

Original Article

Influence of high-dose antithrombin on platelet function and blood coagulation

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Aim: In healthy adults, there are sufficient amounts of antithrombin in the blood to regulate thrombin. However, the effects of high concentrations of antithrombin on dose-dependent anticoagulation and platelet function have not been reported. In this study, we assessed platelet function and blood coagulation following high-dose antithrombin supplementation *in vitro*.

Methods: Blood samples were collected from 10 healthy volunteers, and samples with different antithrombin concentrations were prepared by adding an antithrombin agent (Neuart). Blood coagulation was assessed by the Thrombus-Formation Analysis System (T-TAS) and Rotational Thromboelastometry (ROTEM) using whole blood samples.

Results: The data obtained by the platelet chip, exclusively representing platelet function, revealed that the onset of thrombus formation was significantly delayed in a dose-dependent manner (100%–200%, $P = 0.021$; 100%–500%, $P = 0.011$; 200%–500%, $P = 0.047$). In measurements using the atheroma chip, which enables assessment of blood coagulation, the thrombus formation ability was found to be reduced (100%–200%, $P = 0.022$; 100%–500%, $P = 0.05$). In the ROTEM measurements, clotting time was prolonged in a dose-dependent manner (100%–200%: $P = 0.203$, 200%–500%: $P = 0.005$, 500%–1000%: $P = 0.022$), except when comparing with 100% and 200%. Although antithrombin is reportedly saturated in healthy blood, its anticoagulant ability appears to be enhanced depending on its concentration. Furthermore, data obtained from the platelet chip showed that antithrombin might reduce platelet function.

Conclusions: Antithrombin suppressed platelet function and blood coagulation in a dose-dependent manner.

Key words: Antithrombin, thrombus formation ability, total thrombus formation analysis system

INTRODUCTION

ANTITHROMBIN (AT) IS a 58-kDa plasma glycoprotein and is an important physiological serine protease inhibitor that inhibits thrombin and factors IXa, VIIa, Xa, XIa, and XIIa in the regulation of blood coagulation.^{1–3} Antithrombin has not only anticoagulant effects, but also anti-inflammatory and protective effects on vascular endothelial cells.^{4–7} However, to achieve the anti-inflammatory effect, the AT activity level must be maintained above the physiological activity level.⁸

Antithrombin preparations are available as drugs that replenish AT in the blood. In the KyberSept trial, a large double-blind study, the protocol was to administer a total of 30,000 units of AT over 4 days, which increased the AT activity level from 115% to 180%, but there was no significant improvement in the survival rate.⁹ Therefore, although AT has been found to be effective in patients with congenital AT deficiency and obstetric disseminated intravascular coagulation (DIC), its usefulness in patients with sepsis is becoming questionable.^{10–14}

Standard laboratory tests for blood coagulation include prothrombin time and activated partial thromboplastin time. However, these tests often fail to consistently and accurately reflect the patient's coagulation ability, as they are carried out when thrombin production is only 4% of the total value.¹⁵ Additionally, platelet effects are not reflected, due to the separation of plasma components.¹⁶ Point-of-care testing (POCT), such as ROTEM (TEM International, Munich, Germany), TEG (Haemonetics, Braintree, MA) and Thrombus-Formation Analysis System (T-TAS; Fujimori Industries, Yokohama, Japan), measures coagulation function. These POCTs do not require

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blood component separation and can even capture changes such as hyperfibrinolysis and clot retraction by measuring for a long duration. As POCT enables detailed observation, it is also used as a measurement device in the field of basic research.^{17,18}

In healthy adults, there are sufficient amounts of AT in the blood to regulate thrombin.¹⁹ However, the effects of high concentrations of AT on dose-dependent anticoagulation and platelet function have not been reported. In this study, we used POCT to examine the changes in coagulation characteristics by AT concentration using blood samples collected from healthy volunteers.

The onset of the thrombus formation was significantly delayed in a dose-dependent manner. That means that the platelet function was suppressed by AT.

METHODS

Specimens and drugs

BLOOD SPECIMENS WERE collected from 10 healthy volunteers (aged 24–39 years, mean 31.6 years) who had no detectable abnormalities on examination and no reported young-onset thrombotic events in their families. Neuart was supplied as an AT agent under a material transfer agreement with the Japan Blood Products Organization.

