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Coagulation effects of *in vitro* serial haemodilution with a balanced electrolyte hetastarch solution compared with a saline-based hetastarch solution and lactated Ringer's solution*

A. M. Roche,[1] M. F. M. James,[2] M. P. W. Grocott[1] and M. G. Mythen[3]

1 Research Fellow, Centre for Anaesthesia, University College London, First Floor Crosspiece, Middlesex Hospital, Mortimer Street, London, W1T 3AA, UK
2 Professor and Head, Department of Anaesthesia, University of Cape Town Medical School, Observatory, 7925, South Africa
3 Portex Professor of Anaesthesia, Centre for Anaesthesia, University College London, First Floor Crosspiece, Middlesex Hospital, Mortimer Street, London, W1T 3AA, UK

Summary

The hydroxyethyl starches are a group of compounds that has been associated with impairment of coagulation when large volumes are administered. The thrombelastograph® is commonly used to assess point-of-care whole blood coagulation. Little is known about the dose–response relationships of haemodilution, and it is reasonable to assume that a linear association exists. This may not be the case with altered electrolyte compositions of the fluids used for haemodilution. We have therefore conducted an *in vitro* study of haemodilution of human whole blood using lactated Ringer's solution and two high molecular weight hetastarches, one in a balanced salt solution, the other in a 0.9% saline solution. The thrombelastograph®, commonly used for the assessment of the coagulation effects of synthetic colloids, was used as the coagulation assessment device. Serial haemodilution with hetastarch in a balanced salt solution demonstrated a biphasic response (of r-times and k-times, as well as alpha angles), with haemodilution in the 20–40% range causing enhanced coagulation, and higher degrees of dilution causing a decrease in overall coagulation performance. A similar picture was observed with lactated Ringer's solution, but only significantly so in alpha angles. Hetastarch in saline did not display this initial increased coagulability at mild to moderate dilutions. This biphasic response of lactated Ringer's solution and hetastarch in a balanced salt solution reflects the complex interaction of fluids and the coagulation system, and that these effects cannot be attributed to simple haemodilution. On the other hand, there was a linear decrease in maximum amplitude with haemodilution. Maximum amplitude was particularly affected by both starches, which is an expected finding in view of the known interaction between the hydroxyethyl starches and von Willebrand's factor.

Keywords *Blood*: coagulation. *Fluid balance*: intravenous fluids. *Measurement techniques*: TEG.

Correspondence to: Dr A. M. Roche
E-mail: tonyroche@doctors.org.uk

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Hydroxyethyl starch (hetastarch) intravenous colloid solutions are a group of fluids that provide good volume expansion and replacement, combined with a long intravascular persistence [1]. Deleterious coagulation effects have been documented, especially when administered in large volumes of highly substituted, high molecular weight hetastarch solutions (> 20 $ml.kg^{-1}$) [2, 3].

Hextend® (Abbott Laboratories, Chicago, IL, USA) is a novel formulation of 6% hydroxyethyl starch. It has an average molecular weight of 670 kDa, with a molar substitution ratio of 0.75, and is suspended in a lactate-buffered balanced electrolyte and glucose solution. Phase III clinical studies have demonstrated less derangement of thrombelastograph® variables and improved clinical indices of clotting in patients who received this fluid when compared with a similar 6% hydroxyethyl starch in saline [4]. The reason for this apparent decrease in coagulation derangement is not fully understood. As part of a series of ongoing *in vitro* and *in vivo* experiments investigating the effects of intravenous fluids on clotting, we performed serial *in vitro* dilution of human fresh whole blood with lactated Ringer's solution, 6% hydroxyethyl starch (450 kDa, 0.6 substitution ratio) in 0.9% saline (HS/saline), and 6% hydroxyethyl starch in a balanced electrolyte and glucose solution (HS/bal) in order to characterise the effect of increasing haemodilution on thrombelastograph® variables.

