THE EFFECTS OF CRYSTALLOID SOLUTIONS ON THE HUMAN BLOOD COAGULATION SYSTEM

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DECLARATION

I declare that this thesis is my original work and has not been presented for the award of a degree or any other award in any other University.

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DEDICATION

This work is dedicated to my children: Darlene Lipere, George Nyarumba and Coenraede Ethan Hemker; my wife Caroline Ambata Oluoch; my parents Benjamin Ogweno and Gladyce Onyango.

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DEFINITION OF TERMS

Blood clot	- Clotted blood outside the body.
Crystalloid	- Small solutes with molecular weight less than 30 kilo Daltons, usually salts, sugars or alcohol dissolving completely and forming pure solutions in water. The particles usually permeate biological membranes and crystallize under certain conditions of temperature and concentration. Examples of solutes with therapeutic applications include sodium chloride (salts), dextrose (sugar) and mannitol (polyol alcohol).
Equivalent	- Number of freely moving particles divided by the valence.
Half strength Darrow's solution	- Contains (mMol/L) Na 61, Cl 52, K 17, lactate 27.
Ionic strength	- Description of concentration of charged solutes, dependent on molar concentration and valency.
Normal saline:	- Solution of 0.9% w/v sodium chloride (9g NaCl per Litter solution) having 154 mmol/L each of Na and Cl (308 Osm/L).
Osmolality	- Expression of concentration of a solution in number of osmotically active particles per kg of solvent (Osm/Kg).
Osmolarity	- Expression of concentration of osmotically active particles per Litter of solvent (Osmoles/L)
Plasma	- Blood component without cells, usually separated from anticoagulated whole blood by centrifugation or sedimentation and contains all the coagulation factors.
Ringer's Lactate	- Solution containing (mMol/L) Na 130, Cl 109, Ca 4, K 5, and Lactate 28**.**
Saline	- Solution containing sodium chloride.
Thrombus	- Blood clot in vivo.
Tonicity	- Comparison of osmotic effect of a solution to normal plasma. Graded as isotonic (same as), hypotonic (lower than) and hypertonic (higher than).
Whole blood sample	- Contains cells, including red and white blood cells and platelets suspended in plasma.

LIST OF ACRONYMS / ABBREVIATIONS

ACT	Activated Clotting Time
AIDS	Acquired Immune Deficiency Syndrome
APTT	Activated Partial Thrombin Time
ATIII	Anti Thrombin iii
CAT	Calibrated Automated Thrombogram
CFT	Clot formation time
CT	Clotting time
DM	Diabetes Mellitus
DVT	Deep venous thrombosis
FIX	Factor IX
FPA	Fibrino peptide A
FV	Factor V
FVII	Factor VII
FVIII	Factor VIII
FX	Factor X
FXI	Factor XI
FXII	Factor XII
FXIII	Factor XIII
HIV	Human immunodeficiency virus
HS	Hypertonic Saline
ICIPE	International Centre for Insect Physiology and Ecology
INR	International Normalised Ratio
MCF	Maximum clot formation
NaCl	Sodium Chloride
pH	Measure used to specify acidity or alkalinity of an aqueous solution. It is the negative logarithm to base 10 of the activity of hydrogen ion.
PS	Phospatidyleserine
PT	Prothrombin Time
RL	Ringer's Lactate
roTEG	Rotational Thromboelastography
ROTEM	Rotational Thromboelastometry
TAT	Thrombin Anti Thrombin
TEG	Thromboelastography
TG	Thrombogram
USA	United States of America
WHO	World Health Organisation

ABSTRACT

Crystalloid solutions are used in clinical practice for resuscitation and correction of electrolyte imbalances. However, up to 25% of individuals may develop dysfunctions of haemostasis following fluid infusions, complicating resuscitation and outcome. Studies on the effects of crystalloids solutions on human blood coagulation have produced conflicting results: either suggesting procoagulant effects or impaired coagulation. The mechanisms for these discrepant results remain unclear. However, the role of solute composition remains largely unexplored. The main objective of the study was to determine the effects of crystalloid solutions on human coagulation system under conditions of varying solute type and concentrations. Specifically, the study investigated the effects of ionic crystalloids solutions containing Na+ (sodium gluconate and sodium chloride), Cl- (sodium chloride and choline chloride), and non ionic solutions such as mannitol and dextrose, *in vitro*. The blood samples were obtained from consenting healthy adult human blood donors. The laboratory methods were routine coagulation tests, plasma thrombin generation, and Thromboelastography and Light transmission platelet aggregation. Data analysis was done using STATA version 11.0, Texas, USA. Analysis of variance (ANOVA) and Kruskal Wallis equality of population tests were applied to examine for differences between groups. In routine tests, undiluted control had median prothrombin time 16.1 secs (IQR 15.3-17.1; N=17); median INR 1.2 (IQR 0.99-1.32; N=14); mean activated prothrombin time 33.98secs (SE 0.94; 95% CI 31.94-36.02; N=14) and median fibrinogen level 283.9 mg/dl (IQR 249.9-317.8; N=12). These tests showed significant positive linear increase with NaCl concentration except fibrinogen concentration which decreased with NaCl concentration. Comparison with other crystalloid solutions revealed crystalloid ionic strength had the most influence, and presence and concentration of chloride ions was most significant. In thrombin generation, mannitol had no influence on the thrombin generation parameters either in the extrinsic or intrinsic activation. However, NaCl had most significant effect in intrinsic activation, where there was progressive lengthening of lag time with each concentration though curves remaining qualitatively similar. The median thrombelastography values for undiluted control were: R 12.9(IQR 11.0-17.4); K 5.1 (IQR 3.5-7.0); alpha angle 32.4 (IQR 27.7-46.9); Maximum amplitude 55.8 (IQR 54.3-57). Thrombelastography median parameters had curvilinear relationship with NaCl solutions such that R&K trough inflection point around 0.3 M, as well as alpha angle and Maximum amplitude plateau. Comparison with other ionic crystalloid solutions were qualitatively similar except that from 1200 mOsm, the order of divergence was choline chloride>NaCl> sodium gluconate. Mannitol and dextrose dose dependently decreased thromboelastography alpha and maximum amplitude without much change in R&K. Citrate anticoagulated whole blood samples showed patterns of enhanced coagulation in comparison to neat non-citrate anticoagulated samples. Platelet aggregation was inhibited dose dependently by all the crystalloid solutions irrespective of solute type indicating that platelet activity was sensitive to osmolality rather than ionic strength. This study concludes that the effects of crystalloid solutions on human blood coagulation are dependent on solute content and concentration. Further, increase in chloride concentrations beyond the normal physiological range impairs blood coagulation. It is therefore recommended that the concentrations of solute ions in resuscitation fluids should be within normal plasma levels.

CHAPTER ONE

INTRODUCTION

1.1. Background

Infusions particularly crystalloid solutions are the mainstay of fluid resuscitation post trauma haemorrhage. Depending on formulations, the adverse outcomes associated with their use include plasma electrolyte imbalance, osmotic destruction of blood cells as well as haemodilution of clotting factors (Smorenberg, Ince, & Groeneveld, 2013). In addition, disorders of haemostasis, known as trauma induced coagulopathy (TIC), have been reported in up to 25% among resuscitated trauma patients (MacLeod, 2008; MacLeod, Lynn, McKenney, Cohn, & Murtha, 2003). The significance of coagulopathy is that once it sets in, it contributes to a vicious cycle of coagulopathy, continued haemorrhage and death.

Since the definition of trauma induced coagulopathy, different views have been advanced to explain the role of crystalloid fluids in its causation. Whereas one group postulates that crystalloid haemodilution contributes to pathogenesis of coagulopathy (Daniel Bolliger, Görlinger, & Tanaka, 2010; Cap & Hunt, 2015; Shaz, Winkler, James, Hillyer, & MacLeod, 2011), another group states that coagulopathy develops independent of and before fluid infusion (Brohi, Cohen, & Davenport, 2007; Floccard et al., 2012; Maegele et al., 2007). However, the relationship between crystalloid haemodilution, solute compositions and attendant plasma electrolyte imbalance are still unclear.

Despite the general impression that haemodilution will reduce concentration of clotting factors and therefore intuitively impair their clot forming functions, studies have documented that

crystalloid haemodilution can both accelerate (Heather, Jennings, & Greenhalgh, 1980; T. G. Ruttmann, James, & Viljoen, 1996; Tocantins, Carroll, & Holburn, 1951a) and delay blood clot formation (Timothy John Coats & Heron, 2004; Reed, Johnston, Chen, & Fischer, 1991a; Wilder, Reid, & Bakaltcheva, 2002a). Since many of the crystalloids in current use differ in solute content and concentration, it is not known whether these seemingly contradictory findings are due to general fluid dilutional effects or associated with specific solute constituents. Moreover, why solutions lacking calcium, a well established coagulation cofactor, should promote blood clotting is still unclear. One author has suggested that crystalloid enhancement of coagulation will occur irrespective of nature of type of solutes, concentration or pH (Ruttmann, 2007). However, in this article, the claim was not backed by empirical data. Therefore, given the magnitude of the problem, further investigations are warranted to refute or confirm the claim and guide therapeutic interventions.

A number of postulates have been advanced to explain the association between haemodilution and coagulation. One of the initial theory was that haemodilution reduces the natural anticoagulant antithrombin in plasma out of proportion compared to other constituents thus leaving the procoagulant factor reactions unopposed (Monkhouse, 1959). Others have also made the same observations (Linden, Gibbs, Bremner, Schneider, & Erber, 2004; T. G. Ruttmann, 2003; T. G. Ruttmann, James, & Finlayson, 2002). In this scenario, thrombin generation and action is supposed to be enhanced by reduction of one of its key negative regulatory factor. However, the major weakness of this postulate is that empirical measurement of thrombin activity has never been measured in the same haemodiluted blood specimens so as to demonstrate the relationship. Furthermore, antithrombin is just one of the negative regulators for

thrombin, other plasma constituents such as tissue factor pathway inhibitors (TFPI), and thrombomodulin-protein C-protein S are also at play, and may be reduced as well.

