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Polypropylene and Polycarbonate Containers Have a Varied Effect on Coagulation after Haemodilution, as Judged by TEG® *in vitro*

MMed (Anaesthesia)

Part III Dissertation

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Declaration

I, Anthony Michael Roche, hereby declare that the work on which this thesis is based is my original work, except the work mentioned as the "University College London Study" mentioned in the preface. Neither the whole work, nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the University of Cape Town to reproduce for research either the whole or any portion of the contents in any manner whatsoever.

Date:

To the most long-suffering person in town, Riana.

You, Michael and Lisa are everything to me!

Contents

Preface

The reasons for this study were multi-factorial, but mostly due to some interesting data obtained from a pilot study conducted at University College London (UCL). In that study, the coagulation effects *in vitro* of two hetastarch solutions were compared with two crystalloids by means of thrombelastography (TEG®). The fluids compared were:

1. Hespan® (HES), a high molecular weight hetastarch (450kDa/0.7substitution ratio) in a 0.9% saline solution – Laevosan, Austria.
2. Hextend® (HEX), also a high molecular weight hetastarch (670/0.75 substitution ratio) in a balanced electrolyte, lactate and glucose solution – BioTime Inc, Berkeley, California, USA.
3. Saline 0.9%
4. Hartmann's Solution (Ringer's Lactate)

The crystalloids revealed no surprising differences known from previous published data, but data obtained from the hetastarch solutions revealed contradictory results to known *in vivo* results found in a phase III trial.[17] This previous Phase III *in vivo* trial showed that HEX haemodilution produced a superior coagulation profile to HES, along with a significantly shorter r-time than HES. There was also a significantly smaller transfused volume of blood than HES in the HEX-treated patients.[17] This Phase III study prompted the initial UCL *in vitro* haemodilution study mentioned above. In the UCL study, there were significantly impaired TEG® results, indicating severe hypocoagulability with HEX, when compared with HES. This included prolonged r-and k-times, as well as reduced α-angles and maximum amplitudes in the HEX group, compared with HES and crystalloid groups.

Many theories were discussed for these controversial UCL results, but the thought was that a container-effect could have been responsible, as the *in vitro* UCL study methodology included the use of a polycarbonate container for initial storage, as well as for haemodilution of the blood *in vitro.* In view of the known wettable surface, as well as a strong negative surface charge of polycarbonate, it was suggested that the container surface itself could have affected coagulation. When different ionic compositions of the various fluids and starches were taken into account, it seemed possible that some interaction between the fluids and the material of the containers could have induced or inhibited coagulation at the container surface. The suspicion was that the observed change in TEG® variables was likely due to a methodologic idiosyncrasy.

Previous track record of haemodilution and TEG research at the University of Cape Town made it an obvious setting for exploration of this problem. Preparations were thus made to test container effects with haemodilution *in vitro* at Prof MFM James' anaesthesia laboratory at the University of Cape Town. The hypothesis was that the use of polypropylene and polycarbonate containers, with their different chemical and surface properties, would lead to a variability in TEG® results obtained from fresh whole blood, as well as blood diluted with various fluid solutions.

Choosing TEG® as a monitor of coagulation was essential, as it has a well-established track record in monitoring coagulation effects in trials of haemodilution *(in vitro* and *in vivo).*[9] TEG® produces reliable and quick results, giving a reflection of global coagulation function. It, along with the Sonoclot®, are the only two devices which can reliably diagnose a hypercoagulable state.[6,21] More will be mentioned on the TEG® later.

Introduction

Fluid therapy is an important part of the global medical management of patients. Improved outcomes are attributed purely to rational, appropriate fluid management of patients.[7] Peri-operative fluid optimisation is an important determinant of post-operative morbidity.[4,48] However, apart from monitoring cardiovascular and respiratory function, urine output, routine haematology and biochemistry indices, coagulation status of the patient should be considered. This is often ignored in fluid therapy, but may have disastrous effects if not considered. Blood coagulability can be affected by the volume, speed and type of intravenous fluid being administered.

Crystalloids:

It is well known that crystalloids induce a state of hypercoagulability at mild to moderate haemodilution,[40,41] regardless of the type of crystalloid. This is well observed both *in* vitro, as well as *in vivo.* Two studies are of special relevance to the clinical setting; Janvrin documented an increased deep venous thrombosis (DVT) rate associated with crystalloid administration, and Heather described a DVT predictor test using TEG and crystalloid dilution *in vitro.*[19,23]

Colloids:

Colloids have a more complex effect on coagulation, depending on the type and sub-type of colloid. For example, the dextrans are known to inhibit coagulation by means of von Willebrand factor inhibition, leading to a clinical state similar to von Willebrand disease.[2] The dextrans have been reported as improving the rheologic properties of blood, as well as possessing a mild anticoagulant effect. This has led to the use of dextrans for thrombo-embolic prophylaxis clinically.[2]

The hetastarch (HES) solutions have a very complex range of effects, where the high molecular weight, highly substituted, high C2:C6 ratio starches have the most deleterious effects on coagulation.[50]

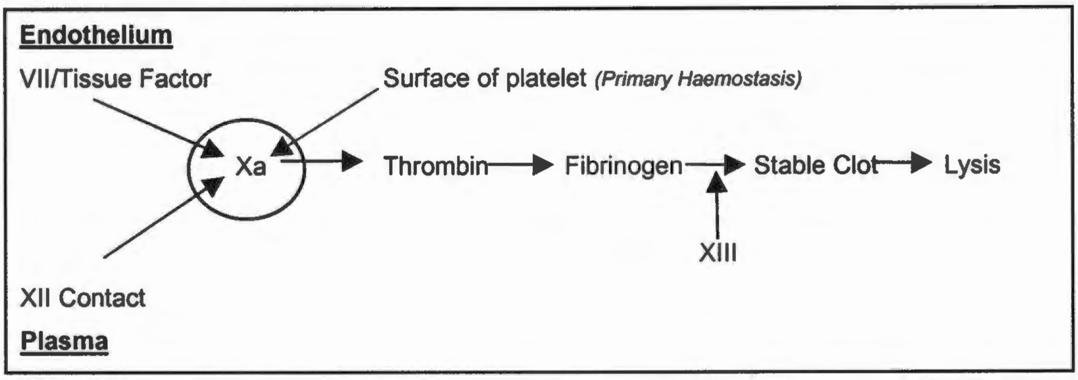
Albumin seems to have minimal or no coagulation effects at mild to moderate haemodilution,[49] but this may well not be the case at high dilutions, where the calcium-binding effects of albumin may leave the ionised calcium level too low to enable normal coagulation.[9]

The gelatins also have minimal coagulation effects at mild to moderate haemodilution, but can inhibit coagulation formation at large doses.[47] This is due to gelatin being a denatured collagen, which binds fibronectin. It is thus incorporated into the forming clot, resulting in reduced total clot strength and weight.[29]

Coagulation:

The important thing to remember about the coagulation system is that there is always a balance between procoagulant and anticoagulant systems (fig 1).

Figure 1: Basic Coagulation



Index: Basic coagulation rests upon stimulation of intrinsic (by contact activation with subendothelial tissues) or extrinsic (activation of factor VII by tissue factor) pathways. These stimulate the final common pathway (factor X) to generate thrombin from prothrombin. Thrombin catalyses the conversion of fibrinogen to fibrin monomers, which then polymerise under the control of factor XIII to form a stable clot. Besides primary haemostasis by platelet adhesion and aggregation, and clotting cascade activation, the third important process is fibrinolysis.

Vascular injury triggers both the coagulation and fibrinolytic systems, and this is essential to maintain homeostasis.

Primary Haemostasis:

The initial physiological responses to vascular injury involve vasoconstriction and mechanical occlusion with an aggregate of platelets (the platelet plug), in addition to initiation of the coagulation system. Coagulation requires the activation of a cascade of enzymes; a biological amplification system where the few initiators sequentially activate a chain of precursor enzymes to generate thrombin. This thrombin activates circulating fibrinogen to form fibrin and this in turn enmeshes platelets at the site of injury producing a firm haemostatic plug, a blood clot, from the previous rather fragile platelet plug.

The scale of amplification is vast with one mole of initially activated enzyme generating up to 2×10^8 moles of fibrin. Local concentration of the circulating clotting factor enzymes is achieved through activation of the cascade by surface mediated reactions on expressed collagen, tissue factors (lipoproteins released from damaged cells) and by platelet factor 3 (PF3) a platelet membrane phospholipid which becomes exposed during platelet aggregation and the formation of the initial plug.

Coagulation factors are mostly either enzyme precursors (all serine proteases, hydrolysing peptide bonds with serine residues at there active site except factor XIII, a transglutaminase) or co-factors, with factor I being fibrinogen, the fibrin monomer subunit precursor.

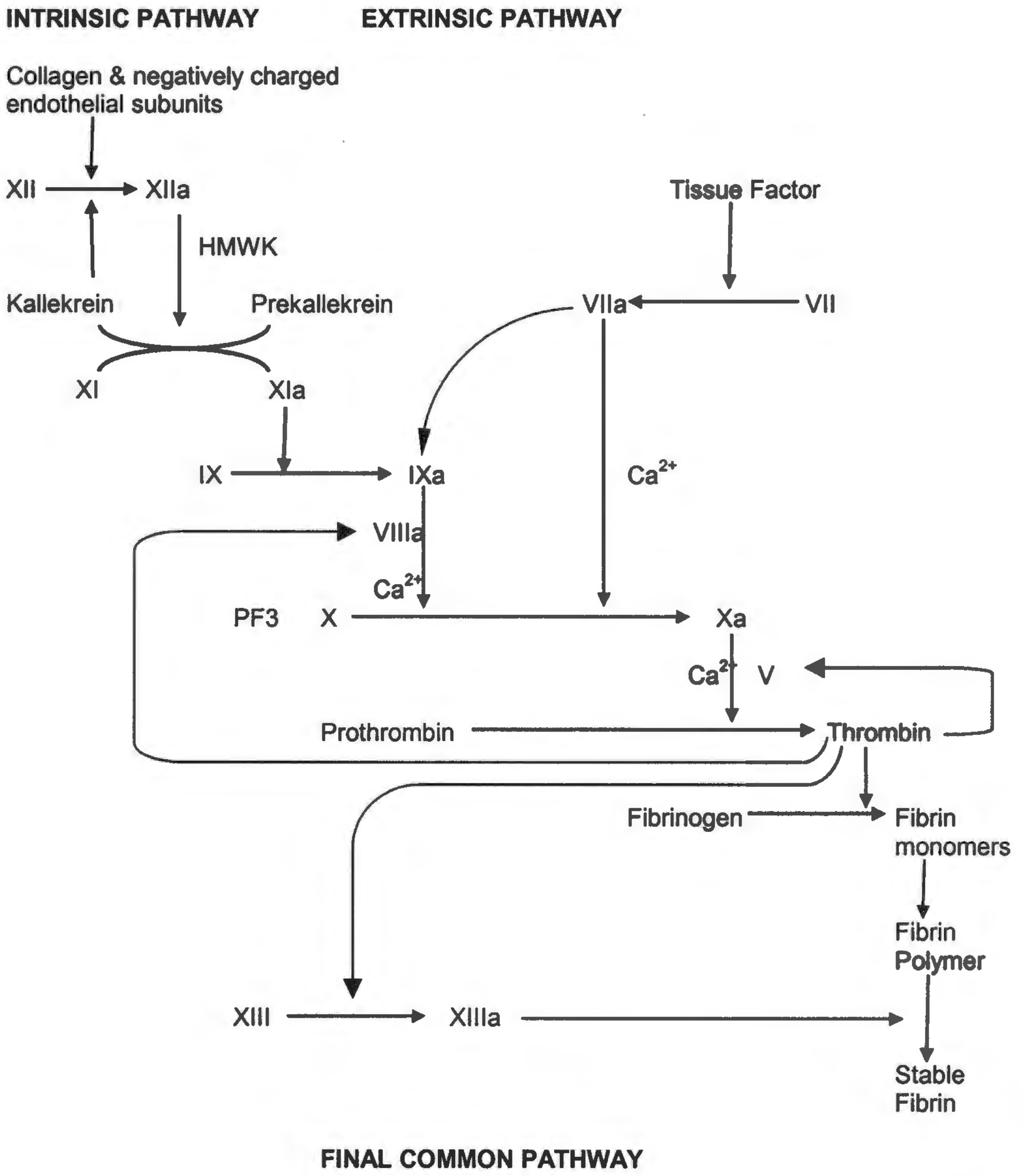
Table 1:

Factor	Name	Active Form
I	Fibrinogen	Fibrin Subunit
II	Prothrombin	Serine Protease
III	Tissue factor (lipoprotein form damaged cells)	Cofactor
IV	Calcium	Cofactor
V	Proaccelerin	Cofactor
VII	Proconvertin	Serine Protease
VIII	Von Willebrand Factor	Cofactor
IX	Christmas factor	Serine Protease
X	Stuart Prower factor	Serine Protease
XI	Plasma Thromboplastin Anticedent	Serine Protease
XII	Hageman (contact) Factor	Serine Protease
XIII	Fibrin Stabilising factor	Transglutaminase
-	Prekallekrein (Fletchers factor)	Serine Protease
-	High molecular weight kininogen	Cofactor

The Intrinsic System:

Exposed collagen and other negatively charged subunits of the damaged endothelium trigger the intrinsic system by activating factor XII. This in turn both activates factor XI and converts prekallekrein into kallekrein, the latter having an autocatalytic effect enhancing further activation of factor XII as well as an indirect vasoactive effect by binding factor XI to High Molecular Weight Kininogen (HMWK) cleaving the vasoactive peptide bradykinin from HMWK.

Figure 2: Coagulation Cascade



Index: Figure 2 provides a diagrammatic representation of the coagulation cascade to the point of stable fibrin clot formation.

Activated factor XI in turn activates factor IX, which, in association with cofactors IV & VIII activates factor X on PF3. This step in the cascade marks the start of the final common pathway where intrinsic & extrinsic systems merge to generate thrombin and, ultimately the final stable blood clot.

The Extrinsic System:

This is activated directly by lipoproteins released from damaged cells, and these activate factor VII, which in turn activates factor X and once more leads to the final common pathway.

