The Effects of Temperature on Clot Microstructure and Strength in Healthy Volunteers

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BACKGROUND: Anesthesia, critical illness, and trauma are known to alter thermoregulation, which can potentially affect coagulation and clinical outcome. This in vitro preclinical study explores the relationship between temperature change and hemostasis using a recently validated viscoelastic technique. We hypothesize that temperature change will cause significant alterations in the microstructural properties of clot.

METHODS: We used a novel viscoelastic technique to identify the gel point of the blood. The gel point identifies the transition of the blood from a viscoelastic liquid to a viscoelastic solid state. Furthermore, identification of the gel point provides 3 related biomarkers: the elastic modulus at the gel point, which is a measure of clot elasticity; the time to the gel point \( T_{GP} \), which is a measure of the time required to form the clot; and the fractal dimension of the clot at the gel point, \( d_s \), which quantifies the microstructure of the clot. The gel point measurements were performed in vitro on whole blood samples from 136 healthy volunteers over a temperature range of 27°C to 43°C.

RESULTS: There was a significant negative correlation between increases in temperature, from 27°C to 43°C, and \( T_{GP} \) \((r = -0.641, P < 0.0005)\). Conversely, significant positive correlations were observed for both the elastic modulus at the gel point \((r = 0.513, P = 0.0008)\) and \( d_s \) \((r = 0.777, P < 0.0005)\) across the range of 27°C to 43°C. When temperature was reduced below 37°C, significant reductions in \( d_s \) and \( T_{GP} \) occurred at ≤32°C (Bonferroni-corrected \( P \leq 0.0317 \)) respectively. No significant changes were observed when temperature was increased to >37°C.

CONCLUSIONS: This study demonstrates that the gel point technique can identify alterations in clot microstructure because of changes in temperature. This was demonstrated in slower-forming clots with less structural complexity as temperature is decreased. We also found that significant changes in clot microstructure occurred when the temperature was ≤32°C. (Anesth Analg 2016;122:21–6)
at attainment of a gel point. The gel point identifies the transition of the blood from a viscoelastic liquid to a viscoelastic solid state, which signifies the first point at which the clot forms an unbroken structure. Taking rheological measurements at the gel point provides 3 important and related measurements: the elastic modulus at the gel point, \( G_{CP} \), which is a measure of clot elasticity; the time to the gel point, \( T_{GP} \), which is a measure of how long it takes to form the clot; and the fractal dimension of the clot at the gel point, \( d_f \), which quantifies the microstructure of the clot. In previous in vitro studies of hemodilution and anticoagulation, we have shown that the measurement of the gel point provides better prediction of coagulation abnormalities than conventional markers.

The aim of this preclinical in vitro study is to determine whether temperature change over a range of 27°C to 43°C will cause alterations in the microstructural properties of the clot. We hypothesize that because temperatures are reduced to the mild and severe hypothermic range, significant changes in the gel point parameters will occur, where the clot will form more slowly and produce reductions in \( d_f \). We also hypothesize that increases of temperature into the hyperthermic range will also cause a significant change in the gel point parameters, where we will see quicker-forming clots with increases in \( d_f \).

**METHODS**

The study was approved by the Wales Research Ethics Committee 6. Informed written consent was obtained from all subjects before enrolment. The exclusion criteria were as follows: (1) individuals with acute or chronic conditions known to effect coagulation (i.e., malignancy, hepatic, and/or renal dysfunction); (2) individuals undergoing antithrombotic treatment; (3) individuals with a family history of bleeding or thromboembolic disorders; and (4) anyone younger than 18 years.

**Blood Sampling**

Before blood sampling, each healthy volunteer was assigned to 1 in vitro test temperature, ranging from 27°C to 43°C increasing in 1°C increments \( (n = 8 \text{ for each temperature}) \). Blood samples were obtained from the antecubital vein through an 18-gauge needle. The first 2 mL of blood was discarded after which 20 mL of blood was collected in a plastic syringe. The blood sample was then immediately divided into 2 aliquots. The first whole-blood aliquot was transferred to the AR-G2 (TA Instruments, New Castle, DE) rheometer for testing. The second sample was used for subsequent standard coagulation screening and full blood counts to ensure that each volunteer had a normal coagulation profile.