An alternative view that Na+ containing solutions allosterically enhance thrombin activity contributing to enhanced coagulation (Enrico Di Cera, 2004, 2008; Enrico Di Cera, Page, Bah, Bush-Pelc, & Garvey, 2007; Wells & Di Cera, 1992a) have been backed by results of experiments performed using pure thrombin and synthetic substrates. Apart from ignoring the fact that plasma contains many components with pro- and anticoagulant functionality, it assumes thrombin's esteratic activity will always correlate with fibrinogen clotting, which is not always the case. The only rational way to investigate this claim is to perform thrombin generation in experiments in haemodiluted plasma samples where all components interact. To the best of knowledge, this has not been done.

1.2 Statement of the Problem

Trauma is the leading cause of death in people aged 18 to 45 years mostly as a result of blood loss. However, coagulopathy or dysfunction of haemostasis is encountered in 25% of individuals (Daniel Bolliger et al., 2010; Wafaisade et al., 2010) though the underlying mechanisms have not been well defined. Since these patients also receive crystalloid fluid volume resuscitation, the development of coagulopathy has been partly attributed to haemodilution (MacLeod, 2008; MacLeod et al., 2003). This notion was reinforced in one simulation study (Hirshberg et al., 2003). The model is not universally supported by many empirical study findings. This is evidenced, over the last 70 years, by investigations on the effects of crystalloid solutions on coagulation that have produced conflicting results; some reporting procoagulant effects (T. G. Ruttmann et al., 2002, 1996; Tocantins et al., 1951a) whereas others report impairment of

coagulation (De Lorenzo, Calatzis, Welsch, & Heindl, 2006; Souza, Klamt, & Garcia, 2010; Souza et al., 2010). Comparability of study results is hampered perhaps by use of crystalloid solutions of different concentrations(Reed et al., 1991a), solute types(Wilder et al., 2002a), degree of haemodilution (Hirshberg et al., 2003; Souza et al., 2010), and methods used to evaluate coagulation (Caballo et al., 2013; Darlington et al., 2011).The potential contribution of crystalloid solutions for development of coagulopathy has come into question arising from findings that haemostatic impairment was observable even before fluid infusion in trauma patients (Maegele et al., 2007; White, Martin, Brophy, & Ward, 2010) and the course was not influenced by the amount of crystalloid fluids infused (Wafaisade et al., 2010). It is quite clear that there is incongruence between clinical observations of bleeding thought to arise from impairment of haemostasis and laboratory investigation's test results. Overall, these laboratory studies have been performed using coagulation factors in isolation having little relevance to whole blood coagulation. Furthermore, little attention has been paid to the influence of the crystalloid fluid osmolality and solute contents in blood clotting especially on whole blood coagulation in humans. It is thus intuitive to speculate that this inability to account for influence of crystalloid fluid solute content and concentration, perhaps accounts for lack of progress in laboratory diagnosis of causative factor(s) in coagulopathy observed alongside fluid resuscitation and thus hampering progress on therapeutic interventions. This study examined the effects of crystalloid solution type and concentration on blood coagulation system of humans. The coagulation test may provide the most sensitive changes induced by crystalloid composition and which blood specimens are suitable to demonstrate the coagulation derangements. This study therefore investigated effects of crystalloid solutions on human coagulation system using a

combination of blood coagulation tests in clinical use with a view to unraveling which is the most sensitive laboratory diagnostic test and possible therapeutic coagulation factor replacement.

1.3 Rationale and Justification for Study

Crystalloids or solutions of small molecules have many therapeutic and diagnostic applications differing in concentration (Homma et al., 2005) and solute content (Shawkat, Westwood, & Mortimer, 2012). Clinicians have used empiric guidelines based on surrogate endpoints of haemodilution, hemodynamic and coagulation modules to determine effectiveness and/or complications following intravenous infusions (Hardy, De Moerloose, Samama, & Groupe d'intérêt en Hémostase Périopératoire, 2004; Rossaint et al., 2010). Of these, coagulation modules have been the most confusing. Most guidelines assume decreased blood coagulation arising from haemodilution of clotting factors and thus have set a cut-off of volume infused. This haemodilutional coagulopathy model (Daniel Bolliger et al., 2010)underestimates the significance of the issues involved as it does not take into account the effect of osmolality and solutes as cofactors in modulating the activity of circulating clotting factors in clot formation. It is possible that particular solute types at critical concentrations interacting with circulating clotting factors and cells may influence quality and kinetics of blood clot formation. Previous investigations of role of crystalloid fluids in association with coagulopathy of trauma have been hampered by multiple factors are at play (Wafaisade, 2010), difficulty in differentiating contribution of trauma inflammatory response (Gando, 2015). Most studies on trauma patients are either retrospective or uncontrolled observational which seldom lead to firm conclusions, given the variable and complex nature of clinical contexts (Hardy, 2004). Injured patients may not be a suitable model since trauma is associated with release of other inflammatory markers whose amount and type may confound the picture (Gando, 2015; Frith, 2011).The trauma

clinical contexts are confounded by early onset of sepsis, and specifically the presence of endothelium. These factors have dual roles of maintaining blood in fluid state, but can also accelerate thrombosis in equal measure depending on the balance. Moreover, the effects of endothelium on the haemostatic system are not fully understood.

In addressing the question of the role of crystalloid haemodilution on the human coagulation system, *in vitro* design involving dilution of human blood using a variety of crystalloid solution types was chosen since it avoids some of the confounding factors associated with tissue trauma as may occur during surgery or accidental trauma.

A pilot study which was conducted prior to this study suggested that the effects of crystalloid haemodilution on coagulation may be a function of concentration and the nature of solute composition (Ogweno, Reed, Dyer, & James, 2009). The specific effects associated with particular solutes/ ions could not be confirmed due to limitations of study design. The current study was therefore designed to answer how particular solutes and their concentration affect blood coagulation, especially clotting time, plasma thrombin generation, and whole blood clot strength and platelet aggregation. Further, it was designed to test hypothesis that crystalloid haemodilution enhances coagulation by promoting thrombin generation. This will increase our understanding of the physiology of blood coagulation under varying conditions and inform on the design of clinical protocols for triggers of coagulation impairment when crystalloid fluids of various types are infused and possible alternative therapeutic interventions.

1.4 Research Questions

I. What are the effects of crystalloid solutions on routine coagulation tests of human blood?

II. What is the influence of crystalloid solutions on thrombin generation?

III. What are the effects of crystalloid solutions on whole blood viscoelasticity?

IV. What are the effects of crystalloid solutions on platelet aggregation?

1.5 Null Hypothesis

The effects of crystalloid solutions on coagulation system are not dependent on solute content and concentration.

1.6 Objectives

1.6.1 General Objective

To investigate the effects of crystalloid solutions on human blood coagulation system

1.6.2. Specific Objectives

To:

1. Investigate the effects of crystalloid solutions on routine coagulation tests of human blood.
2. Determine the influence of various crystalloids on thrombin generation.
3. Investigate the e effects of crystalloid solutions on whole blood viscoelasticity.
4. Establish effects of crystalloid solutions on platelet aggregation.

1.7 Assumptions and Limitations

Since the studies were conducted entirely *in vitro* or *ex vivo*, results may only be used to suggest what may happen *in vivo*. Testing of coagulation *in vitro* avoids the confounding factors associated with surgery or tissue trauma arising from interaction with endothelium. Additionally,

in vitro testing enables identification and focus on circulating clotting factors that may be modified or replaced during coagulopathy. As well, use of blood preservatives/ anticoagulants during sample preparation and storage may introduce non-physiological effects. The collected data may be different if fresh whole blood samples are used during the experiments.

Further, a major limitation was lack of control for sodium and chloride ions since a chelating agent similar to calcium does not exist yet. This hindrance was overcome by substitution at a time. Also, small sample size used in this study may lead to lack of statistical significance in some tests and thus limit general applicability. Owing to the nature of this study (analytical, experimental) cost was an issue.

1.9 Delimitation

The literatures reviewed were mainly from articles written in the English language, mainly due to wide availability and difficulty in getting accurate translations of non-English articles. Although a study of this nature would require literature citations not more than 10 years old, it was imperative to accept some seminal papers published many years back. This is due to the fact that medical problems persist for a long time and scientific progress that specifically address the issues are slow and scientific progress in medicine occurs in leaps in which some decades may elapse without investigations addressing the issue. This historical limitation was acknowledged in literature review and care was taken to include only the most relevant original publications that provided paradigm shift in our understanding of the concepts of crystalloid haemodilution on coagulation.

The crystalloid solutions investigated were chosen to correspond to the molecules applied in formulation of therapeutic fluids at clinical concentrations. In this way, it was envisaged that data generated may be used to design resuscitation fluids with haemostatic competence. Furthermore,

the laboratory tests employed only concentrated on the methods in clinical applications while acknowledging their limitations. Therefore other methods, though scientific and could precisely define the effects were not used. This study neither attempted to assay for coagulation factor concentrations nor cell membrane surface expression of procoagulant molecules. Likewise, other cell derived procoagulant products such as microparticles and nuclear extracellular traps (NETs) were not investigated since the methods of study were not available at study site during the study period. Acknowledgement of this limitation has been given and their use has been recommended for further work.

