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Microparticles and Nucleosomes Are Released From Parenchymal Cells Destroyed After Injury in a Rat Model of Blunt Trauma

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Abstract

We investigated the relationships between circulating procoagulants and trauma severity, including cellular destruction, and the effects of thrombin generation on procoagulants in a rat blunt trauma model. The rats were subjected to tumbling blunt trauma, where they were tumbled for 0, 250, 500, or 1000 revolutions. Creatine kinase, nucleosome, and microparticle plasma levels increased gradually with trauma severity. Strong interrelationships were observed among creatine kinase, nucleosome, and microparticle levels. Time to initiation of thrombin generation shortened with increasing trauma severity. In accordance with trauma severity, prothrombin activity decreased, but the thrombin generation ratio increased. Time to initiation of thrombin generation and the thrombin generation ratio correlated with creatine kinase levels. In an *in vitro* study, a homogenized muscle solution, which included massive nucleosomes and microparticles, showed accelerated thrombin generation of plasma from healthy subjects. Procoagulants, such as microparticles and nucleosomes, are released from destroyed parenchymal cells immediately after external traumatic force, activating the coagulation cascade. The procoagulants shorten the time to initiation of thrombin generation. Furthermore, although coagulation factors are consumed, the thrombin generation ratio increases.

Keywords

blunt trauma, coagulopathy, microparticle, nucleosome, thrombin

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Introduction

For the last several decades, trauma has been a major cause of death worldwide.[1] Recently, advances in the pathophysiological understanding of the acute phase of trauma have clarified that trauma itself induces coagulation and fibrinolytic abnormalities.[2,3] Furthermore, in the acute phase of trauma, trauma-induced coagulation and fibrinolytic abnormalities impair hemostatic functions and increase the use of transfusions and mortality.[4-10] However, 2 different hypotheses have been proposed to explain the trauma-induced coagulation abnormality, including suppression and activation of coagulation.

Many investigations report the presence of various procoagulants in the systemic circulation immediately after trauma.[11-24] Microparticles are well-known procoagulants in the acute phase of trauma.[11-17] Although platelet-derived microparticles are the best known and have been reported frequently, other blood cell- and endothelial-derived microparticles have also been reported.[11-15] Furthermore, recently, coagulation activation by brain-derived microparticles has been reported in the acute phase of brain trauma.[16,17] However,

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other parenchymal organ-derived microparticles have not been reported in trauma.

Extracellular DNA and DNA-binding proteins are released from injured tissue into the extracellular fluid and are also well-known procoagulants observed in the systemic circulation just after trauma.[18-24] Although DNA and DNA-binding proteins are normally located within the nucleus, they are released into the systemic circulation by cellular destruction and death after trauma; moreover, specific extracellular patterns of these proteins in injured tissue are associated with tissue damage.[18-24] Among the various components contained within the nucleus, nucleosomes, which are histone-complexed DNA fragments, are the most frequently reported circulating form of DNA just after trauma.[18-22] However, origins of nucleosomes just after trauma have not been previously investigated or discussed.[18-22]

Although microparticles and nucleosomes have been detected immediately after trauma, the origin and mechanisms of procoagulant release have not been clarified. Considering reports of brain-derived microparticles in brain trauma,[16,17] our hypothesis is that the majority of various procoagulants are released non-specifically and passively from parenchymal cells destroyed by external blunt-force trauma. Using a rat blunt trauma model, the present study aimed to clarify: (i) relationships between circulating procoagulants and trauma severity, (ii) relationships among each circulating procoagulant and cellular destruction, and (iii) the effects of the procoagulants on thrombin generation.

Materials and Methods

Animals

All rats were housed and treated in accordance with the Standards of Animal Experiments of Hokkaido University. All experiments were approved by the Institutional Ethical Review Board at Hokkaido University.