**Gel Point Measurements**

The gel point technique has been validated previously. In this study, 6.6 mL of whole nonanticoagulated venous blood was placed into a double-gap concentric cylinder geometry testing surface mounted on a AR-G2-controlled stress rheometer. Immediately after sample loading, small amplitude oscillatory shear measurements were performed at test frequencies 2, 0.93, 0.43, and 0.2 Hz to obtain measurements of the viscoelastic properties of coagulating blood, and the formation of the gel point was recorded. Identification of the gel point was then used to find (1) the time taken to reach the gel point, \( T_{GP} \); (2) the shear elastic modulus at the gel point, \( G_{CP} \); and (3) the fractal dimension of the clot, \( d_f \). A more detailed description of the gel point technique can be found in the Supplemental Digital Content (http://links.lww.com/AA/B244).

Close temperature control was achieved through an integrated Peltier concentric cylinder system (valid between −10°C and 150°C and is accurate within 0.1°C). The temperatures were checked using a thermocouple (PTL.8013, Portec type K thermometer, Wrestlingworth, United Kingdom). The process was repeated for all temperatures studied. The time taken for whole blood to reach the test temperatures after being loaded onto the testing surface (within 0.2°C) never exceeded 10 seconds.

**Computational Simulation**

In conjunction with the gel point measurements, we also provide a previously published computational simulation. This simulation was used alongside the experimental data collected from the gel point measurements to help illustrate how any changes in incipient clot microstructure (\( d_f \)) will relate to changes in the mass of the clot. This computational simulation will use the established relationship between incipient fibrin clots and their fractal properties, where the mass, \( M \), is related to \( d_f \) by the following power law equation: \( M = \varepsilon^{d_f} \), where \( \varepsilon \) is a length scale value in the range 100 nm to 10 μm.

**Standard Coagulation Markers**

The second aliquot of blood (4.5 mL of the remaining blood) was immediately transferred to a siliconized glass Vacutainer (Becton-Dickinson, Plymouth, United Kingdom; Ref: 367691) for routine coagulation studies, including prothrombin time, activated partial thromboplastin time, and fibrinogen concentration measured using the method of Clauss. These were measured with a Sysmex CA1500 analyzer (Sysmex Co., Milton Keynes, UK) within 2 hours of collection. Fibrinogen concentration was verified against the second International Fibrinogen Standard version 4 (NIBSC code: 96–612; NIBSC, Hertfordshire, UK). All reagents were obtained from Siemens (Frimley, UK). The analyzer was calibrated according to the manufacturer’s instructions.

**Statistical Analysis**

Statistical analysis was performed using Minitab® version 16 software (Minitab Inc., Havertown, PA) and GraphPad Prism® version 6.0 (GraphPad Software Inc., La Jolla, CA). To establish at what temperature significant changes in \( d_f \) would occur compared with the value at 37°C, the number of participants per temperature was calculated by a power calculation. The sample size of this study was based on the previous studies, where the \( d_f \) at 37°C was 1.73 ± 0.035 and expecting a decrease in \( d_f \) of approximately 0.06 as temperature is decreased into the moderate or severe hypothermic range (below 33°C). Assuming an \( \alpha \) of 0.05 and a power of 0.90, we calculated that 8 subjects would be required for each studied temperature, with a total of 17
different temperature graduations (ranging from 27°C to 43°C in 1°C intervals) giving a total of 136 participants. We used the Anderson-Darling method and inspection of the distribution of the residuals to ascertain that $T_{GP}$, $G'_{GP}$, and $d_f$ were normally distributed. One-way analysis of variance was performed to allow for a Bonferroni-corrected multiple comparisons test to be performed post hoc, which was used (for all 17 different groups) to determine the exact temperatures when true differences arose from the values at 37°C. Pearson correlation was used to explore associations among temperature, $d_f$, $T_{GP}$, and $G'_{GP}$.

**RESULTS**

The standard coagulation measurements for all 136 volunteers are reported in Table 1. These results confirmed that all subjects had normal coagulation. At 37°C, the values of $d_f$ (mean ± SD, 1.73 ± 0.039) and $T_{GP}$ (mean ± SD, 231 ± 55) were similar to the values of those reported for healthy volunteers measured in 2 previous studies.\(^{11,14,16}\) Figure 1 shows that increasing or decreasing temperature > or <37°C results in incremental changes in the gel point measurements. Progressive increases in temperature from 27°C to 43°C resulted in a progressive increase in both $d_f$ and $G'_{GP}$, showing significant positive correlations with temperature ($d_f$: $r = 0.777, P < 0.0005$; $G'_{GP}$: $r = 0.513, P = 0.0008$). $T_{GP}$ decreased as temperature increased ($r = -0.641, P < 0.0005$). Moreover, $d_f$ decreased from (mean ± SD) 1.73 ± 0.039 at 37°C to 1.64 ± 0.039 at 27°C with the change becoming significant at $32°C$ ($d_f = 1.67 ± 0.037$; Bonferroni-corrected $P = 0.0093$; 95% confidence interval of the difference, 0.010–0.120). A significant decrease in $T_{GP}$ occurs at <29°C (Bonferroni-corrected $P = 0.0317$; 95% confidence interval of the difference, −285 to −7). By comparison no significant change was observed for $G'_{GP}$ as temperature decreased from 37°C (Bonferroni-corrected $P > 0.75$ for all the different temperature groups in this range). Correlations were found between $T_{GP}$ and $d_f$ ($r = -0.562, P < 0.0005$) and between $d_f$ and $G'_{GP}$ ($r = 0.719, P < 0.0005$).

