学位論文

Effects of arterial hemorrhage speed on blood coagulation/fibrinolysis system and hemodynamics in rats
（ラットの凝固/線溶系及び循環動態における動脈出血速度の影響）

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Effects of arterial hemorrhage speed on the blood coagulation/fibrinolysis system and hemodynamics in rats

Running head: Effects of hemorrhage speed on coagulation

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Abstract

Objectives: The effects of rapid hemorrhage on coagulopathy have been reported. However, the effects of different hemorrhage speeds on the blood coagulation/fibrinolysis system have not been investigated. This study aimed to compare different hemorrhage speeds for clarifying their effects on the coagulation/fibrinolysis system and circulation disorders in rats.

Methods: Male Sprague–Dawley rats (301–396 g) were randomly assigned to five groups depending on hemorrhage speed and length of procedure: 1) rapid (1.4 mL/min, 30-min bleeding), 2) rapid-L (1.4 mL/min, 30-min bleeding and observation until 6 h), 3) slow (0.1 mL/min, intermittently, 6-h bleeding), 4) control (30-min observation), and 5) control-L (6-h observation). Hemorrhage was induced by withdrawing blood until 40% of the estimated blood volume from the femoral artery. We measured vital signs, hematology, general chemistry, blood gas status, coagulation parameters, fibrinolytic markers (tissue-type plasminogen activator [tPA] and plasminogen activator inhibitor 1 [PAI-1]), vascular endothelial damage (syndecan-1), and liver PAI-1 mRNA expression.

Results: Rapid hemorrhage induced elevation of lactate and syndecan-1 levels and prolonged prothrombin time and activated partial thromboplastin time in the rapid group. In contrast, slow hemorrhage did not induce these changes. Hemorrhage speed had no effect on plasma tPA and hematology. Plasma PAI-1 levels were significantly increased in the rapid-L group, while liver PAI-1 mRNA levels were increased in the slow group.

Conclusions: This study shows changes in the circulatory and fibrinolysis systems, depending on the hemorrhage speed. Hemorrhage might promote production of PAI-1,

47 while tissue hypoxia due to rapid hemorrhage might promote release of PAI-1.

48

49 KEY WORDS: hemorrhage speed, plasminogen activator inhibitor-1, lactate

50 ABBREVIATIONS: PAI-1—plasminogen activator inhibitor-1; tPA—tissue-type
51 plasminogen activator; PT—prothrombin time; APTT—activated partial thromboplastin
52 time; MAP—mean arterial pressure; HR—heart rate; RBC—red blood cell; qRT-
53 PCR—quantitative reverse transcription-polymerase chain reaction; mRNA—messenger
54 RNA; cDNA—complementary DNA

55

Introduction

Severe blood loss continues to be the leading cause of mortality despite advances in emergency care [1]. Coagulation disorders, including hypocoagulation and hyperfibrinolysis, are negative effects of hemorrhage, and they are associated with poor outcomes. Previous studies have shown that aggravation of hemodynamics has a risk of not only tissue hypoxia, metabolic acidosis, and tissue damage, but also release of tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) [2, 3, 4, 5]. PAI-1 and tPA are major factors regulating the fibrinolysis system, followed by an imbalance between coagulation and fibrinolysis.

The speed of hemorrhage has an important effect on coagulation and fibrinolysis function. Several studies have regarded rapid hemorrhage as a leading cause of hypocoagulation and hyperfibrinolysis [6, 7]. Zhao et al. focused on the effects of hemorrhage speed and reported that rapid hemorrhage induced a higher risk of hypocoagulation compared with slow hemorrhage in clinical research [8]. However, few studies have observed differences in tissue hypoxia and the fibrinolysis system due to a change in hemorrhage speed. With regard to the speed of hemorrhage, some clinical studies classified rapid short-duration hemorrhage (early hemostasis operation) as rapid hemorrhage and slow long-duration hemorrhage (delayed hemostasis operation) as slow hemorrhage. The above-mentioned study by Zhao et al. classified quick hemorrhage (short-duration surgery) and slow hemorrhage (long-duration surgery) [8]. Bassin et al. investigated patients' physiological responses by classifying rapid blood loss (blood loss occurred in less than 4 h) and slow blood loss (in more than 4 h) [9]. They showed that slow hemorrhage induced redistribution of blood volume toward the central circulation

to maintain cardiac output. In an animal experiment, Scully et al. used a hemorrhagic shock sheep model by randomizing a fast (1.25 mL/kg/min) or slow (0.25 mL/kg/min) speed of hemorrhage [10]. They showed that heart rate (HR) in the slow group was increased, but not as much as in the fast group, which resulted in decreased cardiac output. Therefore, various studies have reported cardiovascular responses to the speed of hemorrhage. These changes in the circulatory state have a risk of affecting production of fibrinolysis regulators according to the effects of hemorrhage speed on the severity of hypoperfusion. Fibrinolysis is regulated by various factors, including tissue hypoxia, inflammation, and sympathetic nerve activity, which are predicted to be affected by circulatory insufficiency [11]. We hypothesized that the speed of hemorrhage affects the coagulation and fibrinolysis system.

The present study aimed to investigate the effect of hemorrhage speed on the coagulation/fibrinolysis system. We used a rat model with two hemorrhage speeds of rapid and slow. Rapid hemorrhage was designed as the speed of a conventional hemorrhagic shock model. Slow hemorrhage was designed as the speed of continuous intermittent hemorrhage. We compared rapid and slow (rapid short-duration hemorrhage vs. slow long-duration hemorrhage) according to previous studies, which defined that rapid hemorrhage was a short duration and slow hemorrhage was a long duration [8, 9]. In this study, we mitigated procedural trauma to rats to focus on the effects of hemorrhage speed because the inflammatory response and sympathetic nerve activity induced by trauma affect the coagulation and fibrinolysis systems.

Materials and methods

Animals

The sample size was calculated using the free sample size calculating software G*Power version 3.1.9.2 (Program written, concept and design by Faul et al., Universitat Kiel, Germany) [12]. With power of 80%, 0.05 level of statistical significance, and effect size of 0.6, the sample size was calculated to be 8 rats per group and a total of 40 rats. Experimental procedures were performed in specific pathogen-free, male Sprague–Dawley rats (301–396 g) from Kyudo Co., Ltd. (Saga, Japan). The rats were housed in cages with soft wood chips as bedding, two rats per cage, under a controlled temperature on a 12-h light/dark cycle with *ad libitum* access to food and water. Rats were allowed to acclimate to conditions for at least 4 days. This study was reviewed and approved by the Animal Care and Use Committee of Sojo University (Permit # 2017-P-020). Maintenance of the rats and the experimental procedures performed on them were carried out in accordance with U.S. National Institutes of Health (NIH) guidelines.

