The effects of temperature on clot microstructure and strength in healthy volunteers

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Short Title: A new rheological biomarker of coagulation

Funding: This translational work was supported by the NISCHR Biomedical Research Unit (BRU) grant (BRO1).

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Abstract

<Background: Critical illness and injury are known to alter the process of thermoregulation causing a change in temperature that will have an impact on coagulation and clinical outcome. This *in-vitro* preclinical study explores the relationship between temperature change and hemostasis using a recently validated rheological technique. In this study we aim to show that temperature change alone can cause a significant alteration in the microstructural properties of the clot.

<Methods: The rheological technique measures the attainment of the Gel Point which provides three related biomarkers of clot quality; elasticity of the clot ($G`_{GP}$), time to the gel point (T_{GP}) and clot microstructure (d_f). Gel Point measurements were performed on whole blood samples from 136 healthy volunteers over a temperature range of 27-43ºC.

<Results: Across the whole temperature range from 27 to 43°C, we see a progressive shortening of T_{GP} with corresponding increases in $G`_{GP}$ and $d_f$. We observed significant correlations between $d_f$, $G`_{GP}$ and T_{GP} across the whole temperature range ($p<0.001$). Furthermore when comparing the changes in d_f to the 'normal' at 37C (1.74 ± 0.035), we observe a significant reduction at temperatures equating to moderate hypothermia (≤32ºC) ($p=0.05$).

<Conclusions: This study shows that temperature change alone is sufficient to have an impact on the formation and properties of the clot. We found that significant changes were observed in d_f *and* T_{GP} when temperatures fell into the moderate and severe hypothermic range.

Number of words: 232

IntroductionExtremes of body temperature occur in approximately 50% of patients admitted to the critical care unit and contribute to morbidity and mortality.[1,2] Changes in the progression of disease and its treatment can alternate between hypothermic or hyperthermic conditions, which result from a wide range of clinical complications.[3] Where hypothermic states are often associated with hypocoagulable effects and hyperthermic states are associated with hypercoagulable conditions.[4-9] However, clinically the isolated effect of temperature change on coagulation is difficult to ascertain as it is often accompanied by other co-morbidities that may also alter coagulation. Previous studies have attempted to investigate the effect of temperature change on coagulation by using a variety of viscoelastic techniques.[ref] These techniques include thromboelastography (TEG), rotational thromboelastometry (ROTEM) and free oscillation rheometry, and show that hypothermia impairs coagulation.[8-10] In general the rate of coagulation and formation of the clot is increased, although the effect on the maximum clot strength is unclear. With some studies showing no change in the maximum clot strength, others showing significant changes at temperatures of 28°C[30], and others showing significant changes at 33°C.[ref] .[27,30,31] 89 free oscillation

Hyper and hypo*coagulable* changes in blood have been shown to affect the fibrin network which forms the basis of the clot microstructure is organized. [10-13] Clots formed under hypercoagulable conditions will have a denser more tightly packed microstructures which are more difficult to breakdown through lysis, when compared to a clot formed under hypocoagulable which would have a more open and loose network.[ref] Furthermore, the microstructure of the clot determines its elastic properties and resistance to deformation.[ref]

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Recent advances in the studies of viscoelastic changes in coagulation have led to the development of a novel rheological test for the analysis of clotting characteristics, including an assessment of clot microstructure.[10,14-16] In contrast to standard coagulation assays, this technique uses unadulterated whole blood and accurately measures coagulation based on the attainment of a Gel Point.[10,14] The rheological measurements at the Gel Point provides three important and related measurements; the elasticity of the clot ($G`_{GP}$), time to the gel point (T_{GP}) and an assessment of clot microstructure (d_f). In previous *in vitro* studies of hemodilution and anticoagulation we have shown that the measurement of the Gel Point provides improved predictors of changes in coagulation compared to conventional markers.refs

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The aim of this pre-clinical *in vitro* study is to determine whether temperature change alone causes alterations in coagulation and identify the exact temperature where these changes become significant, using the Gel Point measurements.

Methods

The study was approved by the Wales Research Ethics Committee 6 with fully informed and written consent obtained from all subjects before enrolment. The inclusion criterion included male and female volunteers who were recruited from a known healthy database. The exclusion criteria were 1) individuals with acute or chronic conditions known to effect coagulation, (i.e. malignancy, hepatic and/or renal dysfunction); 2) individuals taking anti-platelet or anti-coagulation treatment; 3) individuals with a family history of bleeding or thromboembolic disorders; and 4) anyone under the age of 18.

