

Independent Contributions of Hypothermia and Acidosis to Coagulopathy in Swine

Wenjun Z. Martini, PhD, Anthony E. Pusateri, PhD, John M. Uscilowicz, BS, Angel V. Delgado, PhD, and John B. Holcomb, MD

Background: Clinical coagulopathy occurs frequently in the presence of acidosis and hypothermia. The purpose of this study was to determine the relative contributions of acidosis and hypothermia to coagulopathy, as measured by current standard bedside and clinical laboratory analyses (i.e., bleeding time and prothrombin time). In addition, we investigated possible mechanisms of these effects using a modified prothrombin time test, thromboelastography, and thrombin kinetics analyses. An improved understanding of coagulopathy should facilitate hemorrhage control.

Methods: Twenty-four pigs were randomly allocated into normal (pH, 7.4; 39°C), acidotic (pH, 7.1; 39°C), hypothermic (pH, 7.4; 32°C), and acidotic and hy-

pothermic (pH, 7.1; 32°C) combined groups. Acidosis was induced by the infusion of 0.2N hydrochloric acid in lactated Ringer's solution. Hypothermia was induced by using a blanket with circulating water at 4°C. Development of a clinical coagulopathy was defined as a significant increase in splenic bleeding time. Measurements were compared before (pre) and 10 minutes after (post) the target condition was achieved.

Results: Acidosis, hypothermia, or both caused the development of coagulopathy, as indicated by 47%, 57%, and 72% increases in splenic bleeding time ($p < 0.05$, pre vs. post). Plasma fibrinogen concentration was decreased by 18% and 17% in the acidotic and combined groups, respectively, but not in the hypothermic

group. Hypothermia caused a delay in the onset of thrombin generation, whereas acidosis primarily caused a decrease in thrombin generation rates. At 4 minutes' quench time, thrombin generation in the acidotic, hypothermic, and combined groups were 47.0%, 12.5%, and 5.7%, respectively, of the value in the control group. There were no changes in serum tumor necrosis factor- α and interleukin-6 in any group during the study.

Conclusion: Acidosis and hypothermia cause a clinical coagulopathy with different thrombin generation kinetics. These results confirm the need to prevent or correct hypothermia and acidosis and indicate the need for improved techniques to monitor coagulopathy in the trauma population.

J Trauma. 2005;58:1002-1010.

Hypothermia, acidosis, and coagulopathy constitute a "vicious cycle" in trauma patients.¹ The highest mortality has been shown in patients with concurrence of hypothermia, acidosis, and coagulopathy, despite adequate supply of blood, plasma, and platelets.^{2,3} The association of hypothermic coagulopathy with high mortality has been well described.³⁻¹² As many as 66% of trauma patients arrive in emergency departments manifesting hypothermia (temperature < 36°C).⁵ Approximately 80% of nonsurviving patients have had a body temperature of less than 34°C.³ Furthermore, investigators have reported a 2.4-fold increase of blood loss in postlaparotomy patients whose body temperature was $33.8 \pm 0.5^\circ\text{C}$ as compared with that of patients whose temperature was $36.1 \pm$

0.7°C , despite similar injury severity.⁷ The effect of hypothermia on coagulation has been hypothesized to result from inhibition of enzyme activities and platelet function, as well as increased fibrinolysis. The inhibition of the enzyme activity has been estimated by the effect of cold on the prothrombin time (PT) and activated partial thromboplastin time (PTT). Prolonged PT has been found in hypothermic patients and experimental animals and in plasma samples cooled in vitro.¹²⁻¹⁷ Changes in platelet function with cold are less consistent, but shear-induced platelet activation is markedly depressed at temperatures below 34°C.¹⁸ However, no changes in fibrinolysis have been reported under hypothermic conditions.^{12,13,17} These data suggest that the effects of cold on coagulant activity are complex, but general mechanisms related to the development of coagulopathy are known.

The role of acidosis in the development of clinical coagulopathy is poorly described, although it has been implicated.^{1,2,19} With the limited studies performed, inconsistent results have been reported.²⁰⁻²³ Decreased clotting time was observed when lactic acid was added to heparinized human and dog blood in vitro.²⁰ Similar decreases in PT and PTT were reported in dogs after the infusion of 1 mol/L of lactic acid.^{21,22} However, increased PT and PTT and decreased fibrinogen levels and platelet counts were reported by Dunn et al.²³ in dogs after the infusion of hydrochloric acid solution. Decreased activities of factor VIIa-tissue factor (FVIIa/TF) complex and factor Xa-factor Va (FXa/FVa)

Submitted for publication March 23, 2004.

Accepted for publication September 20, 2004.

Copyright © 2005 by Lippincott Williams & Wilkins, Inc.

From the U.S. Army Institute of Surgical Research, Fort Sam Houston, Texas.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

Supported by the U.S. Army Medical Research and Medical Command and the Veterinary Service Support Branch and Laboratory Support Branch at the U.S. Army Institute of Surgical Research.

Address for reprints: John B. Holcomb, MD, U.S. Army Institute of Surgical Research, 3400 Rawley E. Chamber, Fort Sam Houston, TX 78234; email: john.holcomb@amedd.army.mil.

DOI: 10.1097/01.TA.0000156246.53383.9F

Report Documentation Page

Form Approved
OMB No. 0704-0188

Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

| | | | | | |
|---|-----------------------------------|------------------------------------|---|---|------------------------------------|
| 1. REPORT DATE 01 MAY 2005 | | 2. REPORT TYPE N/A | | 3. DATES COVERED - | |
| 4. TITLE AND SUBTITLE Independent contributions of hypothermia and acidosis to coagulopathy in swine | | | | 5a. CONTRACT NUMBER | |
| | | | | 5b. GRANT NUMBER | |
| | | | | 5c. PROGRAM ELEMENT NUMBER | |
| 6. AUTHOR(S) Martini W. Z., Pusateri A. E., Uscilowicz J. M. Delgado A. V., Holcomb J. B., | | | | 5d. PROJECT NUMBER | |
| | | | | 5e. TASK NUMBER | |
| | | | | 5f. WORK UNIT NUMBER | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) United States Army Institute of Surgical Research, JBSA Fort Sam Houston, TX 78234 | | | | 8. PERFORMING ORGANIZATION REPORT NUMBER | |
| 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) | | | | 10. SPONSOR/MONITOR'S ACRONYM(S) | |
| | | | | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) | |
| 12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited | | | | | |
| 13. SUPPLEMENTARY NOTES | | | | | |
| 14. ABSTRACT | | | | | |
| 15. SUBJECT TERMS | | | | | |
| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT SAR | 18. NUMBER OF PAGES 9 | 19a. NAME OF RESPONSIBLE PERSON |
| a REPORT unclassified | b ABSTRACT unclassified | c THIS PAGE unclassified | | | |

complex on phospholipid surfaces were reported by Meng et al. when pH was decreased from 7.4 to 7.0.²⁴ Thus, the effect of acidosis on the coagulation process remains to be clarified.

