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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, 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 analyzed for thrombin generation, fibrinogen consumption, factor (f)XIII activation, and fibrin deposition. Global coagulation potential was evaluated through thromboelastography.
Results: Data showed that thrombin generation in samples at 37°C and 32°C had comparable rates, whereas 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. Fibrin formation as seen through clot weights also followed this trend. Thromboelastography data showed that 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, whereas global clot stability remains intact.

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1. Introduction

Trauma continues to be a leading of cause of death for all ages in the United States and is the major cause of death for those aged 15 to 24 years [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 2 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 patient with trauma 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 patients with trauma who present with core body temperatures less than 32°C [4].

Isolated hypothermia is generally categorized into mild (32°C-36°C), moderate (28°C-32°C), severe (16°C-27°C), profound (6°C-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 is considered mild hypothermia, 34°C to 32°C is moderate hypothermia, and severe hypothermia occurs below 32°C [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 have 40% mortality. Rewarming of hypothermic patients has a significant clinical benefit with decreased resuscitative fluid requirements and mortality after major trauma [7]. Hypothermia may have a 2-fold effect on the coagulation system. Mild to moderate hypothermia appears to result in primarily platelet dysfunction, whereas severe hypothermia seemingly leads to both platelet dysfunction and enzyme (clotting factor) activity reduction [8].

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The coagulation system is a complex yet delicate balance of prothrombotic, anticoagulant, and fibrinolytic processes. In patients with trauma, 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 (in 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 2 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 cross-linked fibrin clot [14]. Interestingly, fibrin clot formation occurs with only 2 nmol/L free thrombin (10 nM TAT) [14,18], with most (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 cross-linked clot [19].

The clinical detection of relevant aberrations in clotting factor activation and consumption leading to coagulopathy in patients with trauma continues to evolve. For the better part of a century, the standard "bedside" tests of coagulation function have primarily been prothombin time and activated partial thromboplastin time. Although they do not measure most of thrombin generation and may underappreciate more subtle clinical coagulopathies, these tests are still ubiquitously used. Thromboelastography (TEG), which has been in existence for nearly 5 decades, has gained wider acceptance as a more reliable bedside tool for the assessment of coagulopathy in patients with trauma [20–24]. Thromboelastrographic profiling has the advantages of providing a comprehensive assessment of blood coagulation and fibrinolysis while using 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 physiologic states that lead to distinct outcomes [26,27]. The diversity and complexity of the etiology of trauma-induced coagulopathy do not lend itself to the simplistic and reductionist approaches often used 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 fXIII activation using a well-established [18,29] in vitro whole blood assay and TEG. This study provides a natural history of alterations that occur to blood coagulation when hypothermia is induced from a normal state.

2. Materials and methods

2.1. Materials

HEPES, Tris-base, ethylenediaminetetraacetic acid (EDTA), trifluoroacetic acid, and benzamidine-HCl were purchased from Fisher (Waltham, Mass). 1-Palmitoyl-2-oleoyl-phosphatidyl serine (PS) and 1-palmitoyl-2-oleoyl-phosphatidylcholine (PC) were purchased from Avanti Polar Lipids, Inc (Alabaster, Ala). Recombinant Tf was a gift from Drs Lundblad and Liu (Hyland Division, Baxter Healthcare Corp, Duarte, Calif) and was relipidated in PCPS (25% PS, 75% PC) vesicles as previously described [30,31]. Corn trypsin inhibitor 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).

2.2. Subjects

Healthy subjects (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 TEG studies, and 3 individuals were evaluated in the whole blood assay with varying temperatures.

2.3. Whole blood assay

Tissue factor–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 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 1 mL 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 for 20 minutes. Clot time was determined visually (by 2 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 and 10 minutes, 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 1100*g*, and insoluble and soluble phases were stored at −80°C for further analysis.

2.4. Thrombin generation

Thrombin generation was measured in complex with antithrombin (TAT; Behring, Westwood, Mass), as per the manufacturer's instructions. Assays were duplicated or triplicated using a minimum of 5 standards, as previously described [32]. Results were analyzed on a Vmax microtiter plate reader equipped with Softmax version 2.35 (Molecular Devices, Menlo Park, Calif).

2.5. 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 to 3 times with 1 mL of 0.15 mol/L NaCl and then allowed to sit in the salt solution (1 mL) for 12 to 15 hours so that the additional soluble material

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