

Acidosis and Coagulopathy

The Differential Effects on Fibrinogen Synthesis and Breakdown in Pigs

Wenjun Z. Martini, PhD, and John B. Holcomb, MD

Objective: Uncontrolled bleeding from coagulopathy signals imminent death in severely injured patients. Acidosis is an important predictor of coagulopathy, but the underlying contributing mechanisms are unclear. This study was designed to investigate the effects of acidosis on fibrinogen metabolism and coagulation function in a swine model. **Methods:** Twelve pigs were randomly divided into the control ($n = 6$) and acid ($n = 6$) groups. Acidosis of pH 7.1 was induced by infusion of 0.2 M HCl in lactated Ringer solution in the acid group. Afterward, an infusion of stable isotope $1\text{-}^{13}\text{C}$ -phenylalanine (6 hours) and d_5 -phenylalanine (4 hours) was performed. Blood samples were withdrawn hourly to quantify fibrinogen synthesis and degradation rates using gas chromatograph and mass spectrometry analysis. To correlate changes in fibrinogen metabolism, coagulation changes were assessed by prolonged prothrombin time, partial activated thromboplastin time, activated clotting time, and thrombelastograph (TEG).

Results: Acidosis caused decreases in mean arterial pressure, arterial bicarbonate concentration, base excess, fibrinogen concentration, and platelet counts. Acidosis increased fibrinogen degradation rate from the control value of 4.3 ± 1.0 mg/kg/h to 11.8 ± 1.4 mg/kg/h ($P < 0.05$), with no effect on fibrinogen synthesis. Prolonged prothrombin time, partial activated thromboplastin time, activated clotting time were consistently prolonged by acidosis (all $P < 0.05$). Clotting rapidity (angle α in TEG) was decreased from a baseline value of 73.3 ± 1.1 degree to 63.0 ± 2.4 degree ($P < 0.05$). Clot strength (maximum amplitude in TEG) was decreased from a baseline value of 72.2 ± 1.4 mm to 56.2 ± 3.1 mm ($P < 0.05$).

Conclusions: Acidosis compromised the clotting process and accelerated fibrinogen consumption with no effect on fibrinogen production, resulting in a deficit in fibrinogen availability.

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Coagulation complications are important contributors to morbidity and mortality in patients after severe injuries.^{1,2} Trauma-related coagulopathy often results from hypothermia, acidosis, hemodilution, and tissue injury-related systemic dysfunction. Among these contributing factors, Cosgriff et al³ found acidosis to be one of the most important predictors of coagulopathy in trauma patients, with the likelihood of death increasing as the severity of acidosis increased.^{3–10}

Impaired clotting enzyme activities have demonstrated the effects of acidosis on coagulation. When pH was reduced from 7.4 to 7.0 in vitro, Meng et al reported that the activities of factor VIIa and FVIIa/TF complex on phospholipid vesicles decreased by more than 90% and 60%, respectively.¹¹ When pH was reduced from 7.4 to 7.1 in pigs, Martini et al showed that thrombin generation decreased to 47% of control values, while fibrinogen concentration decreased by 18%.¹² These decreases were also associated with a 47% increase in spleen bleeding time.¹² Finally, hemostatic analysis of acidotic trauma patients regularly produces prolonged prothrombin time (PT) and partial activated thromboplastin time (aPTT), and decreased coagulation factor levels.^{3–10}

In contrast to clotting enzyme activities, the effects of acidosis on clotting factor availability are unclear. Specifically in our previous acidosis study,¹² we were unable to explain the depletion in fibrinogen levels. The depletion may be due to inhibited synthesis and/or accelerated consumption. To reveal the underlying mechanisms, we recently developed an in vivo technique to quantify fibrinogen synthesis and breakdown, using stable isotope infusion and subsequent gas chromatography and mass spectrometry (GC-MS).¹³ The objective of the current study was to investigate the changes in fibrinogen metabolism following acidotic insult using our newly developed technique. Changes in fibrinogen synthesis and breakdown rates were correlated with coagulation function assessments of PT, aPTT, activated clotting time (ACT), and thrombelastography (TEG).