Method of T-TAS measurement

T-TAS is a POCT product developed in Japan to measure thrombus formation ability. This device can maintain a constant flow of a whole blood sample into a chip with a simulated blood vessel, allowing the observation of thrombus formation in the simulated vessel over time. The rate of thrombus formation and thrombus hardness is reflected in the pressure curve, which is used to evaluate the platelet and coagulation functions of the whole blood sample. There are two types of chips with simulated blood vessels, the platelet (PL) chip and atheroma (AR) chip. The PL chip assesses platelet function and possesses a simulated blood vessel covered with collagen (Fig. 1). The specimen used in this test was anticoagulated using hirudin, a thrombin inhibitor. Platelets bind to collagen in the simulated vessel by von Willebrand factor and generate sliding stress. Platelets are activated by sliding stress, which induces fibrinogen- and von Willebrand factor-mediated aggregation, leading to thrombus formation. As a result of thrombus formation narrowing the simulated vessel, the internal pressure of the pump increases and is reflected in the pressure curve.

The AR chip presents simulated blood vessels covered with collagen and tissue factors (Fig. 2). In simulated blood

vessels, whole blood samples anticoagulated with sodium citrate are activated by collagen and tissue factors following the addition of calcium ions. A thrombus is formed by activated platelets and coagulation factors, increasing the internal pressure of the pump, which is reflected in the pressure curve. In other coagulation tests, including prothrombin time and activated partial thromboplastin time, undertaken in the laboratory or by POCT, the reagents used to induce coagulation were added directly to the specimen and mixed. Conversely, in the T-TAS device, collagen and tissue factors in a simulated blood vessel activate a part of the specimen, triggering thrombus formation similar to that observed in damaged vessels. Furthermore, unlike other POCTs, the use of linear simulated vessels is considered more physiologically representative from a hemodynamic perspective.

Platelet measurement

In brief, specimens anticoagulated with hirudin were injected into three spits of 1 mL each to generate samples with no additional AT, 0.5 units of AT, and 2 units of AT, respectively. Dissolution of the AT drug was standardized to 20 μ L of pure water to avoid specimen dilution. These adjustments produced specimens with AT concentrations of 100%, 200%, and 500%, respectively. For each specimen, PL measurements were carried out using T-TAS and the time to reach 10 kPa (T10) was defined as the onset of thrombus occlusion.

Atheroma measurement

As carried out for PL measurement, specimens anticoagulated with sodium citrate were adjusted to attain AT concentrations of 100%, 200%, and 500%. As AR measurement occurs over a prolonged period when compared with PL measurement, 100% versus 200% and 100% versus 500% were separately performed, considering the possibility of altered specimen coagulation due to incubation time. T10 was defined as the onset of thrombus occlusion, and the time to reach 80 kPa (T80) was defined as the thrombus occlusion time.

ROTEM measurement procedure

Rotational Thromboelastometry (ROTEM) was used to measure clot viscosity. The measured mechanical impedance is plotted over time, as shown in Figure 3, with time (min) on the horizontal axis and thrombus hardness on the vertical axis. The clotting time, clot formation time, alpha angle maximum clot firmness are shown, and fibrinolysis is expressed as maximum lysis (ML). To assess precise clot