The Final Common Pathway:

Here, factor Xa along with factors IV, V and PF3 facilitate conversion of prothrombin to thrombin which in turn both cleaves fibrin from fibrinogen and activates factor XIII. The fibrin monomers spontaneously polymerise, and the polymer is further stabilised with covalent bond cross-links in the presence of XIIIa and calcium. Fibrin enmeshes itself between platelets, anchoring them to the sites of damage. Within 24-28 hours, the platelets autolyse, transforming the plug into a solid fibrinous mass. Thrombin's role in clotting is pivotal as can be seen in figure 2, being responsible for onward conversion of fibrinogen to fibrin and thence to its stable polymer via activation of factor XIII as well as a positive feedback on cofactors VIII & V. Additionally it enhances platelet aggregation and externalisation of PF3, required for factor X activation.

From this brief discussion it will be apparent that the intrinsic and extrinsic clotting systems complement one another. Some authors prefer to see them as one system rather than two complementary pathways. The boundaries between these pathways have become less defined, with cross activation between the systems, for example tissue factor activated factor VII enhancing intrinsic coagulation. Tissue injury will directly stimulate the extrinsic system generating thrombin, which then has a positive feedback on the intrinsic system. Clinically, quite severe bleeding diatheses are seen in individuals deficient in one factor, demonstrating the need for both systems to be functional to achieve homeostasis.

Physiological Limitation of Coagulation:

In order to prevent potentially fatal thrombosis there must be a mechanism for limiting the coagulation cascade and this is provided in 3 ways:

(1) Inhibition of Activated Clotting Factors

Circulating inhibitors directly inactivate enzymes by binding with them to form high molecular weight complexes. The most potent of these is antithrombin III (whose activity is potentiated by heparin), which binds thrombin and other serine protease clotting factors, as do α_2-macroglobulin, α_2-antiplasmin and α_2-antitrypsin.

Another circulating inhibitor is the serine protease Protein C, activated by the bound complex of thrombin and endothelial cell. This destroys factors V & VIII thus preventing further thrombin formation. Protein S is also of endothelial origin and its function is to enhance the action of Protein C.

(2) Blood Flow

Part of the general physiological response to injury includes hyperaemia of the injured part. This facilitates local dilution and dispersal of activated clotting factors from the periphery of the injured site prior to fibrin formation, and these are then carried to the liver and removed by the reticulo-endothelial system.

(3) Fibrin Degradation Products (FDPs)

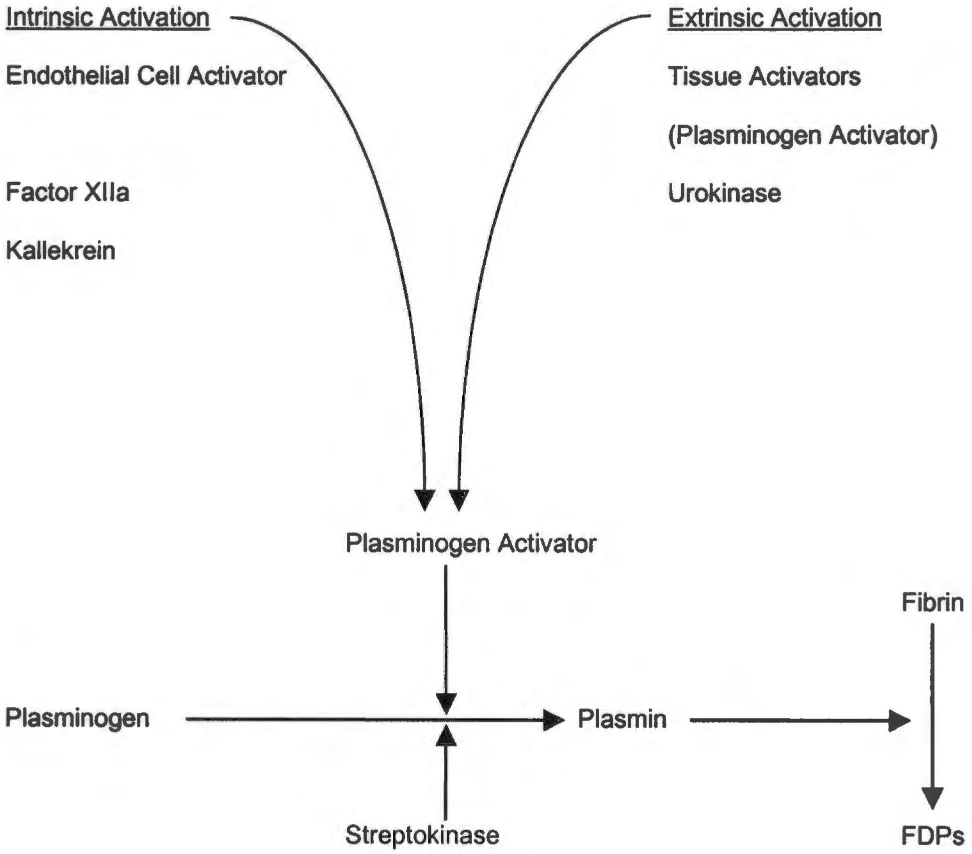
Plasmin (see below) limits the evolving clot by splitting fibrin, fibrinogen and factors V and VIII, creating split products which are, in turn, competitive inhibitors of both thrombin and fibrin polymerisation.

Fibrinolysis:

As with coagulation, fibrinolysis is a normal haemostatic response to vascular injury and may be activated intrinsically (by activators of vessel wall origin) or extrinsically (by activators from tissues). Activation produces the serine protease, plasmin, from its precursor, plasminogen, and this enzyme hydrolyses arginine and lysine peptide bonds, destroying fibrinogen, fibrin, factors V and VIII, and many other proteins. Lysed products of fibrinogen and fibrin (FDPs) themselves have an anticoagulant effect as mentioned earlier (Fig 3).

Circulating plasmin is inactivated by α_2-antiplasmin and α_2-macroglobulin; thus preventing widespread destruction of fibrinogen and clotting factors.[16]

Figure 3: Fibrinolytic Response

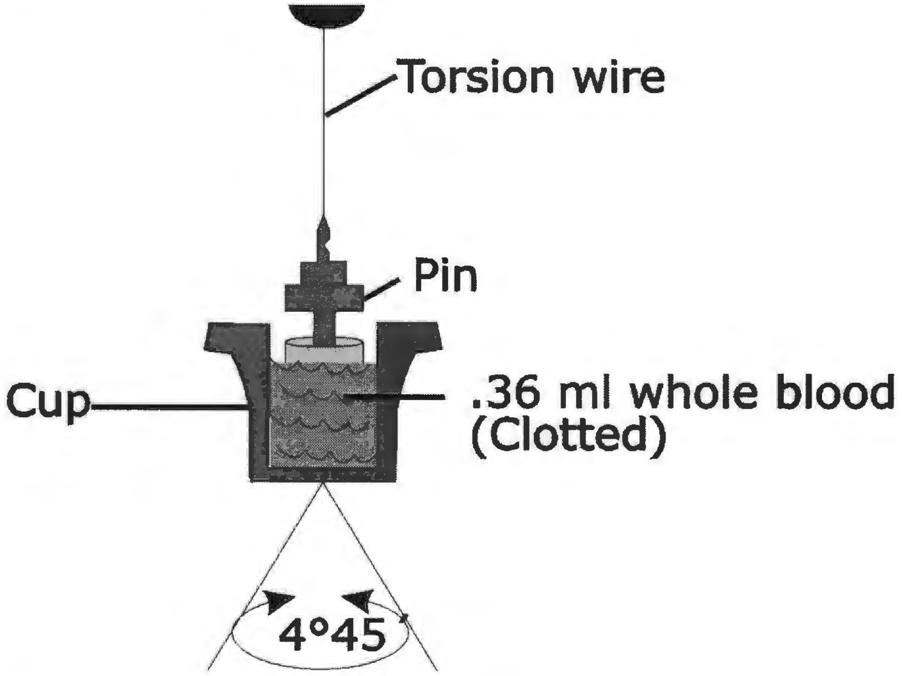


Index: Figure 3 displays the physiologic limitation of coagulation, where intrinsic and extrinsic stimulation of plasminogen occurs. The net result is increased plasmin formation, which cleaves fibrin.

Thrombelastography

The thrombelastograph® (TEG®, Haemoscope®, Skokie, IL) is enjoying a resurgence in use for point-of-care monitoring of coagulation (especially cardiac surgery[13,45] and liver transplantation[24]), in addition to its established track record for research purposes. TEG® monitoring has been associated with reduced blood product usage due to the focussed goal-directed administration of appropriate blood products or drugs during bleeding states.[44] In the research setting it has been used extensively to investigate the coagulation effects of haemodilution, both *in vitro* and *in vivo*.[32,40,42] The benefit is that it provides a quick assessment of global coagulation and the interplay of coagulation systems and pathways.[28] It is possible to perform samples on fresh whole blood, citrated blood, whole plasma, platelet-rich or platelet-poor plasma. Samples can be analysed with or without using coagulation activators, e.g. celite, kaolin, tissue factor. The disadvantages of the TEG® are that it cannot quantify the platelet-endothelial interaction, neither can it diagnose a specific factor abnormality or deficiency.

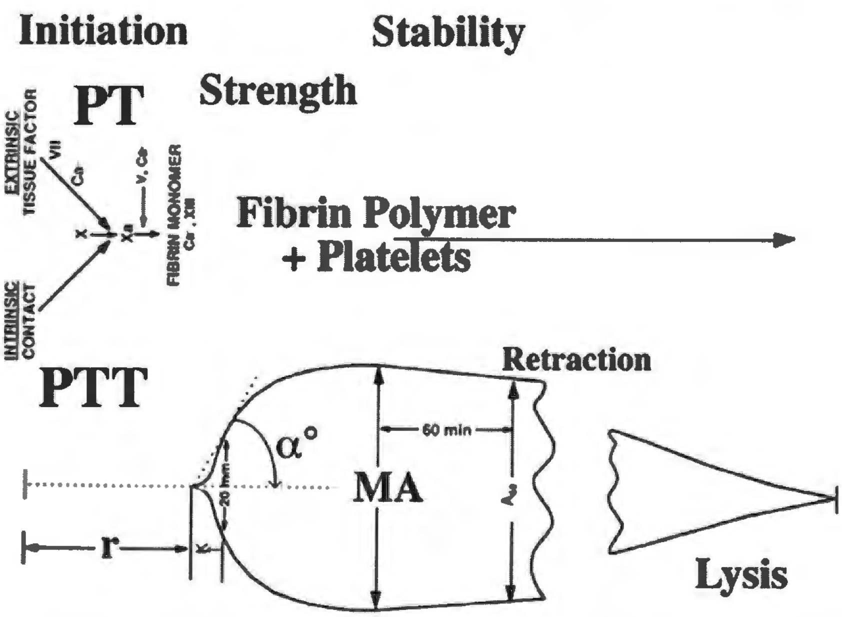
Figure 4: TEG Mechanism of Action



Reproduced with permission: © Haemoscope Corp, Skokie, IL

In performing a TEG® the machine, together with a plastic cup and pin or piston is pre-heated to 37 degrees Celsius . Native, whole blood is then drawn and inserted into the plastic cup, usually as a volume of 0.36 ml (depending on the type of TEG® to be performed) 2-6 minutes later. The blood-air interface is covered with mineral oil to prevent evaporation and increased surface tension. The TEG® is then commenced, running for a time period of 1-2 hours. The cup rotates to and fro through an arc of 4°45' over a 10 second period, with a short pause at either end to overcome kinetic forces. The pin is suspended in the blood by means of a torsion wire, which senses movement of the pin (see figure 4). The trace obtained is due to the oscillating action of the cup being transmitted to the pin as strands of fibrin form within the blood sample. The movement sensed by the torsion wire is then transmitted as a signal to the output.[28] The original machines produced a trace by the oscillating action of a heated stylus on heat-sensitive paper, while the current machines produce a computer output. These traces are then analysed by various times, measurements and angles - r time, K time, r + K time, alpha angle, maximum amplitude (MA) being the baseline measured variables. The relevance of these variables is that they each reflect a different component or relationship within the finely balanced coagulation cascade (see figure 5).

__Figure 5__: **TEG trace and correlation with coagulation pathways and tests**



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Coagulation, being such a dynamic process, balancing pro-coagulant, anticoagulant and fibrinolytic systems to give a final clot formation rate, total strength, as well as fibrinolytic profile, is very susceptible to manipulation of the balance. For example, addition of contact activation factors (e.g. celite) to a sample *in vitro* will produce an accelerated coagulation response.[5] The release of thromboplastins associated with an amniotic fluid embolus in a parturient, will strongly activate the extrinsic coagulation pathway, producing a potentially life-threatening disseminated intravascular coagulopathy (DIC).[27]

Containers

Glass is a strong contact activator, therefore blood specimens stored in non-siliconised glass tubes, albeit for a short period, may produce accelerated laboratory-based coagulation tests.[14]

It is therefore recommended that polypropylene or glass (with a siliconised internal surface) containers should be used for handling of blood samples, as unsiliconised glass containers cause contact activation of the intrinsic coagulation pathway, as well as leucocyte and platelet adhesion.[3,31] Polypropylene prevents this surface activation of factor XII (Hageman Factor),[36] and therefore also causes a minimal thrombogenic response.[26] The TEG manufacturers (Haemoscope Corp, Skokie, IL) also recommend use of polypropylene containers in handling of blood specimens. Polycarbonate surfaces are also implicated in altering coagulation, thought usually to be due to a surface activation, however, there are more issues involved than simple contact activation or inhibition. These issues will be covered in greater detail in the discussion, as it is useful to first review the trial data and results.

Study Outline:

The purpose of this observational study was to determine the TEG® coagulation effects of polypropylene and polycarbonate containers on fresh whole blood and *in vitro* haemodiluted samples. The hypothesis was that significant coagulation variability is found with different polymer containers, especially when fluids with altered ionic compositions are compared. The study was therefore performed diluting fresh whole blood by 50% with:

1. A balanced electrolyte crystalloid similar to Ringer's Lactate (Balsol® - Intramed, South Africa),
2. HES 450 kDa/0.7 in 0.9% NaCl (Sabax Hetastarch 6%® - Adcock-Ingram Critical Care, South Africa), and
3. HES 670 kDa/0.75 in a balanced electrolyte and glucose solution (Hextend® – BioTime Inc, CA).

Control, whole blood samples were also used as a reference for the dilution effects. Dilutions of 50% were chosen as a good starting point, representing severe haemodilution conditions.

The ionic compositions of the fluids used are tabulated below. Note that Balsol®, although similar to a Ringer's type solution, does not contain lactate, but rather bicarbonate, and also has no calcium content.