Experimental protocols

The study design is shown in Fig. 1. Hemorrhage models were prepared as described in a previous report with some modifications [13]. After an acclimation period, we randomly assigned all rats to the rapid (n=8), rapid-L (n=8), slow (n=8), control (n=8), and control-L (n=8) groups. We set the duration of the procedural phase for blood loss as 30 min (rapid) or 6 h (rapid-L and slow). The rapid and slow groups were designed for comparison of rapid short-duration hemorrhage and slow long-duration hemorrhage according to previous studies [8, 9]. The rapid-L group was created to evaluate the effects

of untreated observation (5 h 30 min) after rapid short-duration hemorrhage (30 min). The control group was paired with the rapid group. The control-L group was paired with the slow and rapid-L groups.

Anesthesia was induced with 4% isoflurane/room air (i.e., 20% oxygen and 0.03% carbon dioxide) in a vented anesthesia chamber and sustained by inhalation of 2.5% isoflurane/room air through a nose cone during cannulation using an anesthesia system (NARCOBIT-E; Natsume Seisakusho Co., Ltd., Tokyo, Japan). Rats were placed in the supine position on a surgical heating board at 35°C. The rats received no mechanical ventilation and breathed spontaneously. The hair of the right femur was removed by commercially available depilatory cream (Reckitt Benckiser, Tokyo, Japan), and a skin incision was made on the same position for cannulation. The right femoral artery was cannulated with a polyethylene catheter (PE-50; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) filled with heparinized saline (10 units/mL). The femoral artery was used for invasive arterial pressure monitoring, withdrawing blood, and taking blood samples. Mean arterial pressure (MAP) and HR were monitored through the arterial catheter, which was connected to a pressure transducer coupled with a polygraph system (AD Instruments Inc., Nagoya, Japan). Following catheter insertion, animals were stabilized for 10 min to measure their baseline values. Anesthesia was maintained with 0.7% to 1.2% isoflurane/room air while performing the measurements at baseline and during the procedural phase.

Total blood volume of rats was estimated to be 56 mL/kg [13]. Hemorrhage was induced by withdrawing blood until 40% (22.4 mL/kg) of their estimated blood volume in all bleeding groups. In the rapid and rapid-L groups, the first 75% (16.8 mL/kg) of the

147 total bleeding was withdrawn using a syringe pump (KD Scientific Inc., MA, USA) at a
148 rate of 1.4 mL/min (Fig. 1). Furthermore, the remaining 25% of blood (5.6 mL/kg) was
149 withdrawn manually in two steps to maintain MAP at 20 to 35 mmHg to continue the
150 shock state. Blood withdrawal was completed at 30 min in the rapid and rapid-L groups.
151 The rapid-L group received observation until the end of the procedural phase (at 6 h from
152 the start of bleeding). In the slow group, 1 of 12 equal amounts of the estimated bleeding
153 volume (about 1.87 mL/kg) was withdrawn at a rate of 0.1 mL/min at one time. This
154 bleeding procedure was repeated 12 times at even intervals over 6 h. No resuscitation
155 was provided to any of the bleeding groups. Rats in the control and control-L groups
156 underwent the same anesthesia and catheterization without blood withdrawal for 30
157 min and 6 h, respectively. All rats were sacrificed by exsanguination after observation
158 for 20 min after the procedural phase.

159 A portion of the whole blood was used for arterial blood gas analysis. For serum
160 and plasma preparation, the rest of the blood sample was collected into clot activator BD
161 Microtinar® (Becton Dickinson and Co.) or a 1/10 volume of 3.2% sodium citrate solution.
162 Serum samples were centrifuged at 6000 × g for 90 s at 4°C. Plasma samples were
163 centrifuged at 3000 × g for 10 min at 4°C. The obtained serum and plasma were stored
164 at −80°C until analysis. Liver tissues were harvested and stored at −80°C until analysis.
165

166 **Hematology, blood acid-base status, and blood chemistry**

167 The red blood cell (RBC) count, hemoglobin and hematocrit concentrations, and
168 platelet count were determined using a hematology analyzer (ADVIA 2120i; Siemens
169 Healthineers, Erlangen, Germany) at Kumamoto Mouse Clinic, Institute of Resource

Development and Analysis, Kumamoto University. The arterial blood gas parameters and lactate concentration were determined using an i-STAT analyzer (Abbott Point of Care Inc., Princeton, NJ, USA) according to the manufacturer's instructions. Blood chemistry tests were performed with a chemistry analyzer (JCA-BM6050; JEOL, Tokyo, Japan) at Kumamoto Mouse Clinic.

Coagulation and fibrinolysis parameters

The prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen concentration were measured by a blood coagulation analyzer (COAG2N; A&T Corporation, Fujisawa, Japan) according to the manufacturer's instructions. The following markers were analyzed by ELISA according to the manufacturer's instructions: tPA (Abcam, Tokyo, Japan), PAI-1 (Abcam), and syndecan-1 (MyBioSource, San Diego, CA, USA).

Measurement of mRNA expression of PAI-1 in the liver

Total RNA was isolated from the liver using 1 mL Trizol reagent (Life Technologies, Tokyo, Japan) according to the manufacturer's protocol. First-strand complementary DNA (cDNA) was synthesized from total RNA with random hexamers as primers using SuperScript™ II Reverse Transcriptase (Life Technologies) by following the manufacturer's protocol. To determine PAI-1 mRNA levels of liver tissue, quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was performed using TB Green™ Premix Ex Taq™ II (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Briefly, we made a 25-μL reaction mixture

containing 12.5 μL of 2× TB Green™ Premix Ex Taq™ II, 1.0 μL of 10-μM forward primer, 1.0 μL of 10-μM reverse primer, 8.5 μL of H_2O, and 2.0 μL of cDNA (2400 ng). The primers used for PAI-1 were as follows [14]: forward primer, 5′ ATG AGA TCA GTA CTG CGG ACG CCA TCT TTG 3′ and reverse primer, 5′ GCA CGG AGA TGG TGC TAC CAT CAG ACT TGT 3′. The primers used for β-actin were as follows: forward primer, 5′ GGA GAT TAC TGC CCT GGC TCC TA 3′ and reverse primer, 5′ GAC TCA TCG TAC TCC TGC TTG CTG 3′. All primers in this study were purchased from Hokkaido System Science (Hokkaido, Japan). The PCR reaction was carried out using the Thermal Cycler Dice® Real Time System III (Takara Bio Inc.) under the following conditions: pro-incubation step at 95°C for 30 s, followed by 50 cycles of a 95°C denaturation step for 5 s, 60°C annealing for 30 s, and 72°C extension step for 30 s. At the end of the PCR, melting curve analysis was performed by gradually increasing the temperature from 60°C to 95°C for 30 s to confirm the amplification specificity of the PCR products. A series of standards that were prepared by successive dilutions were also amplified and used to generate a linear standard curve. We calculated relative mRNA expression levels using Thermal Cycler Dice® Real Time System III computer software (v 6.00, Takara Bio Inc.). Specific mRNA expression levels were normalized using the level of β-actin as the reference gene.