The essence of blood coagulation is the production of fibrin from fibrinogen, and thrombin has a central role.²⁵ Thrombin activates platelets, as well as cofactors, enzymes in the clotting process, and inhibitors of the fibrinolytic process. In the initial phase, small amounts of thrombin are produced by the activation of FVIIa/TF complex and factor Xa. Afterward, there is a propagation phase with generation of large amounts of thrombin, which resulted from the production of prothrombinase complex on the surface of activated platelets. At the same time, thrombin generation is subject to inhibition from antithrombin III, thrombomodulin-activated protein C, and tissue factor pathway inhibition. This complex mechanism enables rapid clot formation on tissue injury but inhibition of clot formation away from the site of the injury. In this study, we investigated thrombin generation kinetics during hypothermia, acidosis, and combined hypothermia and acidosis as a possible underlying mechanism contributing to coagulopathy. To the best of our knowledge, changes of thrombin generation kinetics under acid and hypothermic conditions have not been investigated.

Coagulation has been assessed clinically by *in vitro* assays of PT and PTT from citrated plasma. PT measures the time required to form a fibrin clot, starting from the activation of factor VII (extrinsic pathway), and PTT measures the time required to form a fibrin clot, starting from the activation of factor XII (intrinsic pathway). Deficiencies of the factors involved in coagulation pathways may be reflected by the prolongation of PT or PTT. Although PT has been routinely used clinically, the disparities between clinical coagulopathy in patients and near normal PT values have been reported in the literature,^{14,16,26} indicating that PT is not a sensitive index for coagulopathy. A possible explanation is that PT is determined in the clinical laboratory at a standard temperature of 37°C, which masks temperature effects on enzyme activities. A comparison of PT performed at standard temperature and at the patient's body temperatures can clarify the inconsistency. Another *in vitro* measurement for coagulation is use of thromboelastography (TEG) to generate a coagulation curve from citrated plasma or whole blood. This curve provides rapidity of clot formation and fibrinolysis. In addition, measurements of ear bleeding time and splenic bleeding time have been used as *in vivo* assessments in animal studies.^{27,28} With the advantage of including systemic effects and coagulation factor effects, these *in vivo* measurements provide comprehensive assessment of coagulation. An additional increase in understanding the underlying mechanisms of coagulation can be expected with the combination of *in vivo* and concurrent *in vitro* assays.

The primary purpose of this study was to investigate the individual and combined contributions of hypothermia and acidosis to the development of a clinical coagulopathy *in vivo*. We tested the hypothesis that acidosis, hypothermia, or

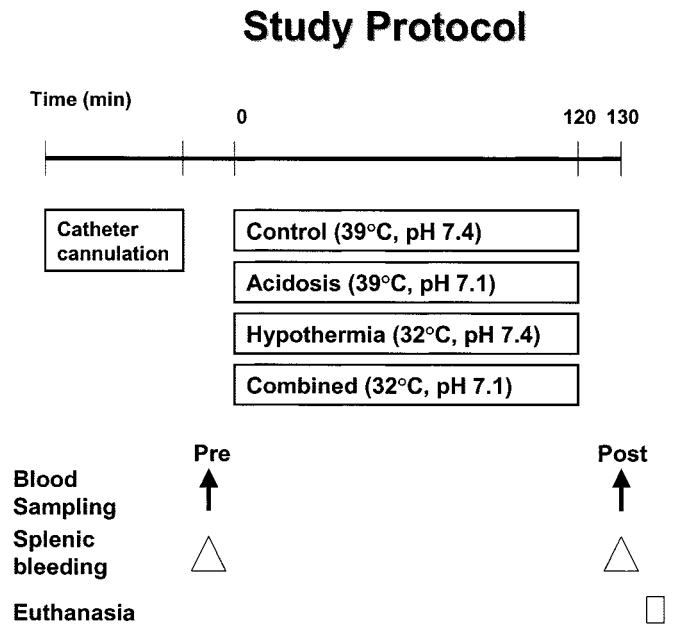


Fig. 1. Study protocol.

both increase blood coagulation time as measured by clinical laboratory analyses and as measured by *in vivo* bleeding time. To understand the mechanisms involved, thrombin generation kinetics was quantified under acidotic, hypothermic, and acidotic and hypothermic combined conditions in swine.

MATERIALS AND METHODS

Experimental Design

This study was approved by the Institutional Animal Care and Use Committee of the U.S. Army Institute of Surgical Research, Fort Sam Houston, TX (A-00-006). A total of 24 crossbred Yorkshire swine (body weight, 40.6 ± 3.9 kg) were randomly allocated into normal control, acidotic, hypothermic, and acidotic and hypothermic combined groups (n = 6 in each group). After baseline blood samples and coagulation measurements were taken (the "pre" sample), hypothermia (32.0°C) was induced using recirculating water at 4°C by means of a water-pumped blanket until the animal's body temperature reached 32.0 to 32.5°C. Acidosis (pH, 7.1) was induced by infusion of 0.2 mol/L hydrochloric acid in lactated Ringer's solution at a rate of 0.4 to 0.8 mL/kg/min. The rate of infusion was slowed below pH 7.3 to facilitate achieving the target pH of 7.1. In the combined group, hypothermia and acidosis were induced simultaneously to reach a temperature of 32.0°C and a pH of 7.1. Normal saline was given to pigs in the control and hypothermia groups to eliminate the dilutional effects between the groups. The fluid infusion did not cause significant changes of mean arterial pressure in any group. Animals were allowed to stabilize for 10 minutes at the target pH and temperature before blood samples and coagulation measurements were taken (the "post" sample). A schematic diagram of the study timeline is shown in Fig. 1.