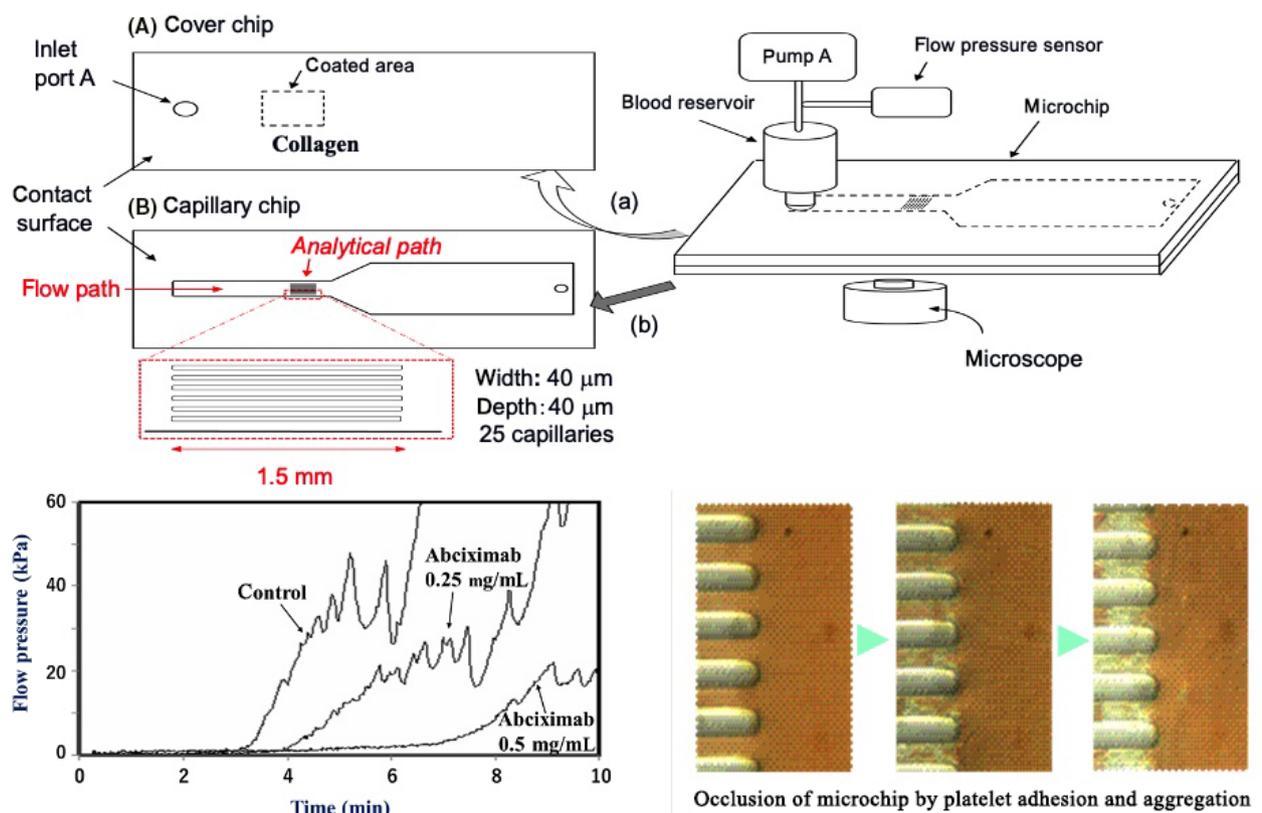


Fig. 1. Platelet chip of the Thrombus-Formation Analysis System (T-TAS) and typical measurement curve of platelet chip. A platelet chip coated with type I collagen was designed to analyze the platelet thrombus formation process. T-TAS is equipped with a pneumatic pump and a flow pressure sensor and analyzes changes in the internal pressure of the chips. Thrombus formation leads to occlusion of the platelet chip, which is analyzed by monitoring the changes in the flow pressure.

formation, we measured NATEM, which was measured without an activator.²⁰

Samples with no additional AT, 0.5 units of AT, 2 units of AT, and 4.5 units of AT were produced. These adjustments produced specimens with 100%, 200%, 500%, and 1,000% AT concentrations, respectively. For each specimen, NATEM was measured using ROTEM, and the obtained parameters were analyzed.

Statistical analysis

All statistical analyses were undertaken using spss version 23 (IBM, Tokyo, Japan). The normality of the distribution of continuous variables was assessed using the Shapiro–Wilk test. The mean and median differences for continuous variables between the two groups were assessed using the independent Student’s *t*-test and Wilcoxon rank–sum test. For all analyses, $P < 0.05$ was established as a significant difference.

RESULTS

A BOX PLOT of the PL measurements of T-TAS is shown in Figure 4. In the present study, the onset of thrombus occlusion time was significantly prolonged depending on the AT concentration (100%–200%, $P = 0.021$; 100%–500%, $P = 0.011$; 200%–500%, $P = 0.047$). A box plot of the results from the AR measurements of T-TAS is shown in Figure 5. On comparing 100% and 200% concentrations, the onset of thrombus occlusion time and thrombus occlusion time was prolonged depending on the AT concentration; similar changes were observed when comparing the 100% and 500% concentrations.

