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Thrombin generation and fibrin clot formation under hypothermic conditions: an in vitro evaluation of tissue factor initiated whole blood coagulation

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Abstract

Background—Despite trauma-induced hypothermic coagulopathy being familiar in the clinical setting, empirical experimentation concerning this phenomenon is lacking. In this study we investigated the effects of hypothermia on thrombin generation, clot formation and global hemostatic functions in an *in vitro* environment using a whole blood model and thromboelastography (TEG) which can recapitulate hypothermia.

Methods—Blood was collected from healthy individuals through venipuncture and treated with corn trypsin inhibitor, to block the contact pathway. Coagulation was initiated with 5pM tissue factor at temperatures 37°C, 32°C, and 27°C. Reactions were quenched over time with soluble and insoluble components of each time point analyzed for thrombin generation, fibrinogen consumption, factor (f)XIII activation and fibrin deposition. Global coagulation potential was evaluated through TEG.

Results—Data showed that thrombin generation in samples at 37°C and 32°C had comparable rates while 27°C had a much lower rate (39.2 ± 1.1 and 43 ± 2.4 nM/min vs 28.6 ± 4.4 nM/min, respectively). Fibrinogen consumption and fXIII activation were highest at 37°C followed by 32°C and 27°C (13.8 ± 2.9 percent/min vs 7.8 ± 1.8 percent/min, respectively). Fibrin formation as seen through clot weights also followed this trend. TEG data showed clot formation was fastest in samples at 37°C and lowest at 27°C. Maximum clot strength was similar for each temperature. Also, percent lysis of clots was highest at 37°C followed by 32°C and then 27°C.

Conclusions—Induced hypothermic conditions directly affect the rate of thrombin generation and clot formation while global clot stability remains intact.

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Keywords

hypothermia; trauma; thrombin generation; fibrin formation; lysis

Introduction

Trauma continues to be a leading cause of death for all ages in the United States and is the major cause of death for ages 15-24 years old.(1) Uncontrolled hemorrhage is the principal cause of death and results from gross vascular injury or disturbances in the coagulation system that leads to a failure to maintain adequate hemostasis. Isolated hypothermia accounts for approximately two deaths per day or 700 deaths per year,(2) and is associated with a 23% mortality rate when the body temperature drops below 32°C.(3) The physiologic responses to hypothermia in the trauma patient are numerous and exacerbate the often already compromised cardiovascular, central nervous, and coagulation systems. These combined effects of severe tissue trauma and hypothermia are deadly, with a reported 100% mortality in trauma patients who present with core body temperatures of less than 32°C.(4)

Isolated hypothermia is generally categorized into mild (32-36°C), moderate (28-32°C), severe (16-27°C), profound (6-15°C) and ultra-profound (<5°C). (5) In the trauma setting, with the combined effect of hypothermia and tissue injury, 36°C to 34°C degrees is considered mild hypothermia, 34°C to 32°C degrees is moderate hypothermia, and severe hypothermia occurs below 32°C degrees.(4, 5) Worsening hypothermia portends greater mortality (4) and increased risk for multiple organ dysfunction syndrome (6).. However, patients presenting with even mild hypothermia have been reported to suffer 40% mortality. Rewarming of hypothermic patients has significant clinical benefit with decreased resuscitative fluid requirements and mortality after major trauma. (7) Hypothermia may have a two-fold effect on the coagulation system. Mild to moderate hypothermia appears to result in primarily platelet dysfunction, while severe hypothermia seemingly leads to both platelet dysfunction and enzyme (clotting factor) activity reduction. (8)