Electrolyte Composition (mmol/l)

Electrolyte	**Hextend®**	**Sabax Hetastarch 6%®**	**Balsol®**
Sodium	143	154	130
Chloride	124	154	109
Lactate	28	0	0
Calcium	2.5	0	0
Potassium	3	0	4
Magnesium	0.45	0	1.5
HCO_3^-	0	0	28

The hypothesis mentioned above was tested by performing two separate studies, after approval of the protocols by the UCT Human Research Ethics Committee. These studies will be described in the following two chapters.

Study 1 Methods

The study was performed using eight healthy volunteers. Exclusion criteria were individuals with a history of bleeding disorders, renal disease, hepatic disease, those who were taking coagulation-altering drugs, or anybody who had received an intravenous infusion of any type of fluid in the preceding 3 months. Informed verbal consent was obtained from all volunteers.

Blood was taken twice (90 minutes apart) by a two-syringe technique from a free-flowing forearm vein (opposite arm for second sampling) into polypropylene syringes (Curity®, South Africa) using 21g butterfly needles (Techno Med cc, South Africa). The first syringe of blood was discarded, and blood from the second syringe was deposited into a 5ml polypropylene container (Infopac, South Africa). Blood from this reservoir container was sampled by pipette for haemodilution purposes (described below). Figure 6 displays the methods and handling of the blood in Study 1.

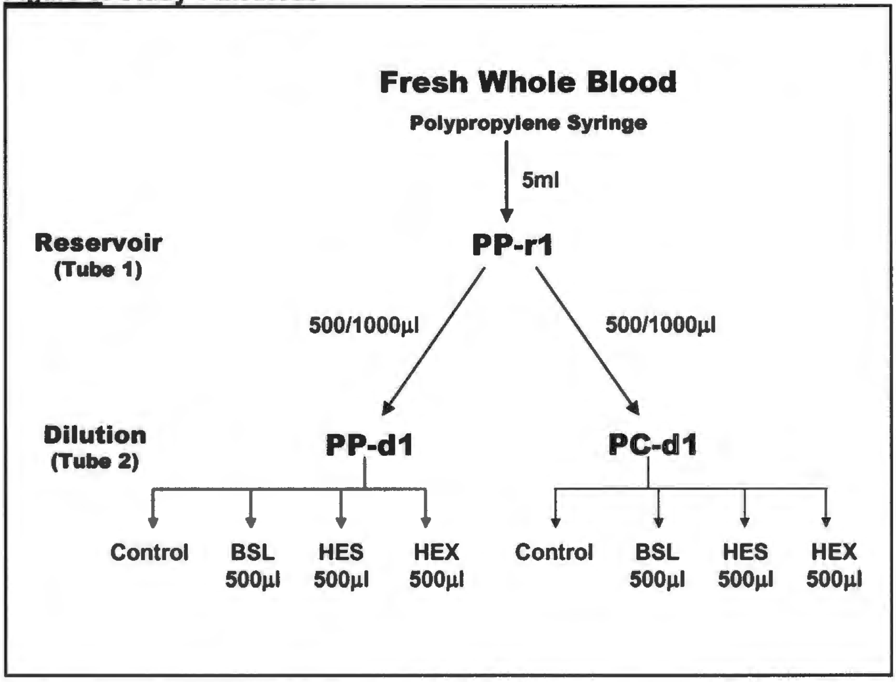
The second step, i.e. the haemodilution, was performed using four polypropylene (Infopac, South Africa) and four polycarbonate (Tool & Carbide, South Africa) tubes. All these tubes were pre-warmed in a 37°C water bath, and will be referred to as the dilution tubes. Preparation of these tubes involved placement of either 500μl test fluid (as described above) or no fluid for the control samples. From the reservoir container mentioned in the paragraph above, 500μl blood was pipetted into each dilution tube containing test fluid, or 1ml was deposited into each control tube. Therefore all dilution tubes contained a total of 1ml (100% blood, or 50% blood with 50% fluid). It follows that "Tube 2" in figure 6 was either a polypropylene or polycarbonate tube as described (PP-d1 or PC-d1).

All tubes, including control samples, were then gently inverted 8 times. Both the polypropylene and polycarbonate tubes had stoppers made of polypropylene. Thereafter 360μl was pipetted out of each of these dilution containers into a calibrated TEG® analyser (Haemoscope Corp, Skokie, IL). The TEG® machines were pre-warmed to a temperature of 37°C, and TEG® analysis was commenced at 5 minutes after venepuncture. Each TEG®

sample surface was then covered with mineral oil. All samples were pipetted into dilution tubes and TEG cuvettes using polypropylene pipette tips.

Control samples as well as all fluids (Balsol®, Sabax Hetastarch 6%®, and Hextend®) were therefore initially briefly stored in polypropylene reservoir containers (PP-r1, Figure 6), and haemodiluted thereafter in either polypropylene and polycarbonate containers (PP-d1 and PC-d1, Figure 6).

Figure 6: Study 1 Methods



Where: PP-r1 and PP-d1 = Polypropylene Tubes (Infopac, South Africa)
PC-d1 = Polycarbonate Tubes (Tool and Carbide, South Africa)
Control = Undiluted fresh whole blood samples
BSL = Balsol® 50% Dilution
HES = Sabax Hetastarch® 50% Dilution
HEX = Hextend® 50% Dilution
The "reservoir" tube was used only for initial storage of blood prior to pipetting for haemodilution purposes in the "dilution" tubes

Study 2 Methods

The second study was also performed using eight healthy volunteers. Exclusion criteria were the same as in study one. Informed verbal consent was obtained from all volunteers.

Sampling methods remained identical to study one, described as follows. Blood was taken twice (90 minutes apart) by a two-syringe technique from a free-flowing forearm vein (opposite arm for second sampling) into polypropylene syringes (Curity®, South Africa) using 21g butterfly needles (Techno Med cc, South Africa). The first syringe of blood was discarded.

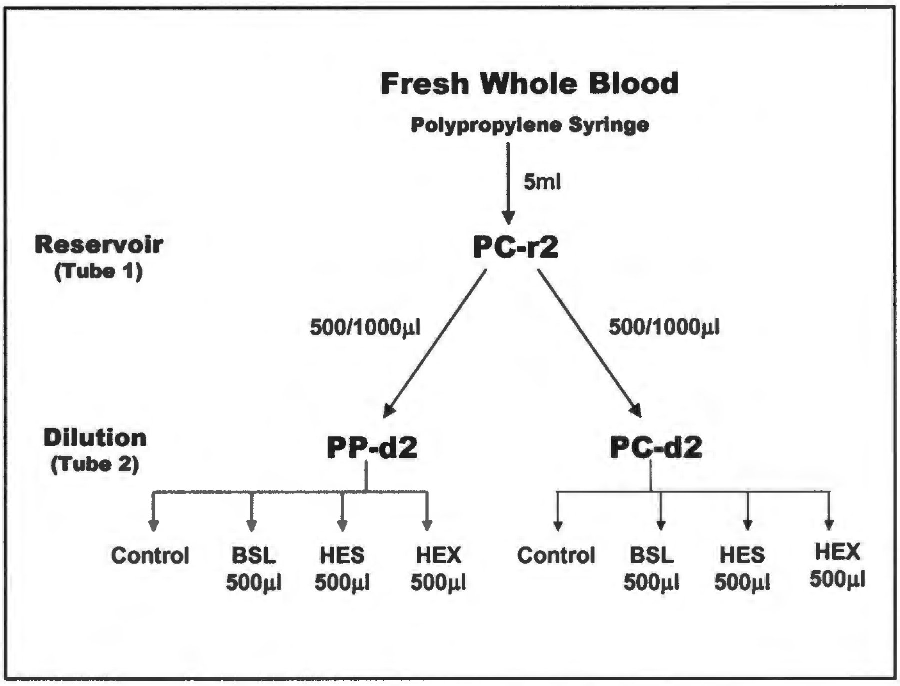
From there, handling differed by storing and diluting in different containers to study one. Blood from the second syringe was deposited into a 5ml polycarbonate container (Bibby Sterilin Ltd, Staffordshire, UK). Out of this reservoir container, it was pipetted for haemodilution purposes (described below). Figure 7 displays the methods and handling of the blood in Study 2.

The second step, i.e. the haemodilution, was performed using polypropylene (Infopac, South Africa) and polycarbonate (Pennine Healthcare, Derby, UK) tubes. Preparation of these tubes involved placement of either 500µl test fluid (as described above) or no fluid for the control samples. All these tubes were also pre-warmed in a 37°C water bath. From the reservoir container, 500µl blood was pipetted into each dilution tube containing test fluid, or 1ml was deposited into each control tube. Therefore all dilution tubes contained a total of 1ml (100% blood, or 50% blood with 50% fluid). It follows that "Tube 2" in figure 7 was either a polypropylene or polycarbonate tube as described (PP-d2 or PC-d2).

The reasons for changing both reservoir and dilution polycarbonate tubes in the second study were due to the need to replicate the exact UCL pilot study methodology.

All tubes, including control samples, were then gently inverted 8 times. Thereafter 360µl was pipetted out of each of these dilution containers into a calibrated TEG® analyser (Haemoscope Corp, Skokie, IL). The TEG® machines were pre-warmed to a temperature of 37°C, and TEG® analysis was commenced at 5 minutes after venepuncture. Each TEG® sample surface was then covered with mineral oil to prevent evaporation. As in trial 1, all samples were pipetted into dilution tubes and TEG cuvettes using polypropylene tips.

Figure 7: Study 2 Methods



Where: PP-d2 = Polypropylene Tubes (Infopac, South Africa)
PC-r2 = Polycarbonate Tubes (Bibby Sterilin Ltd, Staffordshire, UK)
PC-d2 = Polycarbonate Tubes (Pennine Healthcare, Derby, UK)
Control = Undiluted fresh whole blood samples
BSL = Balsol® 50% Dilution
HES = Sabax Hetastarch® 50% Dilution
HEX = Hextend® 50% Dilution

TEG® analysis of each sample continued for a minimum period of 90 minutes. The r-times, k-times, α-angles and maximum amplitude were measured and analysed statistically.

Study two was similar to study one, but with two important changes. The first change was using a polycarbonate container as the initial "reservoir" of the blood (undiluted blood immediately placed in these tubes) for pipetting purposes (PC-r2 in figure 7). This compares with using polypropylene in the first limb. The second change was using a separate type (i.e. manufacturer) of polycarbonate tube for haemodilution (mixing) purposes (PC-d2 in figure 7). There were thus three separate types of polycarbonate tubes used in the study as a whole. On the other hand, only one type of polypropylene container was used throughout the study.

The first change was done to assess the effects of the initial storage (albeit less than a couple of minutes) of the blood prior to pipetting. Our hypothesis for this was that the type of container used in this first step (i.e. storage prior to haemodilution) is of paramount importance to prevent any adverse effects on coagulation. The second change was purely to assess a separate type of polycarbonate container on the mixing of blood and fluid. Attempts were made, unsuccessfully, to establish what types of polycarbonate were used by the different manufacturers. It is likely, however, that small differences would be found, from types of plastic to general manufacturing process, leading to altered laboratory effects.

Statistical Analysis:

One-way ANOVA, multivariate analysis of variance and paired t-tests were used to analyse differences within each limb. Unpaired t-tests were used to analyse differences between limbs. Post hoc LSD testing was used to identify individual differences between limbs where these were demonstrated to exist by the ANOVA technique. These techniques were performed using STATISTICA® software package on an IBM compatible personal computer, running on a Windows®-based platform.

All graphs in the text are presented as means + standard deviations.

Results:

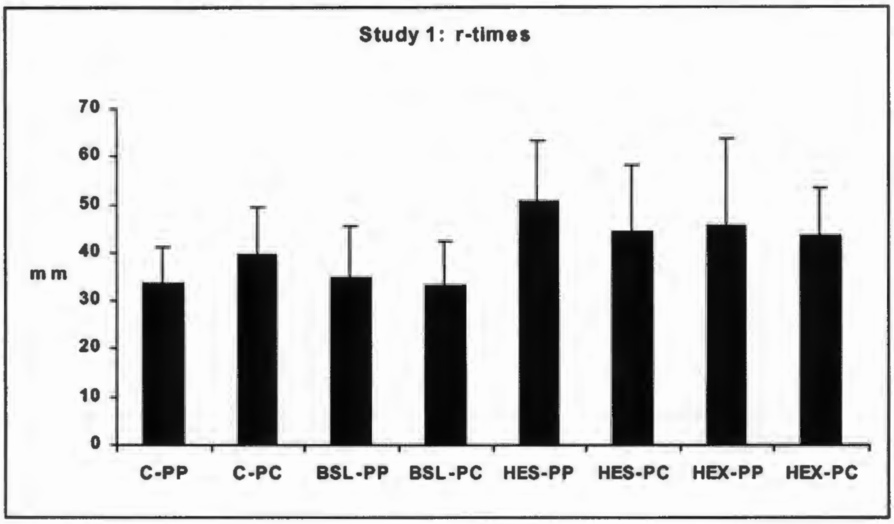
Study 1

Analysis of study 1 revealed no significant differences between the same fluids when diluted in the different plastic containers. Furthermore, there were no differences between the two starches, regardless of the container in which dilution was performed (Figs 8-11). There were also no differences in the whole blood controls.

Differences were found when comparing control samples and the crystalloid samples with the colloids. These results conform with previously described effects of crystalloid haemodilution[10,40] and will not be discussed in this thesis, as they do not add to the understanding of fluid and container effects (Figs 8-11). Briefly examining these results, it was apparent that both HES and HEX produced a hypocoagulable state (in all measured TEG variables) compared with BSL and fresh whole blood control samples. Furthermore, significant differences, reflecting a crystalloid hypercoagulability were found with k-times and α-angles when compared with whole blood controls. Thus, although there were significant coagulation differences attributable to the diluent fluid used, there were no differences attributable to the containers used for either the initial storage or for dilution.