Nine-week-old male Wistar S/T rats were obtained from Japan SLC (Hamamatsu, Japan). Animals were allowed to acclimate for a minimum of 1 day at our animal breeding quarters before being subjected to experimentation. The breeding quarters were maintained at 20°C. Animals were fed a standard diet and provided *ad libitum* access to water. For 1 day before the experiments, animals were fasted and given *ad libitum* access to water.

Experimental procedures

In vivo study. Rats (body weight ~300 g) were anesthetized and restrained in the supine position. Using a small incision, a tracheostomy was performed, and the left carotid artery was exposed. Rats were subsequently placed in a Noble–Collip drum, a plastic wheel 38 cm in diameter with internal shelves, and rotated at 50 rpm.[25,26] Twenty rats were divided randomly into 4 groups of 5 each as follows: (i) the control group was not exposed to Noble–Collip drum trauma; (ii) the D250 group was tumbled for 250 revolutions; (iii) the D500 group was tumbled for 500 revolutions; and (iv) the D1000 group was tumbled for 1000 revolutions.

After induction of Noble–Collip drum trauma, the left carotid artery was immediately catheterized with a 24-gauge SURFLO (Terumo, Tokyo, Japan) catheter to allow mean arterial pressure monitoring and arterial blood sampling. The mean arterial pressure was monitored with a TruWave Disposable Pressure Transducer (Edwards Lifesciences, Irvine, CA, USA) and a Viridia component monitoring system (Hewlett-Packard Japan, Tokyo, Japan). To maintain arterial catheter patency, normal saline (19 mL) with 3.2% sodium citrate solution (1 mL) was constantly infused at 1 mL/h. In addition, a tracheostomy was again performed. During the experimental period, the rectal temperature was maintained at 37–39°C.

After mean arterial pressure measurements, arterial blood was collected via the catheter in the left carotid artery. Blood samples were diluted immediately with 3.2% sodium citrate (1:9 v/v). A portion of whole blood was used for arterial blood gas analysis. The remainder of the blood sample was separated promptly using serial centrifugations (15 min at 1500 × *g* at 18°C, twice). The supernatant was collected and frozen at −80°C until analysis.

In vitro study. After anesthetization, a 24-gauge SURFLO was inserted into the left carotid artery. After an incision was made in the right atrium, the blood was rinsed away through the injection of 400–600 mL of normal saline via the left carotid artery. The right femur muscle was harvested and immediately flash-frozen in liquid nitrogen. The frozen muscle was mixed in normal saline by using a high shear speed homogenizer. The mixture was immediately separated using serial centrifugation (15 min at 1500 × g at 18°C, twice). The supernatant (homogenized muscle solution) was collected and frozen at −80°C until analysis. Blood samples from 5 healthy human volunteers were diluted with 3.2% sodium citrate (1:9 v/v) and separated promptly using serial centrifugations (15 min at 1500 × g at 18°C, twice). The plasma was then frozen at −80°C until analysis.

The homogenized muscle solution was diluted using normal saline to 1,000,000 IU/L of creatine kinase. Furthermore, 25 μL, 5 μL, 1 μL, and 0 μL of homogenized muscle solution and 0 μL, 20 μL, 24 μL, and 25 μL of normal saline were respectively added to 500 μL of plasma to evaluate the effects of procoagulants from destroyed muscle tissue on thrombin generation.

Measurements

Arterial blood gas analyses were performed using an ABL 700 (Radiometer, Tokyo, Japan). Functional assays for the procoagulant activity of microparticles and tissue factor-bearing microparticles were performed using ZYMUPHEN MP-Activity and ZYMUPHEN MP-TF kits (Hyphen Biomed, Neuville-sur-Oise, France), respectively.

Nucleosome antigen levels were measured using the Cell Death Detection ELISA kit (Roche Diagnostics, Mannheim,

Table 1. Blood Pressure and Arterial Blood Gas Analysis.