MATERIALS AND METHODS

This study was reviewed and approved by the Institutional Animal Care and Use Committee. In conducting the research described here, the authors adhered to the “Guide for the Care and Use of Laboratory Animals,” National Research Council (1996). Twelve crossbred Yorkshire swine (33.9 ± 0.8 kg) obtained from a local vendor were randomly divided

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into the control ($n = 6$) and acid ($n = 6$) groups. After an overnight fast, animals were preanesthetized with glycopyrrolate (0.1 mg/kg) and Telazol (tiletamine hydrochloride 4 mg/kg and zolazepam hydrochloride 4 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA). Surgical anesthesia was induced with 5% isoflurane in 100% oxygen by mask and then maintained using 1% to 3% isoflurane after intubation. The right femoral artery was cannulated for blood sampling and the right external jugular vein for fluid infusion. Arterial blood pH was monitored continuously using intra-arterial sensors (Paratrend 7-Trendcare system, Diametrics Medical, Roseville, MN) placed via a 20-gauge carotid artery cannula. Arterial blood pressures and heart rate were monitored using an ex vivo pressure transducer connected to the same cannula during the study.

Study Protocol

Upon the completion of catheter cannulation, blood samples were taken (as baseline) for coagulation and hemodynamics measurements. Afterward, acidosis was induced in the acid group by infusion of 0.2 M HCl in lactated Ringer solution, following the procedures described previously.¹² When the target pH of 7.1 was achieved and stabilized for 15 minutes, blood samples (0 hours) were taken before the 6 hours stable isotope infusion started. Stable isotope 1-¹³C-phenylalanine (0.3 $\mu\text{mol/kg/min}$ with prime dose of 8 $\mu\text{mol/kg}$) and d₅-phenylalanine (0.3 $\mu\text{mol/kg/min}$ with prime dose of 18 $\mu\text{mol/kg}$) were infused via the right jugular vein in both groups (0 hours). Isotope 1-¹³C-phenylalanine was infused for 6 hours (from 0 to 6 hours) and isotope d₅-phenylalanine was infused for 4 hours (from 0 to 4 hours). Blood samples were collected at 0, 1, 2, 3, 4, 4.5, 5, 5.5, and 6 hours (8 mL each). To assess pig plasma volume, a bolus injection of sterile indocyanine green (ICG) was given at 4 hours during the isotope infusion and blood samples (2 mL each) were collected before the ICG injection and at 5, 10, and 15 minutes postinjection. An additional blood sample (6 mL) was collected at 6 hours to assess changes in coagulation and hemodynamics. At the end of the isotope infusion (6 hours), animals were euthanized with sodium pentobarbital (100 mg/kg, IV). The same isotope infusion and blood withdraws were performed in the control group without acidosis induction. Heparin was not used in this study.

Calculations for Fibrinogen Synthesis and Breakdown

Fibrinogen synthesis and breakdown rates were quantified using a primed constant infusion of stable isotope 1-¹³C-phenylalanine and d₅-phenylalanine. Calculations for fibrinogen synthesis and breakdown have been described previously.¹³ Briefly, plasma fibrinogen fractional synthesis rate (FSR) was calculated using the formula:

$$FSR = (EB_{(t_2)} - EB_{(t_1)}) / (EF \times t) \quad (1)$$

where $EB_{(t)}$ is the enrichment of fibrinogen-bound amino acids and EF is the enrichments of plasma amino acids (precursor enrichment). Enrichment is defined as the tracer (labeled amino acids) to trace (unlabeled amino acids) ratio (TTR).

Plasma fibrinogen fractional breakdown rate (FBR) was calculated using the formula:

$$FBR = (EB_{pred} - EB_{act}) / (EB_{(t_1)} \times t) \quad (2)$$

$$EB_{pred} = EB_{(t_1)} + FSR \times EF \times t \quad (3)$$

where EB_{pred} is the predicted fibrinogen-bound d₅-phenylalanine enrichments, EB_{act} is the actual measured fibrinogen-bound d₅-phenylalanine enrichments, $EB_{(t_1)}$ is the starting fibrinogen-bound phenylalanine enrichment when the d₅-phenylalanine infusion is stopped, EF is the enrichment of plasma phenylalanine, and t is the amount of time that has elapsed from the starting fibrinogen-bound phenylalanine measurement. In this study, d₅-phenylalanine was infused from 0 to 4 hours. EB_{pre} is the predicted fibrinogen-bound amino acids enrichment as if d₅-phenylalanine infusion would have continued beyond 4 hours till the end of the study. EB_{act} is the actual measured fibrinogen phenylalanine enrichment with d₅-phenylalanine infusion stopped at 4 hours.