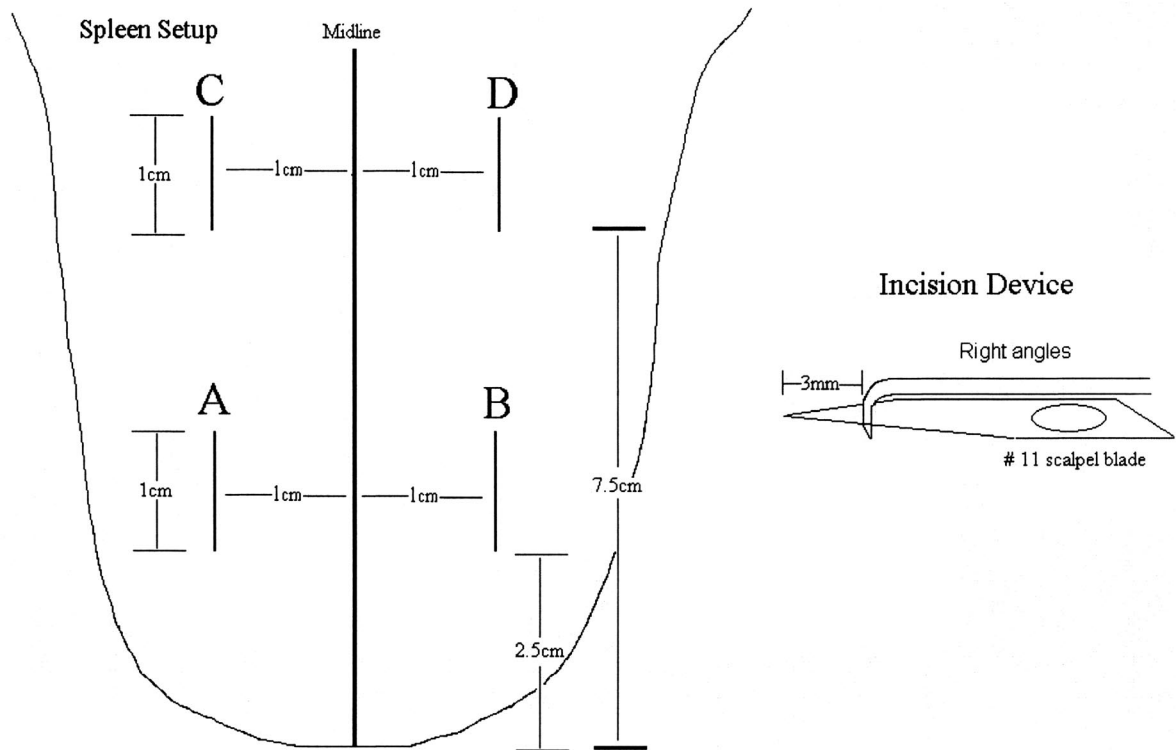


Fig. 2. Splenic bleeding incision diagram.

Animals and Instrumentation

After an overnight fast, animals were preanesthetized with glycopyrrolate (0.1 mg/kg) and Telazol (6 mg/kg), followed by 5% isoflurane in 100% oxygen by mask for the surgical procedures. The right femoral artery and the right external jugular vein were cannulated for blood sampling and fluid infusion, respectively. Arterial blood temperature and pH were monitored in vivo using intra-arterial sensors precalibrated according to the manufacturer's instructions (Paratrend 7 Trendcare System, Diametrics Medical, Inc., Roseville, MN) placed by means of a 20-gauge carotid artery cannula. Artery blood pressure and heart rate were monitored using an ex vivo pressure transducer connected to the same cannula.

Splenic Bleeding Time

An invasive technique was used to directly monitor bleeding time in the spleen. This method measured splenic bleeding time starting from calibrated incision made until no blood appeared from the incision. Briefly, the spleen was exposed, dried, and marked as A, B, C, and D (Fig. 2). The incisions were made using a No. 11 scalpel blades positioned into a right-angle clamp to obtain a 3-mm cutting depth. Bleeding cessation time was recorded and blood from the incision was collected using preweighed gauze. Incisions A and D were made as baseline and incisions B and C were made 10 minutes after the target pH and temperatures were achieved at the end of the study.

Blood Sampling

Blood samples were taken before the induction of acidosis and hypothermia (pre) and 10 minutes after target pH and temperature were achieved (post). Blood samples were collected by inserting a 25-cm single-use catheter made from Tygon tubing (Saint-Gobain Performance Plastics, Akron, OH) into the self-sealing port of the femoral catheter introducer. Blood was gently withdrawn to minimize shear-induced platelet activation. The first 3 mL of blood withdrawn was discarded at each sampling time.

Blood Coagulation and Chemistry Measurements

Hemoglobin concentration, hematocrit, platelet count, total protein concentration, Na^+ , K^+ , fibrinogen concentration, PT, and PTT were measured using standard clinical laboratory analyzers. In addition to standard PT (at 37°C), modified PT analyses were performed with blood maintained at the animal's temperature and pig thromboplastin for comparison.

TEG was determined according to the analyzer manufacturer's procedure (TEG 5000 Hemostasis Analyzer, Hemoscope Corp., Niles, IL) using a whole blood sample obtained from the femoral artery. The TEG coagulation curve was determined at the core temperature and pH of the animal at the time of sampling. In TEG measurements, reaction time (R time) is the latency time for initial clot formation. K-time is the time from the beginning of the initial clot formed to the

time when a fixed level of clot firmness is reached. Angle (α) measures the rapidity of fibrin buildup and cross-linking. Maximum amplitude (MA) represents maximum strength or stiffness of the clot, and CL_{30} indicates the percentage of clot lysis at 30 minutes after maximum amplitude is achieved.

Thrombin-antithrombin III complex (TAT) concentration was measured in plasma using commercially available enzyme-linked immunosorbent assay kits (Enzygnost TAT, Dade Behring, Inc., Deerfield, IL) and a spectrophotometer (Wallac Victor2 1420 multilabel counter, EG&G, Turku, Finland) according to the manufacturer's recommended procedure. Serum tumor necrosis factor (TNF)- α and interleukin (IL)-6 concentrations were determined using commercially available enzyme-linked immunosorbent assay kits (Quantikine porcine TNF- α and IL-6 Immunoassays, R&D Systems, Inc., Minneapolis, MN) and a spectrophotometer (Wallac Victor2).

