

# Proposal of an *in vitro* thrombus-growth model for evaluating anticoagulants

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**SUMMARY** The general anticoagulant evaluation requires high expense equipment, reagents, and space. Therefore, not all laboratories can perform research related to anticoagulant. In this study, we propose a novel simple method "*in vitro* thrombus-growth model" that can evaluate anticoagulant ability by measuring weight. The *in vitro* thrombus-growth model is prepared by creating a "growth-clot" with citrate plasma, calcium chloride, and thrombin, and then pouring new citrate plasma onto it. The prepared growth-clots were increased in volume in citrated human plasma, including surpluses calcium chloride, which was released slowly, leading to clot coagulation around the plasma. As a result of evaluating the anticoagulant ability of direct thrombin inhibitor using this *in vitro* thrombus-growth model, it was confirmed that clot growth was suppressed in a concentration-dependent manner. Therefore, this thrombus-growth model is useful as a primary anticoagulant test that can to discover compounds with anticoagulant activity perform in any laboratory.

**Keywords** Thrombus-growth model, anticoagulants, thrombosis, fibrin clot, *in vitro* model

## 1. Introduction

The first and second leading causes of death in the world are ischemic heart disease and stroke, respectively, with a marked increase in cardiovascular disease, especially in Asian countries (1,2). These diseases have a high prevalence in the elderly, and life expectancy is increasing globally, so it is possible that their prevalence will continue to increase (3). Proper use of antithrombotic drugs to prevent thrombosis is important for extending healthy life expectancy. Recently, the development of various anticoagulants, such as direct oral anticoagulants (DOAC), has significantly improved the prevention of thrombus-related diseases and prognosis of patients (4). However, it may not be enough to prevent blood clot-related diseases; for example, elderly people often have impaired renal function, DOACs are difficult to use because they are excreted by the kidneys, and some DOACs do not have antagonists and cannot be treated to deal with bleeding complications and so on. As such, it is important to improve the case of these anticoagulants and develop new ones.

To accelerate the development of anticoagulants, an experimental system that can easily evaluate the

thrombus formation inhibitory effect is needed. Indeed, the general anticoagulant evaluation requires high expense equipment, reagents, and space. Therefore, not all laboratories can perform research related to anticoagulants. Here, we propose a new experimental system that can evaluate anticoagulant ability without requiring special equipment. Factor IV (Ca<sup>2+</sup>) is involved in blood coagulation cascade. The anticoagulant sodium citrate, which is used for blood collection, exerts an anticoagulant effect by chelating calcium ions. Thus, citrate plasma regains coagulation activity simply by supplementing citrate plasma with calcium ions. Therefore, the proposed model consisted of a fibrin clot containing a high concentration of calcium ions, which release calcium ions in the plasma and coagulate the surrounding plasma, thereby causing the growth of the fibrin clot. In this thrombus-growth model, fibrin clot weight increases in a calcium chloride concentration-dependent manner. The fibrin clot growth was then evaluated by directly adding the direct thrombin inhibitor argatroban to see if the clot stopped growing.

This thrombus-growth model evaluates thrombus growth by weight; thus, it is not suitable for directly elucidating the mechanism of anticoagulants, but it can

clearly evaluate whether thrombus formation can be inhibited.

## 2. Materials and Methods

### 2.1. Growth-clot preparation

Growth-clots were prepared with human citrated plasma, CRYOcheck™ Pooled Normal Plasma (CCN-40) (Precision BioLogic, NS, Canada) 193  $\mu$ L with 2 M calcium dichloride ( $\text{CaCl}_2$ ) (FUJIFILM Wako Chemicals, Osaka, Japan) solution 5  $\mu$ L (final conc. 50 mM), and 0.1 U/ $\mu$ L thrombin (FUJIFILM Wako Chemicals) 2  $\mu$ L (final conc. 1 U/mL) in a latex rubber tube (Finger Cots Unroll Type S) (AS ONE, Osaka, Japan) and incubated at room temperature for 45 min in a moistened box.

### 2.2. Effects of calcium chloride concentration on thrombus-growth

The concentration of the calcium chloride solution for preparing the growth-clots was adjusted, and growth-clots with final concentrations of calcium chloride of 30 mM, 50 mM, and 100 mM were prepared. A total of 3 mL of human citrate plasma was poured into each growth-clot and incubated at 37°C in a water bath for 30 min. Human citrate plasma was removed with a micropipette and growth-clots were weighed by microbalance.

### 2.3. Effects of solvent contamination on thrombus-growth model

A total of 5 or 10 v/v% saline was added to human citrate plasma and poured into growth-clots and incubated at 37°C in a water bath for 30 min. Further, 0.5 or 2.5 v/v% dimethyl sulfoxide (DMSO) was added to human citrate plasma and poured into growth-clots and incubated at 37°C in a water bath for 30 min.

### 2.4. Suppression of growth-clots' growth by argatroban

Argatroban was dissolved in DMSO (20 mg/mL) and added to human citrate plasma at a rate of 2 v/v%. Finally, the plasma was poured into growth-clot and incubated at 37°C in a water bath for 30 min.