Figure 6 presents the results of the ROTEM measurements. Clotting time, a parameter of initiation of enzymatic coagulation reaction, was prolonged in a dose-dependent manner (100%–200%: $P = 0.203$, 200%–500%: $P = 0.005$, 500%–1,000%: $P = 0.022$), except when comparing 100% and 200% (Fig. 6A). Maximum lysis, a parameter of

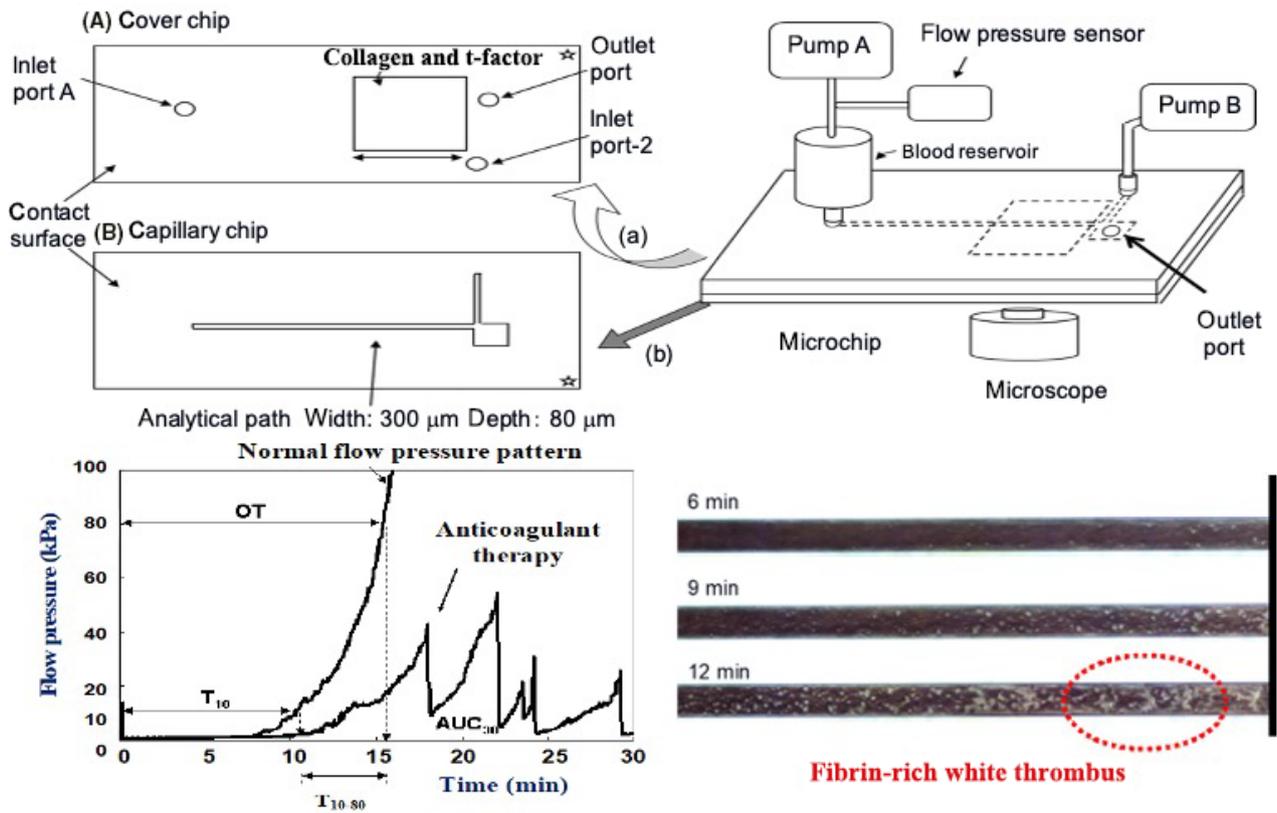


Fig. 2. Atheroma chip of the Thrombus-Formation Analysis System (T-TAS) and typical measurement curve of the atheroma chip. An atheroma chip coated with type I collagen and tissue thromboplastin was designed to analyze the fibrin-rich platelet thrombus formation process. T-TAS is equipped with a pneumatic pump and a flow pressure sensor and analyzes the changes in the internal pressure of the chips. Thrombus formation leads to occlusion of the atheroma chip and is analyzed by monitoring changes in flow pressure. t-factor, tissue factor.