The kinetics of thrombin generation in whole blood was determined by measuring TAT concentration (in milligrams per liter) in aliquots of blood samples by addition of a "quench" agent to sample aliquots at 1-minute intervals after sample withdrawal to stop clot formation and thrombin consumption. The technique was developed by Rand et al.,²⁹ and has been validated in studies of factor VIIa.^{30,31} Briefly, blood samples were withdrawn from the femoral artery using an Eppendorf repeater pipette with a 25-mL syringe barrel. The syringe barrel was wrapped with 500-mL saline bags for insulation. The blood was added (1 mL each) to 24 polystyrene tubes measuring 12×75 -mm with $\frac{3}{16}$ -inch holes in the side at the midpoint of the tubes to start the reaction. All tubes were preloaded with 29 μ L of 20 mmol/L HEPES, 150 mmol/L NaCl, and 5 mmol/L $CaCl_2$ at pH 7.4 (for the control and the hypothermic groups) or pH 7.1 (for the acidotic and combined groups) and 50 μ L of normal saline or a 1:100 dilution of pig thromboplastin. The assay tubes were fixed and rocked continuously in Thermal Rockers (Lab-Line Instruments, Inc., Melrose Park, IL) set to either 39°C or 32°C. Whole blood clotting was stopped at different time points by adding 1 mL of quench solution (50 mmol/L EDTA and 10 mmol/L benzamidine in HEPES-buffered saline). After quench, tape was placed over the holes in the tubes. The tubes were vortexed and centrifuged at 4,000g for 15 minutes. Supernatant was collected and stored at -80°C for TAT analysis. Thrombin generation kinetics is indicated by variation of TAT concentration (in milligrams per liter) with time (quantified as the amount of TAT at different quench time points).

Statistical Analysis

Data were analyzed using SAS statistical software. In each study group, comparisons were made in all measurements on a pre/post basis using one-way analysis of variance. The slope parameter was tested against zero to determine significant changes within a group. Between-group comparisons were made with appropriate adjustments for multiple

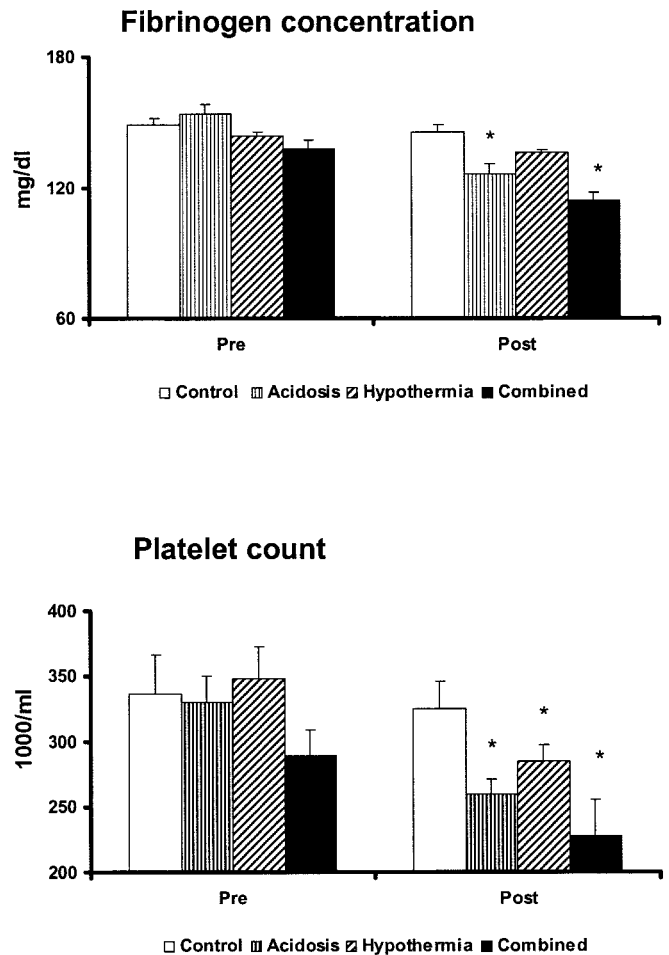


Fig. 3. Changes of plasma fibrinogen concentration (mg/dL) and platelet count ($10^3/\mu$ L) in experimental groups. * $p < 0.05$, pre versus post within each group.

ity using Tukey's adjustment. The statistically significant level was set at $p < 0.05$. Data are expressed as means \pm SEM.

RESULTS

All 24 animals survived the procedures to the end of the experiment. Hypothermia ($32.0 \pm 0.3^\circ\text{C}$) or acidosis (pH, 7.11 ± 0.02), or both, were successfully induced in all animals as intended, within 2 hours. Mean arterial pressure in each group remained unchanged during the study. Heart rate was increased in the acidotic group from 105 ± 5 beats/min to 147 ± 17 beats/min ($p < 0.05$) but was not altered in other groups. The hematocrit, hemoglobin, plasma total protein, and plasma Na^+ and K^+ levels remained unchanged in all groups. Plasma fibrinogen concentration was decreased in the acidotic and combined groups ($p < 0.05$) but was not decreased in the hypothermic group (Fig. 3). The platelet count was decreased in the acidotic, hypothermic, and combined groups ($p < 0.05$) (Fig. 3).

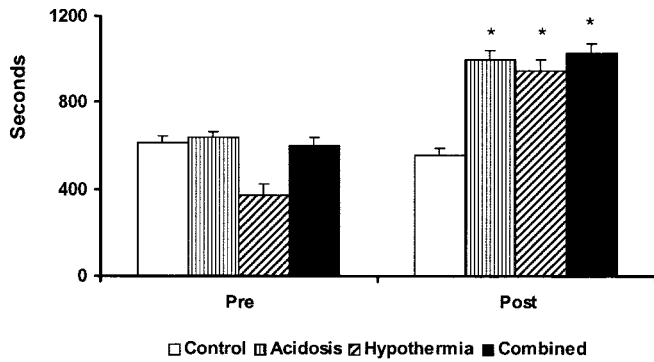


Fig. 4. Changes of splenic bleeding time in experimental groups. * $p < 0.05$, pre versus post within each group.

Splenic bleeding time was significantly prolonged by 41%, 57%, and 72% in the acidotic, hypothermic, and com-

bined groups, respectively ($p < 0.05$) (Fig. 4). However, changes were not demonstrated in the PT measured at 37°C (Table 1). When the assay temperature was adjusted to the pig's body temperature, however, there were 49% and 42% increases in the modified PT in the hypothermic and combined groups ($p < 0.05$), respectively, but no change in the acidotic group. The PTT was prolonged in the acidotic and combined groups ($p < 0.05$) but not in the hypothermic group.