	Control $n = 5$	D250 $n = 5$	D500 $n = 5$	D1000 $n = 5$	P-value
Mean arterial blood pressure (mmHg)	88 (80–100)	100 (84–110)	79 (60–98)	65 (55–85)	0.077
Blood gas analysis					
pH	7.456 (7.436–7.470)	7.429 (7.366–7.462)	7.373 (7.356–7.377)	7.289 (7.269–7.315)	<0.001
$PaCO_2$ (mmHg)	37.6 (33.3–41.2)	35.4 (34.3–37.5)	38.1 (36.2–38.1)	29.7 (21.8–36.3)	0.325
PaO_2 (mmHg)	98 (91.1–105.7)	94.5 (90.0–106.0)	83.6 (82.0–86.3)	108.7 (82.3–139.5)	0.609
Hemoglobin (g/dL)	16.4 (16.0–17.3)	17.7 (15.2–18.4)	17.7 (17.1–17.9)	17.75 (15.4–20.0)	0.325
Sodium (mEq/L)	137 (132–140)	138 (133–139)	136 (135–142)	140 (136–148)	0.287
Potassium (mEq/L)	3.4 (3.1–3.5)	3.0 (2.9–3.1)	3.0 (2.9–3.1)	4.0 (3.9–5.1)	0.131
Chlorine (mEq/L)	103 (95–107)	99 (97–103)	100 (94–100)	97 (89–104)	0.364
Lactate (mmol/L)	0.55 (0.50–0.65)	1.40 (1.20–1.40)	2.20 (2.10–2.30)	3.80 (3.15–4.7)	<0.001

Germany). Prothrombin (coagulation factor II) and antithrombin activity levels were measured using an ACL Top coagulation analyzer (Mitsubishi Chemical Medience, Tokyo, Japan). Measurements of prothrombin activity used human plasma with prothrombin deficiency. Creatine kinase levels were measured using Iatoro LQ CK RATE J II (LSI Medience, Tokyo, Japan).

Thrombin Generation Assay

The method used for the thrombin generation assay was described previously.[26] Briefly, thrombin generation was assessed using a calibrated automated thrombogram (Thermo Trombinoscope; Finggal Link, Tokyo, Japan) and measured using the normal calibrated automated thrombogram method. Plasma samples (80 μL) were supplemented with 20 μL PPP-Reagent (Finggal Link), which contains a mixture of phospholipids and tissue factors. At the start of measurement, 20 μL of FluCa-Kit reagent (Finggal Link), which contains HEPES (pH 7.35), calcium chloride, and fluorogenic substrate, were applied automatically to the plasma samples supplemented with PPP-Reagent. Measurements were recorded every 10 s using a FluoroScan Ascent fluorometer (Finggal Link), and the data were analyzed using Thrombinoscope software. To adjust for inner-filter effects and substrate consumption, each measurement was corrected with respect to the fluorescence curve obtained from a mixture of the sample plasma with a fixed amount of thrombin–α2-macroglobulin complex (Thrombin Calibrator; Finggal Link). The parameters calculated by the software included lag time, time to peak, peak height, and endogenous thrombin potential (ETP). All samples and calibration controls were run at least in duplicate.

Statistical Analyses

Unless otherwise indicated, all measurements are expressed as medians (interquartile range). Comparisons among the 4 groups were made using the Jonckheere–Terpstra test. The Jonckheere-Terpstra test is a rank-based nonparametric test that can be used to determine if there is a statistically significant trend within an independent samples design. Spearman rank correlation coefficients were calculated to determine correlations between 2 variables. SPSS 25 (IBM Japan, Tokyo, Japan) was used for all statistical analyses. The level of statistical significance was set at $P < 0.05$.

Results

In Vivo Study

Mean blood pressure and arterial blood gas analyses of each group are presented in Table 1. Lactic acidosis increased gradually in concert with increased trauma severity. Severe hypotension, hypoxia, anemia, or electrolyte disorders were not observed.