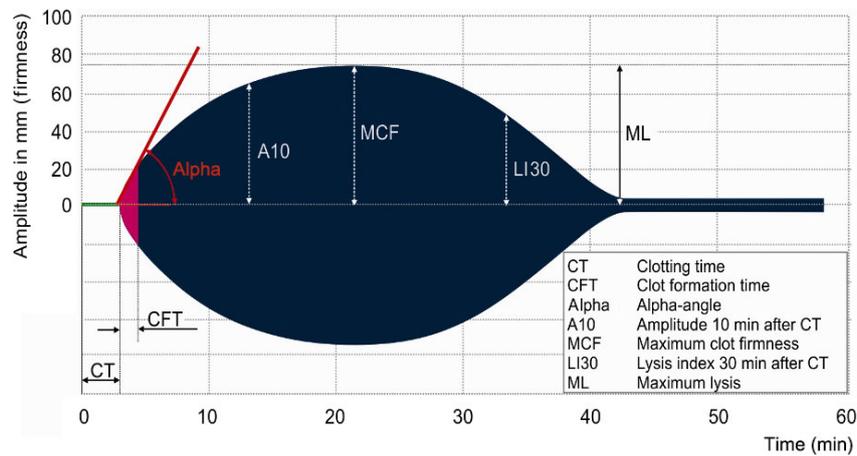


Fig. 3. Diagrammatic representation of Rotational Thromboelastometry (ROTEM). Graphical representation of clot amplitude (mm) over time (min) showing various ROTEM variables.

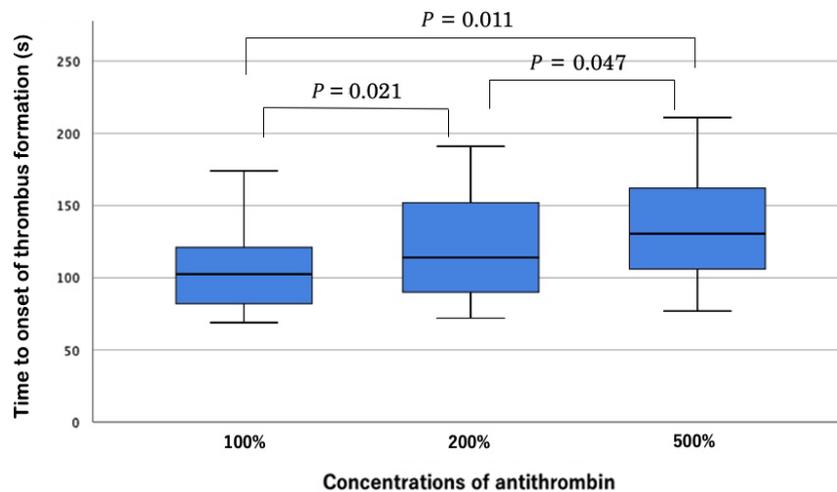


Fig. 4. Box plot diagram of Thrombus-Formation Analysis System (T-TAS) measurement using platelet chip. The onset of thrombus formation was significantly delayed in a dose-dependent manner. This indicates that platelet function is suppressed by antithrombin. Data are shown as box plots with medians (lines inside boxes) and 25th and 75th quartiles (top and bottom of boxes), and whiskers indicate the range. Wilcoxon signed-rank test was applied.