1.10 Significance of the study

While crystalloid solutions are the mainstay of volume resuscitation, they are associated with development of coagulopathy but the mechanisms are as yet not clearly defined. There is need to better understand the human coagulation system and the influence of the crystalloid solutions of varying composition in order to prevent the morbidity and mortality accompanying trauma induced coagulopathy. This research was conducted to find out the role of crystalloid solution solute content and concentration on human blood coagulation *in vitro*. The data obtained would be useful to pharmaceutical industry in formulation of innovative resuscitation fluids for a given patient profile for desired coagulation outcome. It further improves our understanding of the coagulation system, and its interaction with solutes which are important in pathophysiology. In addition, the tests results would assist clinicians and laboratory practitioners in deciding which blood component specimens to collect, and the appropriate coagulation tests to perform on the specimens to demonstrate the effects of crystalloid solutions on the coagulation system. Coagulation factor concentrates are increasingly becoming available for management of coagulopathy, but clinical administrations are based on expert opinion or personal experience

rather on empirical data. This study demonstrates that coagulopathy of crystalloid haemodilution is complex, and therefore not a single coagulation factor concentrate may adequately normalize the derangement. To monitor the effectiveness of these new products, data generated in this study will contribute to the growing body of knowledge in the field of haemodilution and coagulation, to avoid the current viscous cycle of trauma, bleeding, volume replacement, coagulopathy and more bleeding.

1.11 Theoretical framework

Theories regarding blood coagulation have been evolving, depending on period and scientific evidence at the time. Simply, these are predictions of observations of mechanisms that lead to conversion of blood in fluid state to solid or gel like state. The initial theory of coagulation cascade or waterfall (Davie & Ratnoff, 1964; Macfarlane, 1964) (Figure 1-1). This theory postulated that the coagulation factors are activated in a sequence in plasma to generate the active enzyme thrombin. Thrombin eventually converts soluble fibrinogen to fibrin clot. It has progressively been updated to include interaction of many factors in plasma leading to generation of active thrombin, including procoagulants and their inhibitors. Although natural blood coagulation often involve whole blood (plasma together with cellular elements), it was previously assumed that only circulating clotting factors activated by tissue injury are the main actors with the blood cells acting as innocent bystanders.

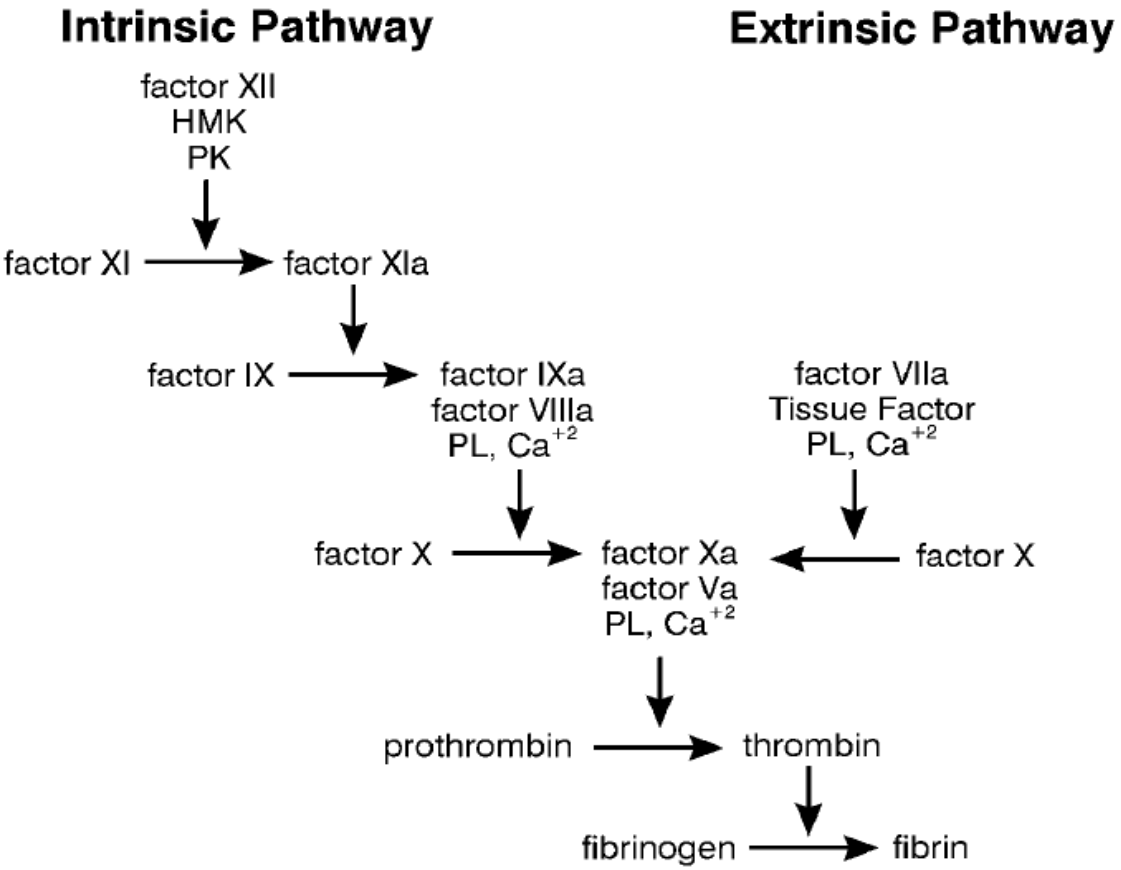

Figure 1-1 Classical coagulation model

Intrinsic coagulation activation pathway initiated by contact activation of FXII-Kallikrein system. Extrinsic activation initiated by TF-FVa. Both systems converge on common pathway, FXa that converts prothrombin to thrombin (Hoffman& Monroe, 2001)

With the observation that blood can clot even without tissue injury, a search for initiators of blood coagulation on circulating blood cells led to identification of tissue factor expression on the surface blood cells. Thus, the current theory of blood coagulation is 'cell based coagulation model' (Hoffman, 2003a, 2003b; Hoffman and Monroe, 2001) (Figure.1-2).

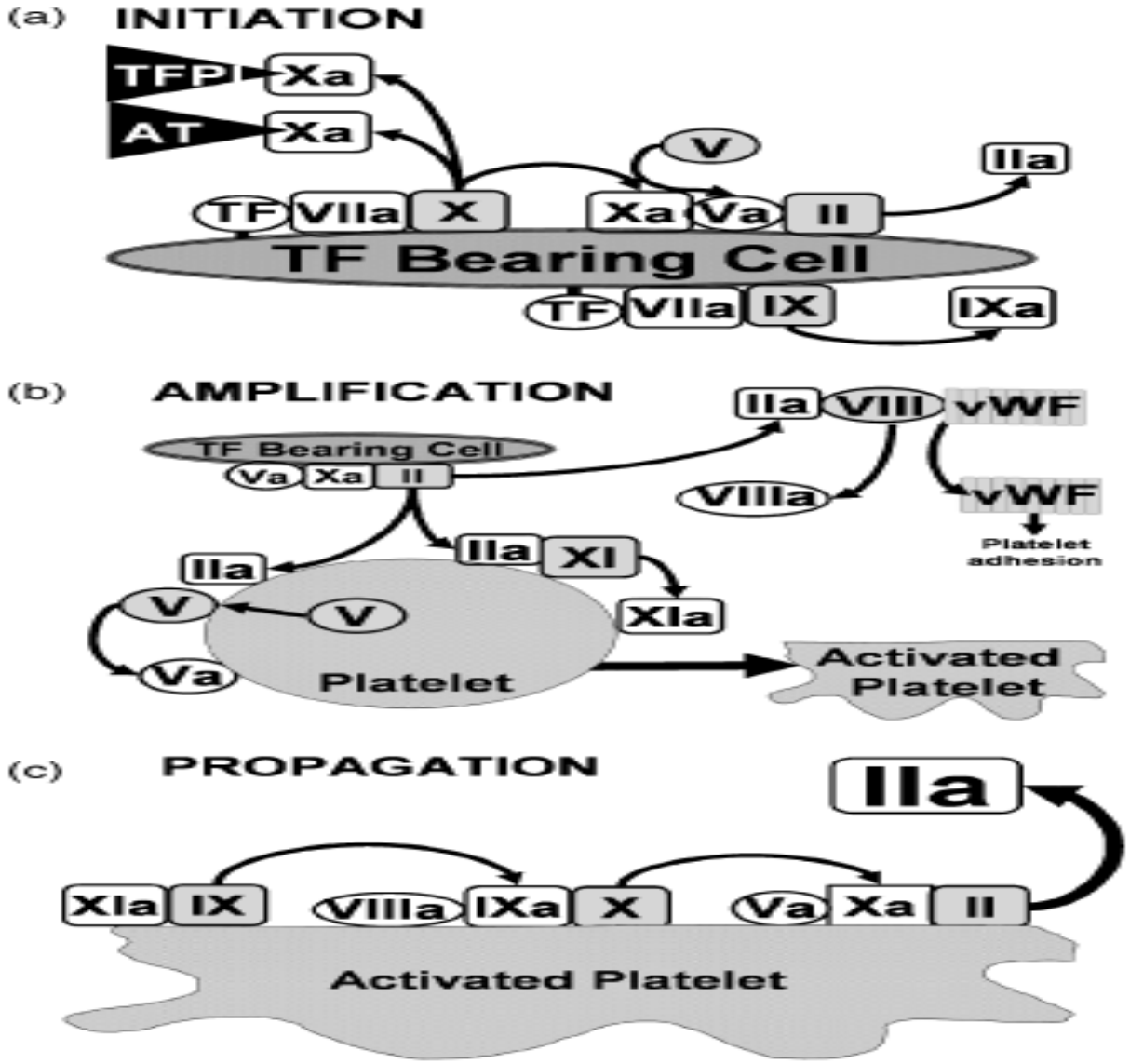

Figure 1-2 Cell based coagulation model

(a) initiation phase-activation of TF bearing cells to express TF that activates coagulation factor reactions on its surface that results in small amounts of thrombin (IIa); (b) amplification phase-small amounts of thrombin participates in positive reactions activating platelets, FVIII, FV, FIX and detaching vWF; (c) Propagation phase-assemply of various enzymes on platelet surface, intrinsic prothrombinase results in massive thrombin burst generation (Smith, 2009).