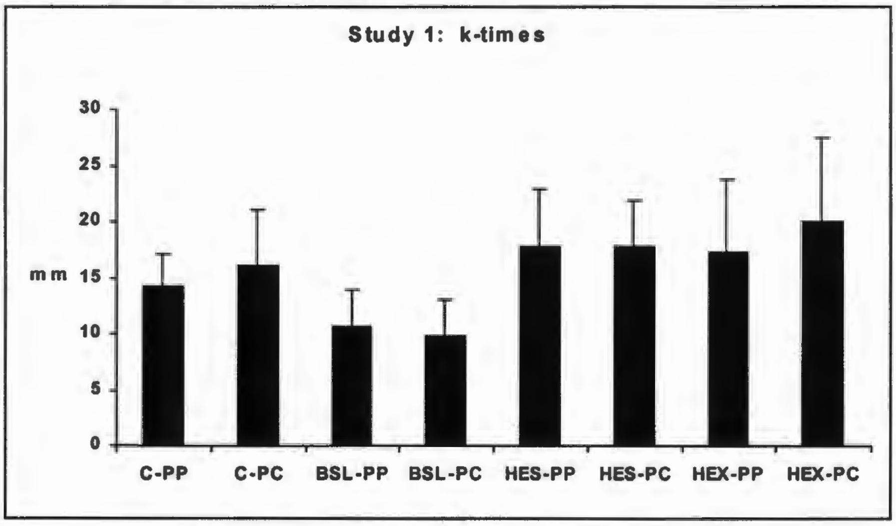
What is relevant to this thesis, is that the results of study one suggest that the paradoxical effects at the blood-container interface were not found with haemodilution using polypropylene reservoir containers and polypropylene or polycarbonate dilution tubes. These results are in stark contrast to the data generated by the UCL pilot study mentioned in the introduction. It follows, therefore, that our second study, using the same tubes as in the pilot study combined with fluids of varying composition (both ionic and crystalloid or colloid), would replicate the paradoxical effects initially observed.

Figure 8: Study 1 r-time means + standard deviations



Index: See below.

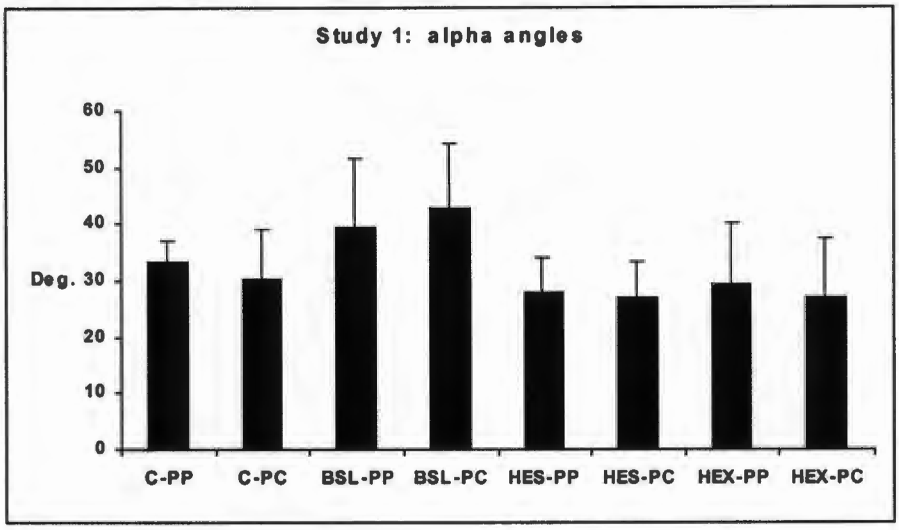
Figure 9: Study 1 k-time means + standard deviations



Figs 8-9:	All reservoir containers PP-r1 (see figure 6), but DILUTIONS subsequently performed in PP-d1 or PC-d1 tubes (Figure 6)

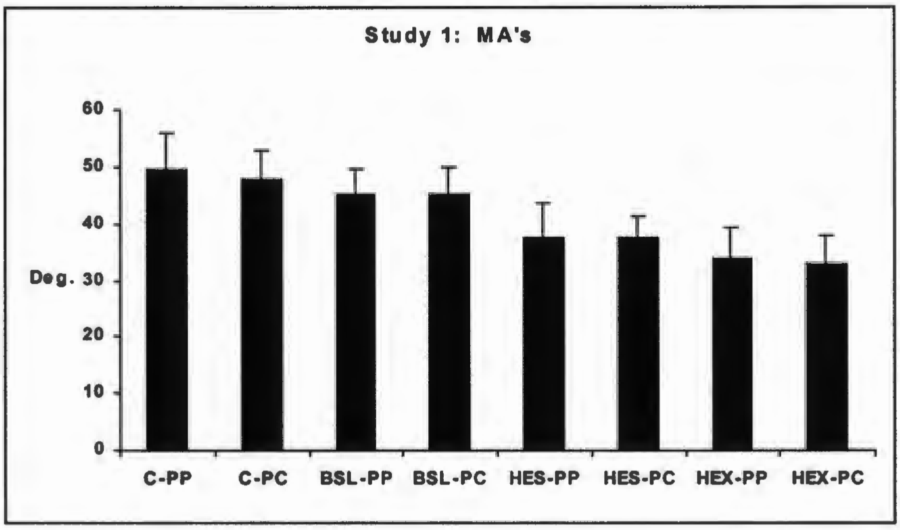
C = Undiluted fresh whole blood controls
PP = Polypropylene dilution tubes
PC = Polycarbonate dilution tubes
BSL = Balsol®
HES = Sabax Hetastarch 6%®
HEX = Hextend®

Figure 10: Study 1 alpha angle means + standard deviations



Index: See below

Figure 11: Study 1 maximum amplitude means + standard deviations



Figs 10-11:	All reservoir containers PP-r1 (see figure 6), but DILUTIONS subsequently performed in PP-d1 or PC-d1 tubes (Figure 6)

C = Undiluted fresh whole blood controls
PP = Polypropylene dilution tubes
PC = Polycarbonate dilution tubes
BSL = Balsol®
HES = Sabax Hetastarch 6%®
HEX = Hextend®

Study 2

The statistically significant results of Study 2 are summarised in Table 2.

Significant differences were already seen between the undiluted control samples placed in either polypropylene or polycarbonate dilution tubes in study 2. The samples were all first placed in a polycarbonate (PC-r2, figure 7) reservoir prior to pipetting into the dilution tubes (PP-d2, and PC-d2, figure 7). The samples placed in polypropylene dilution tubes (PP-d2) displayed shorter k-times, enhanced alpha angles, and enhanced maximum amplitudes when compared with those samples placed in polycarbonate dilution tubes (PC-d2, figure 7).

HES diluted in polypropylene displayed prolonged r-times to HES diluted in polycarbonate, as well as to HEX diluted in polypropylene.

HEX diluted in polypropylene displayed shorter k-times than HEX in polycarbonate dilution tubes.

Table 2: Study Limb 2 Results

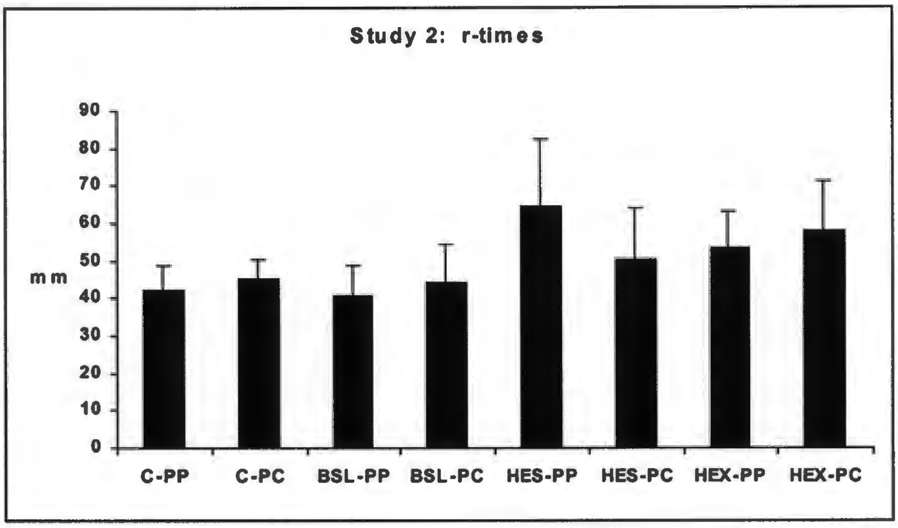
r-time:	HES-PP > HES-PC	$p<0.05$
	HES-PP > HEX-PP	$p<0.05$
k-time:	Control-PP < Control-PC	$p<0.01$
	HEX-PP < HEX-PC	$p=0.01$
α-angle:	Control-PP > Control-PC	$p=0.02$
MA:	Control-PP > Control-PC	$p=0.01$

Where:

PP=Polypropylene
PC=Polycarbonate
Fluid references as in Table 1
p-values as quoted

The graphical representation of the results of study 2 is found in figures 12-15.

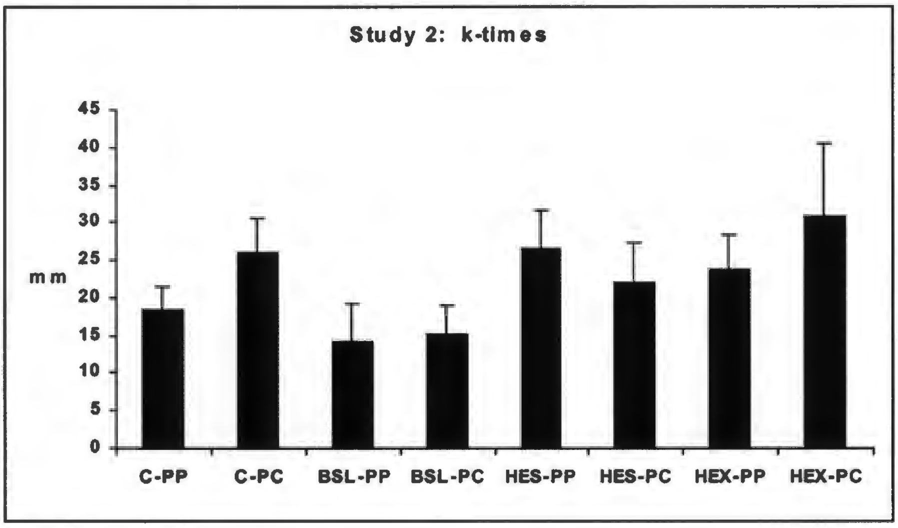
Figure 12: Study 2 r-time means + standard deviations



Where:

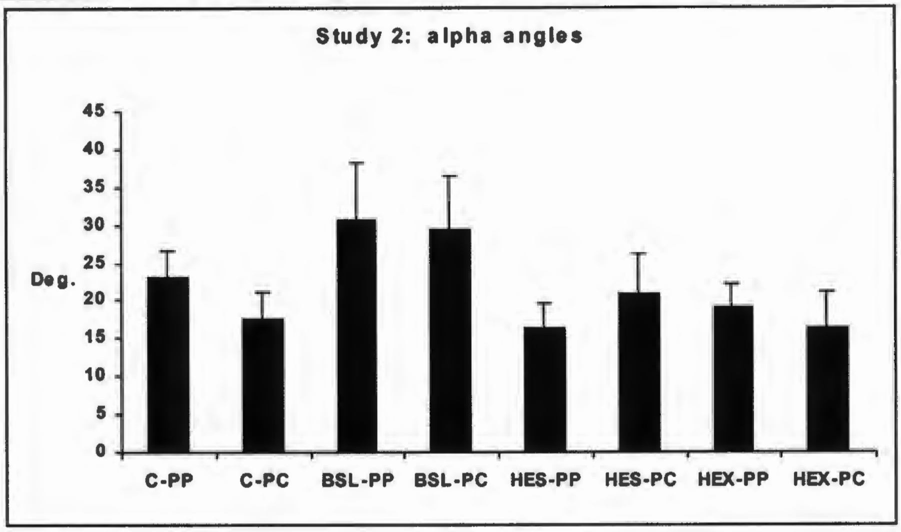
PP=Polypropylene
PC=Polycarbonate
Fluid references as in Table 1
p-values as quoted
r-time: HES-PP > HES-PC $p<0.05$
HES-PP > HEX-PP $p<0.05$

Figure 13: Study 2 k-time means + standard deviations



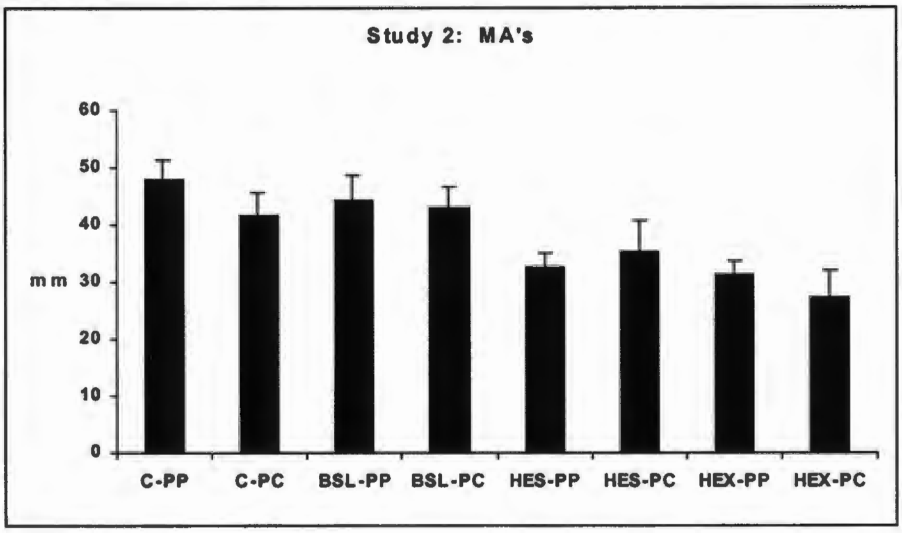
Figs 12-13: All reservoir containers Pc-r2 (see figure 7), but DILUTIONS subsequently performed in PP-d2 or PC-d2 tubes (Figure 7)
C = Undiluted fresh whole blood controls
PP = Polypropylene dilution tubes
PC = Polycarbonate dilution tubes
BSL = Balsol®
HES = Sabax Hetastarch 6%®
HEX = Hextend®
k-time: Control-PP < Control-PC $p<0.01$
HEX-PP < HEX-PC $p=0.01$

Figure 14: Study 2 alpha angle means + standard deviations



α-angle: Control-PP > Control-PC p=0.02

Figure 15: Study 2 maximum amplitude means + standard deviations



Figs 14-15: All reservoir containers Pc-r2 (see figure 7), but DILUTIONS subsequently performed in PP-d2 or PC-d2 tubes (Figure 7)

C = Undiluted fresh whole blood controls
PP = Polypropylene dilution tubes
PC = Polycarbonate dilution tubes
BSL = Balsol®
HES = Sabax Hetastarch 6%®
HEX = Hextend®
MA: Control-PP > Control-PC p=0.01

Reservoir Effects

Initial grouping of all samples (i.e. all fluids and controls) diluted in polypropylene containers only in both studies (i.e. PP-d1 in figure 6, and PP-d2 in figure 7, not PC tubes) showed a shortened r-time (p=0.01), shortened k-time (p=0.0002), and raised α-angle (p<0.0001) in Study 1 when compared with samples stored in polycarbonate reservoir tubes in Study 2. In other words, there was a longer time to initiation, as well as a reduced rate of clot formation in Study 2, regardless of haemodilution. No difference was found in the grouped MA values. The only methodologic difference examined between studies, using this type of grouping analysis, was the initial "reservoir" container used. The samples diluted in polycarbonate containers in both studies (PC-d1 and PC-d2) were thus ignored in this analysis.