Plasma levels of creatine kinase, nucleosomes, and microparticles gradually and significantly increased with trauma severity (all $P < 0.001$) (Figure 1). There were strong correlations among creatine kinase, nucleosomes, and microparticles (all $\rho > 0.7$) (Table 2). The thrombin generation assay revealed that the lag time and time to peak gradually grew shorter with an increase in trauma severity ($P = 0.006$ and 0.002, respectively) (Table 3). Prothrombin (factor II) activity (Figure 2, upper panel) and antithrombin activity (Figure 2, middle panel) decreased gradually with an increase in trauma severity (both $P < 0.001$). However, the thrombin generation ratio, represented by the ETP/prothrombin activity ratio, increased gradually with an increase in trauma severity ($P = 0.006$) (Figure 2, lower panel). Furthermore, the thrombin generation ratio correlated with antithrombin activity ($\rho = -0.538$, $P = 0.018$).

The lag time, time to peak, and thrombin generation ratio from the thrombin generation assay correlated with the creatine kinase level ($\rho = -0.593$, $P = 0.006$; $\rho = -0.701$, $P = 0.001$; and $\rho = 0.554$, $P = 0.014$, respectively).

In Vitro Study

In the homogenized muscle solution, the concentrations of nucleosomes, microparticles, and tissue factor-bearing microparticles were 0.05 AU/ml, 796 nM and 2,680 pg/mL, respectively. The results of the thrombin generation assay from normal plasma samples with different volumes of homogenized muscle solution are presented in Figure 3. With an increasing volume of homogenized muscle solution, lag time and time to peak gradually decreased ($P = 0.002$ and $P = 0.001$,