The independent and combined effects of acidosis and hypothermia on thrombin generation are shown in Figure 5. Each curve represents the average value ($n = 6$ per group) of thrombin content assayed as TAT at 10 different quench times. Acidosis caused a moderate delay in the onset of thrombin generation. This delay was further prolonged in the hypothermic and combined groups. After initial thrombin

Table 1 In vitro clotting time measurements

| | | Control | Acidosis | Hypothermia | Combined |
|-------------------|------|------------|--------------|--------------|--------------|
| PT (sec) | Pre | 10.2 ± 0.1 | 10.1 ± 0.2 | 10.1 ± 0.1 | 10.1 ± 0.2 |
| | Post | 10.0 ± 0.1 | 11.0 ± 0.3 | 10.0 ± 0.1 | 11.1 ± 0.1 |
| Modified PT (sec) | Pre | 31.0 ± 0.8 | 31.8 ± 0.5 | 31.0 ± 0.5 | 31.8 ± 1.2 |
| | Post | 31.8 ± 0.7 | 32.3 ± 0.6 | 46.1 ± 0.9*‡ | 45.2 ± 1.2*‡ |
| PTT (sec) | Pre | 13.8 ± 0.4 | 13.3 ± 0.6 | 13.5 ± 0.5 | 13.3 ± 0.8 |
| | Post | 13.9 ± 0.3 | 19.0 ± 1.5*‡ | 13.5 ± 0.4 | 19.0 ± 0.8*‡ |

PT—prothrombin time measured at 37°C.

Modified PT—prothrombin time measured at the pig's body temperature at the time when blood samples were taken.

PTT—partial activated thromboplastin time.

* pre vs post within each group ($p < 0.05$).

‡ Control vs experimental groups ($p < 0.05$).

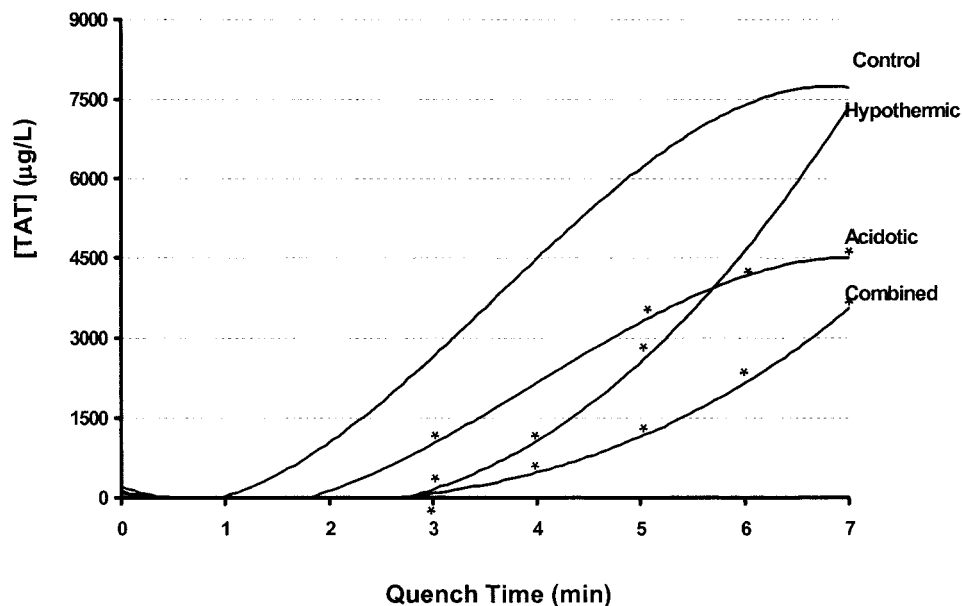


Fig. 5. Thrombin generation kinetics in blood samples obtained with special acquisition procedure from femoral artery measured as thrombin-antithrombin III complex concentration. TAT concentration was measured in sample aliquots at time 0 (sample withdrawal) and at 1-minute intervals thereafter to determine thrombin generation with time in each sample using the method of Rand et al.²⁹ * $p < 0.05$, different from normal value at the same quench time point.

was generated, the thrombin generation rate was primarily inhibited by acidosis. At 4 minutes, thrombin generation in the acidotic, hypothermic, and combined groups was $47.0 \pm 4.9\%$, $12.5 \pm 4.7\%$, and $5.7 \pm 1.3\%$ of the average value in the control group, respectively ($p < 0.05$, control vs. the hypothermia or combined group). At 7 minutes, thrombin generation in the acidotic and combined groups was $60.4 \pm 4.2\%$ and $43.5 \pm 4.0\%$ of the average value in the control group, respectively ($p < 0.05$, control vs. the acidotic or combined group). The inhibitory effects of acidosis and hypothermia on thrombin generation were not simply additive.

In TEG measurements, R time (representing the latency time before initial clot is formed) was prolonged from 3.8 ± 0.7 minutes to 5.4 ± 1.0 minutes in the hypothermia group ($p < 0.05$) and from 3.0 ± 0.5 to 4.3 ± 0.6 in the combined group ($p < 0.05$), with no change in R in the acidotic group. Angle (α , reflecting the rate of fibrin buildup and cross-linking) was decreased from 72.4 ± 1.7 to 64.8 ± 2.6 in the acidotic group ($p < 0.05$) and from 72.6 ± 1.5 to 56.8 ± 2.2 in the combined group ($p < 0.05$), with no change in the hypothermic group. No significant changes of MA, K (both representing platelet function), or CL_{30} (representing fibrinolysis) were observed in any group, although a decreased trend of MA was observed in the acidotic (from 50.2 ± 10.7 to 45.1 ± 8.5) and combined groups (59.4 ± 8.1 to 54.4 ± 5.5) and an increased trend of CL_{30} was observed in the hypothermic (from 75.5 ± 13.0 to 80.1 ± 14.4) and combined groups (from 75.4 ± 13.2 to 81.9 ± 15.0).

There were no changes in serum TNF- α or IL-6 during the study in any group. Plasma TAT was elevated in every group, but there were no differences in the increases between the groups. In addition, all of the measurements in the control group remained constant over time throughout the study.

DISCUSSION

In this study, we used a significant increase of splenic bleeding time to define the development of coagulopathy. This *in vivo* measurement provided an overall assessment of coagulation function, including changes in factors involved in coagulation, blood flow, and other systemic effects. Increases as high as 41%, 57%, and 72% in splenic bleeding time were found in the acidosis, hypothermia, and combined groups, respectively, indicating that acidosis and/or hypothermia caused coagulopathy. These increases were consistent with the decreases in platelet counts and fibrinogen levels. However, the detrimental effects of acidosis and hypothermia on coagulation were not detected in standard PT measurement (assayed at 37°C), because no changes in PT were found in any experimental group. When assay temperature was adjusted to the pig's body temperature at which blood samples were taken, significant increases of PT were shown in the hypothermia and combined groups. Our findings confirm the growing awareness that the standard PT is not a sensitive index of coagulation function in clinical practice. Because current commercially available PT instruments are certified at

37°C, it is important to emphasize that standard PT should be used to assess coagulation factor concentration, but not coagulopathy in hypothermia.¹⁴ Instead, PT measurements obtained at patient core temperatures should be used to assess coagulation.