Of the currently used coagulation tests, the thrombelastograph® (Thrombelastograph®, Haemoscope Corp, Skokie, IL, USA) has gained wide acceptance as a reliable indicator of global dynamic clot formation, reflecting coagulation system interactions, i.e. platelet function, intrinsic and extrinsic pathways, fibrin system, as well as clot stability [5]. The developing trace one observes is a time (x-axis) vs. amplitude (y-axis) graph. The trace continues at 2 $mm.min^{-1}$, while the amplitude is measured by a torsion wire system. The wire is attached to a piston suspended in an oscillating cup (4.75°, 10 s) containing 360 μl of blood. No coagulation results in a straight line trace, while increasing coagulation formation displays an increasing amplitude both sides of the baseline. Its basic variables, among others, are r-time, k-time, alpha angle and maximum amplitude. The r-time reflects the time (measured in mm or min) for the first signs of coagulation (fibrin formation) to appear, in other words, the onset to clot formation. This point is marked when the amplitude of the trace reaches 2 mm, and is largely determined by circulating coagulation factors. The k-time is measured (in mm or min) from the point at which the r-time is marked to the point at which the amplitude reaches 20 mm, and represents the speed of polymerisation of fibrin once coagulation has started. Another commonly cited variable is the alpha angle, which is measured from the r-time as a tangent of the developing curve. It, too, represents polymerisation of fibrin and strengthening of the clot. The maximum amplitude reflects the total strength of the clot, and is measured as the widest amplitude of the trace obtained, i.e. the point at which the clot is the strongest (a function of mostly platelets and fibrin) [5]. Using a thrombelastograph® is cheap and easy, often giving initial results before coagulation and full blood count screens are available. This in itself is of great benefit in early correction of coagulation abnormalities in the clinical setting [6]. The thrombelastograph® is also reliable as a test for the diagnosis of a hypercoagulable state [7].

The thrombelastograph® has been used as the common denominator in many trials of the effects of haemodilution on coagulation. The reasons for this are that it is a standardised test, it provides reliable results, is easy to interpret, and provides a good reflection of the clinical coagulation state of the patient. It is an extremely useful tool for both *in vitro and in vivo* work [5]. As the thrombelastograph® measures dynamic clot formation, usually using whole blood, it is superior to common laboratory tests, e.g. prothrombin time and partial thromboplastin time, in trials investigating the effects of haemodilution on coagulation. Clinical studies have shown that when laboratory tests reflect no difference in coagulation, the thrombelastograph® is still able to reflect significant clotting differences in patients when present. Measured thrombelastograph® variables have been shown to correlate well with indices of bleeding in clinical settings [4].

Our study involves thrombelastograph® analysis of serial *in vitro* haemodilution with three intravenous fluid preparations, two of them high molecular weight hetastarch preparations (one in 0.9% saline, the other in a balanced electrolyte formulation) and lactated Ringer's solution, to assess varied electrolyte and colloid effects on the dose–response profile of haemodilution.

Methods

University of Cape Town Research Ethics Committee approval was obtained before the start of the study. Volunteer exclusions included a history of haematological disease, previous colloid intravenous infusions, or subjects receiving coagulation-altering drugs, including non-steroidal anti-inflammatory drugs. After informed consent, five healthy volunteers donated two samples per day of fresh whole blood on two separate days, i.e. four samples in total, using a two-syringe technique from a free flowing vein. The two samples in each day were separated by 90 min and were taken from different arms. The first 5 ml of each sample were discarded to minimise possible tissue factor or contact activation, and the remainder was used for mixing experiments. The fresh whole blood was immediately mixed with escalating volumes of HS/bal (Hextend®, Abbott Laboratories, Chicago, IL, USA), HS/sal (Sabax Hetastarch®, Adcock Ingram Critical Care, RSA) and

lactated Ringers solution (Intramed, RSA). The dilutional levels were 20, 30, 40, 50, 60 and 75% prepared in polypropylene tubes. The total volume for each dilution was 1 ml. The 20% dilution had 200 μl of test fluid added to 800 μl of blood, the 30% dilution had 300 μl-test fluid added to 700 μl of blood, and so on, such that the final dilution of 75% had 750 μl-test fluid added to 250 μl blood. The fluids and polypropylene tubes were prewarmed in a water bath kept at 37 °C. Each mixed sample was gently inverted eight times. Undiluted fresh whole blood control samples (total volume = 1 ml) were treated in the same way as the diluted samples before each thrombelastograph® run. The mixed samples and controls were placed in calibrated thrombelastograph® analysers (Haemoscope, Skokie, IL, USA) 4 min after venepuncture. Each thrombelastograph® was allowed to run for a minimum period of 60 min, or until the maximum amplitude was reached, as determined by the computer algorithm. The samples were rotated between the thrombelastograph® channels for different volunteers.