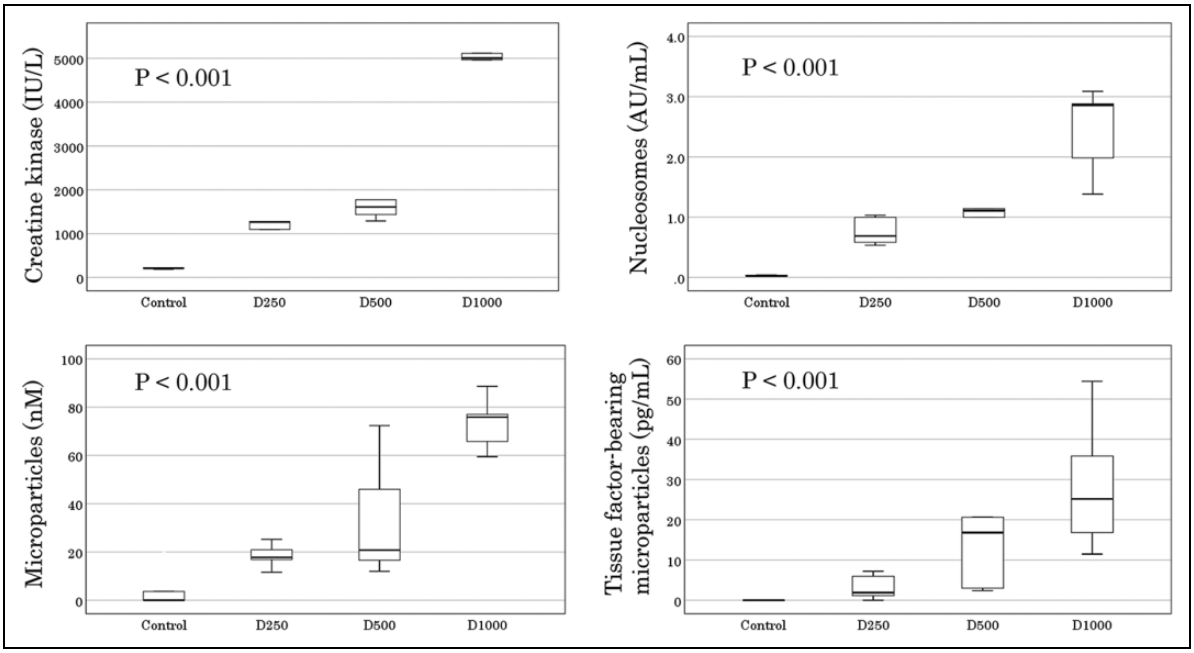

Figure 1. Plasma levels of creatine kinase, nucleosomes, and microparticles by group. Upper left panel, creatine kinase. Upper right panel, nucleosomes. Lower left panel, microparticles. Lower right panel, tissue factor-bearing microparticles. The plasma levels of each indicator increased gradually (P < 0.001, Jonckheere–Terpstra test). Control, not exposed to Noble–Collip drum trauma; D250, tumbled for 250 revolutions; D500, tumbled for 500 revolutions; and D1000, tumbled for 1000 revolutions. Each group included 5 rats.

Table 2. Relationships among Plasma Levels of Creatine Kinase, Nucleosomes, and Microparticles.

	Creatine kinase	Nucleosomes	Microparticles	Tissue factor-bearing microparticles
Creatine kinase	NA	0.916	0.863	0.857
Nucleosomes	0.916	NA	0.907	0.859
Microparticles	0.863	0.907	NA	0.798
Tissue factor-bearing microparticles	0.857	0.859	0.798	NA

The measurment results were in 20 rats of the control, D250, D500, and D1000 groups.
Spearman's ρ between 2 values are presented. All P-values of Spearman's ρ were < 0.001.
NA, not applicable.

Table 3. Results of the Thrombin Generation Assay.

	Control $n = 5$	D250 $n = 5$	D500 $n = 5$	D1000 $n = 5$	P-value
Lag time (min)	2.5 (2.5–2.5)	2.1 (1.8–2.1)	2.1 (1.8–2.3)	2.1 (1.6–2.1)	0.006
Time to peak (min)	4.4 (4.4–4.6)	4.0 (3.3–4.0)	3.5 (3.3–3.8)	3.3 (3.3–3.5)	0.002
Peak height (nM)	166 (149–175)	178 (169–193)	180 (173–223)	137 (115–155)	0.346
ETP (nM·min)	701 (618–711)	607 (524–763)	670 (633–682)	425 (405–485)	0.007

ETP, endogenous thrombin potential

respectively), and peak height and ETP gradually increased (P < 0.001 and P = 0.026, respectively).

Discussion

In the present study, cellular destruction, as indicated by plasma creatine kinase levels, gradually increased in accordance with increased severity of blunt trauma. Nucleosomes and microparticles also increased gradually in conjunction with increased trauma severity. Furthermore, plasma levels of nucleosomes and microparticles significantly correlated with cellular destruction. In the *in vitro* study, the homogenized muscle solution, which included nucleosomes and microparticles from the destructed muscle cells, accelerated thrombin generation in accordance with an increasing volume of homogenized muscle solution. Thrombin production and the rapidity