Another area in modulation of the theory was the appreciation of the role of plasma electrolytes on the clotting process. In brief, changes in concentration of plasma electrolytes modulate

generation of and activity of thrombin, cellular expression of initiators of the clotting factors, and biophysical sequence of fibrin polymerization to a stable clot. In addition, the electrolytes, due to their charge and osmotic effects influence platelet aggregation and their interactions with fibrin in the human coagulation system. Therefore the current working theory of coagulation incorporates classical 'cascade' or 'waterfall'(Davie & Ratnoff, 1964; Macfarlane, 1964) models, 'cell based coagulation model' as well as roles of plasma electrolyte changes, especially sodium(De Filippis et al., 2005; Di Cera, 2007; Huntington, 2008; Page and Di Cera, 2006; Pineda et al., 2004), (Hoffman, 2003a, 2003b; Hoffman and Monroe, 2001) and chloride (Raimondo De Cristofaro et al., 2005a; E. Di Stasio, Nagaswami, Weisel, & Di Cera, 1998; Enrico Di Stasio, 2004). Therefore, the action of crystalloid solutes can be grouped under the so called 'Hoffmeister series' into clot promoters and clot inhibitors/retarders(Baldwin, 1996). Appreciation of the specific roles of crystalloid solutes in the coagulation system abrogates the hypothesis of 'haemodilution induced coagulopathy'(MacLeod, 2008; MacLeod et al., 2003; Maegele et al., 2007; Wohlauer et al., 2012). Arising from the foregoing, this study was grounded on the theory that human blood coagulation system involves a dynamic interaction of plasmatic coagulation factors on the surface of cellular elements that is modulated by the surrounding crystalloid composition of the medium in which the blood clots.

CHAPTER TWO

LITERATURE REVIEW

This review focuses on the theories, models and concepts that have contributed to our understating components of the haemostatic system as well as the physiology of coagulation. A good understanding of these concepts is considered as the basis for the laboratory tests in clinical use and their application in investigating the influence of crystalloid solutions on the human coagulation system. A critical evaluation of pre-analytical and analytical factors are presented, that when not well controlled, may contribute to variability in comparison of study results, and perhaps the basis of controversies in the literature of the effects of crystalloid solutions on human coagulation system.

2.1 Human Coagulation system or Haemostasis

Haemostasis consists of series of processes that maintain blood in fluid state as well as responses to vascular injury leading to formation of a clot with the aim of preventing excessive blood loss from the body. It occurs in stages termed primary, secondary and tertiary involving the interaction of endothelium, sub-endothelial tissues, blood cells and plasma constituents (Loscalzo & Schafer, 2003). The principal actors of the haemostatic system have traditionally been considered as circulating factors acting as procoagulant, anticoagulants and fibrinolytic; and platelets. However, accumulating evidence is increasingly recognising contribution of other blood cells such as red blood cells, leukocytes and plasma electrolyte composition.

2.1.1 Platelet functions or primary haemostasis

Platelets serve the role of primary haemostasis which begins with the formation of platelets plug in which they undergo a variety of reactions during activation. The activated Platelets perform procoagulant functions by releasing their contents and changing shape by formation of pseudopodia that link them to fibrin (Loscalzo & Schafer, 2003) (Loscalzo & Schafer, 2003). In addition, activated platelets recruit more platelets to the growing haemostatic plug by several feedback amplification loops: release of agonists stored in α granules (ADP, and serotonin), and denovo synthesis of pro-aggregatory TXA2. Further, they provide negatively highly charged catalytic surface for activation and binding of coagulation factors as well as release of procoagulant micro vesicles or microparticles (Ni & Freedman, 2003). It is generally known that a variety of Osmolytes affect platelet morphology but the effect on coagulation are less understood.

In summary, platelet thrombus formation involves two separate reactions, all interrelated-tethering to surfaces or adhesion, and platelet to platelet cohesion via fibrin bridges or aggregation (Figure 2-1). Both responses are preceded by platelet activation (S. P. Jackson, 2007; Reininger et al., 2006) (Reininger, 2008).

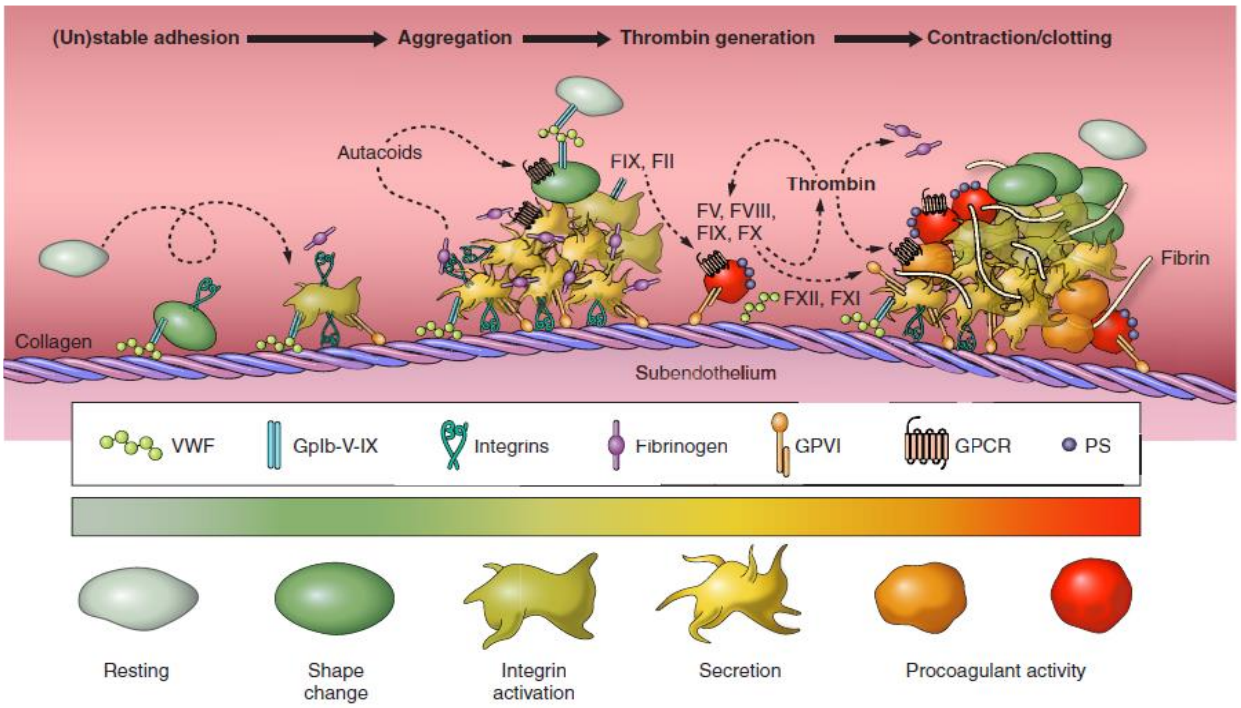

Figure 2-1 Stages of platelet reactions

vWF-von Willebrand factor, GpIb-V-IX-platelet glycoprotein receptor Ib-V-IX, GPVI-platelet glycoprotein receptor VI, GPCR ,PS-phosphatidyle serine, FII,FV,FIX,FX,FIX-coagulation factors. Heat maps with colour codes from green (low Ca++ signals) to red (high Ca++ signals) (Versteeg, Heemskerk, Levi, & Reitsma, 2013)

2.1.2 Plasmatic Coagulation or secondary haemostasis

The reactions of factors in plasma which eventually lead to clot formation, due to limitations of methods of study were the first to be described. Owing to sequence of events in the formation thrombus on a time scale, the plasmatic coagulation reactions are always preceded by platelet reactions *in vivo,* therefore the reactions have been renamed secondary haemostasis. It is important to note that the information in re-naming these reactions were derived from *in vitro* experiments. This consists of a sequence of reactions of factors in plasma that lead formation of active enzyme thrombin which eventually converts soluble fibrinogen to insoluble fibrin clots. The factors are identified using Roman letters (in capital) in the order in which they were

described, but not on the sequence of activation, and suffix ‘a’ indicating active enzyme/factor. Several models have been proposed to outline the interaction of factors. The best known is classical enzyme cascade (Macfarlane, 1964) or waterfall (Davie & Ratnoff, 1964) coagulation pathways (Figure 1-1). The coagulation cascade/waterfall reactions involve interaction of circulating coagulation factors that lead to activation of enzyme thrombin and subsequent fibrin fibre polymerization. These have been considered to occur in stages:

Stage 1: formation of prothrombinase or FXa

The coagulation cascade/waterfall is considered to have 2 main activation pathways in the generation or activation of thrombin (Riddel, Aouizerat, Miaskowski, & Lillicrap, 2007) in the biochemical reaction sequences in the plasma coagulation factors. The components are either extrinsic or tissue factor (TF) initiated, or intrinsic or contact activated. The two pathways converge in activating factor X or prothrombinase.