### 2.5. Statistical analysis

Growth-clot weights were examined using Student's *t*-test. The level of statistical significance was  $p < 0.05$ .

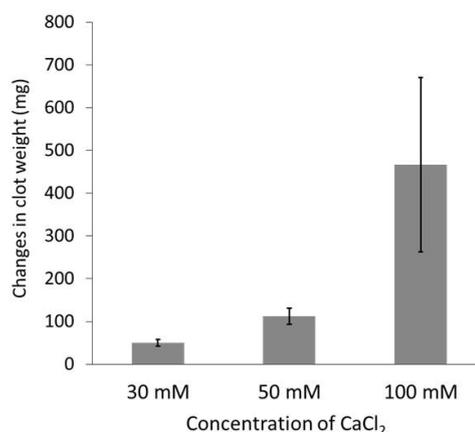
## 3. Results and Discussion

To confirm the growth-clot characteristics of the thrombus-growth model, the effects on growth-clot weight were investigated by changing the calcium

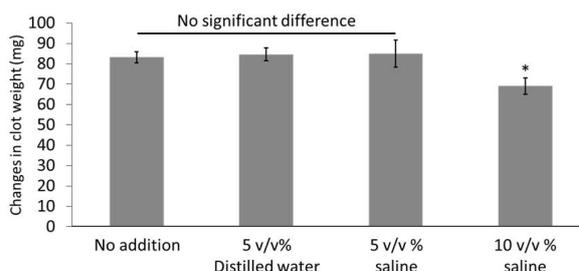
chloride concentration. Growth-clot weight in the 30 mM group was low, making it unsuitable for experiments compared with controls. Growth-clot weight and variability were both large in the 100 mM group. In contrast, in the 50 mM group, the growth-clot weight was relatively large and the variation was small (Figure 1). Thus, the calcium chloride concentration in the thrombus-growth model was set to 50 mM.

When evaluating an anticoagulant, it is conceivable to prepare an aqueous solution of the anticoagulant and add it to the thrombus-growth model. Therefore, the effect on growth-clot weight when plasma was diluted with an aqueous solvent was evaluated. The addition of 5 v/v% distilled water or saline to the thrombus-growth model did not affect the weight of the growth-clot. However, the addition of 10 v/v% saline significantly reduced growth-clot weight (Figure 2). Therefore, it seems that addition of anticoagulant aqueous solution to the plasma should be kept within 5 v/v%.

Many medicines are fat-soluble, and it is difficult to dissolve these medicines in water or plasma; thus, the use of organic solvents is recommended. Therefore, we evaluated the effect of DMSO on the thrombus-growth model, which is widely used as a solvent in biological



**Figure 1. Thrombus-growth weight dependent on CaCl<sub>2</sub> concentration.** Appropriate concentrations of CaCl<sub>2</sub> in thrombus growth were evaluated. mean  $\pm$  standard deviation ( $n = 5$  for each group).



**Figure 2. Effect of plasma dilution on thrombus-growth model.** An aqueous solvent was added to the thrombus-growth model, and the effect on the thrombus growth amount was evaluated. \* $p < 0.05$  vs. no addition (Student's *t*-test), mean  $\pm$  standard deviation ( $n = 4$  for each group).

experiments. The addition of DMSO up to 2.5 v/v% did not affect growth-clot weight, but DMSO at 2.5 v/v% tended to decrease the weight slightly (Figure 3). Therefore, it seems that addition of anticoagulant/DMSO solution to the plasma should be kept below of 2.5 v/v%.

Finally, we evaluated whether clot growth was suppressed when the direct thrombin inhibitor argatroban was added in the thrombus-growth model. Clot growth was suppressed by argatroban in a concentration-dependent manner, and clot growth stopped at 400 µg/mL (Figure 4).

In this study, a thrombus-growth model showed that growth-clots were dependent on calcium chloride concentration and that the mechanism of clot growth was thrombin-dependent as in a living body.

The growth-clots include surpluses of factor IV ( $\text{Ca}^{2+}$ ) of blood coagulation factors. Factor IV was slowly released and coagulated the surrounding plasma into new clots in human citrated plasma. In contrast, growth-clots prepared with 10-fold concentrations (10 U/mL) of thrombin did not change the weight of thrombus growth (data not shown). The fibrin network pore size is affected by thrombin concentration and ionic strength (5), but the pore size is about 4-5 µm (6) and it is unlikely that thrombin cannot be release. We consider the effects of factor XIII to understand why

thrombin cannot be involved in thrombus growth. Factor XIII catalyzes the cross-linking of fibrin and stabilizes the fibrin clot. However, Factor XIII has relatively low substrate specificity, and the substrate of Factor XIII exceeds 140 (7-9). Therefore, we guessed that thrombin forms a complex with the fibrin monomer in fibrin clot and thus cannot release. We would like to examine the possibility of cross-linking between Factor XIII and thrombin in the future. We would like to examine the possibility of cross-linking between Factor XIII and thrombin in the future.