<u>Table 3</u>: Comparison of Reservoir Container Effects

r-times:	HES (S1) < HES (S2)	p=0.025
	HEX (S1) < HES (S2)	p=0.002
k-times:	HES (S1) < HES (S2)	p=0.0002
	HES (S1) < HEX (S2)	p=0.01
	HEX (S1) < HES (S2)	p=0.0001
	HEX (S1) < HEX (S2)	p=0.006
α-angles:	Control (S1) > Control (S2)	p=0.006
	BSL (S1) > BSL (S2)	p=0.01
	HES (S1) > HES (S2)	p=0.002
	HES (S1) > HEX (S2)	p=0.01
	HEX (S1) > HES (S2)	p=0.0006
	HEX (S1) > HEX (S2)	p=0.005
MA:	HES (S1) > HES (S2)	p<0.05
	HES (S1) > HEX (S2)	p=0.01

Where:

S1=Study 1
S2=Study 2
Control = Undiluted fresh whole blood
BSL = Balsol®
HES = Sabax Hetastarch 6%®
HEX = Hextend®
p-values as quoted

Table 3 above shows the statistically significant differences between the various control and fluid diluted samples where the blood was initially placed into a polypropylene (S1 = Study 1) or polycarbonate (S2 = Study 2) reservoir container and subsequently haemodiluted in polypropylene containers only.

Both HES and HEX in study 1 produced significantly shorter r-times than HES in study 2. A similar picture was seen with the k-times, where HES and HEX in study 1 produced significantly shorter k-times to both HES and HEX in study 2.

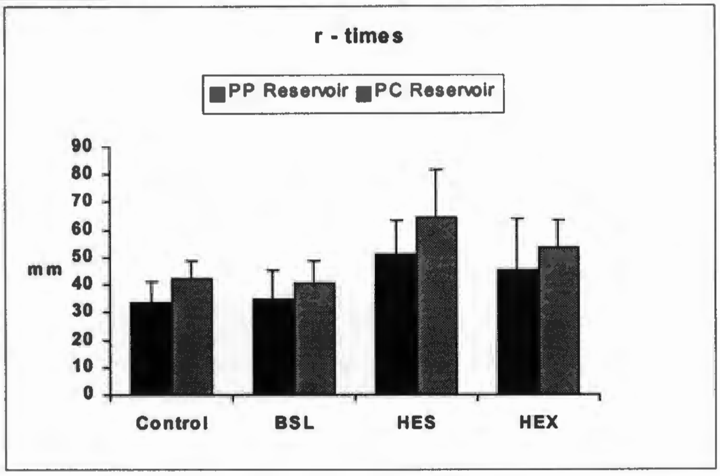
Undiluted control samples had increased alpha angles in study 1 compared with the controls in study 2. BSL also displayed raised alpha angles to itself from study 1 to study 2. Once again, HES and HEX produced significant differences across studies, where they both had raised alpha angles compared to HES and HEX in study 2.

Finally, HES in study 1 had higher maximum amplitudes compared with HES and HEX in study 2.

Thus, the use of polycarbonate containers for initial storage of blood samples resulted in some degree of inhibition of the TEG® regardless of dilution, or the type of diluent used.

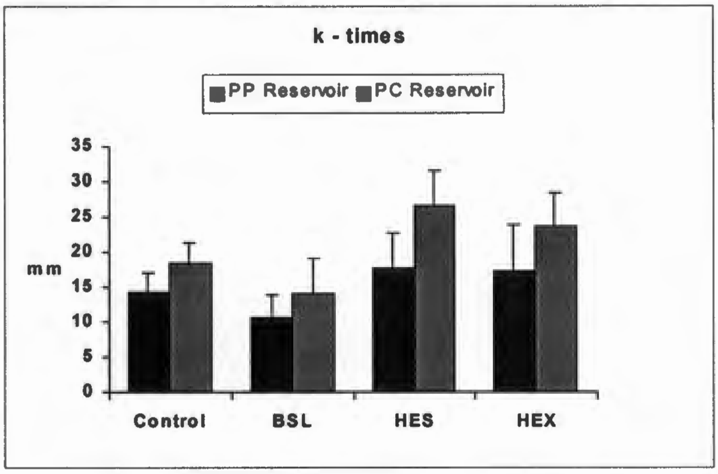
See figures 16-19 for graphical representation of these data comparing reservoir effects. P-values are not quoted in the figures, as they are available in table 3.

Figure 16: r-times Study 1 v Study 2 Reservoir Effects



r-times: HES (S1) < HES (S2) p=0.025
HEX (S1) < HES (S2) p=0.002

Figure 17: k-times Study1 v Study 2 Reservoir Effects

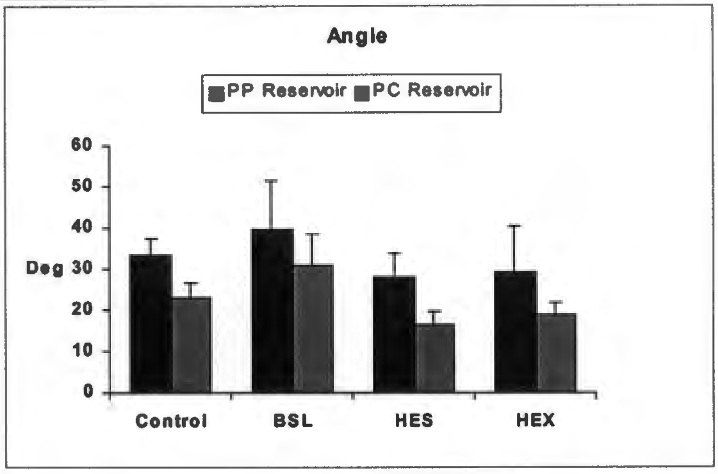


Figs 16-17: Reservoir containers PP-r1 or PC-r2, but all DILUTIONS subsequently performed only in PP (PP-d1 or PP-d2, figures 6 and 7).

Control = Undiluted fresh whole blood
PP = Polypropylene
PC = Polycarbonate
BSL = Balsol®
HES = Sabax Hetastarch 6%®
HEX = Hextend®

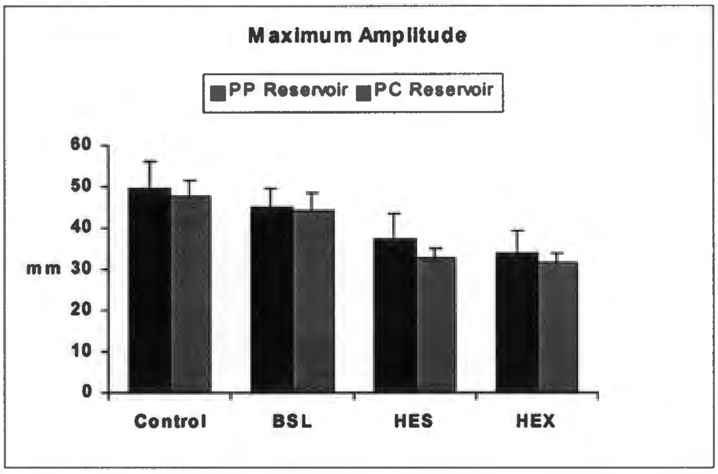
k-times: HES (S1) < HES (S2) p=0.0002
HES (S1) < HEX (S2) p=0.01
HEX (S1) < HES (S2) p=0.0001
HEX (S1) < HEX (S2) p=0.006

Figure 18: **Alpha Angles Study1 v Study 2 Reservoir Effects**



α-angles:	Control (S1) > Control (S2)	p=0.006
	BSL (S1) > BSL (S2)	p=0.01
	HES (S1) > HES (S2)	p=0.002
	HES (S1) > HEX (S2)	p=0.01
	HEX (S1) > HES (S2)	p=0.0006
	HEX (S1) > HEX (S2)	p=0.005

Figure 19: **Maximum Amplitudes Study1 v Study 2 Reservoir Effects**



Figs 18-19: Reservoir containers PP-r1 or PC-r2, but all DILUTIONS subsequently performed only in PP (PP-d1 or PP-d2, figures 6 and 7).

Control = Undiluted fresh whole blood
PP = Polypropylene
PC = Polycarbonate
BSL = Balsol®
HES = Sabax Hetastarch 6%®
HEX = Hextend®

MA:	HES (S1) > HES (S2)	p<0.05
	HES (S1) > HEX (S2)	p=0.01

All Polypropylene versus All Polycarbonate

When examining results of samples initially stored and diluted in only polypropylene (from Study 1) or only polycarbonate (Study 2) containers, the following results were found. Control whole blood samples in polypropylene revealed consistently shorter r-times and k-times, as well as increased α-angles and maximum amplitudes than those in polycarbonate ($p<0.01$). Balsol® (BSL) in polypropylene only produced a significantly shorter k-time ($p=0.02$), but had strong trends in r-times ($p=0.1$) and α-angles ($p=0.06$) compared to polycarbonate handling. HES produced a raised mean α-angle in polypropylene ($p<0.03$), whereas HEX produced shorter k-times, raised α-angles and MA values in the polypropylene versus the polycarbonate group.

There were no significant differences between HES and HEX when blood was stored and diluted in polypropylene containers. On the other hand, k-times were shorter ($p<0.05$) and MA values were raised ($p<0.01$) in HES versus HEX in polycarbonate containers.

When comparing the starches with control samples, in the polypropylene group, the r-times were prolonged ($p\leq0.05$), and MA's were lower ($p<0.001$), with HES and HEX. When samples were stored and diluted in polycarbonate tubes, prolonged r-times ($p<0.025$) were found with HEX, and the MA's were less with HES as well as HEX ($p<0.002$), versus control. These results concur with those found in the UCL pilot study, where decreased coagulation was found with Hextend® dilution (versus the saline based hetastarch solution) after the initial storage of fresh blood in polycarbonate reservoirs.

The differences of performance of crystalloid and starch solutions provided, in some circumstances, paradoxical results when containers were changed. When comparing results with BSL (in PP), the r-times of HES in polypropylene were prolonged ($p<0.01$), whereas only HEX in polycarbonate (and not HES) had significantly longer r-times than BSL polycarbonate ($p<0.01$). HES and HEX produced consistently prolonged k-times, as well as raised α-angles compared with BSL in polypropylene and polycarbonate containers ($p\leq0.05$). MA's were reduced in both HES and HEX versus BSL in both container types ($p\leq0.005$).

See figures 20-23 for graphical representation of all polypropylene or all polycarbonate handling of samples.

Figure 20: Samples Stored and Diluted in ALL PP or ALL PC Tubes

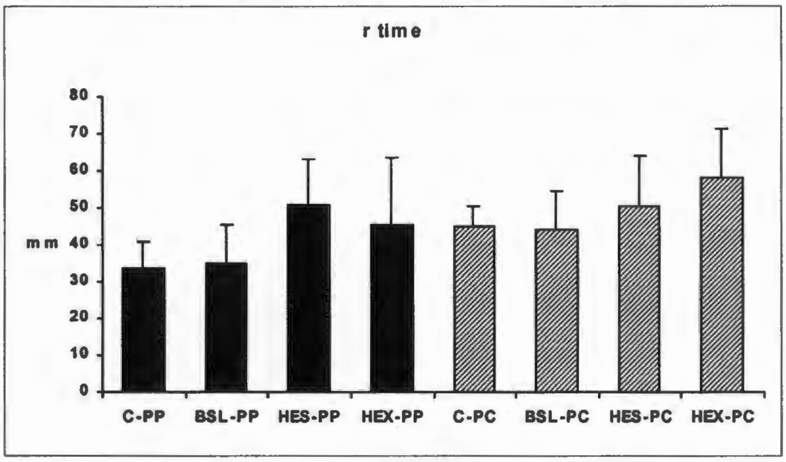
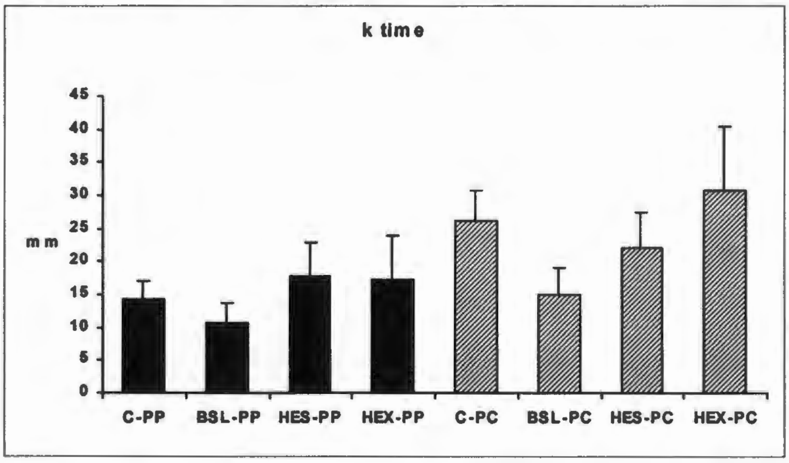


Figure 21: Samples Stored and Diluted in ALL PP or ALL PC Tubes



Figs 20-21:

PP = All Polypropylene – solid bars on graph
PC = All Polycarbonate – diagonal stripes on graph
Control = Undiluted fresh whole blood
BSL = Balsol®
HES = Sabax Hetastarch 6%®
HEX = Hextend®