Analysis of variance (ANOVA) for repeated measures and two-way ANOVA, with post hoc least significant difference testing, were performed on the data obtained. Probability values ≤ 0.05 were considered to be significant. For data not normally distributed, the Kruskal–Wallis non-parametric ANOVA test was used, with the Kolmogorov-Smirnov test used post hoc to identify individual statistical differences.

Results

Significant shortening in r-time relative to the control sample was seen at 30% haemodilution with HS/bal ($p < 0.05$, Fig. 1). Dilutions of ≥ 40% with HS/bal resulted in r-times that were not significantly different from control values, although the power of the test was probably inadequate to detect small changes from control at this level of dilution. Similar shortening of the k-time relative to control at 20, 30 and 40% dilution with HS/bal was seen ($p < 0.05$, Fig. 2). The maximum amplitude obtained at 75% dilution did not reach 20 mm, therefore k-times were quoted as 60 min for 75% dilutions with non-parametric statistical analysis applied to comparisons of this group with control values. Dilutions of 50% and 60% produced k-times that were not significantly different from control. The alpha angle was significantly increased relative to control at 20, 30 and 40% dilutions, with a significant decrease at 75% dilution ($p < 0.05$, Fig. 3). A progressive significant decrease from control in maximum amplitude was seen at 40–75% dilutions ($p < 0.05$, Fig. 4).

Dilution with HS/sal did not produce any decrease in r-time, but increases in r-time relative to control were seen at 50–75% dilutions ($p < 0.05$). The k-times showed a similar increase at 60% and 75% percent relative to control ($p < 0.001$), with alpha angles decreased at 50% ($p = 0.05$), 60% and 75% ($p < 0.001$), and maximum amplitudes also decreased at 40–75% dilutions relative to control samples ($p \leq 0.02$).

Interestingly, lactated Ringer's solution produced no significant differences in r-times from control values, while the k-times showed a prolongation at only 75% ($p = 0.001$). The alpha angles were consistently increased at 30–50% dilutions relative to controls ($p \leq 0.03$), and maximum amplitudes were decreased only at 60% and 75% dilutions ($p < 0.001$).

When comparisons were made at the same dilutional levels across the groups, dilution with HS/sal increased r-times at 40, 60 and 75% dilutions compared to HS/bal and lactated Ringer's solution at the same dilutions ($p < 0.05$). At 50% dilution, r-time was significantly increased with HS/sal compared to HS/bal ($p < 0.05$). The k-times showed a similar pattern: dilution with HS/sal produced longer k-times at 60 and 75% dilution than with lactated Ringer's solution at the same dilutions ($p < 0.001$), and longer k-times than HS/bal at 75%

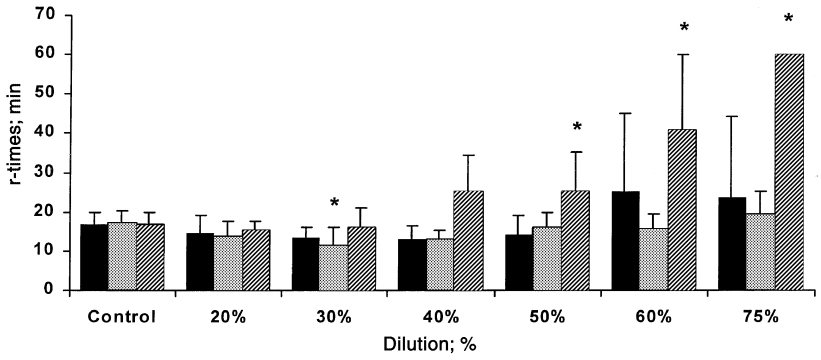

Figure 1 r-times for control (undiluted blood) and diluted samples. Error bars indicate SD. ■ dilution with lactated Ringer's solution. ▨ dilution with 6% hetastarch in a balanced electrolyte and glucose solution. ▨ dilution with 6% hetastarch in saline. * $p < 0.05$ vs. control (undiluted blood). Where no error bars are shown, no clot was formed, therefore r-time assigned as 60 min.