In this study, a latex rubber tube was used as a container for preparing a thrombus-growth model. This is because we used ultrasonic waves in a previous study (10) and decided to use latex rubber because the material does not easily reflect ultrasonic waves. Therefore, the thrombus-growth model does not necessarily have to use latex rubber, and there is no problem with using another container, such as synthetic resin. We also confirmed that growth-clots grow in 5 mL polypropylene and polystyrene test tubes. In addition, although this report proposes a thrombus-growth model using human citrate plasma, similar experiments can be performed using relatively inexpensive bovine citrate plasma (P4639-10ML, Sigma-Aldrich, Tokyo, Japan).

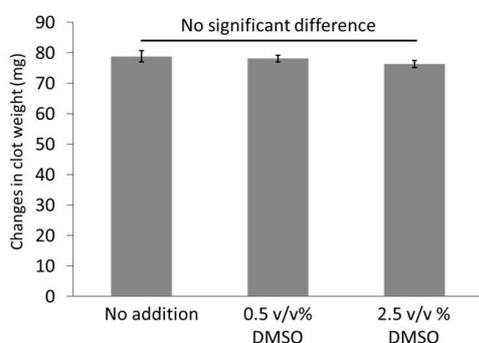
The limitation of this thrombus-growth model is that the weight of growth-clots varies slightly depending on the plasma lot. Therefore, when evaluating the ability of anticoagulants, a comparison target is always necessary. Further, to correct the error between experiments, it is necessary to calculate the thrombus-growth suppression ratio (R) equation as below:

$$R = ((\Delta \text{ control} - \Delta \text{ sample}) / \Delta \text{ control}) \times 100\%$$

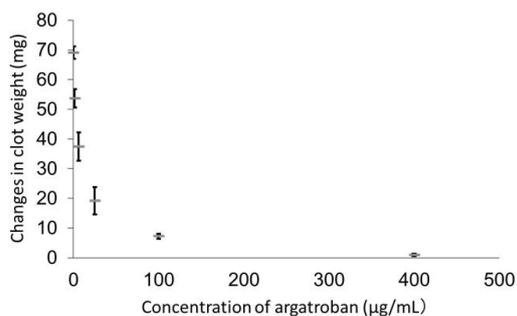
where  $\Delta$  control ( $\Delta$  sample) is change in clot weight (mg).

There are several tips for growing a growth-clot with good reproducibility. When preparing a growth clot, the surface shape of the growth-clots should be hemispherical with a bulge. Clots do not grow much if they are flat or dented. Thus, careful manipulation is required when pipetting thrombin and plasma. However, slow pipetting increases the viscosity of plasma due to the coagulation reaction, thereby increasing the probability of trapping air bubbles in the growth-clots. Moreover, the reagents used to prepare growth-clots should be kept cold to slow down the blood clotting reaction.

The principle of the instrument that measures the parameters of blood coagulation mainly captures changes in plasma based on the coagulation reaction, such as transparency and viscosity. On the other hand, the thrombus-growth model is a novel evaluation method that can evaluate the formation of fibrin clot by clot weight (mg) without the need for expensive equipment or special reagents. Therefore, the thrombus-growth model



**Figure 3. Effect of dimethyl sulfoxide on thrombus-growth model.** Dimethyl sulfoxide as fat-solvent was added to the thrombus-growth model, and the effect on the thrombus growth amount was evaluated. mean  $\pm$  standard deviation ( $n = 4$  for each group).



**Figure 4. Thrombus growth suppression of thrombus-growth model by argatroban.** A thrombus-growth model was tested if it can be suppression of clots growth by direct thrombin inhibitor. mean  $\pm$  standard deviation ( $n = 5$  for each group).

we propose is a utility experimental method that can be performed in any laboratory with common laboratory tool such as microbalance and micropipette.

As a limitation of the evaluation of anticoagulants in this thrombus-growth model, antiplatelet drugs (aspirin, clopidogrel, *etc.*) cannot be evaluated because this model does not contain platelets. It is also not suitable for evaluating vitamin K antagonists such as warfarin. This model is suitable for assessing substances that directly inhibit blood coagulation factors or directly activate anticoagulation factors.

In addition, prothrombin time, activated partial thromboplastin time, and the like are measured in the evaluation of general anticoagulants. Although it is not suitable to analyze these detailed parameters and mechanisms in the thrombus-growth model, it is possible to clearly evaluate the inhibition of thrombus formation. Conversely, even if the mechanism is unclear, it is possible to evaluate the presence or absence of inhibition of thrombus formation.

In conclusion, this thrombus-growth model serves as a primary anticoagulant test that can clearly assess anticoagulant ability even with drugs of both fat-soluble or water-soluble. We hope that this model will contribute to the development of novel antithrombotic drugs.

*Funding:* This work was supported by JSPS KAKENHI Grant Number 20K12679 and in part by the The Uehara Memorial Foundation and Nihon Pharmaceutical University Research Grant.

*Conflict of Interest:* The authors have no conflicts of interest to disclose.

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Received May 19, 2022; Revised June 20, 2022; Accepted June 23, 2022.

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Released online in J-STAGE as advance publication June 26, 2022.