Figure 22: Samples Stored and Diluted in ALL PP or ALL PC Tubes

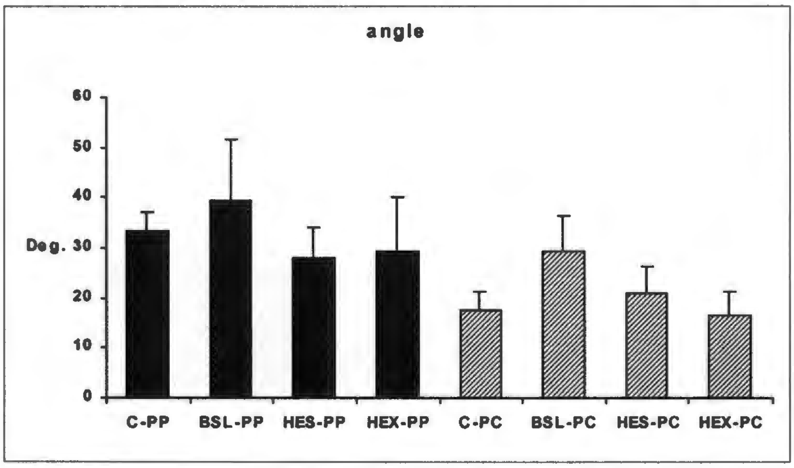
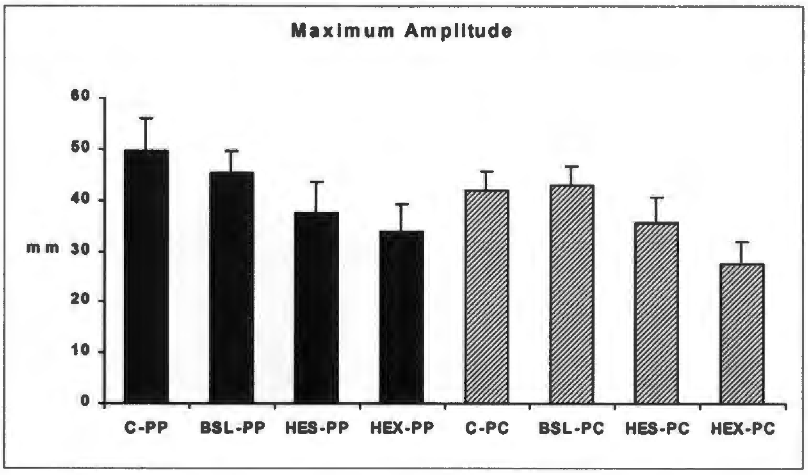


Figure 23: Samples Stored and Diluted in ALL PP or ALL PC Tubes



Figs 22-23:

PP = All Polypropylene – solid bars on graph
PC = All Polycarbonate – diagonal stripes on graph
Control = Undiluted fresh whole blood
BSL = Balsol®
HES = Sabax Hetastarch 6%®
HEX = Hextend®

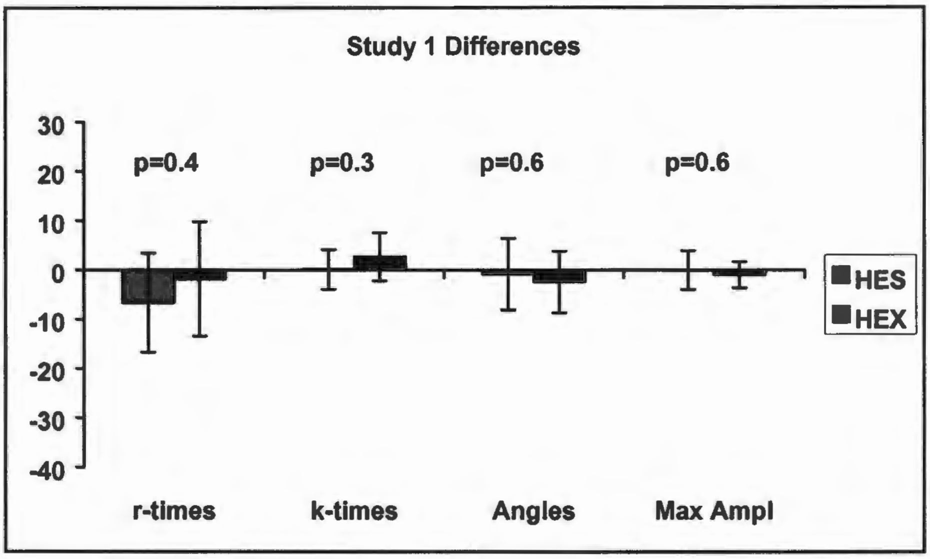
Further graphical representation of all results of both studies can be seen in figures 24-27 (appendix 1).

Polypropylene – Polycarbonate Differences (Study 2)

If one closely examines the graphs of the Study 2 hetastarch fluid results, a fascinating picture emerges (figs 12-15). Firstly HES displays an improved coagulation profile when dilution is performed in polycarbonate dilution tubes ("HES-PC"), relative to being diluted in polypropylene tubes ("HES-PP"), whereas HEX displays a completely opposite profile, reflecting a less coagulable picture in polycarbonate ("HEX-PC") relative to polypropylene ("HEX-PP"). Therefore, a separate analysis was performed of the differences encountered in TEG variables between the same fluids diluted in the two different types of dilution tubes (PP-d1, PP-d2 or PC-d1, PC-d2) in both studies. In other words, the TEG variables of HES diluted in polypropylene were subtracted from those of HES diluted in polycarbonate for each study. The same was done for HEX. The results of these differences were then analysed by t-tests for HES versus HEX.

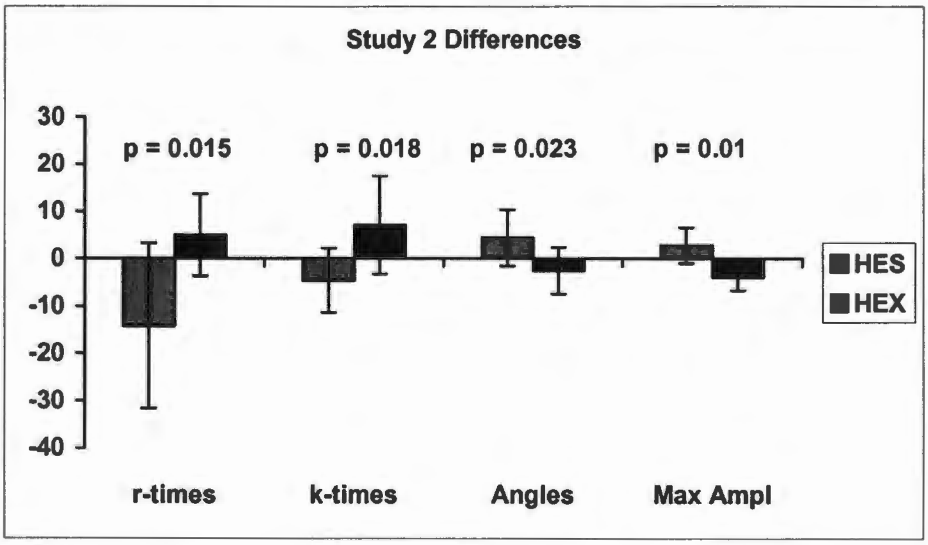
There were no significant changes attributable to the diluent tube used in study 1 between the two fluids, see figure 24 below. Such differences as there were, were all in the same direction. The alteration in all TEG measures attributable to the container in which dilution was performed in study 2 was significantly different between HES and HEX, see figure 25 below. In each case, the use of this specific polycarbonate container enhanced coagulation in the HES diluted samples, and inhibited coagulation in the HEX diluted samples.

Figure 24. Changes in TEG measures due to the diluent container, Study 1.



Index: Figure 24 displays no difference due to diluent container and hetastarch fluid tested in study 1. This is in contrast to that found in study 2, figure 25 below.

Figure 25. Changes in TEG measures due to the diluent container, Study 2.



Index: These data reflect the differences between the results of HES in PP-d2 subtracted from HES in PC-d2, and HEX in PP-d2 subtracted from HEX in PC-d2. P-values of all TEG variables for HES versus HEX are significant.

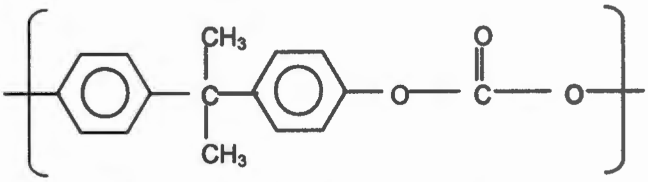
Discussion

Exposure of blood to foreign surfaces is commonplace in clinical and investigative medical practice. Numerous events occur at exposure to these surfaces, be it *in vitro* or *in vivo.* Protein adsorption, coagulation system activation, platelet adsorption, aggregation and activation, leucocyte adhesion, complement activation, as well as stimulation of inflammatory mediators are all known to play a part in this response. The type and extent of this response is highly dependent on many characteristics of the foreign surface being exposed, as well as the blood itself. Much research is being devoted to finding an ideal polymer or surface for implantable use, haemodialysis filtration, cardiopulmonary bypass materials, as well as for the *in vitro* handling of blood, and at the same time causing minimal host inflammatory or coagulation response.

Standardisation of blood sampling and handling thereafter is important in producing reliable, reproducible results. Within an institution, where own reference values are determined and used, it is especially relevant to follow consistent methods for the handling of samples. Where methods and techniques vary between institutions and laboratories for the same investigations, caution must be exercised when results are compared out of the context of their own reference limits.[38] Coagulation is a finely balanced system of pro-coagulation, anticoagulation and fibrinolysis.[22] Without the presence of an intact, working endothelium when blood is sampled for coagulation testing, a vital system in haemostatic control is lost. Correct handling of this blood is essential for reliable laboratory and near-patient assessments to be made. Contact activation by use of a non-siliconised glass tube, for example, will cause activation of the intrinsic pathway, and possibly even the extrinsic pathway.[14] This would thereby produce false results in many tests of coagulation. TEG® is highly susceptible to these effects.

Many of us may be lulled into a false sense of security in believing TEG® is a reliable, robust test in almost all situations. The reality is not far from this, as long as certain precautions and care are taken when using TEG® both for research as well as in the clinical setting. Blood may incorrectly be placed in the nearest tube available for TEG® analysis, this often being of a polycarbonate material. We have shown that this could skew results, leading to incorrect interpretation and actions.

Polycarbonate tubes (usually clear, hard, screw-top plastic tubes) are widely used in medicine. Polycarbonate is a strong, hard, amorphous thermoplastic.[37] Its uses include urine collection for microscopy and culture, small pathology samples, as well as tissues and body fluids for microbiologic investigation. It is probably the most commonly found type of plastic tube we would find on the wards, as well as in theatre. This is why it may commonly be incorrectly used for initial non-citrated storage of fresh whole blood prior to near-patient testing of coagulation.



Polycarbonate generalised chemical structure[1]

The results of our study casts new, interesting light on the issue of blood handling prior to coagulation assessment in haemodilution studies. Not only does the material of the container used for initial storage of blood affect results, but also the container material in which it is haemodiluted. There are significant differences between the same fluids (mostly hydroxyethyl starch preparations) when haemodilution was performed in either polypropylene or polycarbonate tubes. Differences were also found in fresh whole blood control samples when different containers were used.

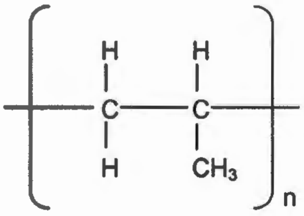
These data show, amongst others, that simply using a polycarbonate tube (in place of a polypropylene tube) for storage of blood prior to haemodilution and TEG® analysis, causes a significantly slower time to clot formation, as well as impairing the rate of clot formation. This is found when all samples were grouped, as well as by individual comparisons of controls, and with various fluid dilutions (especially with hetastarch solutions). We have therefore shown that the use of polycarbonate tubes just for initial storage of blood prior to haemodilution reproduces the anomaly found in the UCL pilot study. The interaction between this different blood-material interface, a physiologic electrolyte fluid composition, as well as the presence of hetastarch (as in Hextend®), produces an impaired TEG coagulation profile. This picture was not observed using polypropylene reservoir containers.

Furthermore, individual differences in the coagulation effects of specific fluids, as well as between fluids of the same group are apparent. Some of these differences are highly significant, suggesting once again that certain polycarbonate surfaces adversely affect coagulation. For example, HES in Study 1 reflects a picture of faster clot formation times and rates, as well as greater total clot strength than the same HES in Study 2. There are also paradoxical differences between two different types of hetastarch solutions in the two limbs (see results listed above). HEX, stored and diluted in polypropylene produces less derangement of especially r-times than HES also stored and diluted in polypropylene. On the other hand, HES in polycarbonate produces less derangement of r-times than HEX treated in the same way. It is known that different groups of fluids produce characteristic coagulation effects, and that even within groups of fluids, coagulability is not necessarily the same.[10,46,50]

What remains interesting in our data is the absence of paradoxical effects with the polycarbonate dilution tubes (Tool and Carbide, South Africa) in study 1. This may be due to the initial storage of blood in polypropylene reservoir containers, but it may also be due to the polycarbonate dilution tubes being of a different nature and composition to those used in the pilot study (and study 2 – UK manufacturers). This remains for further investigation.

The use of polypropylene pipette tips could add an extra dimension to the trials, considering the importance of which material was used for storage and dilution of blood. On the other hand, all the samples were handled in exactly the same way from a pipetting point-of-view (all were pipetted using polypropylene tips). One would expect the resultant effect to be similar for all samples. Also, from our methods, it is clear that initial storage of blood in polycarbonate produces interesting, and sometimes paradoxical coagulation responses. It is likely, therefore, that minimal effect on final TEG coagulation *differences* could be attributed to the pipette tips used. If polycarbonate tips were used, this may have revealed a very different picture, and possibly even with glass tips producing a strong contact activation.

Previous work into biocompatibility implicates leucocyte and platelet adhesion and activation on bio-incompatible surfaces. This starts almost instantaneously, when blood is exposed to a foreign surface. The type of surface, texture and charge, play very important roles in this response seen. This then translates into varied coagulation effects if whole blood coagulation is measured – the direction and extent of response attributable to the factors mentioned. Further work revealed that different inflammatory mediators are released during blood coagulation in contact with different materials.[33] That study showed that complement activation was raised after blood exposure to Teflon and silicon-coated glass, a picture not seen to the same degree with polypropylene or polyurethane. Altered leucocyte reactivity was found in glass versus polypropylene and polyurethane surfaces. Inflammatory mediator release was not uniform across different materials tested. Other work interestingly shows that unsiliconised glass surfaces not only activate coagulation, but also enhance fibrinolysis by plasmin activation.[30] The type of glass used is probably of less importance.[25]



$$\left[-\overset{H}{\underset{H}{C}}-\overset{H}{\underset{CH_3}{C}}- \right]_n$$

Polypropylene basic structure[1]

Polypropylene surfaces are non-wettable (i.e. hydrophobic), and minimal contact- and platelet activation occurs on polypropylene surfaces,[36] as briefly alluded to in the introduction. It is also a long-lasting implantable polymer, used commonly in non-resorbable sutures, with minimal resultant inflammatory response.[20] Polycarbonate has a highly polar, negatively charged, wettable (hydrophilic) surface. Besides its use in medical containers and tubes, it is widely used in haemodialysis filters, as well as in blood oxygenators.