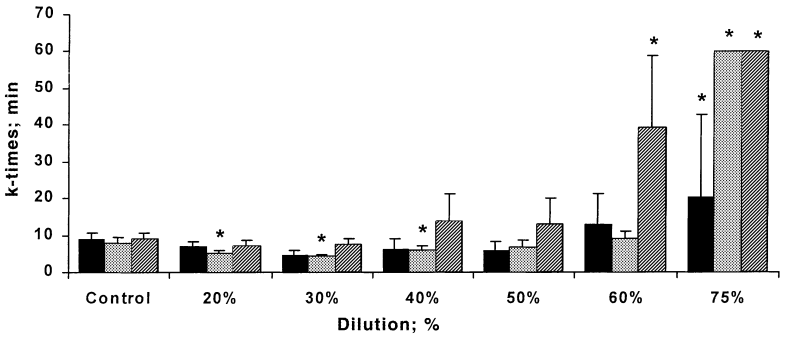

Figure 2 k-times for control (undiluted blood) and diluted samples. Error bars indicate SD. Legend as for Fig. 1. ⋆$p < 0.05$ vs. control (undiluted blood). Where no error bars are shown, maximum amplitude did not reach 20 mm by 60 min, therefore k-time assigned as 60 min.

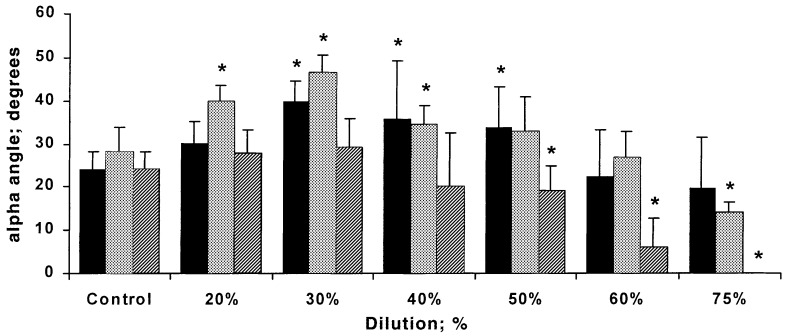

Figure 3 Alpha angles for control (undiluted blood) and diluted samples. Error bars indicate SD. Legend as for Fig. 1. ⋆$p < 0.05$ vs. control (undiluted blood).

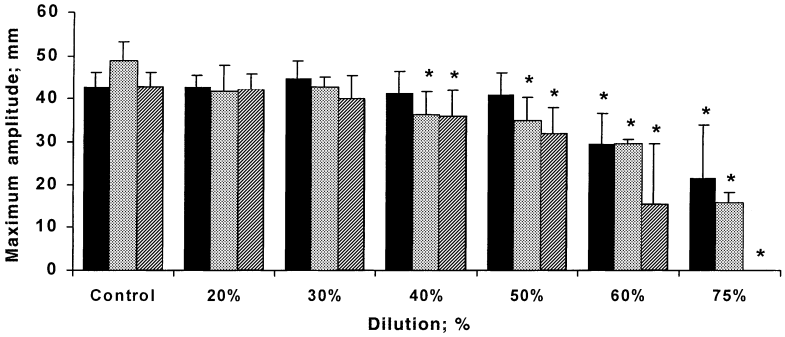

Figure 4 Maximum amplitudes for control (undiluted blood) and diluted samples. Error bars indicate SD. Legend as for Fig. 1. ⋆$p < 0.05$ vs. control (undiluted blood).

dilution ($p < 0.001$). Alpha angles were consistently lower with HS/sal than with HS/bal or lactated Ringer's solution at 30–75% dilutions ($p \leq 0.01$), and lower than HS/bal only at 20% dilution ($p < 0.01$). The maximum amplitudes were decreased in HS/sal vs. lactated Ringer's solution and HS/bal at 60% and 75% dilutions ($p < 0.001$), as well as vs. lactated Ringer's solution at 50% dilution ($p < 0.02$).

Lactated Ringer's solution and HS/bal showed no significant differences between each other in r-times and maximum amplitude, but lactated Ringer's solution had shorter k-times at 75% dilution ($p < 0.001$) and smaller alpha angles at 20% dilution when compared with HS/bal ($p = 0.03$).

Discussion

Haemodilution *per se* is known to cause a disturbance in the balance between procoagulant and anticoagulant systems involved in the coagulation cascade [8]. The most common effect is one of a hypercoagulability associated with mild to moderate haemodilution. This effect occurs with all fluids, but most significantly with crystalloids [9]. This is manifested by decreased r-times and k-times, and increased alpha angles on the thromb-elastograph®, with a varied response in maximum amplitude, depending on the fluid tested. The effect is best seen with *in vitro* haemodilution, but can also be seen *in vivo* [8].