The coagulation system is a complex yet delicate balance of prothrombotic, anticoagulant, and fibrinolytic processes. In trauma patients, tissue damage results in the exposure of massive amounts of tissue factor (Tf) released from the subendothelium into circulation. Membrane-bound Tf binds the circulating enzyme factor (f)VIIa, forming the extrinsic fXase complex which subsequently activates the zymogens fIX and fX to their respective serine proteases, fIXa and fXa.(9-11). Platelet bound fXa subsequently activates a small amount (pM) of the zymogen prothrombin to α -thrombin (12, 13) which in turn activates more platelets (14) and the procofactors fV,(15) and fVIII,(16). Once activated, these cofactors combine with their respective enzymes fIXa and fXa on the surface of activated platelets to form the intrinsic fXase and prothrombinase complexes. The formation of these two essential complexes leads to a massive burst in thrombin generation which simultaneously activates the transglutaminase fXIII,(17) and releases fibrinopeptides A and B to form a stable crosslinked fibrin clot.(14) Interestingly, fibrin clot formation occurs with only 2 nmol/L free thrombin (10 nM TAT) (14, 18) with the vast majority (96%) of thrombin being generated after the initial clot formation has been observed. The subsequent

propagation of thrombin generation is necessary to cleave the remaining fibrinogen and fXIII to form a stable crosslinked clot. (19)

The clinical detection of relevant aberrations in clotting factor activation and consumption leading to coagulopathy in trauma patients continues to evolve. For the better part of a century, the standard “bedside” tests of coagulation function have primarily been prothrombin time (PT) and activated partial thromboplastin time (aPTT). Despite the fact that they do not measure the vast majority of thrombin generation and may under-appreciate more subtle clinical coagulopathies, these tests are still ubiquitously utilized. Thromboelastography (TEG), which has been in existence for nearly five decades, has gained wider acceptance as a more reliable “bedside” tool for the assessment of coagulopathy in trauma patients. (20-24) Thromboelastographic profiling has the advantages of providing a comprehensive assessment of blood coagulation and fibrinolysis while utilizing whole blood samples at defined temperatures and controllable environments. Thus, TEG profiling allows for a thorough evaluation of coagulopathy and is developing into a useful clinical tool (25). Specifically, TEG has been shown to provide a more accurate measure of hypothermic and hemodilution effects on traumatic coagulopathy.(20, 25)

Induced hypothermia and hypothermia secondary to hemorrhagic shock are very different physiological states that lead to distinct outcomes (26, 27). The diversity and complexity of the etiology of trauma-induced coagulopathy does not lend itself to the simplistic and reductionist approaches often employed in clinical research. In fact there are no animal models that can account for all of the variables involved in generating trauma-induced coagulopathy in humans.(28) In this study, we explore the potential effects of a trauma-induced hypothermic situation on the coagulation system with particular focus on thrombin generation, fibrinogen consumption and factor fXIII activation using a well-established (18, 29) *in vitro* whole blood assay and thromboelastography. This study provides a natural history of alterations that occur to blood coagulation when hypothermia is induced from a normal state.

Materials and Methods

Materials

HEPES, Tris-base, ethylenediaminetetraacetic acid (EDTA), trifluoroacetic acid and Benzamidine-HCl were purchased from Fisher (Waltham, MA). 1-palmitoyl-2-oleoylphosphatidyl serine (PS), and 1-palmitoyl-2-oleoyl-phosphatidylcholine (PC) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Recombinant Tf was a gift from Drs. Lundblad and Liu (Hyland division, Baxter Healthcare Corp, Duarte, CA) and was relipidated in PCPS (25% PS, 75% PC) vesicles as previously described.(30, 31) Corn trypsin inhibitor (CTI) was prepared as previously described.(32) D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (FPRck) was supplied by Dr. Jenny (Haematologic Technologies, Essex Junction, VT).

Patients

Healthy patients (n=8) with a mean (SD) age of 35.7 ± 10.2 years (range 25.8-58.5 years) were recruited, and advised according to a protocol approved by the University of Vermont Institutional Review board and Human Studies Committee and consent was obtained. Several individuals were studied multiple times. All donors had no history of thrombosis/hemorrhage, regular aspirin use, drug use, or trauma within the past 30 days. No specific limits were provided regarding diet or behavior. Eight individuals were evaluated in the thromboelastography studies and three individuals were evaluated in the whole blood assay with varying temperatures.