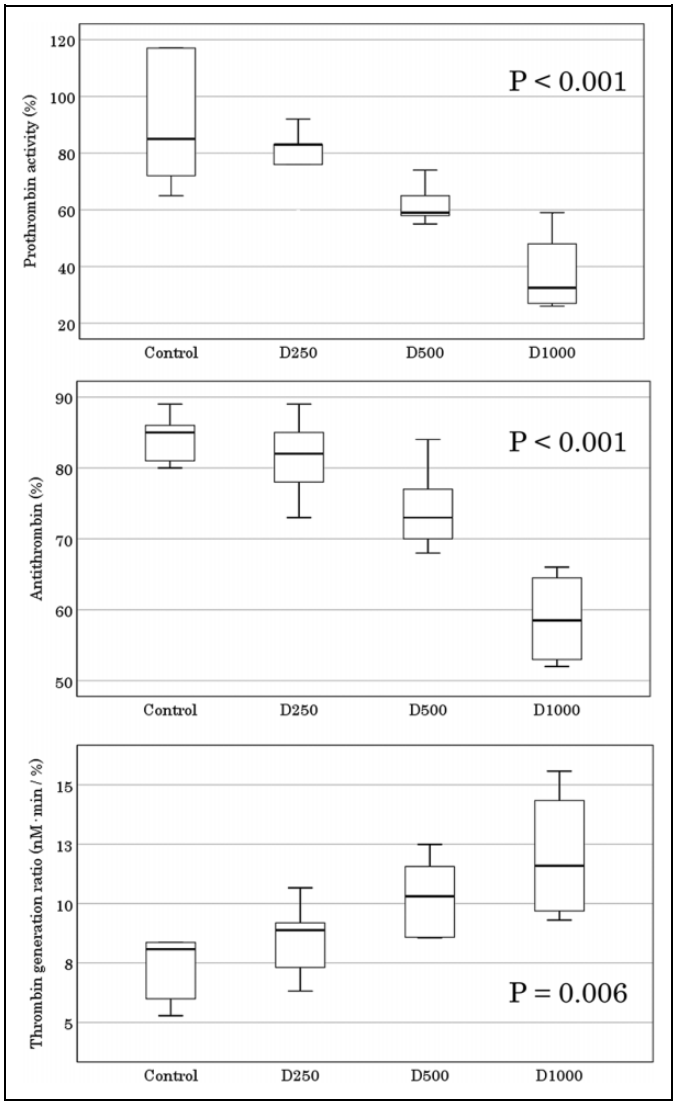

Figure 2. Prothrombin activity, antithrombin activity, and thrombin generation ratio by group. Upper panel, prothrombin (coagulation factor II) activity decreased gradually in accordance with increased trauma severity. Middle panel, antithrombin activity decreased gradually in accordance with increased trauma severity. Lower panel, the thrombin generation ratio, as indicated using the endogenous thrombin potential/prothrombin activity ratio, increased gradually in accordance with increased trauma severity. P-values are derived from the Jonckheere–Terpstra test. Control, not exposed to Noble–Collip drum trauma; D250, tumbled for 250 revolutions; D500, tumbled for 500 revolutions; and D1000, tumbled for 1000 revolutions. Each group included 5 rats.

of thrombin generation, as indicated by lag time and time to peak in the thrombin generation assay, significantly correlated with severity of blunt trauma and cellular destruction.

Platelet- and other blood cell-derived microparticles have been found frequently after trauma.[11-15] However, microparticles derived from parenchymal cells destroyed directly by external force have also been reported, such as brain-derived microparticles in brain trauma.[16,17] In the present study, the plasma levels of microparticles, including tissue factor-bearing microparticles, strongly correlated with creatine kinase and nucleosome levels. Furthermore, the plasma levels of microparticles, nucleosome, and creatine kinase clearly correlated with trauma severity. Because blood cells do not contain creatine kinase, these results imply that the majority of microparticles were released from the damaged parenchymal cells. Moreover, the homogenized muscle solution used in the *in vitro* study contained a high concentration of microparticles and nucleosomes. Furthermore, the *in vivo* study showed that plasma levels of microparticles were elevated just after trauma.

The mechanism underlying microparticle production from activated and apoptotic cells has been well-investigated.[27] After cell activation or apoptosis are triggered, the cytosolic calcium concentration increases and activates calpain, followed by the release of microparticles.[27] Therefore, microparticle release requires some time.[11-15,28] In previous *in vitro* study, platelet-derived microparticles were released several tens of minutes after cell activation.[28] Furthermore, platelet-derived microparticles were detected several tens of minutes after trauma in the previous clinical studies.[11-15] However, in the present study, massive amounts of microparticles were released just after trauma. Therefore, we surmise that most of microparticles were released non-specifically and passively from destroyed parenchymal cells, rather than being actively released from activated or apoptotic cells.