The plasma fibrinogen absolute synthesis rate was calculated by multiplying FSR with plasma fibrinogen concentration and plasma volume quantified by ICG dye. Similarly, plasma fibrinogen absolute breakdown rate was calculated by multiplying FBR with plasma fibrinogen concentration and plasma volume.

Analytical Methods

Hematocrit and platelet count were measured from citrated blood using an ABX Pentra 120 hematology analyzer (ABX Diagnostics, Irvine, CA). Blood chemistries were measured using the Dimension Clinical Chemistry System (Dade Behring, Newark, DE). PT, aPTT, fibrinogen concentration, and D-dimer levels were measured from citrated plasma using the BCS Coagulation System (Dade Behring, Deerfield, IL). ACT was determined in fresh whole blood using HRFTCA 510 Hemochron (International Technique Corp, Edison, NJ).

The coagulation profiles were determined for fresh whole blood with pig thromboplastin using thrombelastography (TEG 5000 Hemostasis Analyzer, Hemoscope Corp, Niles, IL) as previously described.¹² In the TEG measurements, reaction time (R time) is the latency time for initial clot formation. Angle α measures the rapidity of fibrin build-up and cross-linking. Maximum amplitude (MA) represents maximum strength or stiffness of the clot.

To isolate plasma free amino acids, 0.2 mL plasma was acidified by 0.3 mL 15% sulfosalicylic acid and the supernatant was loaded onto a cation exchange column (AG 50W-X8 resin, 200–400 mesh, H⁺ form, Bio-Rad Laboratories, Richmond, CA). Plasma amino acids were separated after elution with 6 N ammonium hydroxide. The extracts were dried under speed vacuum and derivatized by *N*-methyl-*N*-(tert-butyl)dimethylsilyl)-trifluoroacetamide at 100°C for 1 hour. Plasma fibrinogen was isolated by adding 0.5 M CaCl₂ and thrombin to form fibrin clot, following the procedure described by Stein et al.¹⁴ The clot was then washed and hydrolyzed in 6 N HCl at 110°C for 24 hours and dried under speed vacuum. The released amino acids after hydrolysis

were isolated, dried, and derivatized in the same manner as for plasma free amino acids. The enrichments of phenylalanine from the plasma free amino acids and fibrinogen were determined by gas chromatography-mass spectrometry (GC-MS, model 5973, Hewlett-Packard) in the electron impact ionization mode. A selective ion-monitoring method was used at nominal mass-to-charge ratio (m/z) of 336 ($m + 0$), 337 ($m + 1$), 338 ($m + 2$), 339 ($m + 3$), 340 ($m + 4$), and 341 ($m + 5$) for phenylalanine as previously described.¹³

Statistical Analysis

All results are expressed as means \pm SE. Comparisons between the control and acidosis groups in fibrinogen synthesis and breakdown measurements were made with Student *t* test. Comparisons over time in substrate concentrations and coagulation parameters at each time point were made using Bonferroni multiple comparisons test relative to baseline. Statistical significance was set at the 0.05 level.

RESULTS

Baseline sample measurements were similar for the control and acid groups. Acidosis of 7.1 with 0.2 M HCl in lactated Ringer solution was successfully induced in the acid group, and all 6 pigs survived to the end of the study. Following acidosis induction and at the beginning of the isotope infusion in the acid group, mean arterial pressure (MAP) decreased from its baseline value of 92 ± 6 mm Hg to 71 ± 4 mm Hg; arterial bicarbonate concentration from its baseline value of 33.2 ± 0.9 mM to 17.1 ± 0.6 mM; and arterial base excess (BE) dropped from its baseline value of 7.7 ± 0.9 mM to -11.2 ± 0.8 mM (all $P < 0.05$ compared with baseline). During the 6 hours isotope infusion, MAP, bicarbonate concentration, and BE remained unchanged in the acid group. Fibrinogen concentration dropped from a baseline value of 261 ± 9 mg/dL to 221 ± 7 mg/dL at 6 hours ($P < 0.05$) and platelet count decreased from a baseline value of $292 \pm 37 \times 10^3/\mu\text{L}$ to $170 \pm 24 \times 10^3/\mu\text{L}$ at 6 hours ($P < 0.05$). In the control group, there were no significant changes in MAP (86 ± 3 mm Hg), BE (8.2 ± 0.6 mM), fibrinogen concentration (233 ± 17 mg/dL), or platelet counts ($312 \pm 28 \times 10^3/\mu\text{L}$).

