thrombolysis: a forgotten determinant of life or death". *Clinical and Applied Thrombosis/Hemostasis* 12.3 (2006): 358-363.

21. Kovacs IB*., et al.* "Enhanced spontaneous thrombolysis: a new therapeutic challenge". *Journal of Thrombosis and Thrombolysis* 21.3 (2006): 221-227.

22. Gorog DA. "Prognostic value of plasma fibrinolysis activation markers in cardiovascular disease". *Journal of the American College of Cardiology* 55.24 (2010): 2701-2709.

23. Kawano M*., et al.* "Adjuvant effect of argatroban on staphylokinase induced thrombolysis of platelet rich thrombi in rat mesenteric venules *in vivo*". *Thrombosis Research* 86.2 (1997): 115-126.

24. Yamashita T*., et al.* "Conjunctive effects of the 5HT (2) receptor antagonist, sarpogrelate, on thrombolysis with modified tissue plasminogen activator in different laser-induced thrombosis models". *Haemostasis* 30.6 (2000): 321-332.

25. Hashimoto M*., et al.* "Enhanced thrombolysis induced by argatroban or activated protein C in the presence or absence of staphylokinase, measured in an in vivo animal model using mesenteric arterioles". *Haemostasis* 31.2 (2001): 80-89.

26. Hashimoto M*., et al.* "Enhancement of endogenous plasminogen activator-induced thrombolysis by argatroban and APC and its control by TAFI, measured in an arterial thrombolysis model *in vivo* using rat mesenteric arterioles". *Journal of Thrombosis and Thrombolysis* 87.1 (2002): 110-113.

27. Sakariassen KS*., et al.* "A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix, and purified components". *Journal of Laboratory and Clinical Medicine* 102.4 (1983): 522-535.

28. Sakariassen KS*., et al.* "Human blood platelet adhesion to artery subendothelium is mediated by factor VIII-von Willebrand factor bound to the subendothelium". *Nature* 279.5714 (1979): 636-638.

29. Sakariassen KS*., et al.* "Platelet adherence to subendothelium of human arteries in pulsatile and steady flow". *Thrombosis Research* 19.4-5 (1980): 547-559.

30. Sakariassen KS*., et al.* "Factor VIII-von Willebrand factor requires calcium for facilitation of platelet adherence". *Blood* 63.5 (1984): 996-1003.

31. Sakariassen KS*., et al.* "Differentiation of patients with subtype IIb-like von Willebrand`s disease by means of perfusion experiments with reconstituted blood". *British Journal of Haematology* 59.3 (1985): 459-470.

32. Sakariassen KS*., et al.* "Mediation of platelet adhesion to fibrillar collagen in flowing blood by a proteolytic fragment of human von Willebrand factor". *Blood* 67.5 (1986): 1515-1518.

33. Sakariassen KS*., et al.* "Growth and stability of thrombi in flowing blood: Assessment of platelet-surface interactions with computer-assisted morphometry". *Thrombosis and Haemostasis* 60.3 (1988): 392-398.

34. Sakariassen KS*., et al.* "Upstream thrombus growth impairs downstream thrombogenesis in non-anticoagulated blood: Effect of procoagulant artery subendothelium and non-procoagulant collagen". *Thrombosis and Haemostasis* 65.5 (1991): 596-600.

35. Roux SP*., et al.* "Effect of aspirin and epinephrine on experimentally-induced thrombogenesis in dogs: a parallelism between *in vivo* and *ex vivo* thrombosis models". *Arteriosclerosis, Thrombosis, and Vascular Biology* 11.5 (1991): 1182-1191.

36. Fressinaud E*., et al.* "Shear rate-dependent impairment of thrombus growth on collagen in nonanticoagulated blood from patients with von Willebrand disease and hemophilia A". *Blood* 80.4 (1992): 988-994.