Plastics become electrically charged during the manufacturing process, forming electrets on the surface. These electrets are analogous to magnets.[20] This surface charge of the container may be responsible for so-called specific ion adsorption, where certain ions are adsorbed out of solution by the container, providing an altered surface *mileu.*[43] The surface charge is also responsible for the formation of the electric double-layer, which leads to adsorption and accumulation of counter-ions at the interfacial surface. These complex electrokinetic forces can enhance or inhibit platelet and coagulation effects, depending on specific ion effects.[8] The dielectric strengths (the electric potentials at the interface) of polymers are therefore commonly cited in describing their surface properties.[12,18] It is known that a negative surface charge of a container partially resembles the negative charge of vascular endothelium, resulting in an inhibition of coagulation.[37]

Hydrophilic and hydrophobic surfaces selectively adsorb different proteins, also affecting the platelet and coagulation responses. Initial fibrin monolayer formation mediates platelet adhesion, whereas albumin-coated surfaces reduce platelet adhesion.[15] Nygren and colleagues found that, not only are proteins more highly bound to hydrophobic than hydrophilic glass surfaces, but platelets adhere and aggregate more readily on hydrophobic glass surfaces. These platelets displayed morphologic activation (pseudopodia, spreading aggregation) in addition to the adhesion mentioned on the hydrophobic surfaces, whereas, on the hydrophilic surfaces, only adhesion of single platelets were seen, not displaying any aggregation or activation. On the other hand, enzymatically active kallikrein was more highly adsorbed onto hydrophilic surfaces than hydrophobic ones.[34] Proteins adhere to the surface of most polymers within 3 minutes. It has been shown that a good microdomain is one where hydrophilic and hydrophobic surfaces are tightly alternated (such as in block co-polymers), leading to differential protein adsorption, with albumin adhering to hydrophilic microdomains, and fibrinogen and γ-globulin selectively adhering to hydrophobic microdomains. Inhibition of platelet adhesion and/or activation follows, depending on the polymer composition. If these co-polymers are separated into their individual components, the same effect is not observed.[35]

Contact angle, interfacial free energy, critical surface tensions (or "wettability"), and the optimum polar/apolar ratio hypotheses, as well as porosity and texture of the surface, are known to have complex and dynamic effects on coagulation.[20] Various combinations are therefore responsible for the wide variation in coagulation response seen.

Surface energy is a measure of the extent to which bonds are unsatisfied at the surface of a material. A surface energy is created by an asymmetric attraction of atoms into the bulk of the material, resulting in a net depletion of atoms on the surface, thereby leading to a surface tension. Interfacial free energy is analogous to surface free energy, which determines if a liquid placed on a solid or other surface will disperse over the whole surface, or if it will remain as a droplet. This effect is dependent on the interfacial free energies of the two substances.[20]

At equilibrium, the contact angle equation describes the relationship between interfacial free energies of two substances and the angle formed by a droplet on a flat surface. For a liquid on a solid surface, the equation is:

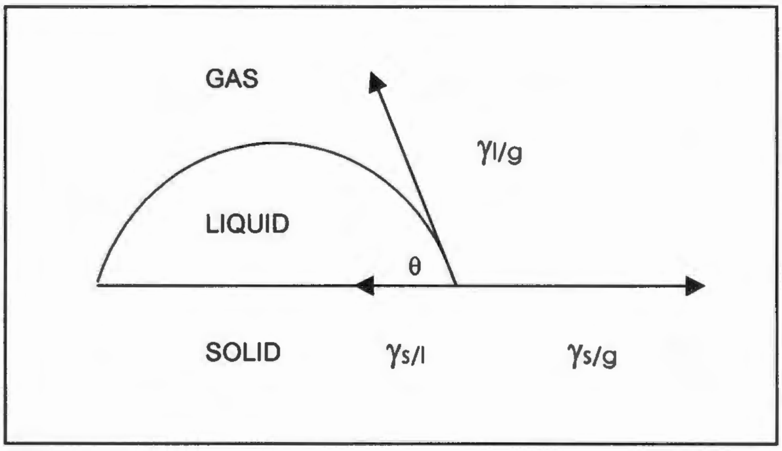
$$\gamma_{s/g} = \gamma_{s/l} + \gamma_{l/g} \cos \theta$$

where $\gamma_{s/g}$ is the interfacial free energy between the solid and gas

$\gamma_{s/l}$ is the interfacial free energy between the solid and the liquid, and

$\gamma_{l/g}$ is the interfacial free energy between the liquid and gas

θ is the contact angle. See diagram below.



It follows that different materials have different contact angles (e.g. Soda-lime-silica glass = 0° and Teflon® = 110°). The higher the contact angle, the more likely droplet-formation will occur on the surface. This can also be altered by surface texture or roughness, as well as dirt contamination and adsorption of polar gases onto the surfaces.[20]

The critical surface tension (γ_c) of a solid surface describes its hydrophilicity or hydrophobicity. This γ_c for a material is determined by measuring the different values of θ formed by liquids with different $\gamma_{l/g}$ values. A high γ_c reflects a relatively hydrophilic surface, and a hydrophobic surface having a low γ_c. It follows that a liquid with an interfacial free energy value higher than the γ_c of a solid ($\gamma_{l/g} > \gamma_c$), will not spread on its surface, but when its interfacial free energy is lower than the solid's γ_c ($\gamma_{l/g} < \gamma_c$), it will spread on the surface of the solid.[20]

The surface tension of water is 72 mN/m, whereas the critical surface tension of polypropylene is only 22.1 mN/m, and that of polytetrafluoroethylene (Teflon®) is less still at 18.5 mN/m.[11] The critical surface tension of polycarbonate is thought to be higher than that of polypropylene, as its surface is apparently more wettable. An exact value for the γ_c of polycarbonate could not be found in extensive literature and Internet searches, however.

The relevance of the critical surface tension of a solid is:

a) Thrombosis *in vivo* increases as γ_c increases,

b) Faster clot formation occurs with increased γ_c,

c) Adhesion of tissue to artificial skin decreases as γ_c increases, and

d) The contact angle formed by cells in tissue culture decreases as γ_c increases.

The γ_c is not perfect when explaining coagulation on the surface of all plastics, but provides a useful guide nonetheless.[20]

The polar/apolar ratio hypothesis is based on the polar contribution of a surface to thrombogenicity. As the polar contribution to surface free energy increases, so the coagulability of the blood in contact increases too. The theory continues that a high dispersion force, low polarity surface favours the formation of a protein film, whereas materials with low dispersion forces and high polarity surface lead to weak adherence of proteins, leading to enhanced coagulability.[8]

Coleman and colleagues disagree, however, with the interfacial free energy and the optimum polar/apolar ratio hypotheses being responsible for coagulation responses, due to the paradoxical effects seen with platelet adhesion and coagulation activation. They found the water content of plastics to cause these paradoxical effects on coagulation, where it was apparent that polymers with high water content inhibited platelet activation, but stimulated the coagulation system, with the low water polymers seeming to produce inverse effects. Increased water content not only changes the surface characteristics of the polymer, but also the electric double-layer.[8] Both polypropylene and polycarbonate materials have low water contents (personal communication with Prof J Andrade, see references). Exact water contents were not available from manufacturers of the various tubes used in our studies.

The coagulation response was not uniform for both the hetastarches we tested. After changing reservoir and/or dilution containers as described, interesting paradoxical responses were observed between the hetastarches tested (see figs 26-29), where one fluid appeared to have less coagulation effects than the other using one set of tubes, and the reverse with a separate methodology. Differences between these hetastarches are related to the molecular weight of the molecule and the carrier fluid of the starch. Sabax Hetastarch 6% ® has a 0.9% Saline suspension fluid, whereas Hextend® is suspended in a buffered, balanced electrolyte solution (similar to Ringer's Lactate).

Worth considering is that the hetastarches, although both high molecular weight hydroxyethyl starches, are not identical. Sabax HES 6%® is a 450kDa, 0.7 substitution ratio starch, whereas Hextend® is a 670kDa, 0.75 substitution ratio starch. Hextend®, being a higher molecular weight starch, could possibly have a different sterical structure, as well as altered surface charge to HES. This could be very important in the interactions with coagulation. Electrolyte compositions differ between the fluids, especially calcium, magnesium and potassium. The interfacial effects of the container on calcium, an extremely important coagulation cofactor, could lead to its relative adsorption to the surface or a starch. When the presence of different plastic surfaces alter hetastarch charge or blood contact reactions further, amplification of previously small coagulation differences could occur due to the "stacking up" of activation or inhibitory coagulation effects. The role of pH is unknown in this study, however, it has been noted previously that pH does not play a significant role in coagulation effects of haemodilution *in vitro.*[42]

Conclusion

It is difficult to single out one specific factor responsible for coagulation changes seen in this trial, as there are so many interactions at the blood-polymer interface. For example, the wettable surface of a polycarbonate container is thought to activate coagulation, whereas the negative surface charge in some circumstances is thought to resemble the endothelium, this causing a degree of thromboresistance. On the other hand, some authors believe that a more polar surface increases coagulability. Ion adsorption out of the blood or solution also affects the electric bi-layer, which has knock-on effects on coagulability, especially if one considers the possible effects on an ion like calcium, essential for coagulation. The resultant coagulation response is due to a combination of all the factors mentioned, including the electrolyte composition of the fluids in contact. Even undiluted whole blood control samples we tested showed differences due to container effects. The different ionic compositions of the two tested hetastarch preparations, may also have a marked, sometimes paradoxical, effect on some of the mentioned electrokinetic and interfacial reactions described above. At this stage, one can only speculate what the exact causes could have been.

It is likely that a marked effect occurs due to the interfacial charges, considering the electric bi-layer. Calcium would probably have a critical role here, and possibly this bi-layer could alter the dynamic relationship of calcium with blood and the starch involved. As mentioned in the discussion, this effect could be magnified when two slightly different starches are used *in vitro.* A strong suspicion would have to centre around the ionic and surface contact effects at the interface, altering dynamic starch, platelet, circulating procoagulant and anticoagulant balance, and calcium interactions.

We observed the most paradoxical results when polycarbonate was combined with blood diluted in a balanced electrolyte hetastarch solution. This picture was not seen with haemodilution using either saline-based hetastarch, or with the balanced electrolyte hetastarch solution in polypropylene. It is evident that a dynamic effect occurs at the polycarbonate interface, especially when combined with Hextend® haemodilution. A significant effect may well occur in the native, undiluted blood prior to haemodilution. These effects are exacerbated by Hextend® haemodilution. It is undoubted from our data that coagulation-changing effects occur, but exactly what they are at the interface is unknown at present. Coagulation proteins may adhere to the surface, thereby reducing their procoagulant effects when diluted or undiluted blood is placed in the TEG. Studies examining the molecular interactions at the interface will help us further explain these data.

Polypropylene and polycarbonate tubes produce different responses on in vitro coagulation testing, especially when haemodilution is performed. Polycarbonate causes the most variation in results. It is thus inappropriate to use these tubes in studies of coagulation. Polypropylene and siliconised glass tubes are deemed to be more innocuous types of storage media.[36,39]

These studies indicate that polycarbonate should not be used under any circumstances for storage of blood or in trials of haemodilution when coagulation testing is to be performed. Too many unknown coagulation effects occur at the polycarbonate-blood interface, especially when considering electrolyte and charge variability. These effects may result in skewed results, possibly incorrectly affecting clinical decision-making.

Further research is certainly also needed to assess the variability in an *in vivo* setting, where accelerated tests (e.g. celite-activated TEG®) are routinely used in assessment of the clinical coagulation status of a patient. It is possible that polycarbonate containers used for initial storage of blood prior to TEG analysis could produce a hypocoagulable picture, purely due to the container effects. Incorrect clinical decision-making regarding the administration of blood products could result, unnecessarily exposing patients to transfusion-associated risks.

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- Dr D Royston, who provided guidance during the planning stages, and still continues to ask honest, probing questions.