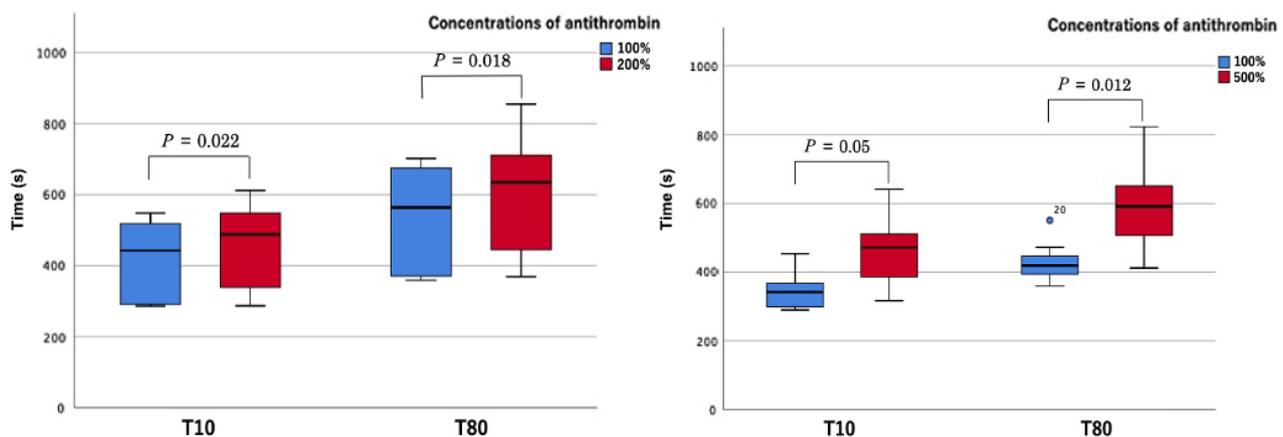


Fig. 5. Box plot diagram of Thrombus-Formation Analysis System (T-TAS) measurement using the atheroma chip. Both the time at which the flow pressure increased by 10 kPa from the baseline due to partial occlusion of the capillary (T10) and the time at which the flow pressure increased by 80 kPa from the baseline (T80, which represents the near-occlusion of the capillaries) were prolonged following antithrombin administration, suggesting that antithrombin suppressed thrombus formation. Data are shown as box plots with medians (lines inside boxes) and 25th and 75th quartiles (top and bottom of boxes), and whiskers indicate the range. Any data not included between the whiskers were plotted as outliers with small circles. Wilcoxon signed-rank test was applied.

fibrinolysis and clot retraction, decreased in a dose-dependent manner, indicating a significant difference (100%–200%, $P = 0.012$; 200%–500%, $P = 0.007$; 500%–1,000%, $P = 0.00$) (Fig. 6B).

DISCUSSION

IN THE PRESENT study, AT suppressed the thrombus formation ability in simulated blood vessels, with an

enhanced anticoagulant effect observed in a dose-dependent manner. By capturing this dose-dependent change, previous studies comparing the effects of AT agents, such as the KyberSept trial, clearly showed that AT treatment causes changes in the coagulation system and platelet function.

The AR chip in T-TAS presents a simulated blood vessel coated with tissue factor and collagen in a chip, simulating an endothelial cell damaged vessel. Therefore, it can be used to determine the effect of AT on damaged endothelial

