

Alterations in Coagulation Induced by Hypothermia and Acidosis in Swine¹

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ABSTRACT

Although clinical coagulopathy is associated with acidosis and hypothermia, the underlying mechanisms by which these factors alter the coagulation process remains unclear. The primary purpose of this study was to investigate the contributory effects of acidosis and hypothermia to the development of coagulopathy. Twenty-four pigs were randomly divided into control (pH 7.4, 39°C), acidotic (pH 7.1, 39°C), hypothermic (pH 7.4, 32°C), and combined acidotic-hypothermic (pH 7.1, 32°C) groups (n=6/group). Acidosis and hypothermia were introduced by infusion of 0.2M HCl and using a blanket with circulating cold water (4°C), respectively. Measurements were compared before (pre) and 10 min after the induction of acidosis and hypothermia (post). Development of coagulopathy was defined as a significant increase in splenic bleeding time in vivo. Coagulopathy developed shortly after the induction of acidosis and/or hypothermia. Splenic bleeding time was prolonged by 41%, 57%, and 72% in acidotic, hypothermic, and the combined groups (p<0.05, pre vs post in each group), respectively. Hypothermia caused a delay in the onset of thrombin generation, whereas acidosis caused both a delay in the onset of thrombin generation and an impairment in thrombin generation rate. The reaction time (latency time for initial clot formation) of the thromboelastogram (TEG) was prolonged in the hypothermia and the combined groups, but not in the acidotic group. The α -angle (the rapidity of fibrin build up and cross linking) of the TEG was reduced in the acidosis and combined groups, but not in the hypothermia group. We conclude that acidosis and hypothermia cause coagulopathy via different mechanisms.

1.0 INTRODUCTION

The association of hypothermic coagulopathy with high mortality has been well described [1-10]. As many as 66% of trauma patients arrive in emergency departments manifesting hypothermia (temperature < 36°C) [2]. Approximately 80 % of non-surviving patients have had a body temperature of less than 34°C [9]. Furthermore, investigators have reported a 2.4-fold increase of blood loss in post-laparotomy patients whose body temperature was 33.8±0.5°C as compared to that of patients whose temperature was 36.1±0.7°C, despite similar injury severity [4]. 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

¹ 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.

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thromboplastin time (PTT). Prolonged PT has been found in hypothermic patients and experimental animals, as well as in plasma samples cooled *in vitro* [10-15]. Changes in platelet function with cold are less consistent, but shear induced platelet activation is markedly depressed at temperatures below 34°C [16]. However, no changes in fibrinolysis have been reported under hypothermic conditions [10, 11, 15]. 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, though it has been implicated [17-19]. 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 following the infusion of 1M lactic acid [21, 22]. However, increased PT and PTT, as well as decreased fibrinogen levels and platelet counts were reported by Dunn et al [23] in dogs after the infusion of HCl solution. Decreased activities of factor VIIa - tissue factor (FVIIa/TF) complex and factor Xa -factor Va (FXa/FVa) 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 result 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 upon tissue injury, but inhibition of clot formation away from the site of the injury. Thus, changes of thrombin generation kinetics under different circumstances provide information about the mechanisms of underlying alterations in coagulation. To the best of our knowledge, changes of thrombin generation kinetics under acid and hypothermic conditions have not been investigated.

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*. To understand the mechanisms involved, thrombin generation kinetics were quantified under acidotic, hypothermic, and acidotic and hypothermic combined conditions in swine.

2.0 METHODS

2.1 Animals

This study was approved by the Institutional Animal Care and Use Committee of the U.S. Army Institute of Surgical Research, Fort Sam Houston, Texas (A-00-006). A total of 24 crossbred Yorkshire swine (body wt. 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 an overnight fast, animals were pre-anesthetized with Glycopyrrolate (0.1 mg/kg) and Telazol (6mg/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 intraarterial sensors precalibrated according to manufacturer's instructions (Paratrend 7- Trendcare system, Diametrics Medical Inc, Roseville MN) placed via 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.