Whole blood assay

Tf-initiated whole blood assays were performed as previously described(14, 18) in which 3 temperatures were investigated: normothermia 37°C, moderate hypothermia 32°C, and severe hypothermia 27°C. Experiments were performed in polystyrene tubes placed on a rocking table enclosed in a temperature-controlled glove box at 37°C, 32°C, or 27°C. Contact pathway inhibitor (CTI- 100 µg/ml), which blocks fXIIa, and relipidated Tf at 1:2000 protein/lipid (functionally 5pM) were preloaded into tubes. Blood was collected by venipuncture at either the Fletcher Allen Health Care Clinical Research Center (Burlington, VT) or at the Colchester Research Facility (Colchester, VT) with a 19-3/4 gauge Vacutainer drawn into a 60-ml repeater syringe and the tube was immersed while swirling in a water bath at 37°C, 32°C, or 27°C for a specific time calculated to reach each individual temperature. The blood was then removed from the water bath and 1ml aliquots were placed into tubes at the appropriate temperatures containing the CTI and Tf. A control tube containing CTI and no Tf was used each time. Whole blood was allowed to rock at each temperature during a set time course over 20 minutes. Clot time was determined visually (by two observers: K.B.Z and M.W.). The reaction of dynamic thrombin generation was stopped by the addition of inhibitors to a final concentration 25 mmol/L EDTA and 10 mmol/L benzamidine-HCL in HBS (HEPES [buffered saline], 0.15 mol/L NaCl and 0.02 mol/L HEPES), pH 7.4, and 50 µmol/L FPRck in 10 mmol/L HCl at every minute between 0-10 followed by 12, 14, 16 and 20 minutes. The 0 time point contained the inhibitors before the addition of blood. After quenching the coagulation process, samples were centrifuged for 15 minutes at 1100g, and solid and solution phases were stored at -80°C for further analysis.

Thrombin generation

Thrombin generation was measured in complex with antithrombin (TAT, Behring, Westwood, MA) as per the manufacturer's instructions. Assays were duplicated or triplicated using a minimum of five standards as previously described.(32) Results were analyzed on a Vmax microtiter plate reader equipped with Softmax version 2.35 (Molecular Devices, Menlo Park, CA).

Fibrin clot analysis

The insoluble clotted material contained within the whole blood tubes at each time point was analyzed as previously described.(19) Briefly, the insoluble clotted samples were washed 2-3 times with 1 mL of 0.15 mol/L NaCl and then allowed to sit in the salt solution (1 mL)

for 12-15 hours so that additional soluble material within the clots could diffuse into solution. The clots were rinsed with H₂O to remove salt, lyophilized and weighed.

Fibrinogen consumption and fXIII activation

SDS-PAGE (4-12%) was performed on quenched whole blood serum samples according to the modified Laemmli procedure.(33) High molecular weight standard mixtures (12-200 kDa) were loaded along with fibrinogen standards (300ng/lane), fXIII (50ng/lane), or activated fXIII (fXIIIa) to allow for comparison on the immunoblots. The gels were transferred to nitrocellulose membranes (Bio-Rad) and subjected to semi-dry transfer for 3 h at 250 mA as described by Towbin *et al.* (34) The primary antibodies used were a mouse anti-Fbgn-2E (*in house*) or a polyclonal rabbit anti-fXIII (D4679, ZymoGenetics, Seattle, WA) for fibrinogen and FXIII detection respectively. The blots were developed as previously described.(18) Comparisons of fXIII conversion to fXIIIa, and fibrinogen levels in solution were analyzed on immunoblots via densitometry. A relative fraction was calculated from fibrinogen or fXIIIa present at each time point divided by total fibrinogen or total fXIII A-chain detectable.

Thromboelastography (TEG)

Thromboelastography studies were performed using phlebotomy blood with 5pM Tf and CTI (100 µg/ml) at 37°C, 32°C, and 27°C. Four TEG units (eight reaction cups; Haemoscope Corporation) were used simultaneously. A single cup containing Tf without CTI was used as control. TEG profiles are analyzed in terms of R, K, alpha, MA, TMA and LY60. “R” is the time from the start of measurement until initial fibrin formation, which again results from fibrinogen cleavage by thrombin. Prolongation of the R-time indicates clotting factor deficiency or enzymatic inhibition. “Alpha” measures the rapidity of fibrin build-up and cross-linking by fXIII, which is the speed of clot strengthening. “K” measures the time necessary to reach 20 mm of clot strength and “MA” or maximal amplitude measures the maximum strength of clot. Together, the K-time and MA measure clot formation and strength, and aberrations more likely represent platelet dysfunction and to a lesser extent fibrin build-up. “TMA” is the time required to reach maximum clot strength. Fibrinolysis is measured by the fibrinolytic index or “LY”. LY60 represents the percent change in clot strength at sixty minutes, respectively.(35, 36)