Blood Sampling

Prior to blood sampling each healthy volunteer was assigned to one test temperature, ranging from 27ºC to 43ºC increasing in 1° increments (n=8 for each temperature). Blood samples were obtained from the antecubital vein via an 18 -gauge needle, the first 2mls of blood were discarded following which 20ml was collected in a plastic syringe. The blood sample were then immediately divided into 2 aliquots. The first whole blood aliquot was transferred immediately to the AR-G2 (TA instruments, New Castle, DE, USA) 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 previously validated for use with blood.[14] A more detailed description of the Gel Point technique can be sound in the online supplemental (S1). In the

present study 6.6 ml of whole non-anticoagulated venous blood was immediately after bloodletting placed into a double-gap concentric cylinder geometry testing surface amounted on a TA Instruments AR-G2 (TA Instruments, New Castle, DE, USA) controlled-stress rheometer. The testing surfaces of the double-gap concentric cylinder geometry was set and controlled to one of the specified temperatures mentioned above. Immediately after loading measurements of viscoelastic properties of coagulating blood were taken and the formation of the Gel Point recorded, which identifies the transition of the blood from a visco-elastic liquid to a visco-elastic solid. The Gel Point provides three separate but related markers of coagulation; (i) the time taken to reach the Gel Point (the incipient clot formation time), T_{GP}; (ii) the shear elastic modulus at the Gel Point, G'_{GP}, which is a measure of clot elasticity; and (iii) the fractal dimension of the clot, d_f, which is a quantification of the clot structure.

Close temperature control was achieved in the AR-G2 rheometer via an integrated peltier concentric cylinder system. The PCC is valid for use between -10 to 150°C and is accurate to within 0.1°C. To ensure its accuracy the temperature of the PCC was checked by loading the testing surface with a fluid (normal saline), setting to the required temperature then immersing a thermocouple (P.I.8013, Portec type K theromometer, Wrestlingworth, UK) and recording the temperature. The process was repeated for all temperatures studied, in all cases the temperature reported by the PCC unit was within ±0.1°C of the temperature set on the AR-G2 rheometer. The time taken for whole blood to reach the test temperatures after being loaded onto the testing surface (within 0.1°C) never exceeded 10 seconds.

Computational Simulation

In conjunction with the Gel Point measurements we also provide a previously published computational simulation. This simulation will be 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.[17] This computational simulation will use the established relationship between incipient fibrin clots and there fractal properties, where the mass, M, is related to d_f by the following power law equation [$M \approx \varepsilon^{df}$, where ε is a length scale value in the range 100nm to 10μm].[18]

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, UK Ref: 367691) for routine coagulation studies, including Prothrombin Time (PT), activated Partial Thromboplastin Time (aPTT), and fibrinogen concentration measured using the Clauss method.[ref] These were measured using a Sysmex CA1500 analyzer within 2 hours of collection. Fibrinogen concentration was verified against the 2nd International Fibrinogen Standard Version 4 (NIBSC code 96-612). All reagents were obtained from Siemens, (Frimley, UK). The analyzer was calibrated according to manufacturer's instructions.

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Statistical Analysis

Statistical analysis was performed using Minitab® version 16 software (Havertown, PA) and GRAPHPAD PRISM® version 6.0 (GraphPad software Inc., La Jolla, CA, USA). Data was confirmed as normally distributed using Anderson-Darling method and Tukey`s pairwise analysis used to determine when true differences arose. Pearson correlation was undertaken to

explore associations between temperature, d_f, T_{GP} and $G`_{GP}$. Data was assumed to be significant when $p<0.05$ unless otherwise stated. The primary aim was to establish at what temperature significant changes in d_f would occur compared to the value at 37°C. To determine the number of participants we would need in each of the temperatures we performed a power calculation. When temperature is decreased into the moderate or severe hypothermic range (below 33°C) we would expect a decrease in d_f or around 0.06, reductions similar to those seen in other in vitro studies and where the d_f at 37°C seen in a previous study was 1.73±0.035. ref Taking an $\alpha = 0.05$ and a power of 0.90, we calculated that we would need an n of 8 for each temperature, giving a total of 136 participants.

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Results

Blood was obtained from 136 healthy volunteers. The standard coagulation measurements reported in Table 1 were normal for all subjects and were performed at 37°C. At 37ºC the value of d_f = 1.73 (±0.039) and T_{GP} = 231(±55), were commensurate with those reported for healthy volunteers measured at 37°C in two previous studies.[10,14] Figure 1 shows that increasing or decreasing temperature above or below 37°C results in incremental changes in the Gel Point measurements. Progressive increases in temperature from 27 to 43°C resulted in a progressive increase in both d_f and $G`_{GP}$, showing significant positive correlations with temperature of ?? and ?? respectively (see Figure 1). T_{GP} was seen to decrease as temperature increased (??). We found that d_f significantly decreased from its baseline value (37ºC) ($p<0.05$) when temperatures reached 32°C (see Figure 1). A decrease in T_{GP} occurs at 27°C ($p=0.05$). Significant correlations were found between T_{GP} and d_f ($r= -0.562$, $p<0.001$) and between d_f and $G`_{GP}$ ($r = 0.719$, $p<0.001$).