Extrinsic pathway is initiated when tissue factor from injured tissues binds and forms a complex with factor VII, which thereafter leads to activation of factor X. It is currently considered the main pathway for initiation of coagulation. However, since TF-FVIIa is sensitive to inhibition by circulating tissue factor pathway inhibitor (TFPI), therefore FX activation by extrinsic pathway is short lived unless augmented by intrinsic pathway(K. G. Mann, Brummel, & Butenas, 2003; Orfeo, Butenas, Brummel-Ziedins, & Mann, 2005a).This model assumes that coagulation can only be initiated when blood gets into contact with collagen in injured tissues, which is not always true. Clinically, blood coagulation or thrombus formation have been observed even without evidence of tissue injury.

The intrinsic pathway is stimulated when blood, in contact with negatively charged surfaces such as kaolin in the presence of calcium and platelet phospholipids facilitates spontaneous and sequential activation of high-molecular weight kininogen, prekalikrein and factors XII that activates factor XI to FXIa. Three physiological triggers have been discovered, namely; collagen, linear phosphate polymers termed polyphosphate, and nuclear extracellular traps (NETs) (Veersteg, et al, 2013). Activated factor IX, being the product of FXIa, in the presence of active factor VIII activates factor X that serves the common pathway. However, the physiological significance of intrinsic coagulation pathway activation is currently questionable and is probably a test tube phenomenon. Nevertheless, its reactions act to amplify the coagulation apart from linking it to immune system and also with fibrinolysis (Tanaka, Key, & Levy, 2009a). Since the extrinsic or intrinsic activation pathways end up in generation of FXa, they are also referred to as extrinsic or intrinsic Tenase respectively.

Stage 2: Conversion of Prothrombin to Thrombin

The common pathway involves the FX-mediated conversion of prothrombin to active thrombin (factor II) which converts fibrinogen (factor I) to fibrin monomers. FVa, Ca++ and phospholipid acting as cofactors in prothrombinase complex amplifies the reaction 30,000 times (Tans, Janssen-Claessen, Hemker, Zwaal, & Rosing, 1991).

Prothrombin (FII) is a 72 kDa single chain protein, and together with FV, FVII, FX and Prot C are members of vit K-dependent factors synthesized in the liver. The vit K-dependent factors are characterized by having NH_2-terminal domain of γ-carboxylic acid (Gla). Vit-K is required by carboxylase to form Gla from glutamic acid. The Gla domain mediates salt bridge formation

with Ca++ and membrane binding, thus positioning for enzymatic activity (Licari & Kovacic, 2009).

Prothrombin cleavage by prothrombinase occurs in stepwise reactions that generate small amounts of active α- thrombin and non-active intermediate fragments such as prethrombin1.2 and meizothrombin. Whereas α-thrombin is the active form of thrombin enzyme, meizothrombin lacks clotting and platelet activating activity and is thus converted into α-thrombin in the next steps (Frenkel, Shen, & Haley, 2005)(Carlisle, Bock, & Jackson, 1990; C. T. Esmon, Owen, & Jackson, 1974; C. M. Jackson, 2007; Rabiet, Blashill, Furie, & Furie, 1986). The γ and β thrombin fragments lack exosites for full procoagulant activities (J. C. Fredenburgh, Stafford, & Weitz, 1997; James C. Fredenburgh, Stafford, Pospisil, & Weitz, 2004; James C. Fredenburgh et al., 2004). The initial prothrombinase yields less than 4% of active thrombin, while most thrombin (96%) are formed from thrombin positive feedback activities leading to subsequent 300,000 times greater prothrombinase activity and thereafter further activation of prothrombin fragments (Kathleen E. Brummel, Paradis, Butenas, & Mann, 2002; K. G. Mann, Jenny, & Krishnaswamy, 1988).

Stage 3: Conversion of Soluble Fibrinogen to Fibrin Clot

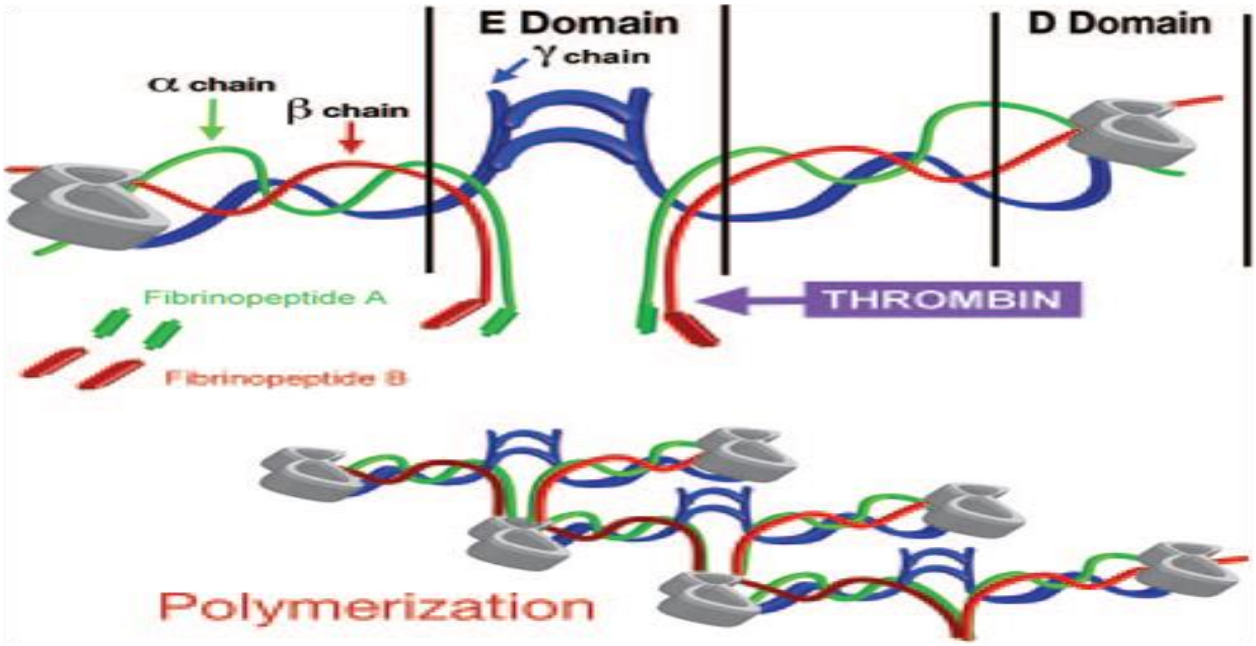

Figure 2-2: Structure of fibrinogen molecule (Standeven, Ariëns, & Grant, 2005)

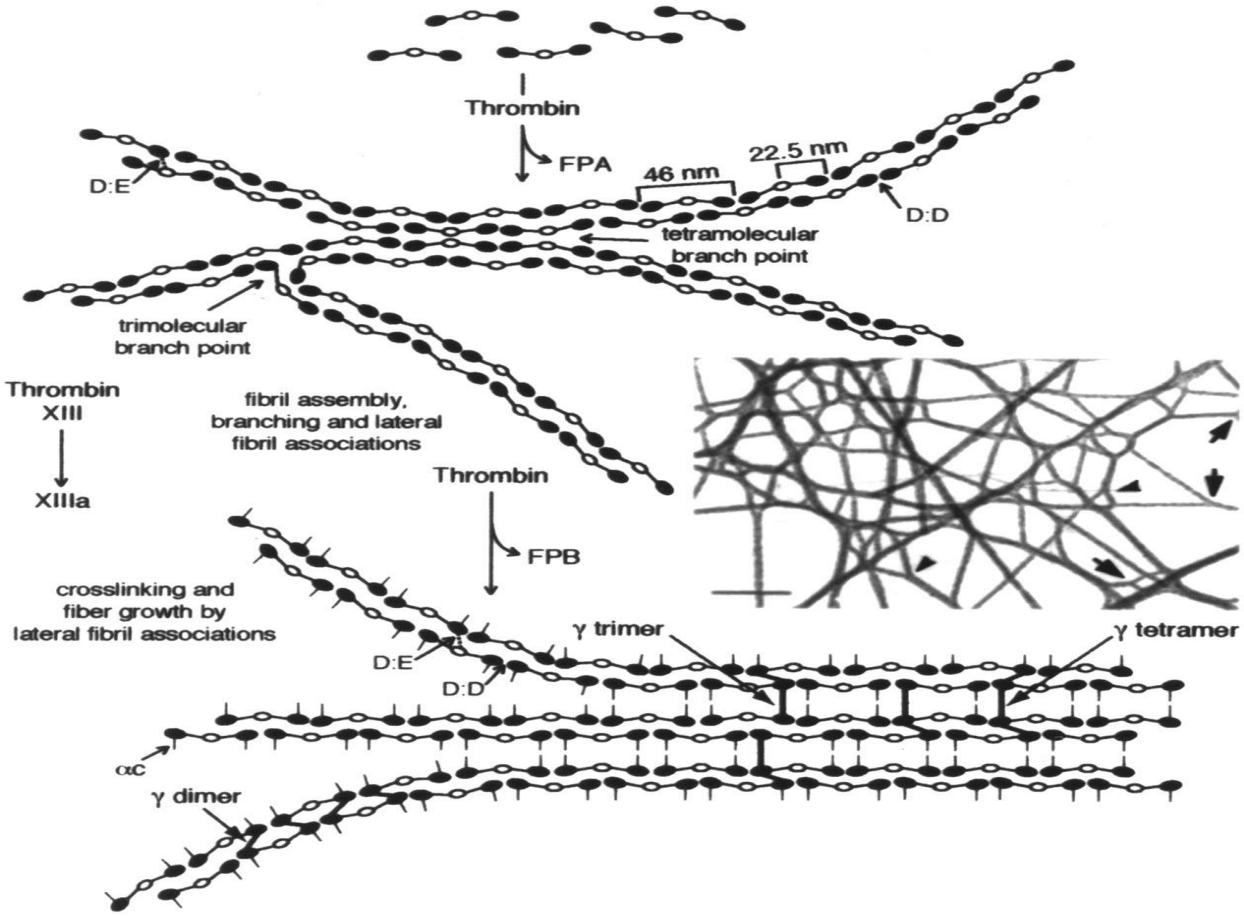

Figure 2-2 Schematic diagram of fibrin formation by thrombin, assembly and cross linking to form protofibrils and polymers (Mosesson, Siebenlist, & Meh, 2001)

Fibrinogen is a dimeric glycoprotein of 340 kDa synthesized in the liver and circulates in plasma at a concentration of 1.5-3 mg/ml. Blood clotting occurs when soluble fibrinogen is converted to insoluble fibrin by thrombin.It is composed of three polypeptide chains: (Aα, Bβ, γ); each half of the dimeric molecule consists of three non-identical polypeptide chains: Aα (610 amino acids), Bβ (461 amino acids) and γ (411 amino acids) held by a network of 29 disulfide bonds (Standeven et al., 2005) (fig 2-2).