The ultimate outcome of the coagulation process is clot formation from precursor fibrinogen. Decreased fibrinogen levels have been described in trauma patients, and the decline of fibrinogen levels have been considered as one of the two most sensitive measures of clinical coagulopathy (the other being platelet counts).³² Consistently, we observed decreases of approximately 20% in fibrinogen concentration shortly after induction of acidosis. This 20% drop can be amplified by hemorrhage and resuscitation, as occurs in patients after a trauma injury or after surgery. The underlying mechanism of the depletion is not clear. Because fibrinogen synthesis in normal pigs is approximately 1% to 3% of the total pool size per hour,^{33,34} the rapid 20% decrease of the fibrinogen level observed in this study is not likely the result of altered synthesis. Instead, it is more likely caused by altered sequestration or degradation. Additional investigation is required to clarify the underlying mechanism.

Thrombin generation regulates various biochemical and physiologic processes involved in coagulation and inflammation. In blood coagulation, thrombin plays a central role in activating cofactors, platelets, enzymes, and inhibitors and in cleaving fibrinogen to fibrin monomer. This study, for the first time, investigated thrombin generation kinetics under hypothermia and/or acidosis induced *in vivo*. We found that both hypothermia and acidosis impaired thrombin generation. Furthermore, we identified that hypothermia and acidosis inhibited thrombin generation with different kinetics.

Hypothermia primarily caused a delay in the onset of initial thrombin generation, indicating that the inhibition was located primarily in the FVIIa/TF pathway. In acidosis, initial thrombin generation was moderately delayed. After initial thrombin was generated, the thrombin generation rate at the propagation phase was persistently and drastically inhibited by acidosis. Consistent with our findings, Meng et al. reported that the activities of the FVIIa/TF complex and the FXa/FVa complex on phospholipid surfaces were decreased by 55% and 70%, respectively, at pH 7.0 compared with that at pH 7.4.²⁴ Because of the persistent inhibition at the propagation phase and moderate inhibition at the initial phase, acidosis might be more detrimental than hypothermia in the development of coagulopathy. In addition, we found that the acidotic inhibition on the thrombin generation rate was amplified when hypothermia was present, which correlates with clinical findings of a high mortality rate in trauma patients complicated with acidosis, hypothermia, and coagulopathy.² Thus, correcting blood pH (i.e., by administration of bicarbonate solution) should be considered a potentially important strategy in reversing clinical coagulopathies.

Our measurement of thrombin generation kinetics is consistent with the coagulation profiles obtained from TEG.

Reaction time in TEG is the latency time for initial clot formation. A prolonged R time represents a deficiency or dysfunction in coagulation factors. In this study, R time was found to increase in the hypothermic and combined groups (but not altered in the acidotic group), which was consistent with the prolonged delay of initial thrombin generation found in the hypothermic and combined group. Angle (α) measures the rapidity of fibrin buildup and cross-linking. It is affected by the availability of fibrinogen and platelets, but mostly by thrombin activity. In this study, α was found to decrease in the acidosis group and combined group but did not change in the hypothermic group. The decreases of α in those groups were consistent with the decreased thrombin generation rates and the decreased fibrinogen concentration observed in the acidotic and combined groups. In addition, these similarities indicate that initial thrombin generation is an essential step in clot formation.

Similar consistency is shown between thrombin generation kinetics and measurements of PT and PTT. The PT assay provides an estimate of the activation of FVIIa/TF complex. In the modified PT assay, we found prolonged PTs in the hypothermic and combined group but not in the acidotic group. Consistently, a more profound delay of initiation of thrombin generation (from activation of FVIIa/TF complex) was shown in the hypothermic and combined groups. In contrast, a prolonged PTT (an assay that estimates the activation of the intrinsic pathway) was induced by acidosis, which correlated well with the significant impairments of the thrombin generation rate (in the propagation phase) that occurred in acidosis.

Together with procoagulation enzymes, fibrinolysis and platelets play important roles in blood coagulation. However, in the setting of this study, we did not observe significant changes in TEG CL₃₀ measurements (fibrinolysis). Similar findings have been reported by Watts et al.¹³ in hypothermic trauma patients and by Kurrek et al.¹⁸ in hypothermic pigs. In addition, we did not observe significant changes in TEG MA and K measurements, although platelet counts were decreased under conditions of acidosis and hypothermia. Using a different approach, Staab et al. reported that temperature changes in minipigs did not cause alterations in platelet aggregation.¹² However, Watts et al. showed significant alterations in platelet function (by TEG) in hypothermic patients.¹¹ One possible explanation for the discrepancy shown in this study could be that the decreases in platelet counts were not large enough to cause detectable functional changes. Additional investigation is, therefore, required to clarify the effects of acidosis and hypothermia on platelet function. Nonetheless, it is reasonable to conclude that the primary detrimental effect of acidosis and hypothermia is the inhibition of thrombin generation.

Recently, recombinant activated factor VII (rFVIIa) has been used as therapy in patients bleeding uncontrollably, with beneficial effects in some of these patients.³⁵⁻³⁷ However, rFVIIa has not been effective in some acidotic trauma pa-

tients (U. Martinowitz, personal communication). These different clinical outcomes may be explained in relation to the findings from the present study. Because acidosis affects thrombin generation on both initial and subsequent propagation steps, supplementation with rFVIIa alone may not release the inhibition of propagation. Thus, a better alternative in acidotic patients may be to supplement rFVIIa in conjunction with pH correction (i.e., bicarbonate infusion). In contrast, improvement can be expected from supplementation with rFVIIa alone in hypothermic patients, because hypothermia primarily inhibits the activation of FVIIa/TF complex.

In summary, we investigated the independent and combined contributions of hypothermia and acidosis in the development of a clinical coagulopathy. Acidosis and/or hypothermia caused significant increases in splenic bleeding time and decreases in platelet counts and fibrinogen concentration. As a possible mechanism contributing to coagulopathy, we found that the kinetics of thrombin generation impairment by acidosis and hypothermia were different. Further understanding of the mechanisms underlying the development of coagulopathy induced by different means may facilitate hemorrhage control in trauma patients.

ACKNOWLEDGMENTS

We thank John Hess for reviewing the article and Kathy Ryan for editing the article; Michael Scherer, Suzanne Christensen, and James Martinez for technical assistance; and the Veterinary Service Support Branch and Laboratory Support Branch at the U.S. Army Institute of Surgical Research.