Fibrinogen Synthesis and Degradation

After 1-hour infusion of $1\text{-}^{13}\text{C}$ -phenylalanine, plasma-free phenylalanine TTR ($m + 1$) reached plateau values in the control ($21.23\% \pm 0.58\%$) and acid ($24.70\% \pm 1.50\%$) groups. Fibrinogen-bound phenylalanine enrichments TTR ($m + 1$) increased linearly during the 6 hours infusion of $1\text{-}^{13}\text{C}$ -phenylalanine. The fibrinogen-bound phenylalanine enrichment TTR ($m + 1$) at 6 hours was $2.71\% \pm 0.20\%$ in the control and $3.15\% \pm 0.20\%$ in the acid groups ($P < 0.05$). FSR calculated from the increasing slope of fibrinogen-bound phenylalanine enrichments ($m + 1$) was $2.6\% \pm 0.1\%/h$ in the control group and $3.4\% \pm 0.2\%/h$ in the acid group ($P < 0.05$). The plasma volume measured at 4 hours was 49.4 ± 1.6 mL/kg in the control group and 54.3 ± 2.7 mL/kg in the acid group. Fibrinogen absolute synthesis rate, calculated by multiplying FSR with plasma volume and fibrinogen concentration, was 3.7 ± 0.3 mg/kg/h, which was

not significantly different from the control value of 3.2 ± 0.2 mg/kg/h.

Isotope d_5 -phenylalanine was infused from 0 to 4 hours for fibrinogen breakdown quantification. From 4 to 6 hours, fibrinogen-bound phenylalanine enrichment TTR ($m + 5$) in the control group was $1.15\% \pm 0.13\%$ at 4 hours and $1.68\% \pm 0.19\%$ at 6 hours, respectively; fibrinogen-bound phenylalanine enrichment TTR ($m + 5$) in the acid group was $1.66\% \pm 0.10\%$ and $1.90\% \pm 0.10\%$, respectively. FBR, calculated based on the changes of fibrinogen-bound phenylalanine enrichment TTR ($m + 5$) using formulae (2) and (3) in the Methods section, was $3.6\% \pm 1.0\%/h$ in the control group and $11.4\% \pm 2.0\%/h$ in the acid group ($P < 0.05$). Fibrinogen absolute breakdown rates, calculated by multiplying FBR with plasma volume and fibrinogen concentration, was elevated by 1.8-fold in the acid group, compared with the control group ($P < 0.05$, Fig. 1).

Coagulation Changes

Coagulation changes were assessed at baseline (before acidosis induction) and the end of the study. There were no changes in any parameters in the control group during the study. In the acid group, PT and aPTT were significantly prolonged as compared with baseline values (Fig. 2). Similarly, ACT from fresh whole blood was prolonged from the baseline value of 106 ± 2 to 130 ± 6 seconds at 6 hours in the acid group ($P < 0.05$), but remained unchanged in the control group (102 ± 6 seconds at baseline and 97 ± 3 seconds at 6 hours). Clotting rapidity (angle α) was decreased by acidosis from baseline value of 73.3 ± 1.1 degree to 63.0 ± 2.4 degree ($P < 0.05$). Clot strength (MA) was decreased by acidosis from baseline value of 72.2 ± 1.4 to 56.2 ± 3.1 ($P < 0.05$). The initial clotting time *R* remained unchanged (3.2 ± 0.3 minutes at baseline and 3.3 ± 0.3 minutes at the end). D-dimer levels were not changed in either the control group (1.3 ± 0.3 mg/dL at baseline and 1.4 ± 0.4 mg/dL at 6 hours) or the acid group (1.8 ± 0.4 mg/dL at baseline and 1.9 ± 0.5 mg/dL at 6 hours).