Statistical analysis

This study is descriptive in nature. The data were evaluated by unpaired t-tests with STATA version 10.0 software (College Station, TX). Significance is indicated as levels of $p < 0.05$. Data are presented as the mean and standard error of the mean (SEM).

Results

Thrombin generation

Thrombin generation profiles from whole blood time courses measured with TAT ELISA are displayed in Figure 1. Samples assessed at 37°C produced thrombin at a maximum rate of 39.2 ± 1.1 nM/min, while samples analyzed at 32°C produced thrombin at a maximum rate of 43 ± 2.4 nM/min. Maximum thrombin levels were 727 ± 79 nM for 37°C and $801 \pm$

67.3 nM for 32°C. Whole blood samples at 27°C reflected both lower rates and levels of thrombin generation, respectively: 28.6 ± 4.4 nM/min and 541 ± 77 nM.

Visual inspection of clot times is an imperfect method for measurement of the clot time and onset of thrombin generation. Therefore, thrombin generation was assessed based on the time to 10 nM TAT which typically coincides with the clot time (Figure 1 inset).(14, 37) At lower temperatures, the time to 10 nM TAT was markedly delayed (37°C: 4.0 ± 0.2 min; 32°C: 4.6 ± 0.5 min; 27°C: 6.3 ± 0.7 min). Samples analyzed at 27°C demonstrated significantly ($p=0.02$) delayed early thrombin generation when compared to normothermia (Figure 2).

Fibrinogen consumption and fibrin deposition

Consumption of fibrinogen in healthy individuals is a parallel marker of the onset of thrombin generation (14, 19). Fibrinogen analysis through western blots and densitometry analysis is displayed in Figure 3 and shows a significantly ($p=0.01$) higher maximum rate of consumption at 37°C (-18.7 ± 2.3 percent/min) when compared to samples at 27°C (-10.7 ± 0.87 percent/min). Whole blood analyzed at 27°C also demonstrated a 57% reduction in initial (5 min) fibrinogen consumption when compared to blood at 37°C (27°C 7.8 ± 1.8 percent/min vs. 37 °C 13.8 ± 2.9 percent/min). While, fibrinogen consumption appeared to be complete between 6 and 20 minutes at 37°C, both the hypothermic time courses (32°C and 27°C) failed to completely deplete their respective stocks of fibrinogen by 20 minutes.

Alternatively, fibrin clot formation is another important marker of adequate thrombin generation. Fibrin generation as determined through clot mass varied significantly ($p<0.003$) between normothermic and hypothermic temperatures. Normothermic samples had an average mass of 1.2 ± 0.00 . Samples at 32 °C had a mass of 0.88 ± 0.01 mg while samples at 27°C had a mass of 0.82 ± 0.01 mg.

Factor XIII activation

Factor XIII activation (Figure 4) did not vary significantly between mild hypothermic and normothermic temperatures. However, samples analyzed at 27°C (19.1 ± 0.7 percent/min) demonstrated a reduced activation when compared to 32°C (29.9 ± 0.3 percent/min) and 37°C (30.3 ± 0.4 percent/min).