Statistical analysis

Data in this study are expressed as the mean ± standard deviation (SD) or shown as box and whisker plots. Differences between groups were analyzed by one-way analysis of variance and Tukey's multiple comparison test. All statistical analyses were performed with GraphPad Prism 7 software (GraphPad Software Inc., San Diego, CA,

USA). A P value < 0.05 was considered to be statistically significant.

Results

Hemodynamic changes induced by hemorrhagic shock

All rats survived the complete protocol. In the rapid, rapid-L, and slow groups, rats were bled by a mean final volume of 7.7 ± 0.6 mL (actual measurements). Serial changes in MAP and HR are shown in Figs. 2 and 3, respectively. The control groups showed no significant change over time (data not shown). Rats in these bleeding groups had the lowest blood pressure at the end of withdrawing the target volume of blood (rapid and rapid-L, 0.5 h; slow, 6 h from the start of bleeding). After withdrawing blood, MAP in the rapid and rapid-L groups was immediately decreased compared with the control and control-L groups, respectively, and then MAP in the rapid-L group recovered to baseline levels at the end of the procedural phase. However, in the slow group, MAP gradually decreased as blood was withdrawn, and the lowest values appeared at 6 h from the start of bleeding. HR behaved similarly to MAP in each group.

Effects of hemorrhage speed on hematology, acid-base status, and blood chemistry

Table 1 shows the results of hematology and blood gas analysis in each group. The RBC count, hemoglobin, and hematocrit were significantly lower ($P < 0.05$) in each of the bleeding groups (rapid, rapid-L, and slow) compared with the control groups (control and control-L). These hematological parameters showed no significant difference among the bleeding groups. The platelet count showed no significant change after hemorrhage. The platelet count in the control group was 951 ± 101 × 10^3 /μL when we excluded a rat that had an abnormally high platelet level (8487 × 10^3 /μL). Although the abnormal value was removed, there was no difference in the platelet count among the

five groups.

In the rapid group, base excess (BE), bicarbonate (HCO_{3-}), and the partial pressure of carbon dioxide (pCO_2) were significantly lower (all $P < 0.05$) and lactate levels were higher ($P < 0.05$) compared with the control group. The partial pressure of oxygen (pO_2) also rose ($P < 0.05$) in the rapid group compared with the control group, suggesting hyperventilation against metabolic acidosis. However, pH was not decreased in the rapid group, but it was significantly higher in the rapid-L group ($P < 0.05$) compared with the control-L group because of respiratory alkalosis by hyperventilation. In contrast, the slow group showed no significant differences in blood gas analysis.

Table 2 shows the results of blood chemistry in each group. Blood urea nitrogen (BUN) levels were significantly higher ($P < 0.05$) in the rapid-L and slow groups compared with the control-L group. Creatinine levels were significantly higher in the rapid group and slow groups compared with the control and control-L groups, respectively. Sodium (Na) levels were significantly lower ($P < 0.05$) in the rapid-L and slow groups compared with the control-L group. Glucose levels were significantly higher ($P < 0.05$) in the rapid group compared with the control and slow groups, whereas no change was observed in the slow and rapid-L groups. Aspartate transaminase (AST) and alanine aminotransferase (ALT) levels were significantly higher (both $P < 0.05$) in the rapid-L group compared with the slow group. Amylase levels were significantly higher ($P < 0.05$) in the slow and rapid-L groups compared with the rapid group. This finding suggested the onset of tissue damage, including acute liver failure and pancreatic damage. Alkaline phosphatase (ALP) levels were significantly lower ($P < 0.05$) in the slow group compared with the control-L, rapid, and rapid-L groups. Total cholesterol (T-

CHO) and triglyceride levels were significantly lower ($P < 0.05$) in the rapid group compared with the control and rapid-L groups, whereas no change was observed in the slow group. High-density lipoprotein cholesterol (HDL-C) levels were significantly lower ($P < 0.05$) in the rapid-L group compared with the control-L group, whereas no change was observed in the slow group. Levels of potassium (K), lactate dehydrogenase (LDH), creatine kinase (CK) and low-density lipoprotein cholesterol (LDL-C) were not significantly different among any of the five groups.

Effects of hemorrhage speed on coagulation parameters

Fig. 4 shows the results of coagulation tests, including the PT, the APTT, and fibrinogen concentrations. The PT and APTT were significantly higher (both $P < 0.05$) in the rapid group compared with the control group (Fig. 4a, b). There were no significant differences in the PT and APTT in the rapid-L and slow groups. Fibrinogen concentrations were significantly lower ($P < 0.05$) in all of the bleeding groups compared with the control groups, but there was no significant difference among the bleeding groups (Fig. 4c).

Effects of hemorrhage speed on fibrinolytic parameters and PAI-1 mRNA levels in the liver

Fig. 5 shows plasma/serum concentrations of the fibrinolysis markers tPA and syndecan-1. There was no significant difference in plasma tPA levels among any of the five groups (Fig. 5a). Plasma D-dimer levels in the five groups were below the detection limit (5 ng/mL) (data not shown). Serum syndecan-1 levels were significantly higher (P

287 < 0.05) in the rapid group compared with the control group (Fig. 5b).