The computational simulation results (Fig. 2A) showed that for $d_f$ decreases with corresponding decreases in temperature (specifically ≤32°C), large decreases in clot mass are observed. Figure 2B shows that for $d_f$ of 1.65 (corresponding to a temperature of 28°C) would produce a clot that contains approximately 40% the mass from a clot formed with a $d_f$ of 1.73 (37°C; see Fig. 2C). Figure 2A shows that the small incremental increases in $d_f$ would require exponential increases in mass. Figure 2D shows that for a $d_f$ of 1.80 (some of the highest single values observed) would require

**DISCUSSION**

In this study, we observed significant changes in the gel point measurements when temperatures fell into the hypothermic range but not when they increased into the hyperthermic range. Furthermore, we found that the structural organization of the clot ($d_f$) is related to the kinetics of clot formation ($T_{GP}$) and its elastic properties ($G'_{GP}$) documented as significant correlations found between $d_f$ and $T_{GP}$, and $d_f$ and $G'_{GP}$.

Hypothermia is common in critical illness, trauma, and during surgery, and it has been linked with excessive bleeding, blood transfusion, and poor outcomes.\(^{1,33}\) Previous in
vitro studies have shown that temperatures ranging from 33°C to 35.9°C (mild hypothermia) have a minimal effect on the enzymatic activity of coagulation factors; however, reduced platelet adhesion and aggregation are observed. Temperatures below a threshold of 36.4°C and 34°C decrease the activation of glycoprotein IIb/IIa and P-selectin, respectively, processes important for platelet adhesion. Because temperature is decreased below 33°C, into the moderate (32.9°C–28°C) and severe (<27.9°C) hypothermic ranges, a further inhibitory effect on platelet activity is observed. The inhibitory effect on platelets is coupled with a progressive decrease in the enzymatic activity of coagulation factors, where at 25°C, enzyme activity ranges from 0% (FVIII and FXI) to 5% (FII and FVII).

In this study, we report a progressive decrease in $d_f$ and $G'_{CP}$, or $G''_{CP}$, with a prolongation of $T_{CP}$ as temperature is reduced below 37°C (see Fig. 1). The progressive changes observed in $d_f$ and $T_{CP}$ with hypothermia are indicative of a worsening hypocoagulable state, producing $d_f$ and $T_{CP}$ values more commonly associated with anticoagulated or diluted blood. However, the changes in $d_f$ do not become significant until a temperature of 32°C is reached, where $d_f$ decreases from 1.73 ± 0.040 at 37°C to 1.67 ± 0.037 at 32°C ($P = 0.0093$). In contrast to standard coagulation testing, the gel point technique includes the vital contribution of platelets and cellular components of coagulation. However, over the temperature range from 33°C to 35.9°C, the observed inhibitory effect of reduced temperature on platelet adhesion does not seem sufficient to produce significant changes in the gel point parameters. When temperatures reach those synonymous with moderate hypothermia (<33°C), the additional inhibition of platelet and coagulation enzyme activity is likely to be the cause of this significant reduction in $d_f$. We observed a significant change in $T_{CP}$ only when the temperature decreases to 29°C (245 ± 44 at 37°C to 391 ± 69 at 27°C, $P = 0.0317$), and no significant change was observed in $G'_{CP}$ ($P > 0.75$).

Hyperthermia is associated with hypercoagulability, where increases in temperature result in an increase in thrombin generation. The results of this study, however, show that increases in temperature >37°C and up to 43°C (temperatures synonymous with hyperthermia) had no significant effect on $d_f$, $G'_{CP}$, or $T_{CP}$ (Fig. 1). This finding suggests that, in hyperthermic conditions, temperature alone does not significantly affect coagulation.