Thrombin catalyzed cleavage of fibrinogen occurs at 4 sites on the E region to form soluble fibrin monomers (Standeven et al., 2005).However, transformation of fibrin out of solution is a biophysical polymerization process independent of thrombin to form a rigid gel. Fibrin monomers spontaneously aggregate in a regular array to form weak fibrin clots in a two stage reaction. Cleavage of fibrinopeptides from parent protein unmasks self assembly potential built into fibrinogen molecule exposing necessary fibrin-fibrin contact sites. Finally, protofibrils cross link and form strong covalent bonds aided by FXIIIa that confers mechanical strength. Fibrin fiber cross linking, in addition to FXIIIa, is aided by its multiplicity of available polymerization and calcium and plays important roles in resisting fibrinolysis and determining viscoelastic properties of fibrin clot (Ryan, Mockros, Weisel, & Lorand, 1999).The fibrin monomers aggregate in a biophysical process to form polymers that gel out of solution to form the solid clot (fig 2-3).

A major weakness of the cascade/waterfall hypothesis is that it ignores the contribution of other cells like platelets while assuming that the reactions occur in sequence. It neither does not explain the positive amplifications observed nor account for the other regulatory proteins or of roles of plasma solutes on their actions.

Owing to the limitations of the classical cascade/waterfall model in explaining modern understanding of blood coagulation, a number of modifications have been added. Originally, thrombin feedback reactions in activating factors' X, IX, VIII and V which were not part of the coagulation cascade have since been added as new information are made available. Thus there is also incorporation of natural anticoagulants- Thrombomodulin (TM), protein S (PS), protein C

(PC) and antithrombin (AT), and cross talk between extrinsic and intrinsic activation-the 'Josso Loop'(fig 2-4).

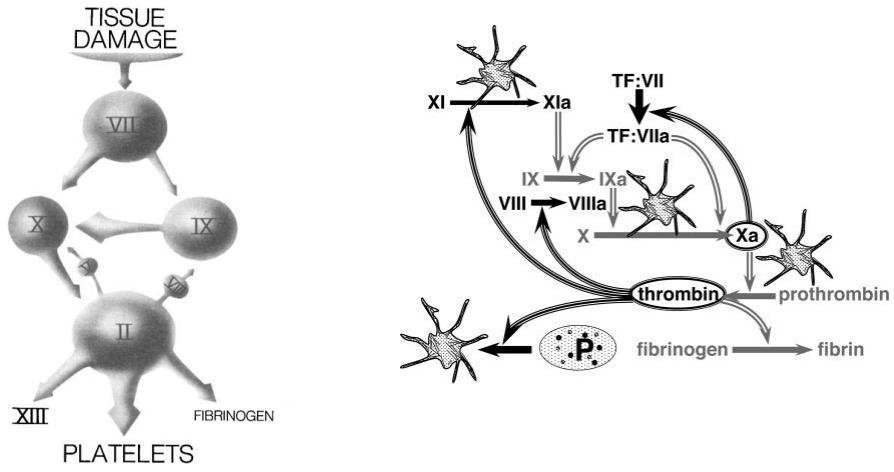

Figure 2-3: TF-FVII participation in Josso-Loop (H C Hemker, 1984)

The Josso Loop, a term coined by Hemker to describe clinical observations by Francois Josso in 1965 (H C Hemker, 1984)(Xi, Béguin, & Hemker, 1989a) recognises the contribution of extrinsic TF-FVII complex in not only activating FX directly, but also indirectly via FIX. It has subsequently been confirmed in a number of experimental studies (Altman & Hemker, 1967; Maureane Hoffman, 2003b; Lu, Broze, & Krishnaswamy, 2004) that explain observations of slow onset but sustained generation of FXa on activation with TF-FVII (Kenneth G. Mann, Brummel-Ziedins, Orfeo, & Butenas, 2006)and mechanism of action of high dose factor VIIa in the treatment of haemophilia (Augustsson & Persson, 2014; Maureane Hoffman, 2003a).

Role of factor XIII

Plasma FXIII or transglutamase, previously called 'fibrin stabilising factor of Laki-Lorand', is recognized essential for providing the clot with structural stiffness and plasticity for wound closure, and resistance against lytic enzymes (Lorand, 2005)..

Whereas the presence of FXIIIa does not affect clot morphology significantly (fibre density and branch point), it increases clot stiffness 5 fold (Falvo, Gorkun, & Lord, 2010; Lorand, 2005). Thus, FXIIIa merely performs ligation of half staggered fibrin units without interfering with clotting time. Hence, bleeding due to FXIII deficiency is not due to lack of clot formation, but arises out of mechanical weakness and proneness to lyses.

2.1. 3 Regulation of Coagulation

In vivo, during haemostasis in order to prevent clotting inside blood vessels , small amounts of active coagulation factors which are produced in an idling fashion are neutralized as soon as they are formed (fig 2-5) which is in balance to reactions that break down clots that do form(C. T. Esmon, 2000). Becker (2005) has described three phases Vis: termination of coagulation factor activation/activities, elimination of formed clots (fibrinolysis) and stabilization of thrombus. Tissue factor pathway inhibitor (TFPI) from endothelium mediates negative feedback inhibition of TF-FVii complex thereby limiting activation of FIX and FX. Activities of TFPI are potentiated by small amounts of FXa and heparin(Mackman, 2009). The noticeable effect of TFPI is to prolong initiation phase of extrinsic coagulation system. In the presence of thrombomodulin, which binds thrombin, there is increased activation of protein C that subsequently inhibits plasminogen activator inhibitor, leading to increased fibrinolysis.

Thrombin regulates its own generation by having two isoforms. The procoagulant or fast form has a greater affinity for sodium ions (Bah, Garvey, Ge, & Di Cera, 2006; Orthner & Kosow,

1980; Wells & Di Cera, 1992b). The anticoagulant or slow form (without Na+) is stabilized by endothelial bound Thrombomodulin (TM) and activates circulating protein C to its active form (APC) (E. Di Cera, 2007; Enrico Di Cera et al., 2007; Page & Di Cera, 2006)(Dang, Vindigni, & Di Cera, 1995). APC anchored to its endothelial receptor (EPCR), in the presence of protein S denatures FVa, FVIIIa as well as activation of fibrinolysis through dis-inhibition of thrombin activable fibrinolysis inhibitor (TAFI). This pathway therefore prevents thrombin burst at the injured sites(C. T. Esmon, 2000). The activities of TM, Prot S and prot C are synergistic and lead to decrease in velocity or propagation phase of thrombin generation.

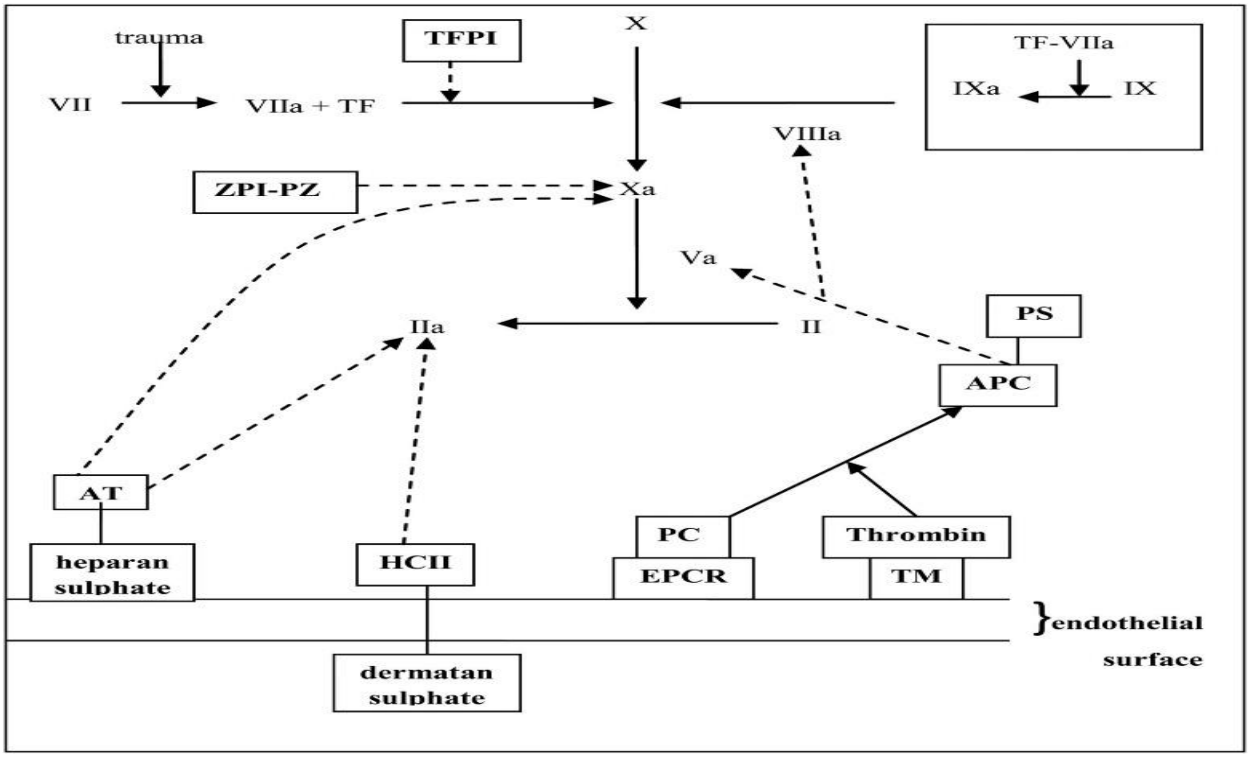

Figure 2-4 Thrombin anticoagulant regulatory mechanisms

Activated protein C (APC), antithrombin (AT), and tissue factor pathway inhibitor (TFPI) are the most important. The importance of protein Z-dependent protease inhibitor (ZPI) with protein Z (PZ), and heparin cofactor II (HCII) is not known. Protein S (PS, cofactor of APC); endothelial protein C receptor (EPCR); thrombomodulin (TM). (Ezihe-Ejiofor JA& Hutchinson N, 2013