288 Plasma PAI-1 levels were significantly higher ($P < 0.05$) in the rapid-L group
289 compared with the control-L group (Fig. 6a). Plasma PAI-1 levels were not significantly
290 different between the slow and control-L groups. In contrast to plasma levels of PAI-1,
291 surprisingly, PAI-1 mRNA expression levels were significantly higher ($P < 0.05$) in the
292 slow group compared with the control-L group, but there was no difference in the rapid-
293 L group (Fig. 6b).
294

Discussion

In this study, we examined the effect of different speeds of hemorrhage on coagulation, fibrinolysis, and tissue damage in a rat model. In a previous hemorrhage rat model, some experiments required a rapid decrease in blood pressure (e.g., MAP fell to 35–40 mmHg within 5 min [15]). In another study, the speed of hemorrhage was set to approximately 1.0 mL/min [16]. The rapid and rapid-L groups in our study were designed to hemorrhage faster than in previous models and to achieve a prompt decrease in blood pressure. We set the duration of the procedural phase for blood loss as 30 min (rapid) or 6 h (rapid-L and slow) according to previous studies. Bassin et al. classified the speed of hemorrhage as rapid (blood loss occurred in less than 4 h) and slow (more than 4 h). However, Zhao et al. reported that the duration of slow hemorrhage was approximately six times as long as rapid hemorrhage (2.3 h vs. 0.4 h) [8, 9]. We performed not only comparison between rapid short-duration hemorrhage and slow long-duration hemorrhage (rapid vs. slow), but also evaluated the effects of elapsed time (slow vs. rapid-L).

In contrast to previous findings, which showed that hemorrhage increased tPA levels in blood [7], tPA levels did not increase in our study. The lack of change in tPA and D-dimer levels in our study showed that apparent degradation of fibrinogen did not occur [17]. The reason for this lack of change could be because our rat model received a minimum amount of injury by using a femoral incision to insert a catheter to mitigate the effects of trauma on fibrinolysis [18]. This procedure is in contrast to previous experiments where animals received severe trauma, such as tracheotomy, laparotomy, bone fracture, and organ injury [7, 15, 16, 18]. Severe trauma might be a major factor for

318 overactivity of the sympathetic nervous system, followed by endothelial injury and
319 release of tPA.

320 Comparisons between the rapid and slow groups reflected the biological
321 response just after MAP fell to the lowest level in each group. In our study, a rise in
322 creatinine levels followed a decrease in MAP (rapid and rapid-L, 0.5 h; slow, 6 h from the
323 start of bleeding). This result indicated that blood pressure affected filtration of the
324 kidney [19]. Furthermore, hemorrhage in the rapid group induced metabolic acidosis,
325 tissue hypoxia (elevation of lactate levels), temporary hypocoagulability (prolongation of
326 the PT and APTT), and endothelial injury (examined by syndecan-1 levels). However,
327 slow hemorrhage did not induce these circulatory disorders, despite the same amount of
328 bleeding. Brohi et al. reported that tissue hypoxia activated protein C, which is a factor
329 that regulates anticoagulation, to prolong the PT and APTT [20]. Additionally,
330 endothelial injury is related to the coagulation system because of platelet function [21].
331 Rapid hemorrhage might affect coagulability by inducing tissue hypoxia and endothelial
332 injury.

333 Lactate is considered as an indicator of systemic hypoperfusion because an
334 increase in blood lactate levels is caused by anaerobic glycolysis [22]. In this study, rapid
335 hemorrhage increased blood lactate levels, while slow hemorrhage had no effect on
336 lactate levels. Additionally, hematocrit levels showed no significant difference with the
337 speed of hemorrhage. This finding indicates that there was no change in hemodilution
338 due to normal autoresuscitation after hemorrhage among the bleeding groups [23]. These
339 results suggested that the cause of increased lactate levels might not be only due to a
340 decrease in circulating blood volume. At completion of blood withdrawal, MAP in the

rapid group was lower than that in the slow group. Rapid hemorrhage tended to decrease blood pressure more rapidly compared with slow hemorrhage, followed by more serious circulation disorders. However, assessments of lactate have been controversial. We collected blood samples from the femoral artery to measure blood lactate levels. Blood lactate levels are mainly produced in skeletal muscle and are not reflected in ischemia of internal organs and local tissue [24]. Bassin et al. showed that gradual blood loss tended to reduce the central blood circulation, suggesting that slow hemorrhage might not decrease the blood circulation in skeletal muscle [9]. To evaluate the effects of slow hemorrhage, assessment of tissue hypoxia might be necessary, especially in local tissue or in each organ.

Comparisons of the measurements in the rapid-L and slow groups might reflect the response of keeping them exposed to blood loss. In the rapid-L group, rats experienced a prompt decrease in MAP and subsequent recovery accompanied by tissue hypoxia and tissue damage. In the slow group, MAP of rats kept falling, although without apparent signs of a circulatory disorder.

With regard to fibrinolysis markers, plasma PAI-1 levels were higher in the rapid-L group compared with the control-L group, while PAI-1 mRNA expression levels in the liver were higher in the slow group compared with the control-L group. Rapid hemorrhage induced endothelial injury, which may lead to release of PAI-1 from vascular endothelial cells [25, 26]. In contrast, slow hemorrhage increased PAI-1 mRNA expression levels in the liver without increasing plasma lactate levels at the time of lowest blood pressure. Tissue hypoxia is thought to be an important factor in regulating PAI-1 expression [11]. As mentioned above, plasma lactate levels might not reflect

hypoperfusion of local tissue or organs.

In this study, rapid hemorrhage was assumed to be a manifestation of major vascular injury. In contrast, slow hemorrhage was assumed to manifest as continuous bleeding following minor trauma such as gastrointestinal bleeding, retroperitoneal bleeding, or hemostatic failure after trauma. Slow hemorrhage is considered to be less dangerous than rapid hemorrhage because an obvious shock state is often absent. In the present study, however, slow hemorrhage induced an increase in PAI-1 expression in liver, indicating that slow hemorrhage also induces organ ischemia. This finding suggests that the character of hemorrhage, including the hemorrhage speed, must be evaluated with caution to ensure correct assessment of complications and prognosis in a bleeding patient.

Release of PAI-1 caused by hemorrhage inhibits fibrinolysis, which assists with hemostasis [27]. However, a large amount of PAI-1 leads to an excess of clot formation [28]. In clinical medicine, an unchanged lactate level appears likely to lead to misjudgment of the risk of coagulopathy [29]. Predicting fluctuation of PAI-1 production and release due to the influence of various factors might be difficult [30]. Previous studies of hemorrhage showed that there are two types of elevation in PAI-1 levels. In the first type, PAI-1 promptly increases after hemorrhagic shock and gradually decreases [7]. In the second type, PAI-1 gradually increases and reaches a peak at 5 to 8 h after hemorrhagic shock [31]. These studies suggest that release and production of PAI-1 in tissues depend on hemorrhage and conditions of trauma. In the present study, the speed of hemorrhage induced a difference in hypoperfusion, including tissue hypoxia and tissue damage. Further studies are required to clarify associations between tissue

hypoperfusion and elevation of PAI-1 levels in plasma and in organs.