DISCUSSION

As the precursor for clot formation, fibrinogen plays an important role in the coagulation process. Alterations in

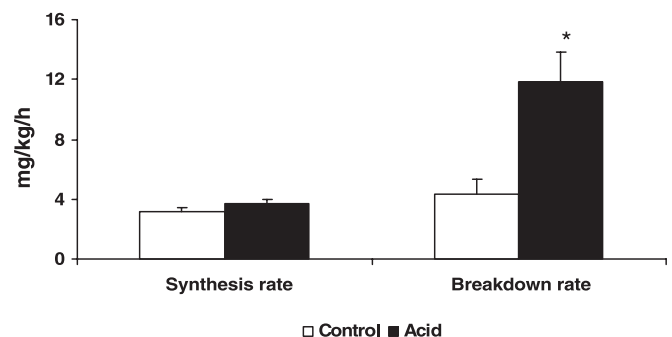


FIGURE 1. Effects of acidosis on fibrinogen synthesis and breakdown rates. * $P < 0.05$ compared with values in the control group.

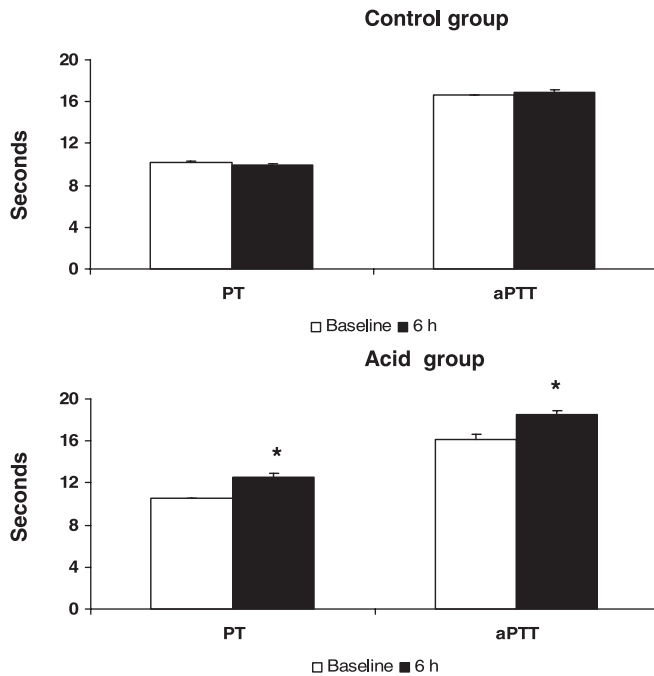


FIGURE 2. Effects of acidosis on prothrombin time (PT) and activated partial thromboplastin time (aPTT). * $P < 0.05$ compared with baseline values within the group.

fibrinogen levels are associated with coagulation complications. Fibrinogen deficiency puts patients in danger of uncontrolled hemorrhage, whereas overly abundant fibrinogen levels predispose patients to thrombotic risks. Acidosis has been found to be associated with decreased fibrinogen levels and a compromised coagulation process.^{12,15,16} Using a swine model and stable isotope technique, this study revealed that acidosis caused a near 2-fold increase in fibrinogen degradation but no changes in fibrinogen synthesis. These kinetic data provide explanations to the depletion of fibrinogen levels shown in previous acidosis studies.^{12,15,16} Furthermore, we recently reported that bicarbonate pH neutralization failed to replenish fibrinogen levels or correct coagulation function impaired by acidosis.¹⁷ The depletion of fibrinogen availability by acidosis and the lack of improvement following bicarbonate pH neutralization reflect the complexities in acidosis-induced coagulopathy.

Our observations of accelerated fibrinogen degradation without significant changes in fibrinogen synthesis indicate that acidosis induced by HCl has differential effects on fibrinogen synthesis and degradation. Similar differential effects of acidosis on thrombin generation were observed in our previous study.¹² Thrombin generation consists of the initiation phase and the propagation phase. The initiation phase involves the activation of factor VIIa/tissue factor complex and factor X to form small amounts of thrombin. The propagation phase involves the production of prothrombinase complex on the surface of activated platelets to generate large amounts of thrombin. We found that acidosis caused about 50% inhibition in the propagation phase of thrombin generation, with no effects shown in the initiation phase.¹² The

differential effects of acidosis on fibrinogen kinetics and thrombin generation emphasize the challenges in correcting acidosis-associated coagulopathy.

Changes in fibrinogen degradation under various pathophysiological states have been investigated previously using a radioactive technique.^{18–23} By injecting ¹²⁵I-labeled fibrinogen and subsequent daily blood samplings for 4 to 6 days, Tytgat et al reported that fibrinogen degradation was elevated in patients with disseminated intravascular coagulation, liver diseases, polycythemia, thrombocytosis, cardiovascular instability, sepsis, major surgery, and trauma.^{21,22} Consistently, using a 6 hours stable isotope infusion and subsequent GC-MS analysis, the present study showed that fibrinogen degradation was accelerated by acidosis insult. Thus, accelerated fibrinogen degradation appears to be a generalized response to stressed states.