The computational simulation (Figure 2a) shows that for values of d_f in the lower temperature range, large decreases in clot mass are observed. Figure 2b shows that a 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 Figure 2c). Figure 2a shows that the small incremental increases in d_f would require exponential increases in mass. Figure 2d shows that a d_f of 1.80 (some of the highest single values observed) would require a structure with 120% additional mass compared to one formed at a d_f of 1.73.

Discussion

This study shows that changes in temperature of the blood has a significant impact on the formation of the clot. We found that significant changes were observed in the Gel Point measurements of clot formation when temperatures fell into hypothermic range. Hypothermia is common in critical illness, trauma and surgery and has been linked with excessive bleeding, blood transfusion, and poor outcomes.[1] In the literature it has been divided into mild (32° to 35.9°C), moderate (28° to 32.9°C) and severe (less than 27.9°C) [25-27]. Previous *in vitro* studies have shown that temperatures in the range 33° to 35.9°C cause a minimal effect on the enzymatic activity of coagulation factors[6,7], however reduced platelet adhesion and aggregation is present. Where temperatures below a threshold of 36.4ºC and 34ºC have been shown to slow GPIIb/IIa and P-selectin activation respectively, both important in platelet adhesion.[7,19] As temperature is reduced beyond 33°C, into the moderate and severe hypothermic ranges, a further inhibitory effect on platelet activity is observed,[5,7] coupled with a progressive decrease in the enzymatic activity of coagulation factors, where at 25ºC enzyme activity ranges from 0 % (FVIII and FIX) to 5% (FII and FVII).[5,7,21]

In the present study we report a progressive decrease in d_f and $G`_{GP}$ with a prolongation of T_{GP} as temperature is reduced below 37°C (see Figure 1). The progressive changes observed in d_f and T_{GP} at temperatures below 37°C are indicative of a worsening hypocoagulable state, producing d_f and T_{GP} values more commonly associated with anticoagulated or diluted blood.[10,14] Whilst changes are observed in the range of 33°C to 36°C, the change from the values at 37°C do not become significant until a temperature of 32°C is reached and only in the case of d_f (1.73±0.040 at 37°C to 1.67±0.037 at 32°C $p<0.05$). One advantage of this

rheological technique is that it includes the vital contribution of platelets and cellular components of coagulation, one major disadvantage of current routine coagulation laboratory tests. However, over the temperature range 33° to 35.9°C, the stated inhibitory effect on platelet adhesion does not seem sufficient to produce significant changes in the Gel Point parameters alone. When temperatures reach those synonymous with moderate hypothermia (below 33°C), the additional platelet and coagulation enzyme activity inhibition are likely to be the cause of this significant reduction in d_f. We only observe a significant change in T_{GP} when the temperature falls to 27°C (??±?? at 37°C to ??±?? at 27°C $p<0.05$) and no significant change is observed in $G`_{GP}$. This suggests that clot microstructure is more sensitive to the changes in temperature.

Hyperthermic conditions have been associated with hypercoagulablity, where increases in temperature result in a net increase in thrombin generation.[21] In the present study when temperature was increased *above* 37ºC (up to 43ºC) we observe no significant changes in d_f $G`_{GP}$ and T_{GP} (Figure 1). These findings when compared to the changes in d_f observed in the hypothermic range, suggests that clot microstructure is more susceptible to reductions in temperature than increases. Across the whole range of temperatures studied (27-43°C) we record significant correlations between temperature and the three gel point parameters (d_f, T_{GP} and $G`_{GP}$). Where an increase in temperature has a profound effect on the formation and characteristics of the clot, creating faster forming, more compact clots with increased elasticity. Where, the significant correlations found between $d_f$ & $T_{GP}$ and $d_f$ & $G`_{GP}$ suggesting the structural organization of the clot (d_f) is related to the kinetics of clot formation (T_{GP}) and its elastic properties ($G`_{GP}$),