We examined the effect of hemorrhage speed on coagulation, fibrinolysis, tissue damage, and tissue hypoperfusion. Our findings of rapid hemorrhage supported those obtained from previous animal models hemorrhagic shock, which showed tissue hypoxia and elevation of plasma PAI-1 levels. In contrast, slow hemorrhage increased liver mRNA expression of PAI-1 without significant systemic hypoperfusion. For a better understanding of activity of the coagulation/fibrinolysis system, the relation between hemorrhage and local hypoperfusion needs to be better understood.

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Conflicts of interest

There are no conflicts of interest.

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491 **Figure Legends**

492

493 Fig. 1

494 Illustration of the experimental models. Rats were assigned to five groups depending on
495 the speed of hemorrhage and length of the experimental time as follows: 1) rapid (1.4
496 mL/min), 2) slow (0.1 mL/min, intermittently), 3) rapid-L (1.4 mL/min), control, and
497 control-L. The amount of bleeding in the bleeding groups was 40% of their estimated
498 blood volume. Total blood volume was estimated to be 56 mL/kg.

499

500 Fig. 2

501 Serial changes in mean arterial pressure (MAP) in (a) the rapid (n=8), (b) slow (n=8),
502 and (c) rapid-L (n=8) groups. Values are shown as mean ± SD. *P < 0.05, control groups
503 (control and control-L) vs. bleeding groups (rapid, slow, and rapid-L).

504

505 Fig. 3

506 Serial changes in heart rate (HR) in (a) the rapid (n=8), (b) slow (n=8), and (c) rapid-L
507 (n=8) groups. Values are shown as mean ± SD. *P < 0.05, control groups (control and
508 control-L) vs. bleeding groups (rapid, slow, and rapid-L).

509

510 Fig. 4

511 Effects of hemorrhage speed on (a) the prothrombin time (PT), (b) the activated partial
512 thromboplastin time (APTT), and (c) fibrinogen levels in the rapid (n = 8), slow (n = 8),
513 rapid-L (n = 8), control (n = 8), and control-L (n = 8) groups. Data are presented as box

514 and whiskers plots where the box represents the interquartile range with a horizontal
515 line at the median and whiskers are plotted using the Tukey method. *P< 0.05.
516
517 Fig. 5
518 Effects of hemorrhage speed on (a) tissue-type plasminogen activator (tPA) and (b)
519 syndecan-1 levels in the rapid (n = 8), slow (n=8), rapid-L (n=8), control (n=8), and
520 control-L (n=8) groups. Values are shown as mean ± SD. *P< 0.05.
521
522 Fig. 6
523 Effects of hemorrhage speed on (a) PAI-1 and (b) liver mRNA expression levels in the
524 rapid (n = 8), slow (n = 8), rapid-L (n = 8), control (n = 8), and control-L (n = 8) groups.
525 Values are shown as mean ± SD. *P< 0.05.
526

Figure 1

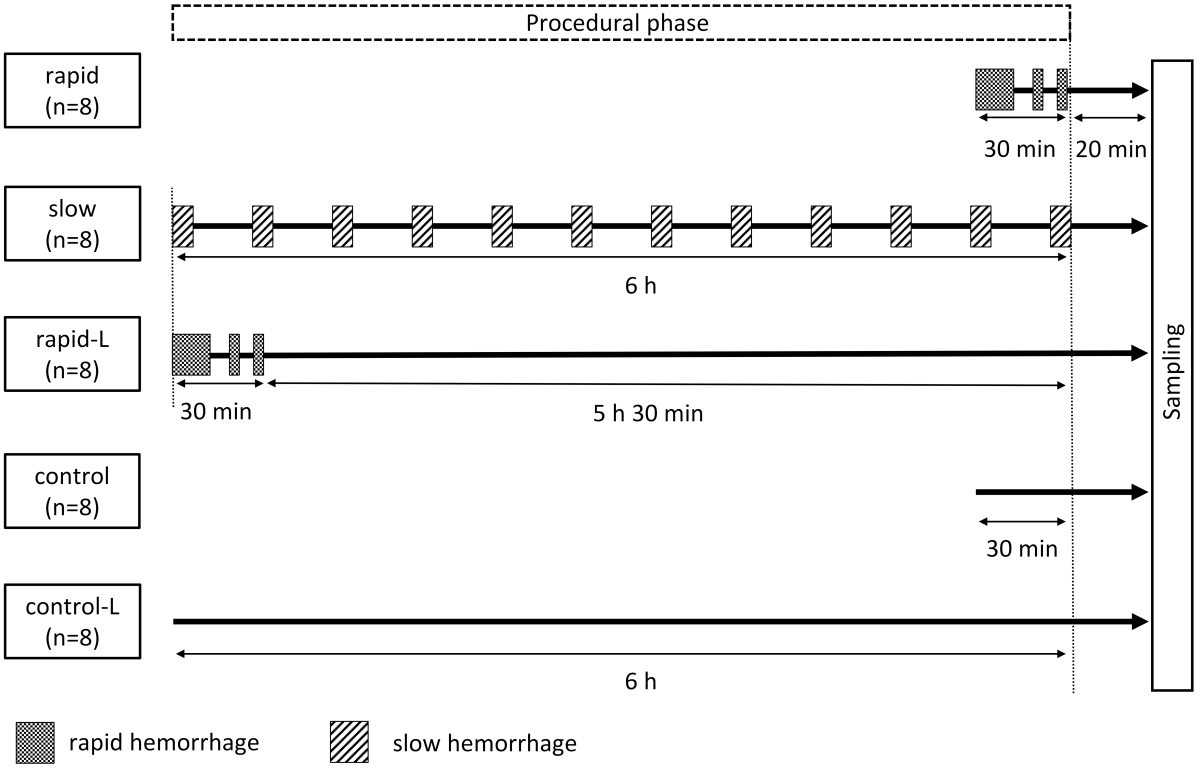
Procedural phase
rapid (n=8)
30 min
20 min
slow (n=8)
6 h
rapid-L (n=8)
30 min
5 h 30 min
control (n=8)
30 min
control-L (n=8)
6 h
Sampling
rapid hemorrhage
slow hemorrhage