Antithrombin III (ATIII) or simply antithrombin (AT) being the most potent thrombin inhibitor is the best known circulating natural anticoagulant. AT neutralizes non clot bound thrombin in a stochiometric ratio of 1:1, thus preventing it from participating in clot formation. AT, potentiated by heparin inactivates serine proteases, specifically; FIIa, FXIIa, FXIa, FIXa and FXa. Other antithrombins are fibrin or antithrombin I (ATI), α2 or antithrombin II (ATII) but their effects are considered minimal (Tanaka, Key, & Levy, 2009b).

2.1. 4 Fibrinolysis or Tertiary Haemostasis

Enzymatic dissolution of the clot or fibrinolysis has also been referred to as tertiary haemostasis. The purpose is to keep the vascular lumen patent by limiting further thrombus formation.

Fibrin also plays a key role in the subsequent dissolution of the clot. Fibrinolysis is initiated when tissue-type plasminogen activator (tPA) and plasminogen bind to fibrin. The activation of plasminogen to plasmin by tPA is stimulated by its substrate, fibrin. Plasmin then digests the fibrin mesh, thereby, dissolving the clot(Hunt & Segal, 1996).Plasminogen can be activated endogenously by either tissue-type plasminogen activator (t-PA) or urokinase-type plasmonogen activator (u-PA), or by exogenous bacterial products streptokinase. Other activators are tissue kallikrein system which also initiates the contact coagulation pathway (fig 2-6).

During fibrinolysis, plasminogen, an inactive proenzyme, is converted to plasmin that degrades fibrin to fibrin degradation products (FDP). Plasmin activity is non-specific and capable of degrading not only fibrin but its precursor fibrinogen and other extracellular matrix proteins (Hunt & Segal, 1996). Plasmin puts a break on coagulation activation by degrading FVa and FVIIIa thus abrogating the amplification and burst phase of thrombin generation.

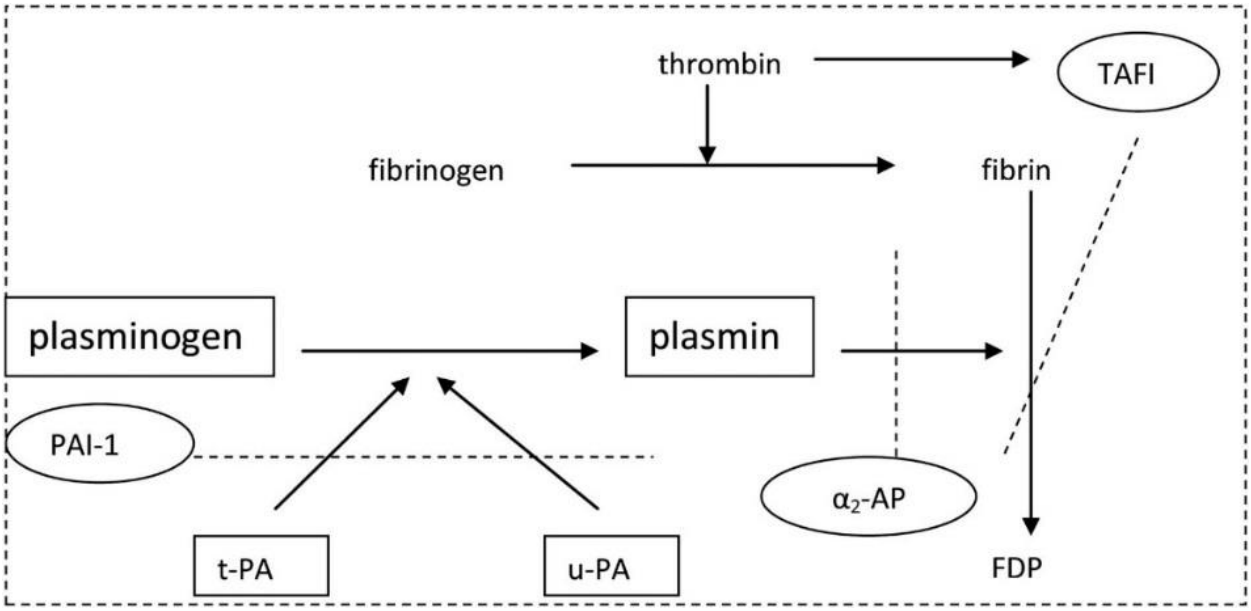

Figure 2-5 Fibrin degradation by the fibrinolytic system and its regulation

Plasmin degrades fibrin into soluble fibrin degradation products (FDP) and is regulated by α2-antiplasmin (α2-AP), also called plasmin inhibitor. Plasminogen is activated by tissue-type plasminogen activator (t-PA) or urokinase-type plasminogenactivator (u-PA). These enzymes are regulated by plasminogen activator inhibitor-1 (PAI-1).Thrombin not only converts fibrinogen into fibrin but also activates thrombin-activatable fibrinolysis inhibitor (TAFI), which inhibits fibrinolysis by modifying the fibrin substrate. (Ezihe-Ejiofor and Hutchinson, 2013)

Fibrinolysis is regulated by a number of inhibitors in plasma. Plasminogen activator inhibitors (PAI-1, 2, 3) and complement-1 esterase inhibitor (C1-inh) control t-PA activation of plasminogen. A2 macroglobulin is an antiplasmin. Other inhibitors are thrombin activable fibrinolysis inhibitor (TAFI), which links coagulation with fibrinolysis (Mosnier & Bouma, 2006). Down regulation of these inhibitors may result in pathological hyperfibrinolysis (Hunt & Segal, 1996; Stump et al., 1990). Experimental studies have suggested that chloride ions may inhibit fibrinolysis (Chibber & Castellino, 1986; Raimondo De Cristofaro et al., 2005b; Gaffney et al., 1988; Urano, Sator de Serrano, Gaffney, & Castellino, 1988; Yarzábal, Serrano, & Puig, 1999), but the clinical significance *in vivo* is still undetermined.

2.1.5 Cell Based Coagulation Model

Due to the inadequacies of cascade/waterfall hypothesis, a cell based model has been developed (Becker, 2005; M. Hoffman & Monroe, 2001; Maureane Hoffman, 2003a, 2003b). In this model, there is recognition of cellular elements (fig 2-7). A major proposition is that the coagulation reactions proceed on cell surfaces in three overlapping steps: initiation, amplification and propagation. Increased amounts of thrombin are generated from the positive feedback reactions set in motion as formation of factors on the platelet surface prevents inactivation by plasma inhibitors (Borissoff & ten Cate, 2011).

Initiation is primarily a function of cell membrane bound TF which is localized to cell surfaces in encrypted or non-active form (Butenas, 2012). Upon stimulation, TF bearing cells, especially circulating monocytes increase TF decryption or expression a thousand times (Saulius Butenas, Orfeo, & Mann, 2009). TF stimulates FVII to FVIIa which activates FX to FXa as well as FV to FVa. The FXa-FVa complex or prothrombinase formed acts on FII to produce FIIa or active thrombin. At this stage, the small amounts of thrombin produced participate in positive feedback reactions to amplify further thrombin generation. Although the reactions may proceed as described in plasma, availability of membrane surface facilitates efficient prothrombinase assembly and protects it from inhibitory effects of tissue factor pathway inhibitor (TFPI) and antithrombin (AT) in solution. Empirical evidence adduced in support of this phenomenon arises from demonstration of TF expression and prothrombinase on monocytes (Osterud, 1998; Østerud & Bjørklid, 2006)which are increased several fold by stimulation (Hiller, Saal, Ostendorf, & Griffiths, 1977).Prothrombinase (FXa, FVa and ca++) has a high affinity for membrane surfaces. In fact, FVa binding onto its receptors on platelet membranes provides receptor sites and

structural support for FXa(K. G. Mann et al., 2003, 1988; Kenneth G. Mann, Butenas, & Brummel, 2003).