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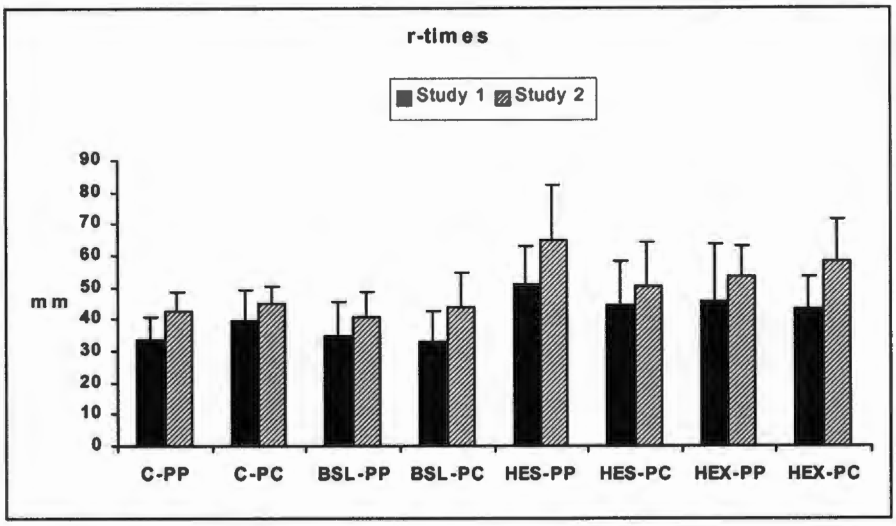
Appendix 1:

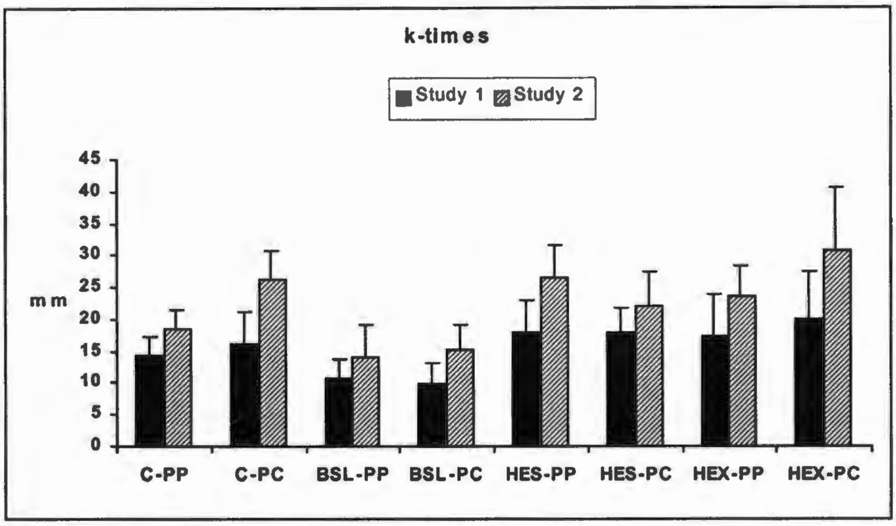
Figure 26: r-times



Where:

C = Undiluted fresh whole blood
PP = Polypropylene
PC = Polycarbonate
BSL = Balsol®
HES = Sabax Hetastarch 6%®
HEX = Hextend®

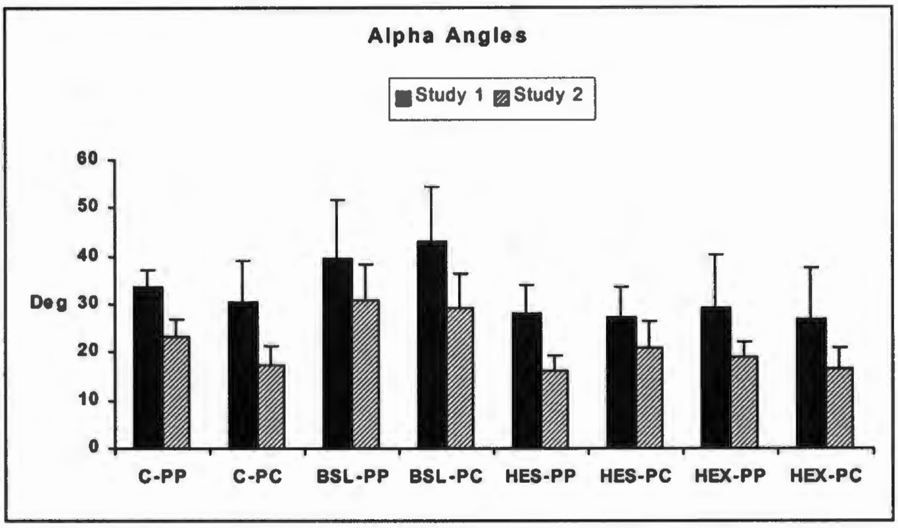
Figure 27: k-times



Where:

C = Undiluted fresh whole blood
PP = Polypropylene
PC = Polycarbonate
BSL = Balsol®
HES = Sabax Hetastarch 6%®
HEX = Hextend®

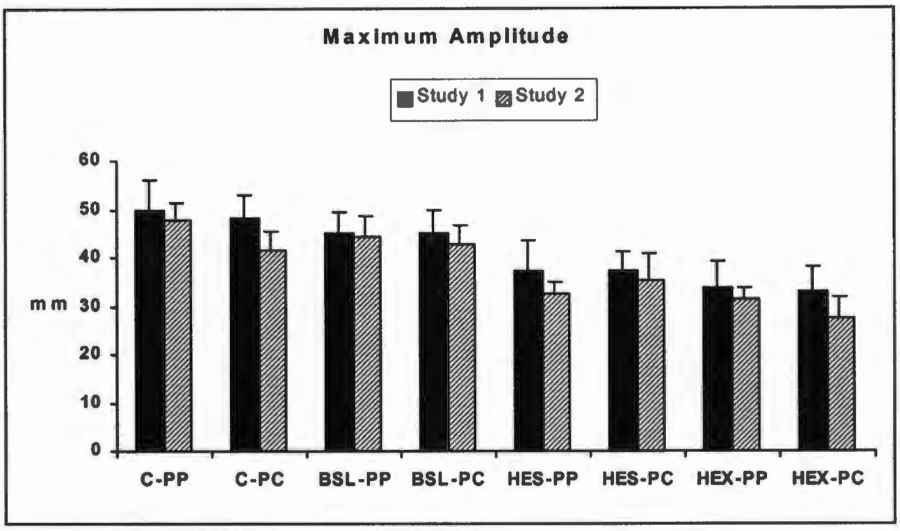
Figure 28: Alpha Angles



Where:

C = Undiluted fresh whole blood
PP = Polypropylene
PC = Polycarbonate
BSL = Balsol®
HES = Sabax Hetastarch 6%®
HEX = Hextend®

Figure 29: Maximum Amplitudes



Where:

C = Undiluted fresh whole blood
PP = Polypropylene
PC = Polycarbonate
BSL = Balsol®
HES = Sabax Hetastarch 6%®
HEX = Hextend®

Appendix 2:

Table 4: Raw Data Means (and Standard Deviations)

	r-time		k-time		α-angle		Max Ampl	
	Study 1	Study 2	Study 1	Study 2	Study 1	Study 2	Study 1	Study 2
C-PP	33.7(7.3)	42.3(6.5)	14.3(2.8)	18.5(2.9)	33.4(3.8)	23.3(3.4)	49.8(6.4)	47.9(3.5)
C-PC	39.6(9.7)	45.2(5.3)	16.1(4.9)	26.1(4.6)	30.4(8.6)	17.6(3.6)	48.1(4.9)	41.8(3.9)
BSL-PP	35.0(10.4)	40.8(8.0)	10.6(3.2)	14.1(5.0)	39.5(12.2)	30.7(7.6)	45.2(4.3)	44.3(4.2)
BSL-PC	33.1(9.4)	44.1(10.5)	9.9(3.2)	15.1(3.8)	42.9(11.3)	29.3(7.0)	45.1(4.8)	42.9(3.7)
HES-PP	50.8(12.4)	64.6(17.5)	17.8(5.1)	26.6(5.1)	27.9(6.1)	16.4(3.1)	37.4(6.0)	32.6(2.5)
HES-PC	44.2(13.8)	50.4(13.6)	17.9(3.9)	22.0(5.4)	27.1(6.3)	20.8(5.5)	37.4(3.7)	35.4(5.2)
HEX-PP	45.3(18.4)	53.2(10.1)	17.3(6.5)	23.7(4.7)	29.3(10.9)	19.0(3.1)	33.8(5.6)	31.4(2.4)
HEX-PC	43.5(9.7)	58.1(13.3)	19.9(7.4)	30.8(9.7)	26.9(10.5)	16.4(4.6)	32.8(5.1)	27.5(4.4)

Raw Data: Study 1

Tube	Fluid	r	k	a	ma	STUDY
PP-d1	Control	21	10	38	54.5	1
PP-d1	Control	28.5	12	39	60.5	1
PP-d1	Control	34.5	14.5	35.5	43	1
PP-d1	Control	40	18	31.5	45.5	1
PP-d1	Control	37	14.5	29	49.5	1
PP-d1	Control	32	12	33.5	55.5	1
PP-d1	Control	31.5	16	31.5	45.5	1
PP-d1	Control	45	17.5	29.5	44	1
PC-d1	Control	24	9.5	45	49.5	1
PC-d1	Control	32.5	11	38.5	55	1
PC-d1	Control	41.5	18	21.5	43	1
PC-d1	Control	35.5	15	30.5	46	1
PC-d1	Control	47.5	20.5	25	44	1
PC-d1	Control	35	12	35.5	54.5	1
PC-d1	Control	47.5	20.5	21.5	49.5	1
PC-d1	Control	53.5	22.5	26	43	1
PC-d1	BSL	18	7.5	48.5	47.5	1
PC-d1	BSL	30	7.5	54	49.5	1
PC-d1	BSL	33	12.5	37	37.5	1
PC-d1	BSL	47.5	14	28	41.5	1
PC-d1	BSL	36.5	9.5	40	46.5	1
PC-d1	BSL	28	6.5	56.5	52	1
PC-d1	BSL	28	7.5	51.5	45.5	1
PC-d1	BSL	43.5	14	28	41	1
PP-d1	BSL	19	6.5	55	47.5	1
PP-d1	BSL	27	9.5	46.5	50	1
PP-d1	BSL	44	13	28	40	1
PP-d1	BSL	42	11	35	42.5	1
PP-d1	BSL	40.5	13.5	31	44	1
PP-d1	BSL	25.5	7	56	51.5	1
PP-d1	BSL	33.5	9	41	46	1
PP-d1	BSL	48.5	15.5	23.5	40	1
PC-d1	HES	21.5	11	42	41	1
PC-d1	HES	48.5	17	23	40.5	1
PC-d1	HES	44	15.5	28	36.5	1
PC-d1	HES	65.5	21.5	25	32	1
PC-d1	HES	53.5	22	24	35	1
PC-d1	HES	31	16	27	42	1
PC-d1	HES	38.5	17.5	22.5	38.5	1
PC-d1	HES	51	22.5	25.5	33.5	1
PP-d1	HES	31.5	14.5	31	38.5	1
PP-d1	HES	51	13	30.5	44.5	1
PP-d1	HES	51.5	18	28	33.5	1
PP-d1	HES	50	17.5	29.5	35	1
PP-d1	HES	69.5	24.5	16.5	34	1
PP-d1	HES	36.5	10	37.5	48.5	1
PP-d1	HES	54.5	22	26	34	1
PP-d1	HES	62	22.5	24.5	31.5	1
PC-d1	HEX	26.5	11.5	40.5	37.5	1
PC-d1	HEX	40.5	16	31.5	35.5	1
PC-d1	HEX	40.5	16	31.5	29.5	1
PC-d1	HEX	46.5	23	18	28	1
PC-d1	HEX	48.5	33	13.5	29.5	1
PC-d1	HEX	38	13	40.5	42	1
PC-d1	HEX	47.5	27.5	19	32.5	1
PC-d1	HEX	60	19.5	20.5	28	1
PP-d1	HEX	24	11	41	38.5	1
PP-d1	HEX	33.5	10	43.5	40	1
PP-d1	HEX	36	15	28.5	32	1
PP-d1	HEX	39.5	17.5	25	30	1
PP-d1	HEX	52	21	23.5	32	1
PP-d1	HEX	33	12	40	42	1
PP-d1	HEX	74	28.5	15	28	1
PP-d1	HEX	70.5	23	18	28	1

Index

All reservoir tubes = PP
-d1 = dilution tubes
PP-d1 = POLYPROPYLENE
PC-d1 = POLYCARBONATE (SA)

Raw Data: Study 2

Tube	**Fluid**	**r**	**k**	**a**	**ma**	**STUDY**
PP-d2	Control	51	19	22	47	2
PP-d2	Control	38.5	16	26	49.5	2
PP-d2	Control	35	15	28	53.5	2
PP-d2	Control	34	18	23	48	2
PP-d2	Control	48	23.5	17	42	2
PP-d2	Control	45.5	16	26	49.5	2
PP-d2	Control	39	19	21.5	44.5	2
PP-d2	Control	47.5	21.5	22.5	49	2
PC-d2	Control	51	23.5	18	41.5	2
PC-d2	Control	39	30	14.5	39.5	2
PC-d2	Control	38.5	18	25	46.5	2
PC-d2	Control	43.5	26	17.5	42	2
PC-d2	Control	48	30.5	15	39	2
PC-d2	Control	52	22	20	35.5	2
PC-d2	Control	48	30	14.5	43	2
PC-d2	Control	41.5	29	16	47	2
PC-d2	BSL	48	16	29.5	42	2
PC-d2	BSL	35	14.5	29	38	2
PC-d2	BSL	33	8.5	44	51	2
PC-d2	BSL	32	13	31.5	41.5	2
PC-d2	BSL	54	22	19.5	41	2
PC-d2	BSL	52	14	30	43	2
PC-d2	BSL	39.5	16	26	43	2
PC-d2	BSL	59	17	25	44	2
PP-d2	BSL	38.5	11.5	34.5	44.5	2
PP-d2	BSL	34.5	11.5	35	42	2
PP-d2	BSL	29.5	9	42	54	2
PP-d2	BSL	39	11.5	34	41.5	2
PP-d2	BSL	49	16	24.5	41	2
PP-d2	BSL	52	25	18	42	2
PP-d2	BSL	36	12.5	32.5	45	2
PP-d2	BSL	48	16	25	44.5	2
PC-d2	HES	52.5	15.5	27	39.5	2
PC-d2	HES	37.5	23	19.5	33.5	2
PC-d2	HES	36	14.5	29.5	45.5	2
PC-d2	HES	47.5	27	15	29.5	2
PC-d2	HES	48	18.5	23.5	35.5	2
PC-d2	HES	64	29.5	14.5	30	2
PC-d2	HES	42	23	20	36.5	2
PC-d2	HES	76	25	17.5	33.5	2
PP-d2	HES	59.5	22.5	18.5	35.5	2
PP-d2	HES	46	26.5	17	32	2
PP-d2	HES	54	21	19.5	37	2
PP-d2	HES	60.5	21.5	20.5	32	2
PP-d2	HES	91	35.5	11	31.5	2
PP-d2	HES	93	27	15.5	31	2
PP-d2	HES	54	27.5	14.5	29.5	2
PP-d2	HES	59	31.5	14.5	32	2
PC-d2	HEX	57	30.5	15	29.5	2
PC-d2	HEX	43	25.5	17	26.5	2
PC-d2	HEX	47	23	22	34	2
PC-d2	HEX	61	44	11	23	2
PC-d2	HEX	70	46	12.5	21.5	2
PC-d2	HEX	69	28.5	15.5	25.5	2
PC-d2	HEX	41.5	18	24.5	33	2
PC-d2	HEX	76.5	31	14	27	2
PP-d2	HEX	52	26	16.5	30	2
PP-d2	HEX	44	22.5	21	29.5	2
PP-d2	HEX	53	23	19	35.5	2
PP-d2	HEX	58	33	13	28.5	2
PP-d2	HEX	56.5	17	22.5	30.5	2
PP-d2	HEX	47.5	24	19	31	2
PP-d2	HEX	41	24	19.5	34.5	2
PP-d2	HEX	73.5	20	21.5	31.5	2

Index

All reservoir tubes = PP
-d1 = dilution tubes
PP-d2 = POLYPROPYLENE
PC-d2=POLYCARBONATE (UK)