Figure 2

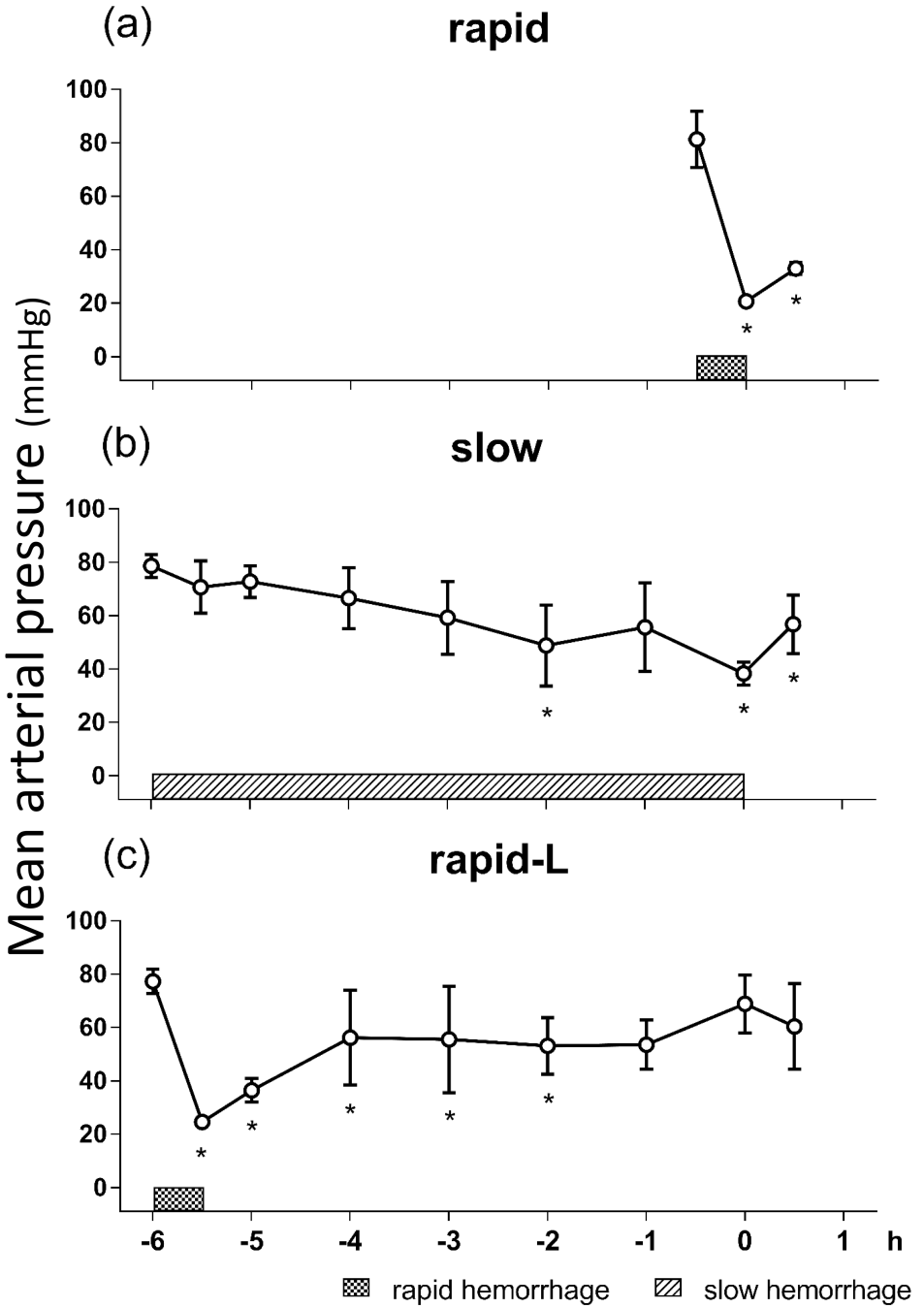
(a)
rapid
(b)
slow
(c)
rapid-L
Mean arterial pressure (mmHg)
100
80
60
40
20
0
-6
-5
-4
-3
-2
-1
0
1
h
rapid hemorrhage
slow hemorrhage

Figure 3

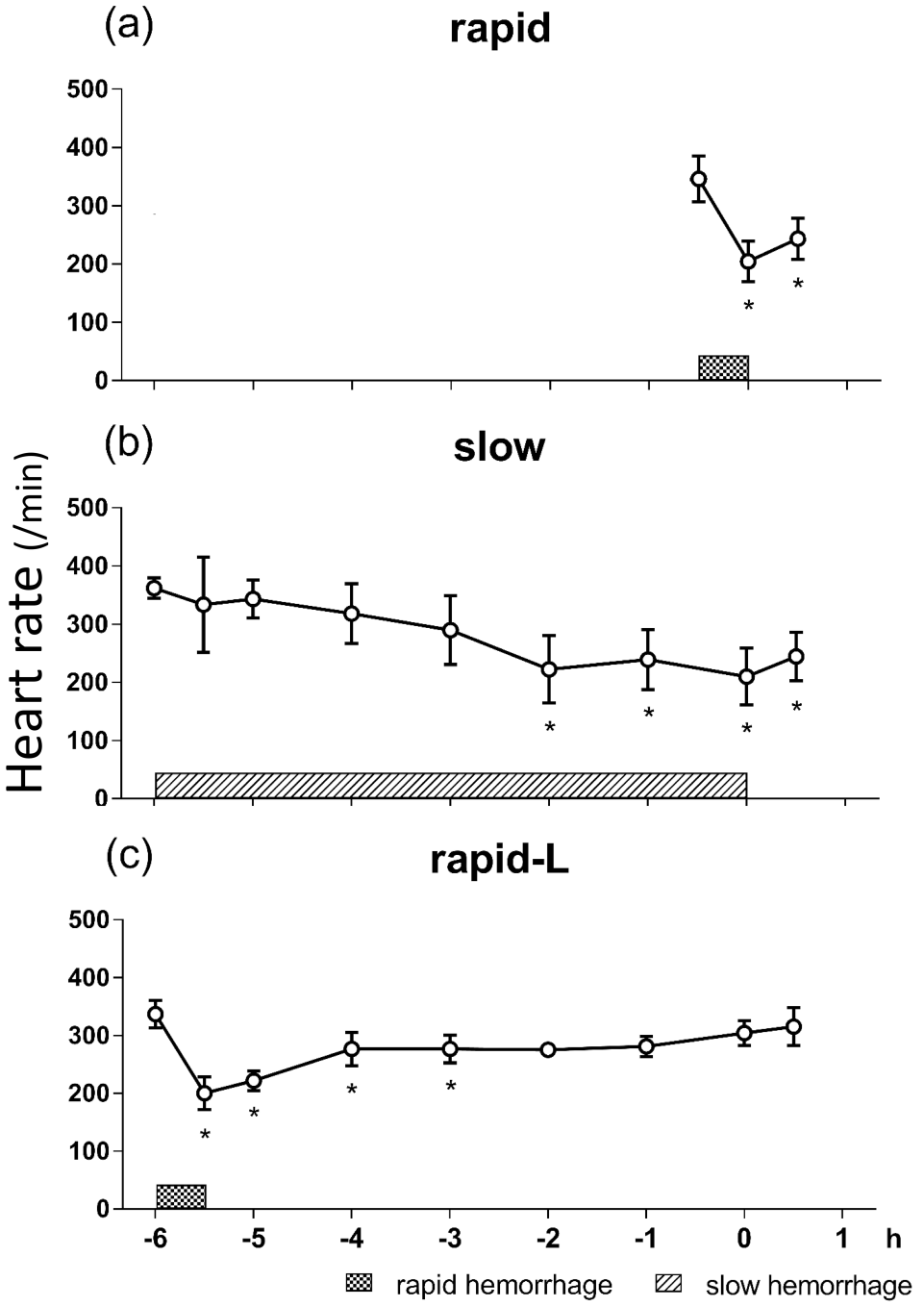
(a)
rapid
500
400
300
200
100
0
*
*
(b)
slow
Heart rate (/min)
500
400
300
200
100
0
*
*
*
*
(c)
rapid-L
500
400
300
200
100
0
*
*
*
*
-6
-5
-4
-3
-2
-1
0
1
h
rapid hemorrhage
slow hemorrhage