REFERENCES

1. Mikhail J. The trauma triad of death: hypothermia, acidosis, and coagulopathy. *AACN Clin Issues*. 1999;10:85-94.
2. Cosgriff N, Moore EE, Sauaia A, Kenny-Moynihan M, Burch JM, Galloway B. Predicting life-threatening coagulopathy in the massively transfused trauma patient: hypothermia and acidosis revisited. *J Trauma*. 1997;42:857-862.
3. Ferrara A, MacArthur JD, Wright HK, Modlin IM, McMillen MA. Hypothermia and acidosis worsen coagulopathy in the patient requiring massive transfusion. *Am J Surg*. 1990;160:515-518.
4. Jurkovich GJ, Greiser WB, Luteran A, Curreri PW. Hypothermia in trauma victims: an ominous predictor of survival. *J Trauma*. 1987;27:1019-1024.
5. Luna GK, Maier RV, Pavlin EG, Anardi D, Copass MK, Oreskovich MR. Incidence and effect of hypothermia in seriously injured patients. *J Trauma*. 1987;27:1014-1018.
6. Gregory JS, Flancaum L, Townsend MC, Cloutier CT, Jonasson O. Incidence and timing of hypothermia in trauma patients undergoing operations. *J Trauma*. 1991;31:795-800.
7. Bernabei AF, Levison MA, Bender JS. The effects of hypothermia and injury severity on blood loss during trauma laparotomy. *J Trauma*. 1992;33:835-839.
8. Prakash O, Jonson B, Bos E, Meij S, Hugenholtz PG, Hekman W. Cardiorespiratory and metabolic effects of profound hypothermia. *Crit Care Med*. 1978;6:165-171.
9. Britt LD, Dascombe WH, Rodriguez A. New horizons in management of hypothermia and frostbite injury. *Surg Clin North Am*. 1991;71:345-370.
10. Steinemann S, Shackford SR, Davis JW. Implications of admission hypothermia in trauma patients. *J Trauma*. 1990;30:200-202.

11. Gentilello LM, Jurkovich GJ, Stark MS, Hassantash SA, O'Keefe GE. Is hypothermia in the victim of major trauma protective or harmful? A randomized, prospective study. *Ann Surg.* 1997; 226:439–449.
12. Staab DB, Sorensen VJ, Fath JJ, Raman SB, Horst HM, Obeid FN. Coagulation defects resulting from ambient temperature-induced hypothermia. *J Trauma.* 1994;36:634–638.
13. Watts DD, Trask A, Soeken K, Perdue P, Dols S, Kaufmann C. Hypothermic coagulopathy in trauma: effect of varying levels of hypothermia on enzyme speed, platelet function, and fibrinolytic activity. *J Trauma.* 1998;44:846–854.
14. Reed RL II, Johnson TD, Hudson JD, Fischer RP. The disparity between hypothermic coagulopathy and clotting studies. *J Trauma.* 1992;33:465–470.
15. Reed RL II, Bracey AW Jr, Hudson JD, Miller TA, Fischer RP. Hypothermia and blood coagulation: dissociation between enzyme activity and clotting factor levels. *Circ Shock.* 1990;32:141–152.
16. Rohrer MJ, Natale AM. Effect of hypothermia on the coagulation cascade. *Crit Care Med.* 1992;20:1402–1405.
17. Kermod JC, Zheng Q, Milner EP. Marked temperature dependence of the platelet calcium signal induced by human von Willebrand factor. *Blood.* 1999;94:199–207.
18. Kurrek M, Reed R. Effect of hypothermia on enzymatic activity of thrombin and plasmin. *Surg Forum.* 1987;38:221–223.
19. Moore EE, Thomas G. Orr Memorial Lecture: staged laparotomy for the hypothermia, acidosis, and coagulopathy syndrome. *Am J Surg.* 1996;172:405–410.
20. Hardaway RM, Elowitz MJ, Brewster WR Jr, Houchin DN. Clotting time of heparinized blood: influence of acidosis. *Arch Surg.* 1964; 89:701–705.
21. Broersma RJ, Bullemer GD, Mammen EF. Acidosis induced disseminated intravascular microthrombosis and its dissolution by streptokinase. *Thromb Diath Haemorrh.* 1970;24:55–67.
22. Crowell JW, Houston B. Effect of acidity on blood coagulation. *Am J Physiol.* 1961;201:379–382.
23. Dunn EL, Moore EE, Breslich DJ, Galloway WB. Acidosis-induced coagulopathy. *Surg Forum.* 1979;30:471–473.
24. Meng ZH, Wolberg AS, Monroe DM III, Hoffman M. The effect of temperature and pH on the activity of factor VIIa: implications for the efficacy of high-dose factor VIIa in hypothermic and acidotic patients. *J Trauma.* 2003;55:886–891.
25. Mann KG, Brummel K, Butenas S. What is all that thrombin for? *J Thromb Haemost.* 2003;1:1504–1514.
26. Bunker JP, Goldstein R. Coagulation during hypothermia in man. *Proc Soc Exp Biol Med.* 1958;97:199–202.
27. Stewart D, Kong M, Novokhatny V, Jesmok G, Marder VJ. Distinct dose-dependent effects of plasmin and TPA on coagulation and hemorrhage. *Blood.* 2003;101:3002–3007.
28. Delaporte-Cerceau S, Samama CM, Riou B, Bonnin P, Guillosson JJ, Coriat P. Ketorolac and enoxaparin affect arterial thrombosis and bleeding in the rabbit. *Anesthesiology.* 1998;88:1310–1317.
29. Rand MD, Lock JB, van't Veer C, Gaffney DP, Mann KG. Blood clotting in minimally altered whole blood. *Blood.* 1996;88:3432–3445.
30. Butenas S, Brummel KE, Branda RF, Paradis SG, Mann KG. Mechanism of factor VIIa-dependent coagulation in hemophilia blood. *Blood.* 2002;99:923–930.
31. Butenas S, Brummel KE, Bouchard BA, Mann KG. How factor VIIa works in hemophilia. *J Thromb Haemost.* 2003;1:1158–1160.
32. Counts RB, Haisch C, Simon TL, Maxwell NG, Heimbach DM, Carrico CJ. Hemostasis in massively transfused trauma patients. *Ann Surg.* 1979;190:91–99.
33. Fu A, Sreekumaran Nair K. Age effect on fibrinogen and albumin synthesis in humans. *Am J Physiol.* 1998;275(6 pt 1):E1023–E1030.
34. Stoll B, Burrin DG, Henry J, Yu H, Jahoor F, Reeds PJ. Dietary amino acids are the preferential source of hepatic protein synthesis in piglets. *J Nutr.* 1998;128:1517–1524.
35. Allen GA, Hoffman M, Roberts HR, Monroe DM III. Recombinant activated factor VII: its mechanism of action and role in the control of hemorrhage. *Can J Anaesth.* 2002;49:S7–S14.
36. Dutton RP, Hess JR, Scalea TM. Recombinant factor VIIa for control of hemorrhage: early experience in critically ill trauma patients. *J Clin Anesth.* 2003;15:184–188.
37. Lynn M, Jeroukhimov I, Klein Y, Martinowitz U. Updates in the management of severe coagulopathy in trauma patients. *Intensive Care Med.* 2002;28(suppl 2):S241–S247.