Thromboelastography

Clot behavior, determined through TEG assays, reflected a delayed onset of clot formation and clot lysis in hypothermic blood samples (Table 1 and Figure 4). On average, the 32 °C samples displayed shorter R times than the 27 °C with the R time being significantly slower at 32°C (10.1 ± 2.3 min) and 27°C (12.5 ± 2.8 min) when compared to normothermia (8.1 ± 0.6 min). The Alpha or rapidity of fibrin build-up and cross-linking by fXIIIa was also significantly decreased at 32°C ($32.5 \pm 8.9^\circ$) and 27°C ($26.6 \pm 6.7^\circ$) when compared to normothermia ($38.2 \pm 4.4^\circ$). Maximum clot strength or MA was not significantly different at any temperature (37°C: 55.4 ± 4.5 mm; 32°C: 55.5 ± 5.6 mm; 27°C: 53.6 ± 5.1 mm). However, the time to maximum clot formation (TMA) was significantly longer at hypothermic temperatures 32°C (44.0 ± 5.6 min) and 27°C (51.2 ± 9.3 min) when compared

to normothermia (34.5 ± 3.4 min). In addition, fibrinolysis was significantly delayed at hypothermic temperatures 32°C ($0.8 \pm 1.0\%$) and 27°C ($0.2 \pm 0.3\%$) when compared to normothermia ($3.8 \pm 2.8\%$).

Discussion

Significant derangements in the coagulation system are known to occur at hypothermic temperatures and effect both enzyme and platelet function. We used our *in vitro* whole blood model to evaluate how blood coagulation changes from normothermia to hypothermia after a Tf stimulus. Our results show that severe hypothermia (27°C) leads to significant delays in early thrombin generation rate ($p=0.02$) and maximum rate of fibrinogen consumption ($p=0.01$). Severe hypothermia also caused a marked 57% reduction in initial (5 min) fibrinogen consumption. Fibrin generation as measured by fibrin clot mass also varied significantly ($p<0.003$) between normothermic and hypothermic samples. Both severe (27°C) and mild (32°C) hypothermia significantly affected global hemostasis as measured by TEG. In general, hypothermic conditions caused delays in initial thrombin generation, as well as decreases in the rate of clot formation and lysis. Interestingly, hypothermia did not affect the ultimate strength of the formed clot but did prolong the time required to reach the maximum strength.

Thrombin generation occurs in two fundamental steps; initiation and propagation. Using a standard ELISA assay (TAT) on cooled healthy human blood samples activated by a standard 5 pM Tf stimulus in the presence of CTI, we have demonstrated that moderate and severe hypothermia result in decreased early thrombin generation as a consequence of delayed thrombin initiation. Interestingly, we observed no significant effect on thrombin propagation. These findings have also been previously described in a swine hypothermia model, where hypothermia primarily effected thrombin generation by impeding thrombin initiation.(38) Notably, total thrombin generation in our experimental system was not affected by hypothermia. TEG analysis corroborated these findings, as hypothermic samples revealed significantly prolonged R-time, decreased α -angles and no significant difference in MA when compared to normothermic samples. These data further support previous findings, where isolated hypothermic platelets have also been shown to exhibit a temperature dependent decrease in thrombin generation; with hypothermic platelet samples exhibiting 25% (33°C) and 68% (23°C) less thrombin generation when compared to normothermic platelet samples.(8) Therefore, in the setting of hypothermia with traumatic tissue injury (i.e. Tf exposure to circulating blood and activation of the extrinsic pathway), appropriate microvascular hemostasis may be initially delayed from the postponement of the thrombin initiation phase. In the context of beneficial/induced hypothermia as in the case of surgery, we would expect a similar coagulation response to that observed in this study. The lack of traumatic tissue damage combined with the controlled hypothermic environment should yield a proportionate reduction in thrombin generation rate while maintaining adequate clot formation. The effects of anticoagulants under hypothermic conditions, however, remain to be investigated.

In addition to the detrimental effect on thrombin initiation, fibrinogen consumption was also reduced by hypothermia. Fibrinogen is cleaved by thrombin to form fibrin, which is