2.2 Experimental Design

After baseline blood samples and coagulation measurements were taken (the “pre “ sample), hypothermia (32.0°C) was induced using recirculating water at 4°C via a water-pumped blanket until the animal’s body temperature reached 32.0-32.5°C. Acidosis (pH 7.1) was induced by infusion of 0.2 M HCl in Lactated Ringers solution at a rate of 0.4-0.8 ml/kg/min. The rate of infusion was slowed below pH 7.3 to facilitate achieving the target pH of 7.1. In combined group, hypothermia and acidosis were induced simultaneously to reach 32.0°C temperature and pH 7.1.

Blood samples were taken before the induction of acidosis and hypothermia (pre) and 10 min 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, Ohio) 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 were discarded at each sampling time.

Splenic bleeding time was measured before the induction of acidosis and hypothermia and after target pH and temperature were achieved using #11 scalpel blades positioned into a right-angle clamp to obtain a 3 mm cutting depth. Splenic bleeding cessation time was recorded and blood from the incision was collected using pre-weighted gauze.

2.3 Analyses

Hemoglobin (Hgb), Hematocrit (Hct), and Platelet (PLT) counts from citrated blood were measured using a Pentra 120 hematology analyzer (ABX Diagnostics, Inc., Irvine, CA). **Prothrombin time (PT), Partial Prothrombin Time (PTT), and Fibrinogen** concentration from citrated plasma were determined at 37°C using the ACL Futura Coagulation System (Instrumentation Laboratory, Lexington, MA).

Thrombelastography (TEG): Whole blood (300 µl) was drawn from the femoral artery into an Eppendorf repeater pipet (Brinkmann Instruments Inc. Westbury, NY) and transferred directly into saline preloaded cups of Model 5000 TEG’s (Haemoscope, Skokie, IL). TEGs were run under the same pH and temperature conditions as the pig from which the blood samples were taken following the manufacture’s instructions.

Thrombin generation kinetics was determined from thrombin-antithrombin III complex (TAT) concentration using minimally altered whole blood, following the procedure described by Rand et al.[26]. This approach has been validated in previous reports [25, 27]. Briefly, blood samples were withdrawn from the femoral artery using an Eppendorf repeater pipet with a 25ml syringe barrel. The syringe barrel was wrapped with 500 ml saline bags for insulation. The blood was added (1ml each) to twenty-four 12 x 75 mm polystyrene tubes with 3/16-inch holes in the side at the midpoint of the tubes to start the reaction. All tubes were preloaded with 29 µl of 20 mmol/L HEPES, 150 mmol/L NaCl, and 5 mmol/L CaCl₂ at pH 7.4 (for control and the hypothermic groups) or pH 7.1 (for the acidotic and combined groups) and 50 µl 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, Illinois) set to either 39°C or 32°C. Whole blood clotting was stopped at different time points by adding 1ml of quench solution (50mmol/L EDTA and 10mmol/L L-benzamidine in HEPES-buffered saline). After quench, tape was placed over the holes in the tubes. The tubes were vortexed and centrifuged at 4000 g for 15 min. Supernatant was collected and concentrations of TAT was measured using Enzygnost TAT micro enzyme immunoassay kits (Dade Behring, Marburg, Germany) following the manufacture’s instructions.

2.4 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 ANOVA. The slope parameter was tested against zero to determine significant changes within a group. Between group comparisons were made with appropriate adjustments for multiplicity using Tukey adjustment. The statistical significant level was set at $p < 0.05$. Data are expressed as Means \pm SEM.

3.0 RESULTS

All 24 animals survived the procedures to the end of the experiment. Hypothermia ($32.0 \pm 0.3^\circ\text{C}$) or acidosis ($\text{pH } 7.11 \pm 0.02$), or both, were successfully induced in all animals as intended, within 2 hours. Mean arterial pressure (MAP) in each group remained unchanged during the study. Heart rate (bpm) was increased in the acidotic group from 105 ± 5 to 147 ± 17 ($p < 0.05$), but 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.1). The platelet count was decreased in the acidotic, hypothermic, and combined groups ($p < 0.05$, Fig. 1).