We considered it essential to perform an observational dose–response study of HS/bal as part of ongoing work into explaining the apparent improved coagulation profile when compared with hetastarch in 0.9% saline. It is perhaps surprising that few differences were found between HS/bal and lactated Ringer's solution, as a greater hypercoagulability response would be expected with the crystalloid solution than with a hetastarch colloid [10]. Hetastarch in saline produced increasing differences as the dilutions increased, and it is possible that the electrolyte differences between HS/sal (suspended in 0.9% saline, i.e. an unbalanced electrolyte solution), and HS/bal and lactated Ringer's solution (both balanced electrolyte solutions containing calcium) were responsible. We are currently investigating the effects of electrolyte differences on the coagulation-altering effects of intravenous fluids.

The interesting significant biphasic pattern seen in r-times, k-times and alpha angles, with serial haemodilution with HS/bal (Figs 1, 2 and 3), as well as with alpha angles of lactated Ringer's solution dilution, reflects a hypercoagulability at mild to moderate haemodilution *in vitro.* This is contrary to what would commonly be expected especially of a hetastarch solution. The progressive decrease in maximum amplitude of the starches over the dilution range (Fig. 4) can be explained by the documented dose-related inhibition of von Willebrand's factor (antigen and coagulant) by HS/sal solutions [11]. Since the maximum amplitude is largely dependent on platelet function, the different behaviour of maximum amplitude with dilution in comparison to the other thrombelastograph® variables is entirely predictable. This is in contradiction to the findings of Bick, who found no significant differences in Factor VIII/von Willebrand's factor activity or structure between HS/bal dilution and saline dilution of plasma [12]. Admittedly, it was an *in vitro* study, and plasma was used rather than whole blood. Tobias *et al.* performed a serial haemodilution study, using saline, 5% albumin and a hetastarch [13]. They showed that a hypercoagulable trend developed at mild to moderate haemodilution, even in the colloid groups. They found albumin to have more hypocoagulable effects than HS/sal. Petroianu *et al.* also performed a dose–response type study, where significant decreases in r-times were found after mild to moderate haemodilution with two gelatin preparations, a dextran and certain hydroxyethyl starches [14]. A biphasic response was found in their r-times, although it was not discussed in their paper. This effect was offset by an increase in k-times and a decrease in maximum amplitudes.

Investigating the coagulation effects of a range of dilutions is necessary in explaining the varied clotting profiles that may be seen after large or small volume infusions. This must be taken into account when the results of studies are compared, as the degrees of dilution may well be different, providing conflicting results.

It is important to remember that hydroxyethyl starches, being synthetic colloids, are polydisperse fluids, i.e. they have a range of molecular sizes in a single preparation. The 'performance' and profile of these starch preparations change with time *in vivo,* as the smaller molecules (usually < 55 kDa) are rapidly filtered and excreted through the kidneys [1]. The large molecules often undergo a rapid hydrolysis, therefore leaving a colloid in the circulation with a smaller mean molecular weight and different osmotic and volume-expanding effects. In the intact subject, the coagulation system has considerable reserve capacity, both in terms of release of stored, preformed elements, and in terms of the synthesis of new coagulation proteins. Together with the metabolism of the starch particles to smaller units, this may account for the lesser effects of starches on coagulation that have been observed *in vivo* [15]. More, well-constructed, *in vivo* studies are needed to explain further the effects of intravenous fluids on coagulation.

Interactions between hydroxyethyl starches and the endothelial system are largely unexplored from a coagulation point of view. It is likely that endothelial-related coagulation plays a significant role, as the starches diminish leucocyte adherence to the endothelium, as well as modify endothelial porosity in states of capillary leak [16, 17]. However, these experiments throw no light on this aspect of coagulation, as thrombelastographic measurements exclude any consideration of platelet–endothelial interactions.

Our results therefore support the importance of performing progressive haemodilution in an attempt to determine the effects of fluids on dynamic blood clot formation. The clot onset time (r-time), as well as the clot formation rate (k-time) first decrease with mild to moderate haemodilution, then increase with gross haemodilution in the two balanced electrolyte preparations we tested. A gradual decrease in total clot strength (maximum amplitude) with progressive haemodilution was found. There was little difference between HS/bal and lactated Ringer's solution in our study, but numerous differences were found between HS/sal and the two balanced electrolyte preparations (HS/bal and lactated Ringer's solution) at the studied dilutional levels. This may be due to electrolyte composition, a subject of our research into fluids and coagulation. It is also essential to consider the dilutions used when comparing thrombelastograph® results between fluid therapy and haemodilution coagulation studies.

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