To better understand the changes we observed with $d_f$, we performed computational analysis that illustrate the nonlinear relationship between $d_f$ and clot mass at the incipient clot stage of development. When temperature is reduced, a progressive decrease in $d_f$ is observed, indicating a decrease in the complexity of the clot microstructure. This is illustrated in Figure 2, where a clot with $d_f = 1.67$ (33°C) has approximately 60% of the mass of a clot formed at 37°C. Substantial increases in mass occur when temperatures reach those synonymous with moderate hypothermia (<33°C), where $d_f$ increases from 1.73 ± 0.040 at 37°C to 1.67 ± 0.037 at 32°C ($P = 0.0093$). In contrast to standard coagulation testing, the gel point technique includes the vital contribution of platelets and cellular components of coagulation. However, over the temperature range from 33°C to 35.9°C, the observed inhibitory effect of reduced temperature on platelet adhesion does not seem sufficient to produce significant changes in the gel point parameters. When temperatures reach those synonymous with moderate hypothermia (<33°C), the additional inhibition of platelet and coagulation enzyme activity is likely to be the cause of this significant reduction in $d_f$. We observed a significant change in $T_{CP}$ only when the temperature decreases to 29°C (245 ± 44 at 37°C to 391 ± 69 at 27°C, $P = 0.0317$), and no significant change was observed in $G'_{CP}$ ($P > 0.75$).

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the viscoelasticity of cross-linked polymeric gel networks in which $G'$ is a function of polymer chain length between cross links. For idealized polymer systems with fixed cross links, increases in both $G'$ and $d_i$ will result from an increased cross link density.\(^{34}\)

The major limitation of the study was that gel point measurements at each of the different temperatures for each of the volunteers were not performed. The test is designed to be used in a near-patient setting, and it is performed with the use of whole nonanticoagulated blood. Hence, because of the limited numbers of AR-G2 instruments available and to avoid multiple blood samplings in each volunteer, not all 17 temperatures were studied in each subject. Thus, blood from different healthy volunteers was used at each temperature testing condition. To limit any possible variations between the testing conditions, all participants were determined to have normal coagulation. The gel point measurements are taken at the initial or incipient stage of clot development and are not measures of the mature clot. This has the advantage of providing a very rapid measure of the clotting properties. In addition, we have shown previously how the incipient clot acts as a template of mature clot growth\(^{28}\) and that $d_i$ is significantly correlated with other viscoelastic measurements of the mature clot.\(^{16}\)

Regardless, this study highlights the potential of the gel point technique to measure the effects of temperature on coagulation. The gel point measurements have now been validated in the models of anticoagulation, dilution, and temperature.\(^{14,16}\) The results of this study suggest that preventing moderate-to-severe hypothermia ($<32^\circ$C) and maintaining normothermia could be important for maintaining normal clot microstructure formation. However, the changes in temperature $>37^\circ$C (hyperthermic conditions) do not seem to have such a pronounced effect on clot microstructure. Our results highlight the need for further clinical investigations, such as studying the role of temperature in critical illnesses such as sepsis and in patients with burn injury.

**DISCLOSURES**

**Name:** Matthew James Lawrence, MEng, PhD.
**Contribution:** This author helped design the study, conduct the study, collect the data, analyze the data (rheology), and prepare the manuscript.
**Attestation:** Matthew James Lawrence approved the final manuscript and attests to having reviewed the original data reported in the manuscript.
**Conflicts of Interest:** The author declares no conflicts of interest.
**Name:** Nick Marsden, BSc, MBBSch, MRCS.
**Contribution:** This author helped recruit the subjects, collect the data, design the study, and prepare the manuscript.
**Attestation:** Nick Marsden approved the final manuscript.
**Conflicts of Interest:** The author declares no conflicts of interest.
**Name:** Rangaswamy Mothukuri, MBBS, MRCS(Edin), FCEM, PG Cert.
**Contribution:** This author helped recruit the subjects, collect the data, design the study, and prepare the manuscript.
**Attestation:** Rangaswamy Mothukuri approved the final manuscript.
**Conflicts of Interest:** The author declares no conflicts of interest.

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**Contribution:** This author helped design the study, statistical design and analysis, and prepare the manuscript.
**Attestation:** Roger H. K. Morris approved the final manuscript and the statistical analysis reported in the manuscript.
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**Contribution:** This author helped collect the data (rheology), revise the article for scientific and intellectual content, and prepare the manuscript.
**Attestation:** Gareth Davies approved the final manuscript.
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**Name:** Karl Hawkins, MEng, PhD.
**Contribution:** This author helped revise the article for scientific and intellectual content, interpret the data, and prepare the manuscript.
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A New Rheological Biomarker of Coagulation