Splenic bleeding time was significantly prolonged by 41%, 57%, and 72% in the acidotic, hypothermic, and combined groups, respectively ($P < 0.05$, Fig 2). However, changes were not demonstrated in the PT measured at 37°C (Table 1). The PTT was prolonged in the acidotic and combined groups ($p < 0.05$), but not in the hypothermic group (Table 1).

Table 1: In Vitro Clotting Time Measurements

		Control	Acidosis	Hypothermia	Combined
PT (sec)	Pre	10.2 \pm 0.1	10.1 \pm 0.2	10.1 \pm 0.1	10.1 \pm 0.2
	Post	10.0 \pm 0.1	11.0 \pm 0.3	10.0 \pm 0.1	11.1 \pm 0.1
PTT(sec)	Pre	13.8 \pm 0.4	13.3 \pm 0.6	13.5 \pm 0.5	13.3 \pm 0.8
	Post	13.9 \pm 0.3	19.0 \pm 1.5*‡	13.5 \pm 0.4	19.0 \pm 0.8*‡

PT- prothrombin time measured at 37°C .

PTT – partial activated thromboplastin time measured at 37°C .

* pre vs post within each group ($p < 0.05$). ‡ Control vs experimental groups ($p < 0.05$).

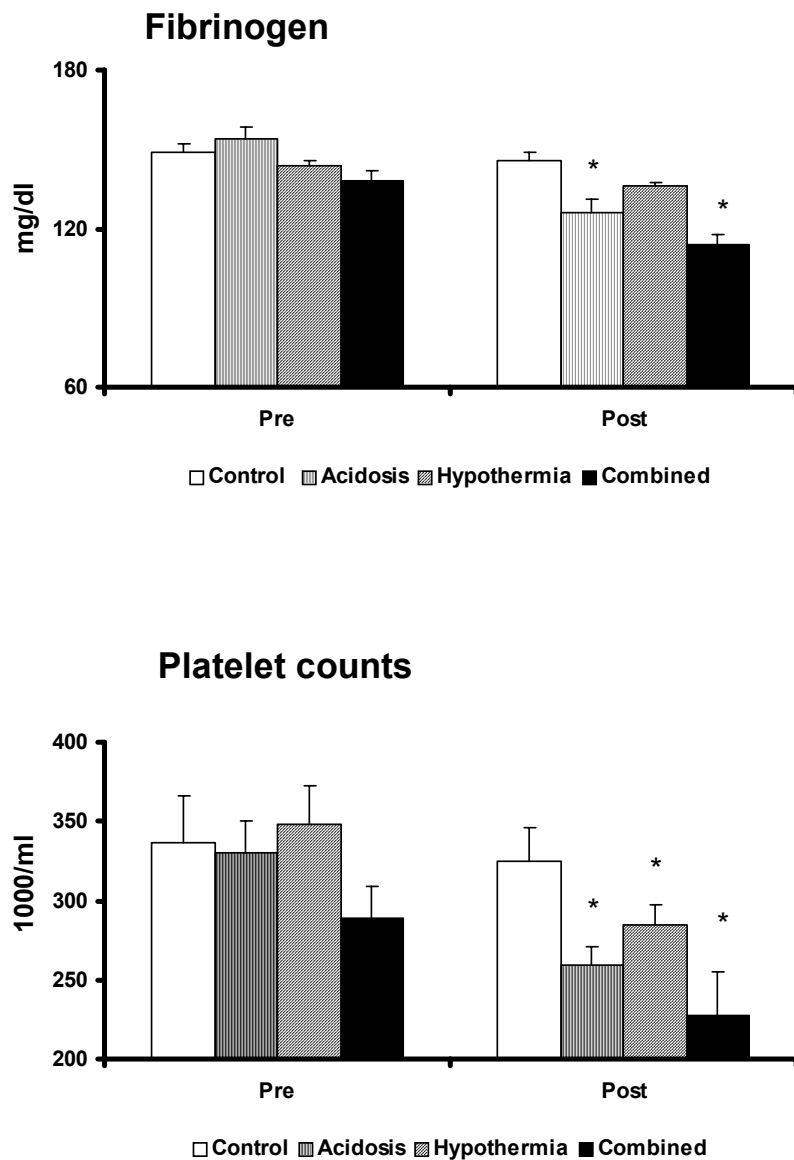


Figure 1: Changes of plasma fibrinogen concentration (mg/dl) and platelet count ($10^3/\mu$) in experimental groups. * $P < 0.05$, pre vs post within each group

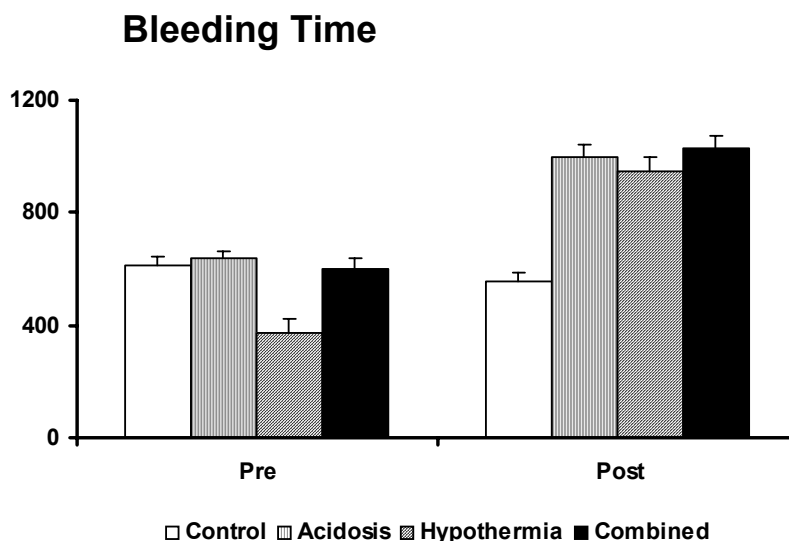


Figure 2: Changes of splenic bleeding time in experimental groups. * $p < 0.05$, pre vs post within each group.

The independent and combined effects of acidosis and hypothermia on thrombin generation are shown in Fig. 3. Each curve represents the average value ($n=6$ /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 was generated, the thrombin generation rate was primarily inhibited by acidosis. At 4 min, thrombin generation in the acidotic, hypothermic, and the 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 min, 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 apparent additive inhibitory effect in thrombin generation rate in the combined group was not statistically significant.

In TEG measurements, R time (representing the latency time before initial clot is formed) was prolonged from 3.8 ± 0.7 min to 5.4 ± 1.0 min in the hypothermia ($p < 0.05$) and from 3.0 ± 0.5 to 4.3 ± 0.6 in combined groups ($p < 0.05$), with no change in R in the acidotic group. Angle (α , reflecting the rate of fibrin build up 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 combined group ($p < 0.05$), with no change in the hypothermic group.