ultimately cross-linked by fXIIIa, which is also activated by thrombin. Therefore, not surprisingly, we found using immunoblotting techniques that early (5 min) fibrinogen consumption and overall fXIII activation were similarly depressed with severe hypothermia when compared to normothermic samples. In particular, our data shows that under mildly hypothermic conditions (32°C), fibrinogen consumption is comparable to that observed under normothermic conditions. However, under severe hypothermic conditions (27°C) fibrinogen consumption was drastically delayed and TAT assay data corroborates these findings. Under mild hypothermic conditions (32°C), thrombin generation is slightly delayed during the initial phase. This delay becomes visible after four minutes (see Figure 1A insert). Note however, that the time to reach 2nM thrombin is approximately 3 minutes in both 37°C and 32°C samples. As mentioned earlier, initial fibrin clot formation occurs and can be detected with this low nanomolar amount of active thrombin. (9, 13) This explains the results for fibrinogen consumption in 37°C and 32°C samples; since both samples were able to generate 2nM thrombin at comparable times, their fibrinogen consumption profiles can be expected to be quite similar during the early stages of coagulation. The 27°C samples did not generate 2nM thrombin until 4 minutes, and thus also show more pronounced delays over the entire 20-minute time course (Figure 1A). This substantiates the significant reduction in fibrinogen consumption for the 27°C samples observed in Figure 2A.

Notably, total fibrinogen consumption and fXIII activation were not appreciably different at the conclusion of our experiments (20 minutes). This suggests that like thrombin generation, hypothermia affects the rate of fibrin clot formation while clot stability and strength are unaltered. This again is supported by our TEG analysis which revealed prolonged R-time and TMA with hypothermia but similar MA amongst all samples. Decreases (up to 60%) in fXIII activation have been previously implicated in post-operative neurosurgical (39) and intra-operative general surgical bleeding. (40) Our data demonstrates a decrease in fXIII activation in the setting of induced hypothermia that may also contribute to these clinical findings.

Clinically, hyperfibrinolysis has been defined as having a maximal clot lysis at 60 minutes of 10% or higher that is reversible by aprotinin. (41) When we assessed the coagulopathy of clot lysis/fibrinolysis in our system, our data demonstrates that hypothermia results in significantly delayed clot lysis. This can be seen in Table 1, as denoted by the percentage of clot lysis after 60 minutes (LY60%). LY60% in the context of this study, is a measurement of the blood's intrinsic ability to activate fibrinolysis. In the absence of a standardized physiologic plasminogen activator (such as tPA) or the addition of aprotinin, conclusions based on these results are speculative at best. However, this finding of delayed clot lysis in combination with delays in the initiation of thrombin generation, fibrin consumption, and fXIII activation is particularly interesting as it describes a phenomenon where thrombin generation is delayed in the early stages (2-6 minutes) but is pathologically insignificant in later stages of clot formation (>60 minutes).

Clinically, these findings may correlate with the uncontrolled bleeding diathesis in the early phases of trauma care, followed by venous thrombosis upon return to therapeutically achieved normothermia. Furthermore, in line with the obvious biophysics of enzymatic

reaction mechanisms, these data support the hypothesis that induced hypothermia primarily affects the rate of thrombin generation and clot formation. Although these data are not directly translatable to the case of prophylaxis in the trauma patient, in the context of induced hypothermia, it suggests that reconstitution to a normothermic state will maintain adequate thrombin generation and clot formation.

We acknowledge certain limitations of this study. Namely, we have utilized a small number of “healthy” control samples in a controlled hypothermia trauma-induced environment and this may not reflect the actual changes in blood samples from hypothermic trauma patients. More specifically, in our model we have not accounted for changes in coagulation factors that are likely occurring in the trauma patient (both consumption and increased production). Future studies need to be performed that describe changes in the hemostatic profile of hypothermic trauma patients applying a similar approach. Our whole blood assay is a compromise between biological authenticity and experimental opacity. However, in the current format, it is impractical to perform in a hospital setting. Therefore, in an attempt to align our research with clinically relevant methodologies, we employed the use of TEG analysis. The use of both of these models provides us with multiple experimental approaches to analyzing coagulation dynamics. While both of these techniques are pertinent to our hypothermia study, there are fundamental differences in their respective methodologies which do not allow them to be directly compared. For example, the ubiquitous use of 5 pM Tf in the TEG experiments was chosen to give an approximate clot time where changes and alterations could be easily observed and noted (see Table 1). We have 15 years experience using 5pM Tf in our *in vitro* assay and have applied the same concentration to our TEG line of experimentation expecting equal results. Interestingly equal Tf activation in separate models resulted in unique coagulation parameter outcomes per model. Significant differences were found in TEG parameters when comparing whole blood coagulation at various temperatures. Lower concentrations of Tf were not pursued in our *in vitro* model because of the loss of comparative analysis power that would be realized with a divergence from our extremely well described history of whole blood investigation within this model. We speculate the fundamental differences in assay mechanics plays the largest role in the discrepancies found between models. Due to these differences the clot times and other parameter comparisons between these two experimental methods solely involve general trends. In summary, hypothermia causes a multifactorial delay in clot formation and breakdown, which may lead to both initial problems with bleeding in the trauma patient followed by clot stasis and development of thrombosis (DVT or PE). Our results suggest that induced hypothermia produces only temporal changes to thrombin generation and clot formation.