37. Sakariassen KS*., et al.* "The impact of blood flow on thrombogenesis". *Journal of Blood Rheology* 7 (1993): 33-36.

38. Roald HE*., et al.* "Clopidogrel - a platelet inhibitor which inhibits thrombogenesis in human non-anticoagulated blood independently of the blood flow conditions". *Thrombosis and Haemostasis* 71.5 (1994): 665-672.

39. Diquélou A*., et al*. "Effect of blood flow on thrombin generation is dependent on the nature of the thrombogenic surface". *Blood* 84.7 (1994): 2206-2213.

40. Barstad RM*., et al.* "A perfusion chamber developed to investigate thrombus formation and shear profiles in flowing native human blood at the apex of well-defined stenoses". *Arteriosclerosis, Thrombosis, and Vascular Biology* 14.12 (1994): 1984-1991.

41. Sakariassen KS*., et al.* "The impact on blood shear rate on arterial thrombus formation". *Future Science OA* 1.4 (2015): FSO30.

42. Sakariassen KS. "Thrombus formation on apex of arterial stenoses: the need for a fluid high shear diagnosis device". *Future Cardiology* 3.2 (2007): 193-201.

43. Taka T*., et al.* "Platelet reactivity in spontaneously diabetic rats is independent from blood glucose and insulin levels". *Platelets* 13.5-6 (2002): 313-316.

44. Taka T*., et al.* "Thrombotic tendency of a non-insulin dependent diabetic rat OLETF". *Research Conference on OLETF Rats* 2 (1996): 75-79.

45. Iwasaki M*., et al.* "Are all wines made from various grape varieties beneficial in the prevention of myocardial infarction and stroke?" *Future Science OA* 7.2 FSO649 (2021).

46. Yamamoto J*., et al.* "The antithrombotic effects of carrot filtrates in rats and mice". *Blood Coagulation and Fibrinolysis* 19.8 (2008): 785-792.

47. Noguchi T*., et al.* "Enhanced thrombogenicity and altered hemodynamics in the cerebral microvasculature of stroke-prone spontaneously hypertensive rats". *Haemostasis* 27.5 (1997): 237-245.

48. Taka T*., et al.* "Impaired flow-mediated vasodilation *in vivo* and reduced shear-induced platelet reactivity *in vitro* in response to nitric oxide in prothrombotic, stroke-prone spontaneously hypertensive rats". *Pathophysiology of Haemostasis and Thrombosis* 32.4 (2002): 184-189.

49. Tamura Y*., et al.* "Impaired endothelial function may be due to decreased aortic tetrahydrobiopterin, assessed by a new flow-mediated vasodilation *in vivo* in hypercholesterolemic/atherogenic mice". *Blood Coagulation and Fibrinolysis* 20.8 (2009): 699-705.

50. Ijiri Y*., et al.* "Dietary diacylglycerol extenuates arterial thrombosis in apoE and LDLR deficient mice". *Thrombosis Research* 117.4 (2006): 411-417.

51. Ijiri Y*., et al.* "Mechanism of the antithrombotic effect of dietary diacylglycerol in atherogenic mice". *Pathophysiology of Haemostasis and Thrombosis* 35.5 (2006): 380-387.

52. Noma K*., et al.* "Cilostazol for treatment of cerebral infarction". *Expert Opinion on Pharmacotherapy* 19.15 (2018): 1719-1726.

53. Seong-Joon Lee*., et al.* "Cilostazol improves endothelial function in acute cerebral ischemia patients: a double-blind placebo controlled trial with flow-mediated dilation technique". *BMC Neurology* 17.1 (2017): 169.

54. Hiroyoshi M*., et al.* "The Effect of Cilostazol on Endothelial Function as Assessed by Flow-Mediated Dilation in Patients with Coronary Artery Disease". *Journal of Atherosclerosis and Thrombosis* 23.10 (2016): 1168-1177.

55. Takase B*., et al.* "Combined therapeutic effect of probucol and cilostazol on endothelial function in patients with silent cerebral lacunar infarcts and hypercholesterolemia: a preliminary study". *Medical Principles and Practice* 23.1 (2014): 59-65.