Nucleosomes form the basic organizational unit of nuclear DNA and consist of DNA wrapped around a core histone octamer.[29] Circulating nucleosomes are released from dying and destroyed cells.[30-32] Many previous investigations reported elevated plasma levels of nucleosomes occurring immediately after trauma.[18-22] However, these reports did not evaluate the cellular source of post-trauma nucleosomes.[18-24] Recently, neutrophil extracellular traps, which are extracellular web-like structures containing nucleosomes released from activated neutrophils, have been investigated in various diseases.[32-34] However, in the present study, similar to findings with microparticles, massive amounts of nucleosomes were observed just after trauma. Furthermore, plasma levels of nucleosomes strongly correlated with creatine kinase levels. This evidence suggests that, similar to findings in microparticles, nucleosomes are released non-specifically and passively from the destroyed parenchymal cells and not from activated neutrophils.

Microparticle- and nucleosome-activated coagulation has been widely reported.[27,32,35] In this process, phosphatidylserine and tissue factors on the microparticle surface play important roles.[27] In the present study, we measured the plasma levels of microparticles and tissue factor-bearing microparticles by using phosphatidylserine and tissue factors on the surface of microparticles, along with thrombin generation ability. Therefore, plasma levels of microparticles directly reflect their coagulation activation ability. Because nucleosomes do not typically contact blood, they act as the body's "natural foreign surface" to promote blood coagulation.[32,35] Accordingly, nucleosome-activated coagulation has been reported repeatedly.[32,35-37] Furthermore, although we only measured plasma levels of nucleosomes, the nucleus contains various other components and releases them after