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Figure 1. Thrombin generation in whole blood

Whole blood was drawn into 0.1 mg/mL CTI and activated by 5 pM Tf at $t=0$. Samples were quenched at varying intervals with EDTA and FPR-chloromethyl ketone prior to being analyzed by TAT ELISA. **Main figure.** α TAT generation profiles for healthy individuals at 37°C (●), 32°C (■) and 27°C (▲) (N=3). **Inset.** α TAT generation profiles at 37°C (●), 32°C (■) and 27°C (▲) (N=3) for the first 6 minutes of the time course. Data are shown as the mean $\pm$ SEM.

Figure 2. Early thrombin generation in whole blood

Time (min) to 10nM TAT formation is shown for 37°C, 32°C, and 27 °C (N=3). Data are shown as the mean±SEM.

Figure 3. Fibrinogen consumption in whole blood

Whole blood sera were diluted with 1x SDS-PAGE sample buffer, and run out on 4–20% SDS-PAGE under non-reducing conditions followed by Western blotting probing with an anti-Fibrinogen-2a mouse monoclonal antibody. Densitometry was performed on the blots for the 37°C (●), 32°C (■) and 27°C (▲) time courses (N=3). Data are shown as the mean $\pm$ SEM of the normalized percent activation.

Figure 4. Factor XIII activation in whole blood

Whole blood sera were diluted with 1x SDS-PAGE sample buffer, and run out on 4–20% SDS-PAGE under non-reducing conditions followed by Western blotting probing with a FXIII polyclonal antibody. Densitometry was performed on the blots for the 37°C (●), 32°C (■) and 27°C (▲) time courses (N=3). Data are shown as the mean±SEM of the normalized percent activation.

Figure 5. Thromboelastography (TEG) in whole blood

TEG data are shown for CTI-inhibited whole blood samples collected at 37°C, 32°C, and 27°C. Coagulation was initiated with 5pM Tf. **A.** R-time (min): time to initial clot formation. **B.** K-time (min): duration from initial clot formation to maximum clot. **C.** Alpha (degrees): rapidity of fibrin build up and cross-linking. **D.** Maximum Amplitude (MA) (mm): maximum clot strength – fibrin and platelets. **E.** TMA (min): time to max clot strength. **F.** LY 60: percent of clot lysis at 60 minutes. (N=8) Data are shown as mean $\pm$ SEM.

Table 1

Summary of thromboelastographic parameters in whole blood.

TEG Parameter	27C	32C	37C	P<0.05
R (min)	12.5 (2.8)	10.1 (2.3)	8.1 (0.6)	* (32,27 vs 37)
K (min)	8.4 (3.4)	6.4 (2.5)	5.0 (1.0)	* (32,27 vs 37)
α (deg)	26.6 (6.7)	32.5 (8.9)	38.2 (4.4)	* (32,27 vs 37)
MA (mm)	53.6 (5.1)	55.5 (5.6)	55.4 (4.5)	
TMA (min)	51.2 (9.3)	44.0 (5.6)	34.5 (3.4)	* (32,27 vs 37)
LY60 (%)	0.2 (0.3)	0.8 (1.0)	3.8 (2.8)	* (32,27 vs 37)

R-time: duration to initial clot formation; K-time: duration from initial clot formation to max clot; Alpha: rapidity of fibrin build up and cross linking; MA: maximum clot strength – fibrin and platelets; TMA: time to maximum clot strength; LY60: percent lysis at 60 minutes. Data are presented as mean (SD).