Figure 4

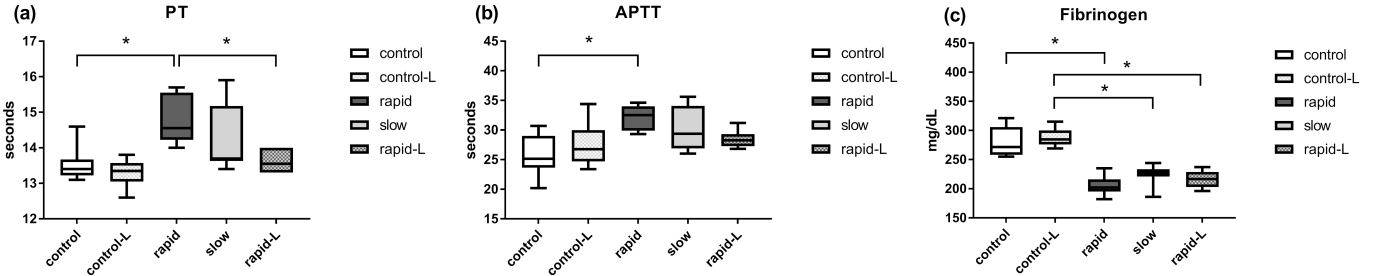
(a)
PT
seconds
17
16
15
14
13
12
*
*
control
control-L
rapid
slow
rapid-L
(b)
APTT
seconds
45
40
35
30
25
20
15
*
control
control-L
rapid
slow
rapid-L
(c)
Fibrinogen
mg/dL
450
400
350
300
250
200
150
*
*
*
control
control-L
rapid
slow
rapid-L

Figure 5

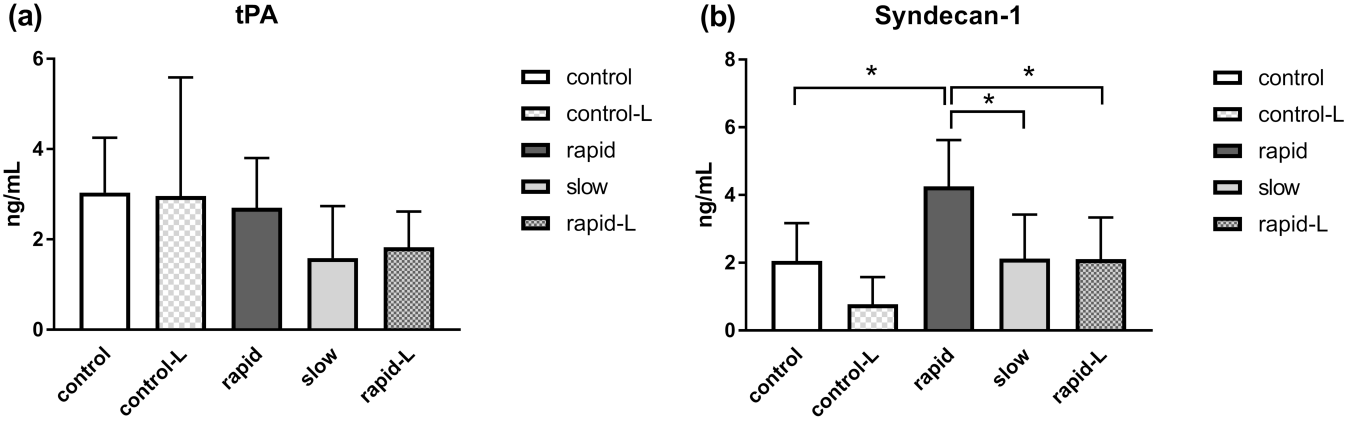
(a)
tPA
ng/mL
6
4
2
0
control
control-L
rapid
slow
rapid-L
(b)
Syndecan-1
ng/mL
8
6
4
2
0
*
*
*
control
control-L
rapid
slow
rapid-L

Figure 6

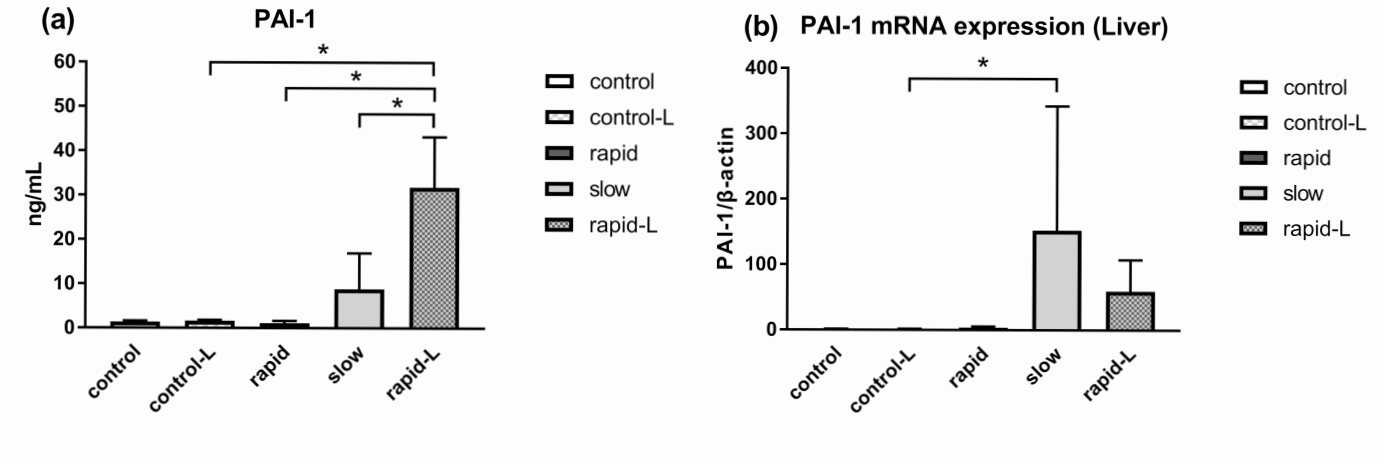
(a)
PAI-1
ng/mL
60
50
40
30
20
10
0
*
*
*
control
control-L
rapid
slow
rapid-L
control
control-L
rapid
slow
rapid-L
(b)
PAI-1 mRNA expression (Liver)
PAI-1/β-actin
400
300
200
100
0
*
control
control-L
rapid
slow
rapid-L
control
control-L
rapid
slow
rapid-L