The mechanisms contributing to the stimulation of fibrinogen degradation, however, remain unclear. Fibrinogen catabolic pathways include the conversion of fibrinogen to fibrin clots (the coagulation process), binding of intact fibrinogen to activated platelet surface, and the degradation of intact fibrinogen (fibrinogenolysis). The stable isotope technique used in this study allows the quantification of the total degradation rate from all of the catabolic pathways. However, it cannot differentiate the contribution from a specific pathway. Additional information is needed to reveal the changes from a specific pathway. For example, the degradation of fibrin clots is catalyzed by the enzyme plasmin, producing specific degradation product, ie, D-dimer. Since the coagulation process was impaired by acidosis and there were no significant changes in D-dimer levels in either group in this study, it appears that the conversion of fibrinogen to fibrin clots followed by clot degradation was unlikely to be a significant contributor to the increase of fibrinogen degradation. Intact fibrinogen binding to activated platelet via glycoprotein IIb-IIIa complexes is a prerequisite for platelet aggregation.²⁴ In this study, platelet count in the circulation was decreased to 60% of the baseline value by acidosis. The mechanisms contributing to the decrease are unclear. We speculate that the decrease of platelet count by acidosis might be due to an increase of systemic platelet aggregation. Thus, the pathway of intact fibrinogen binding to activated platelet was likely to be a significant contributor to the increase of fibrinogen degradation observed in this study. Additional studies on effects of acidosis on platelet aggregation and fibrinogenolysis are needed to prove or disprove this speculation.

In contrast to changes in fibrinogen degradation under various pathophysiological states, changes in fibrinogen synthesis, and consequently changes in fibrinogen availability, remain unclear due to the fact that the previous ¹²⁵I technique did not allow the quantification of fibrinogen synthesis. The stable isotope technique used in this study allows simultaneous and independent quantifications of the dynamic changes in fibrinogen synthesis and degradation. Our technique is advantageous especially when changes in synthesis and degradation are different, as in this study. The kinetic data from this study showed that acidosis caused a near 2-fold increase in fibrinogen degradation but fibrinogen synthesis

remained unchanged. It should be stressed that these findings were only a snapshot of fibrinogen kinetics at 4 to 6 hours after acidosis insult. Changes in fibrinogen metabolism by acidosis may or may not be the same in other time intervals. Nonetheless, data from this study may support the approach of replenishing fibrinogen levels to restore coagulation function after acidosis.

The changes in fibrinogen metabolism following acidosis insult were correlated with compromised coagulation function in this study. Acidosis compromised clotting rapidity and clot strength (determined by TEG measurements) and prolonged clotting times of PT, aPTT, and ACT. These consistent changes agreed with the decreased fibrinogen availability in this study. Thus, to restore coagulation function, it may be necessary to replenish fibrinogen levels as part of the acidosis correction. Since endogenous fibrinogen synthesis is slow (2% to 4% of total fibrinogen pool/h), administering a fibrinogen product would appear to be the most effective way to replenish fibrinogen levels and restore coagulation function.

Both lactic acid and HCl have been used to induce intravascular acidosis to investigate acidosis effects on coagulation function.^{12,15,25,26} Since life-threatening acidosis in trauma is often a result of lactic acidosis, we initially tried lactic acid infusion to induce acidosis but encountered the technical difficulties of hemolysis and high mortality. Therefore, 0.2 M HCl was used in this study. While physiological differences between the 2 models remain to be clarified, both models result in intravascular pH changes. Since the primary purpose of this study was to investigate changes in fibrinogen kinetics under different intravascular pH, it is reasonable to consider that the effects observed in this study are valid. Future studies will address the effects of endogenous acidemia on changes in fibrinogen metabolism and coagulation.

In summary, the effects of acidosis on fibrinogen metabolism were investigated in a swine model. Acidosis accelerated fibrinogen consumption with no significant effects on fibrinogen production, resulting in a deficit in fibrinogen availability. These kinetic changes were associated with a prolonged and compromised coagulation process. Our data support fibrinogen supplementation to restore coagulation function in acidosis-associated coagulopathy.

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