56. Jacobsen AP*., et al.* "Lifelong aspirin for all in the secondary prevention of chronic coronary syndrome: still sacrosanct or is reappraisal warranted?". *Circulation* 142.16 (2020): 1579-1590.

57. Peng S*., et al.* "A long-acting PAI-1 inhibitor reduces thrombus formation". *Thrombosis and Haemostasis* 117.7 (2017): 1338-1347.

58. Noguchi N*., et al.* "Fibrinolytic potential of DS-1040, a novel orally available inhibitor of activated thrombin-activatable fibrinolysis inhibitor". *Thrombosis Research* 168 (2018): 96-101.

59. Sansilvestri-Morel P*., et al.* "S62798, a potent TAFIa inhibitor, accelerates endogenous fibrinolysis in a murine model of pulmonary thromboembolism". *Thrombosis Research* 204 (2021): 81-87.

60. Violi F*., et al.* "Statins as antithrombotic drugs". *Circulation* 127.2 (2013): 251-257.

61. Bonetti PO*., et al.* "Statin effects beyond lipid lowering--are they clinically relevant?". *European Heart Journal* 24(3 (2003): 225-248.

62. Oppelt P*., et al.* "Approach to chemotherapy-associated thrombosis". *Vascular Medicine* 20.2 (2015): 153-161.

63. Phillippe HM. "Overview of venous thromboembolism". *The American Journal of Managed Care* 23 (2017): S376-S382.

64. Morishita M*., et al.* "Mechanism of the experimental antithrombotic effect of some apple varieties involves enhanced endogenous thrombolytic activity". *Interventional Medicine and Applied Science* 4.3 (2012): 115-124.

65. Kondo K*., et al.* "Trans-fatty acid promotes thrombus formation in mice by aggravating antithrombogenic endothelial functions via toll-like receptors". *Molecular Nutrition and Food Research* 59.4 (2015) 729-740.

66. Ikarugi H*., et al.* "Impaired spontaneous thrombolytic activity in elderly and in habitual smokers, as measured by a new global thrombosis test". *Blood Coagulation and Fibrinolysis* 14.8 (2003): 781-784.

67. Yamamoto J*., et al.* "Tomatoes have natural anti-thrombotic effects". *British Journal of Nutrition* 90.6 (2003): 1031-1038.

68. Naemura A*., et al.* "An experimentally antithrombotic strawberry variety is also effective in humans". *Pathophysiology of Haemostasis and Thrombosis* 35.5 (2006): 398-404.

69. Ijiri Y*., et al.* "Diet of fruits and vegetables with experimental antithrombotic effect may be beneficial to humans in the prevention of arterial thrombotic diseases". *International Journal of Drug Development and Research* 8.3 (2016): 12-16.

70. Ikarugi H*., et al*. "High intensity exercise enhances platelet reactivity to shear stress and coagulation during and after exercise". *Pathophysiology of Haemostasis and Thrombosis* 33.3 (2003): 127-133.

71. Baba Y. "Effects of exercise and diet interventions modeled on Healthy Japan 21 on thrombus formation". Master's Thesis, Kobe Gakuin University (2012).

72. Baba Y*., et al.* "Changes in platelet reactivity, thrombolytic activity and other physical activities by habitual exercise". Presented at the at the 51st Japan Society of Nutrition and Food Science Kinki Meeting. Hyogo, Japan (2012).

73. Ikarugi H*., et al.* "The exercise paradox may be solved by measuring the overall thrombotic state using native blood". *Drug Discoveries and Therapeutics* 11.1 (2017): 15-19.

74. Gorog DA*., et al.* "First direct comparison of platelet reactivity and thrombolytic status between Japanese and Western volunteers: possible relationship to the "Japanese paradox". *International Journal of Cardiology* 152.1 (2011): 43-48.

75. Gue YX*., et al.* "Thrombotic profile and oral anticoagulation in Asian and non-Asian patients with nonvalvular atrial fibrillation". *Journal of the American College of Cardiology* 74.22 (2019): 2822-2824.