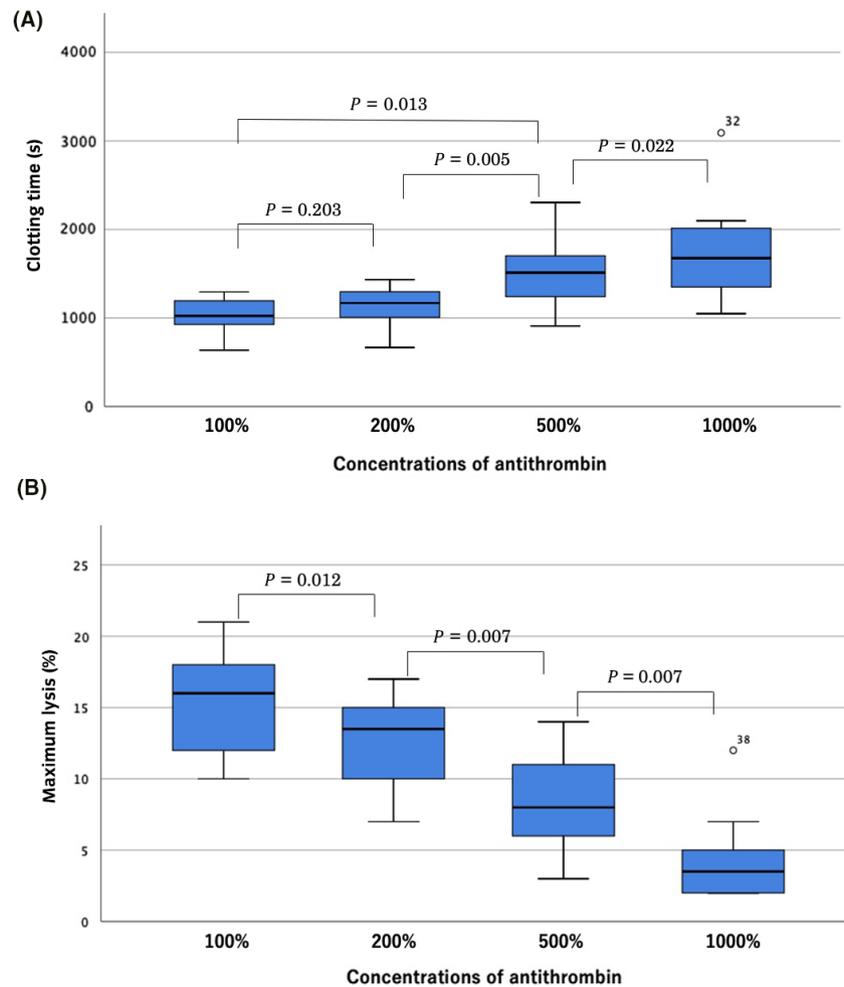


Fig. 6. (A) Box plot diagram of clotting time. The clotting time was significantly delayed in a dose-dependent manner. (B) Box plot diagram of maximum lysis. Maximum lysis was significantly decreased in a dose-dependent manner, suggesting that clot retraction was inhibited by antithrombin. Data are shown as box plots with medians (lines inside boxes) and 25th and 75th quartiles (top and bottom of boxes), and whiskers indicate the range. Any data not included between the whiskers were plotted as outliers with small circles. Wilcoxon signed-rank test was applied.

vessels, such as DIC, unlike other devices that measure viscosity. By utilizing simulated blood vessels, the present study revealed that AT exerts its anticoagulant effect in a dose-dependent manner, without the need for heparinoid in the vascular endothelium.

To measure T-TAS with the PL chip, we used specimens anticoagulated with hirudin. Hirudin inhibits blood coagulation by potently inhibiting thrombin without affecting platelet function. Therefore, the results obtained from PL chip measurements indicated that AT showed a dose-dependent suppressive effect on the platelet thrombus formation process. However, the mechanism underlying the suppression of platelet thrombus formation remains unclear. This could

be attributed to the suppression of ADP-induced platelet granule secretion through direct AT action on platelets, as reported by Doi *et al.*²¹

Typically, ROTEM reflects not only the coagulation factors but also the blood cell components by using whole blood and it can measure the coagulation cascade over a prolonged period. In the present study, we carried out ROTEM measurements in combination with T-TAS, as ROTEM does not require plasma separation, and it is possible to observe fibrinolysis and clot retraction by undertaking measurements over a prolonged period. Accordingly, the dose-dependent prolongation of clotting time showed no ceiling effect, even at an AT concentration 10 times that of the control. Clotting

time reflects the initiation time of the enzymatic coagulation reaction, which begins in the ROTEM cuvette and is similar to the onset of thrombus occlusion time in the AR chip of T-TAS. On undertaking ROTEM measurements over a prolonged period, we observed that AT decreased ML in a dose-dependent manner; this phenomenon could not be identified by T-TAS measurements. In the present study, by utilizing specimens from healthy volunteers, we observed high ML due to clot retraction in several control cases. Conversely, clot retraction was significantly reduced in specimens treated with AT, which could be attributed to the inhibition of thrombin-induced platelet activation by AT, resulting in suppressed platelet factor 3 expression and reduced clot retraction. Alternatively, AT could suppress the activation of factor XIII through thrombin inhibition, which suppresses actomyosin function in platelets and reduces clot retraction.

Using two POCTs, T-TAS and ROTEM, we identified the dose-dependent effect of AT. Regarding the anticoagulant effect, up to a concentration of 1,000%, which is 10 times higher than that of normal specimens, the anticoagulant effect was enhanced in a dose-dependent manner without any ceiling effect. Furthermore, AT inhibited platelet thrombus formation in a dose-dependent manner and decreased clot retraction, which increased the stiffness of the thrombus.

LIMITATIONS

THE findings of this study are based on *in vitro* experiments using specimens from healthy volunteers to prevent variation due to disease effects. Therefore, not all phenomena are applicable to patients with septic DIC. Further studies are needed to clarify the effects of AT on septic DIC.

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DISCLOSURES

Approval of the research protocol: This observational retrospective study was approved by the Institutional Review Board of Saga University Hospital (IRB study no. 2019-10-02).

Informed consent: Informed consent was obtained from all subjects.

Registry and registration no. of the study/trial: N/A.

Animal studies: N/A.

Conflict of interest: None.

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