Table 1. Hematology and blood gas analysis

Parameter	Groups				
	control (n=8)	control-L (n=8)	rapid (n=8)	slow (n=8)	rapid-L (n=8)
Hematology					
RBC (10^6/μL)	6.19 ± 1.28	6.67 ± 0.13	5.24 ± 0.28[a]	5.35 ± 0.30 [a]	4.94 ± 0.11 [a]
Hemoglobin (g/dL)	13.6 ± 0.8	13.4 ± 0.3	10.8 ± 1.0 [a]	10.7 ± 0.2 [a]	10.0 ± 0.3 [a]
Hematocrit (%)	34.9 ± 5.9	37.3 ± 1.0	29.2 ± 1.7 [a]	28.6 ± 1.1 [a]	27.6 ± 0.6 [a]
Platelet (10^3/μL)	1,893 ± 2,666	899 ± 56	852 ± 93	978 ± 105	840 ± 53
Blood gas analysis					
pH	7.43 ± 0.02	7.45 ± 0.01	7.44 ± 0.04	7.46 ± 0.03	7.48 ± 0.01 [d]
BE (mmol/L)	4.88 ± 1.64	5.37 ± 0.92	-1.13 ± 2.36 [a]	6.13 ± 3.00 [b]	5.50 ± 1.20 [d]
HCO_3^- (mmol/L)	29.2 ± 1.60	29.3 ± 1.01	23.0 ± 2.10 [a]	30.0 ± 2.40 [b]	29.1 ± 1.0 [d]
pCO_2 (mmHg)	43.9 ± 2.80	42.3 ± 2.26	34.2 ± 3.20 [a]	42.2 ± 2.00 [b]	38.8 ± 1.7 [d]
pO_2 (mmHg)	93.1 ± 4.60	88.0 ± 4.40	110.5 ± 4.80 [a]	92.1 ± 4.10 [b]	94.5 ± 5.0 [d]
Lactate (mmol/L)	1.20 ± 0.56	0.97 ± 0.44	4.59 ± 0.89 [a]	0.77 ± 0.18 [b]	0.98 ± 0.37 [d]

RBC, red blood cell; BE, base excess; HCO_3^-, bicarbonate; pCO_2, partial pressure of carbon dioxide; pO_2, partial pressure of oxygen

Values represent means ± SD. [a]$P < 0.05$, control groups (control and control-L) vs. bleeding group (rapid, slow, and rapid-L); [b]$P < 0.05$, rapid vs. slow; [c]$P < 0.05$, rapid-L vs. slow; [d]$P < 0.05$, rapid vs. rapid-L.

Table 2. Blood chemistry

Parameter	Groups				
	control (n=8)	control-L (n=8)	rapid (n=8)	slow (n=8)	rapid-L (n=8)
AST (IU/L)	55.4 ± 15.0	55.3 ± 35.7	59.3 ± 20.4	41.0 ± 11.5 [c]	81.3 ± 29.2
ALT (IU/L)	26.8 ± 5.0	23.0 ± 5.9	25.6 ± 5.4	18.0 ± 3.2 [c]	46.4 ± 28.9 [a]
BUN (mg/dL)	14.8 ± 2.4	14.6 ± 3.3	20.3 ± 1.8	33.6 ± 2.5 [a,b]	36.4 ± 9.6 [a,d]
Creatinine (mg/dL)	0.23 ± 0.05	0.26 ± 0.04	0.50 ± 0.05 [a]	0.47 ± 0.12 [a,c]	0.32 ± 0.05 [d]
ALP (IU/L)	594 ± 141	477 ± 134	469 ± 114	283 ± 55 [a,b,c]	465 ± 63
Glucose (mg/dL)	185 ± 18	173 ± 9	287 ± 78 [a]	200 ± 22 [b]	198 ± 21 [d]
Na (mEq/L)	136 ± 2	144 ± 1	135 ± 2	135 ± 3 [a]	138 ± 3 [a]
K (mEq/L)	6.87 ± 1.11	6.11 ± 0.57	6.33 ± 1.11	6.04 ± 0.70	5.93 ± 0.75
LDH (IU/L)	214 ± 212	102 ± 79	346 ± 282	107 ± 56	114 ± 53
CK (IU/L)	62.8 ± 15.7	132.9 ± 252.3	69.3 ± 36.5	44.6 ± 15.7	144.4 ± 104.8
Amylase (IU/L)	1,288 ± 149	1,379 ± 170	1,241 ± 194	1,505 ± 128 [b]	1,622 ± 160 [d]
T-CHO (mg/dL)	56.5 ± 7.3	61.6 ± 11.0	43.3 ± 8.0 [a]	51.6 ± 4.3	58.9 ± 10.0 [d]
HDL-C (mg/dL)	25.2 ± 3.3	34.6 ± 5.1	21.1 ± 3.5	27.0 ± 1.6	25.2 ± 8.1 [a]
LDL-C (mg/dL)	10.5 ± 1.9	9.3 ± 2.7	9.6 ± 3.3	9.1 ± 1.1	8.6 ± 1.0
Triglyceride (mg/dL)	155.3 ± 49.7	73.5 ± 35.8	58.3 ± 36.1 [a]	117.3 ± 31.4	191.9 ± 81.6 [a,d]

AST, aspartate transaminase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; CK, creatine kinase; T-CHO, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. Values represent means ± SD. [a] $P < 0.05$, control groups (control and control-L) vs. bleeding group (rapid, slow, and rapid-L); [b] $P < 0.05$, rapid vs. slow; [c] $P < 0.05$, rapid-L vs. slow; [d] $P < 0.05$, rapid vs. rapid-L.