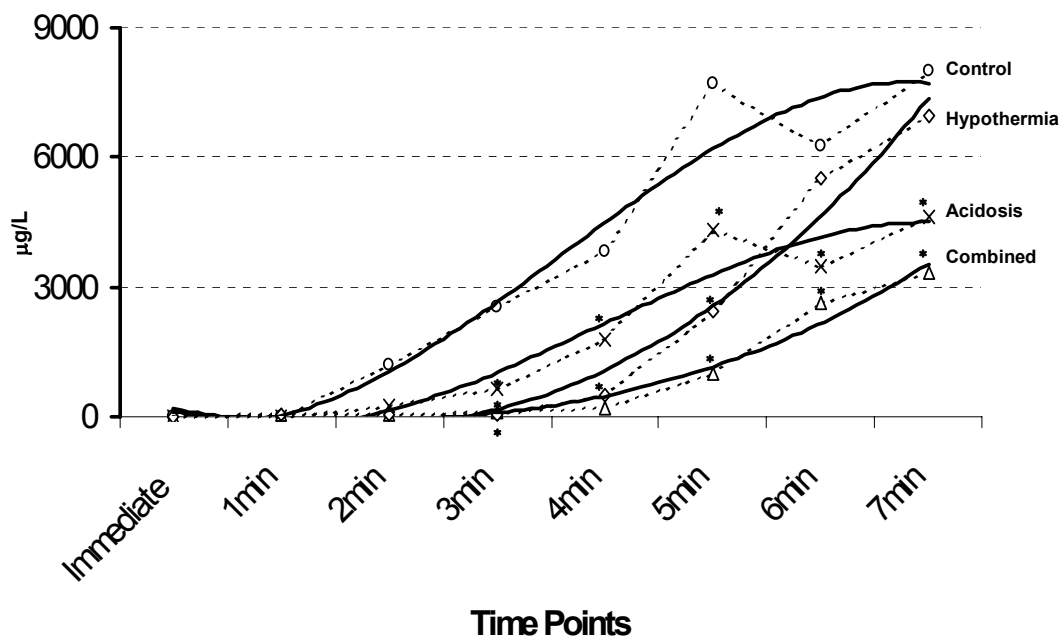


Figure 3: Thrombin generation kinetics in blood samples obtained with special acquisition procedure from femoral artery measured as thrombin-antithrombin III complex concentration ([TAT]). [TAT] was measured in sample aliquots at time 0 (sample withdrawal), and at 1 min intervals thereafter to determine thrombin generation with time in each sample using the method of Rand et al. [26].

* $p < 0.05$ different than normal value.

4.0 DISCUSSION

Thrombin generation regulates various biochemical and physiological 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. Further, we identified that hypothermia and acidosis inhibited thrombin generation with different kinetics. These findings were well correlated with an increase in splenic bleeding times and results of TEGs.

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. Consistently, 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. This point is worth emphasizing because acidotic effects on coagulopathy have been under-appreciated. In addition, we found that the acidotic inhibition on the

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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 [18]. Thus, correcting blood pH should be considered a potentially important strategy in reversing clinical coagulopathies.

In this study, the development of coagulopathy was defined as a statistically significant increase of splenic bleeding time. This measurement provided an overall estimate of coagulation, as it included all of the factors involved in coagulation, such as blood flow and 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. However, these detrimental effects were not detected in standard PT measurement (assayed at 37°C), since no changes in PT were found in any experimental group. Our findings confirm the widening appreciation that the standard PT is not a sensitive index of coagulation function in clinical practice. Since 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 [12].

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) [28]. Consistently, we observed decreases of about 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 post-surgery. The underlying mechanism of the depletion is not clear. Additional investigation is required to clarify the underlying mechanism.

Our measurement of thrombin generation kinetics is consistent with the coagulation profiles obtained from TEG. Reaction time (R) 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 built up 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, as well as decreased fibrinogen concentration, observed in the acidotic and combined groups.

Recently, recombinant activated factor VII (rFVIIa) has been used as therapy in patients bleeding uncontrollably, with beneficial effects in some of these patients [29-31]. However, rFVIIa has not been effective in some acidotic trauma patients (U. Martinowitz, personal communication). These different clinical outcomes may be explained in relation to the findings from the present study. Since 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, since hypothermia primarily inhibits the activation of FVIIa/TF complex.

5.0 SUMMARY

In summary, we investigated the independent and combined contributions of hypothermia and acidosis in the development of a clinical coagulopathy. Acidosis and /or hypothermia impair blood coagulation process. As a possible underlying mechanism, acidosis and hypothermia inhibit thrombin generation for clot formation by different kinetics. Further understanding of the mechanisms underlying the development of coagulopathy induced by different means may facilitate hemorrhage control in trauma patients.

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