DISCUSSION

Dr. James G. Tyburski (Detroit, Michigan): Good afternoon, Dr. Pasquale, Dr. Frankel, members, and guests of the Eastern Association for the Surgery of Trauma. I want to thank the Association first for allowing me to review this article, and I also want to thank the authors for getting it to me in a very timely fashion.

Dr. Martini and her coauthors should be congratulated on investigating a tremendously important clinical topic. The authors, with a clear and tight experimental design, present their data on independent contributions of hypothermia, acidosis, and the combinations in a porcine model.

I highly recommend the article. It is thought-provoking, very good reading, and like all good articles, raises many more questions than it answers. I'll try to ask some of them now.

First, for inducing metabolic acidosis, the authors used an infusion of hydrochloric acid. As noted in the article, previous work has shown that introduction of lactic acid actually decreases the prothrombin time, the partial thromboplastin time, and clotting times.

However, when hydrochloric acid was used in previous studies, the PT and PTT were increased with the decrease of fibrinogen levels and platelet counts. Knowing this, why did you choose hydrochloric acid over lactate? Why not use lactate, because you know that that had different effects, and it may be more clinically relevant?

Second, the platelet count was decreased in the acidotic/hypothermic-combined groups but not in the control group. The simple question is, why? Saline was given to the pigs in the control hypothermic groups to eliminate any dilutional or homodynamic effects of giving hydrochloric acid in the acidotic group.

Although the infusion did not cause significant changes in the mean arterial pressure, was it enough fluid to account for any dilatational effects? How much fluid was given per kilogram to the pigs in each group? Third—a related question—is it possible the increase in splenic bleeding time was attributable just to the decreased platelet count mentioned above when we talk about bleeding time?

Fourth, do you have any data on the partial arterial pressure of carbon dioxide in these animals? Was the ventilation manipulated in any way to control the pH, as most clinical acidosis is a mixed bag of metabolic and respiratory

entities. Thus, do you have any blood gas data on this, talking about their P_{CO_2} ?

Finally, there were no differences noted in the article between TNF- α or IL-6 between groups. This could be easily attributed to the lack of hypotension and the lack of significant tissue damage. Can you speculate on how the addition of hypotension and more extensive tissue damage may have affected the results if these parameters were added to the experimental model?

Indeed, in immediate proinflammatory cytokine and tissue factors, the coagulates may have behaved differently, particularly in some of the antithrombin-III activated factors. Once again, I do want to credit the authors and especially Dr. Martini and colleagues in attempting to tease out the independent contributions of acidosis and hypothermia coagulopathy.

Although these two factors are often stated both in the literature and in teaching grounds as important effects and significant clinical bleeding in the trauma patient, there are precious few data to support or refute these statements. This study is one step toward elucidating these mechanisms.

Dr. Peter M. Rhee (Los Angeles, California): Very nice study, which is elegant, and I don't necessarily disagree with your findings. I would like to point out a very important issue with this model. One is that spontaneous hypothermia and acidosis in trauma is not really that much of an issue, and induced hypothermia and acidosis are quite different if you don't have blood loss.

Blood loss, causing shock and ischemia, is what causes loss of adenosine triphosphate, which then causes lactic acidosis, and then that lactic acidosis and the minimal amount of adenosine triphosphate produces what makes your body temperature go to ambient temperature and therefore hypothermia. Then you've got blood loss. So you start resuscitating with cold fluids, which are devoid of factors, and you lose whole blood onto the ground, and that's why you get delusional coagulopathy as well and open body cavities and what not.

I think for this model to be relevant to trauma, you really have to have blood loss and resuscitation before you test the independent factors of acidosis and hypothermia. Thank you.

Dr. Martin A. Schreiber (Portland, Oregon): Thrombin is an extremely unstable molecule and very difficult to measure, causing us to have to use alternative parameters such as the thrombin-antithrombin complex and the prothrombin fragments. Could you please comment on your assay for thrombin generation?

Dr. Wenjun Z. Martini (closing): Thanks, everyone for all these interesting questions. I will try to answer them in order in which they were asked.

The first question is, why use hydrochloric acid and not a lactate acid, because the lactate has more clinical relevance. It's a very good question.

We did try lactate first. However, we faced the problem of why the pig didn't take it well. If we infused lactate alone, just lactate, hemolysis occurred in most of the pigs; and the second thing is when we infused lactate, the amount and concentration was hard to control. Some pigs died at one concentration, some didn't.

As an alternative, we tried hydrochloric acid at low concentrations and dissolved it in lactated Ringer's solution. In this way, it's in clinical relevant range as lactate was involved. In contrast, we avoided the hemolyses problem. So that's the answer for the question.

The second question is, what caused the decrease? The answer for this question is that we don't know. We don't know whether it's because of degradation or the secretion or whatever.

The third question, if the splenic bleeding time decreased, it's purely because of the platelet count decrease. Well, because many things happened, and as I showed in the data here, we see that the essential enzyme thrombin generation is inhibited in all of this group. We don't know what the mechanism is for platelet count decrease, so I cannot say the decrease is purely attributable to the platelets or to some other reason.

However, on the basis of the data I've been showing here, I think it's reasonable to say that the splenic bleeding time decrease is primarily attributable to the essential enzyme of the coagulation process, thrombin generation impairment.

In response to the question about complicated clinical setting, when there is a trauma injury in which the IL-6 is involved, what kind of result you would expect? This is a step forward question. Right now, I don't have solid data to answer the question. This study is just bringing up a very initial point for you to think about, and what the answer is for the complicated situation. So I can stand here and speculate that the situation will be getting worse, or in other words, the effects that we see here may become worse.

I think the next question is the similar situation, like you ask what is going to happen in a clinical situation because of the hypotension profusion and what is going to happen. Once again, the purpose of this study was to try to see the independent contribution of hypothermia and acidosis effects on coagulopathy. To test this, we have to use acidosis and hypothermia as independent variables. Thus, this study was initiated for this purpose, and when you apply this study to a clinical situation, I think we can just consider this study as a good starting point.

On the basis of the findings from this study, now we can build our next study; that is, to add blood loss and tissue injury to determine the cause for coagulopathy. In fact, we are currently doing this study. That is all. Thanks.