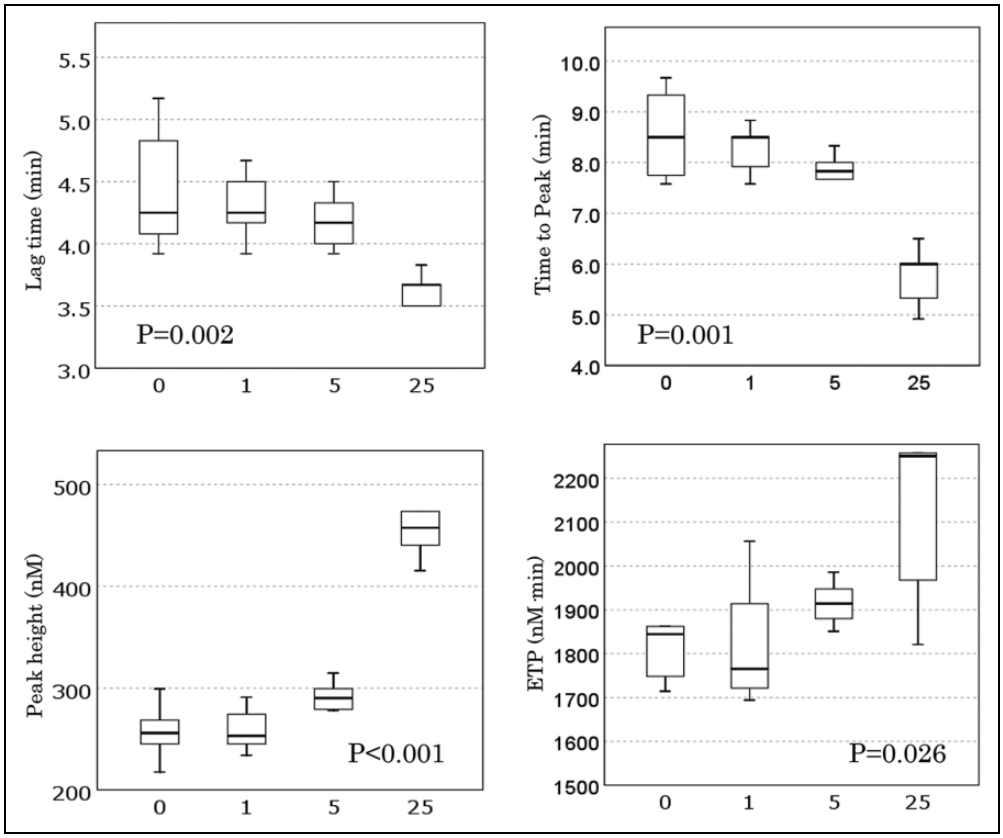

Figure 3. Thrombin generation assay of normal plasma with various volumes of homogenized muscle solution Upper left panel, lag time. Upper right panel, time to peak. Lower left panel, peak height. Lower right panel, endogenous thrombin potential (ETP). The sample groups 0, 1, 5, and 25 indicate 500-μL plasma samples to which 0 μL (control), 1 μL, 5 μL, and 25 μL of homogenized muscle solution were added, respectively. Five samples were included in each sample group. In accordance with an increasing volume of homogenized muscle solution, lag time and time to peak decreased gradually ($P = 0.002$ and $P = 0.001$, respectively; Jonckheere–Terpstra test), and peak height and ETP gradually increased ($P < 0.001$ and $P = 0.026$, respectively; Jonckheere–Terpstra test).

trauma.[19,21,38] Therefore, in the present trauma model, other procoagulants, such as individual histones and high mobility group box 1, would also be released from destroyed cells.

In the present study, the following 2 points of coagulation activation after trauma were observed: (i) a shorter time until the start of thrombin generation, as represented by a shorter lag time and time to peak in the thrombin generation assay (Table 3) and (ii) an increased thrombin generation ratio (lower panel in Figure 2). In the thrombin generation assay, measurements of the lag time and time to peak begin after mixing specific amounts of phospholipids, tissue factors, and calcium chloride into the plasma samples. However, in the present study, the plasma samples after trauma contained procoagulants such as microparticles and nucleosomes. Consequently, the total procoagulants in plasma samples were increased compared with the specified amounts, resulting in a shorter lag time and time to peak. Furthermore, because the amount of procoagulant increased with trauma severity, the coagulation activation also increased with trauma severity. In the present *in vitro* study, the homogenized muscle solution, which included nucleosomes and microparticles from the destructed muscle cells, accelerated thrombin generation in accordance with the increasing volume of homogenized muscle solution (Figure 3).

Consistent with previous studies,[39,40] we observed the consumption of coagulation factors just after trauma (upper panel in Figure 2). Therefore, the ETP for thrombin generation decreased after trauma (Table 2) because prothrombin (coagulation factor II) is a substrate of thrombin. However, the thrombin generation ratio, indicated by the amount of thrombin generated per 1% of prothrombin activity, increased after trauma. This increased thrombin activity was affected by the post-trauma decrease in antithrombin. However, in the present *in vitro* study, peak height and ETP for thrombin generation gradually increased in accordance with an increasing volume of homogenized muscle solution (lower panels in Figure 3), and this differed from the results of the present *in vivo* study. The plasma samples in the *in vitro* study contained normal levels of coagulation factors, which differ from the trauma models used in the *in vivo* study. Therefore, thrombin generation gradually increased in accordance with stimulation by procoagulants in the homogenized muscle solution.

The primary and significant limitation of our study is that we could not directly indicate the origin of the microparticles and nucleosomes in the *in vivo* study. Therefore, we additionally performed *in vitro* study and found that homogenized muscle released microparticles and nucleosomes and induced coagulation activation. These results of the *in vitro* study support our speculations based on the results of the *in vivo* study. However, further study is needed to clarify the origin of the microparticles and nucleosomes in blunt trauma models.

Conclusions

Following trauma, parenchymal cells that are destroyed by the external traumatic force immediately release microparticles and nucleosomes, which then initiate the coagulation cascade. Therefore, circulating microparticles and nucleosomes were corelated with trauma severity and parenchymal cell distraction. As a result, the time until the start of thrombin generation shortens, and the thrombin generation ratio increases, in accordance with the amount of procoagulants. However, this is accompanied by a simultaneous consumption of coagulation factors.

Abbreviations

ACoTS, acute coagulopathy of trauma shock; DIC, disseminated intravascular coagulation; ETP, endogenous thrombin potential.

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Declaration of Conflicting Interests

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