To better understand the impact of changes in d_f it is important to recognize what they represent in terms of the fibrin mass incorporated within the clot. To achieve this we use a previously published computational analysis that illustrates the non-linear relationship between d_f and clot mass at the incipient clot stage of development (see Figure 2). When temperature is reduced a progressive decrease in d_f is observed, Figure 2 reveals that a clot with d_f=1.67 (33°C) has approximately 60% of the mass of a clot for which d_f = 1.73 (37°C). This value is reduced further when considering the lowest temperature investigated in this study (27°C) which has a mean d_f of 1.62 and would contain approximately 40% mass. Small increases in d_f will result in an exponential increase in the mass of the network. A change from 1.73 to 1.80 requiring some 120% extra mass. From a clinical perspective, these substantial changes in the clot mass are significant, given its role as a microstructural template for clot development.[17] The consequences of increased mass will make the clot more difficult to lyse due to increased polymerized fibrin in a more tightly packed structure resulting in increases in clot elasticity. This is supported by the positive significant correlation between d_f and $G`_{GP}$ herein. This may be understood in terms of the viscoelasticity of cross-linked polymeric gel networks in which $G`$ is a function of polymer chain length between cross links. For idealized networks with fixed cross links, increases in both $G`$ and d_f will result from increased cross link density.[22]

The major limitation of the study was not being able to perform Gel Point measures at all 17 different temperatures for all volunteers. This was due to the test being performed using whole non-anticoaglated blood, where only a limited number of AR-G2 instruments were available and not wanting to bleed the volunteers multiple times. As such blood from different healthy volunteers was used at each temperature interval. To limit any possible variations between the

groups all participants were drawn from a known healthy database and did not exceed the limits of the healthy range for d_f. 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. Additionally we have shown previously how the incipient clot acts as a template of mature clot growth[ref] and that d_f is significantly correlated with other viscoelastic measurements of the mature clot.[ref]

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This is the first study that quantifies how alterations in temperature result in significant changes in the quality of the clot. This study highlights the potential of the Gel Point technique as an improved measure of coagulation, furthermore we demonstrate the importance of performing coagulation analysis at the patient's body temperature. Maintaining normothermia may be important, the results of this study would suggest that preventing moderate to severe hypothermia (<32ºC) could be important. The Gel Point measurements have now been validated in models of anticoagulation, dilution and temperature.[10,14] These studies identify that the Gel Point measurement can potentially be used in monitoring the pathophysiological progression, regression and treatment of an individual, to improve the management of critically ill patients with abnormal thermal conditions or undergoing surgery. The results of this *in vitro* study highlight the need for further clinical studies, such as studying the role of temperature in critical illness for example, sepsis and burns.

Acknowledgements

We would like to acknowledge the outstanding support provided to us by the ABMU Health Board. Special thanks to Steve Atherton, Medical Illustrations Department, Morriston Hospital for their help with the illustrations.

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Table 1: The normal coagulation profile of the study participants with normal ranges for reference.

Coagulation profile	Result	Normal range
PT (sec)	10.4 ± 0.4	9 – 12.5
APTT (sec)	25.7 ± 1.9	22.1 – 30.9
Fibrinogen Clauss (g/l)	2.9 ± 0.5	1.5 – 4.2
Haemaglobin (g/l)	149 ± 15	130 – 180
Platelets ($x10^9$/l)	263 ± 58	150 – 400
Haematocrit (vo/vol)	0.441 ± 0.039	0.40 – 0.52

Figure 1: Graphs of the changes in d_f (a), T_{GP} (b) and $G`_{GP}$ (c) with progressive alterations in temperature. a) At lower temperatures a reduced value of d_f is observed, which represents a poorly formed, weak, porous clot. The reverse is seen at high temperatures, with a stronger, more densely formed clot produced. **b)** At lower temperatures a progressive prolongation of T_{GP} is observed indicating a hypocoagulable response, conversely at higher temperatures a shortening of T_{GP} is present indicating a hypercoagulable response. **c)** A progressive increase in $G`_{GP}$ is observed as temperature is increased, indicating a general increase in clot strength at higher temperatures. (*p<0.05 1-way ANOVA Tukey's pairwise analysis). Dotted lines represent the mean and standard deviation at 37°C.

Figure 2 – Computational simulation of d_f *vs.* mass: 2a) Graph showing the relationship between d_f and mass incorporated into the structure. Substantial increases in mass are required to generate small increments of d_f. The mass value on the y-axis is normalized for the healthy index value of d_f (=1.73). **Figure 2 (b), (c) and (d):** Illustrations of different incipient clot microstructures at particular values of d_f are provided (cross = 1.65, circle = 1.73 and square = 1.80 respectively). The color of each node (unit sphere) within the fractal represents the local density of constituent nodes within a sphere of radii 5 units, the color ranges from green (1 neighboring node) to red (20 neighboring nodes).