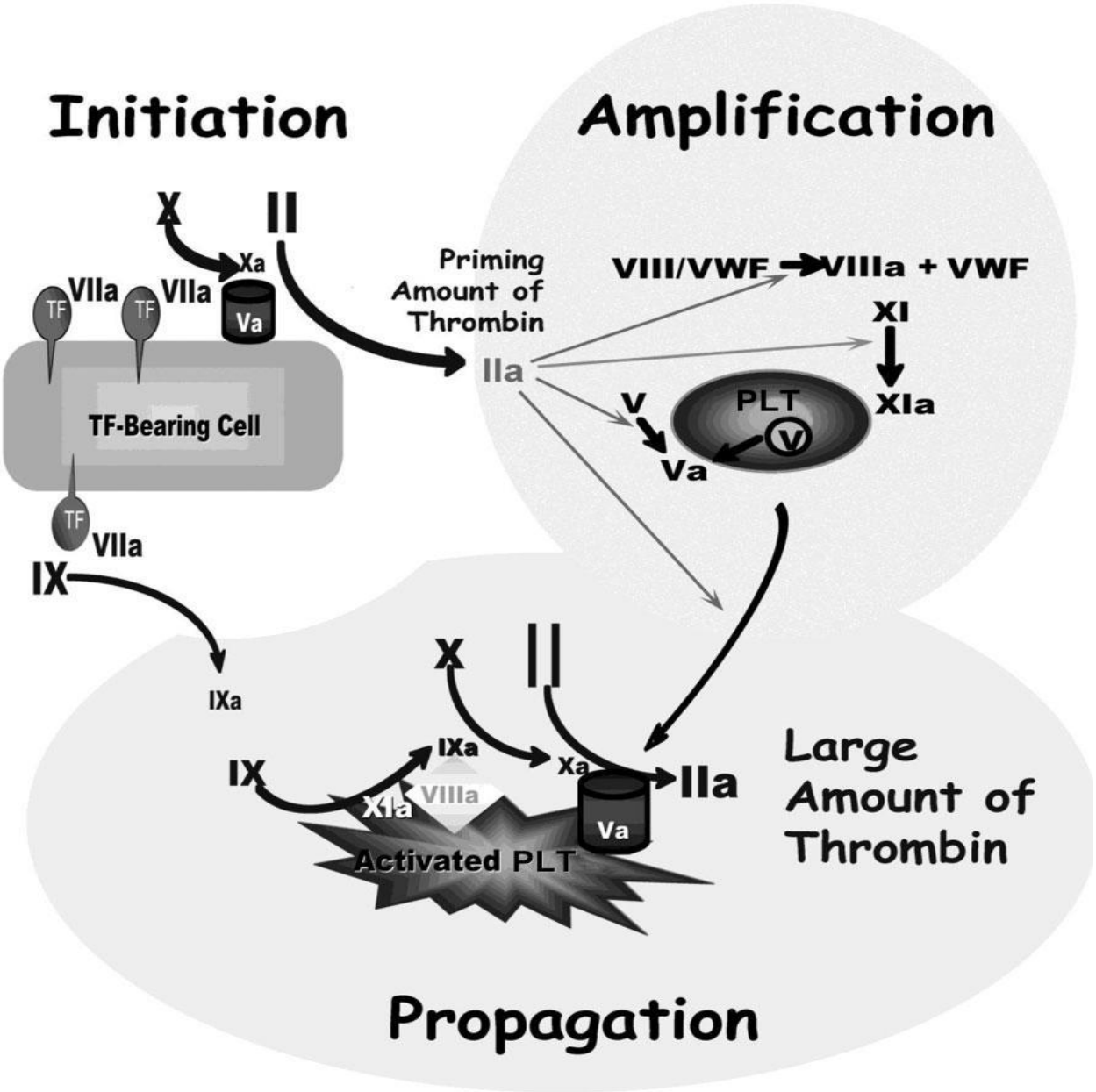

Figure 2-6 the phases of a cell based model of coagulation (Maureane Hoffman & Cichon, 2013)

In the amplification or priming phase, thrombin generation occurs on surface of platelets. Small amounts of thrombin from TF bearing cells are passed onto platelets that enhances their reactions-adherence, secretion, shape change and expression of procoagulant receptors. During this process, thrombin activated platelets release procoagulant factors Ca++, FIX, FVIII and FV which are activated by thrombin setting in motion chain reactions. In the process, von Willebrand factor gets detached from FVIII, which together with FV remains bound to activated platelet surface setting stage for large scale thrombin generation. A major hallmark of amplification phase is thrombin initiated positive reactions on the surface of platelets producing FVIIIa free from vWF, FVa, FIXa and activated platelets(M. Hoffman & Monroe, 2001; Maureane Hoffman, 2003a). However, it must be noted that deficiencies of factors such as in haemophilia can not be compensated by the extrinsic system since TF tissue distribution is asymmetric and selectively expressed, being abundant in monocytes, platelets, leukocytes and endothelium but deficient in muscles and synovial membranes (Tanaka et al., 2009b) that explains selective tissue bleeding

The propagation phase is accompanied by massive thrombin burst that cleaves fibrinogen to fibrin clot. This is due to membrane bound Tenase and prothrombinase complexes and thus protected from inhibitory factors, display enhanced catalytic activity more than ten thousand times. Prothrombinase complex forms and results in a burst of thrombin generation directly on the platelet(Stephanie A. Smith, 2009). FXa or Tenase alone in solution is very slow as a prothrombinase but with the combination of factors on membrane surface (FXa, Ca++, phospholipid, FVa), the relative prothrombin activation rate increases to 20,000 times (H. C. Hemker et al., 1983).

Activated platelet membrane surface therefore performs unique functions in coagulation: facilitates Tenase and prothrombinase complex assembly, fixation and protection from inactivation(M. Hoffman & Monroe, 2001; Maureane Hoffman, 2003a).

Termination phase of thrombin generation ensures thrombus is limited to sites of vascular leaks. The participants are: TFPI, from endothelial cells and platelets, which inhibits TF-FVIIa complex and FXa; circulating antithrombin which is fixed and stabilized on cell surfaces to inhibit thrombin, FIXa, FXIa, and TF-FVIIa complex; TM stabilized on the membrane surfaces activates PC that on fixation on membrane surfaces together with cofactor PS cleaves FVIIIa and FVa. The overall effect is to terminate further thrombin generation. At the same time activated platelets release anexin II that blocks soluble-phase FXIa. Also at the same time, activated thrombin acting on platelets, which together with endothelium expresst-PA that converts circulating plasminogen to plasmin that cleaves both fibrinogen and fibrin thus regulating further fibrin deposition(M. Hoffman & Monroe, 2001; Maureane Hoffman, 2003a).

The cell based model closely resembles *in vivo* situations and correlates with many clinical observations. New coagulation tests that use whole blood are better explained by this paradigm. Moreover, it provides explanations for factors that are either procoagulant or antihaemostatic when present in whole blood but not in plasma.

2.1.5.1 Role of Erythrocytes in coagulation

Although many coagulation studies were carried in plasma or with purified factors, this gave the impression that only soluble circulating factors participate in clot formation, and that blood cells have no role in the clotting process. However, *in vivo* blood clots and thrombi contain platelets,

RBC and leukocytes. Appreciation of role of RBC on the clotting process began from observations that: anaemic patients had prolonged bleeding times (Hellem, Borchgrevink, & Ames, 1961). Patients with polycythaemia (Landolfi, Rocca, & Patrono, 1995) or haemoglobinopathies such as sickle cell disease (R. F. A. Zwaal, Comfurius, & Bevers, 2005; R. F. Zwaal & Schroit, 1997) suffer from increased incidence of stroke and thromboses than control normal individuals (Ataga, 2009; Ataga & Orringer, 2003); infusion of old stored blood cells lead to poor coagulation performance (Reinhart, Zehnder, & Schulzki, 2009). But these were indirect evidence.

Previous mechanism of roles of RBC in thrombosis were that, during flow, differential velocity profiles force RBC at the luminal centre leaving plasma close to the luminal wall while platelets lie in between owing to skimming effects. But this paradigm could not wholly account for incorporation of RBC within fibrin networks when blood is left to clot under no flow. Numerous investigations have revealed that presence of RBC within blood clots increases fibrin fiber (M. E. Carr, 1988; M. E. Carr & Hardin, 1987), and their ordered polyhedral packing array (Cines et al., 2014)influences blood clot viscoelastic performance (Gersh, Nagaswami, & Weisel, 2009). These data suggest a role of RBC in fibrin fibre formation and thrombin generation. With development of sophisticated laboratory methods that overcame the earlier technical difficulties it has been possible to demonstrate clearly that thrombin generation occurs on surface of RBC (Marisa Ninivaggi et al., 2012), (Whelihan & Mann, 2013).

Normally, RBCs do not support coagulation but the process is initiated in pathological states when TF on RBC membrane which exist in encrypted or quiescent state (Bach, 2006) are

decrypted or activated (Rao, Kothari, & Pendurthi, 2012). The events leading to decryption are TF dimer formation, association with lipid rafts rich in cholesterol, oxidation of cysteinene thiols and disulfide formation, and membrane phosphatidyle exposure from inner leaflet to outer leaflet (Spronk, ten Cate, & van der Meijden, 2014). Disturbance of this arrangement attenuates the enzyme activities resulting in PS exposure on the outside which not only facilitates TF decryption but also assembly of vitamin K dependent coagulation factors by salt bridge formation (Bach, 2006). The net effect is initiation of and dramatic acceleration of enzymatic velocity of Tenase and prothrombinase (R. F. A. Zwaal et al., 2005) (Kuypers et al., 1996). Furthermore, release of ADP from RBCs leading to platelets aggregation has been known for a long time (Reimers, Sutera, & Joist, 1984; N. Wohner, 2008; Nikolett Wohner et al., 2011).

In brief, normal RBCs being the most abundant formed elements, when stimulated provide membrane surface area for anchoring the coagulation factors, decryption of membrane bound TF and negatively charged phospholipids (PS) for triggering reactions and augmentation of thrombin generation a thousand fold. In addition, haemolysis releases ADP, haemoglobin and Fe++ acting as platelet agonists as well as resisting fibrinolysis therefore stabilising thrombi. It seems that conditions that promote RBC haemolysis, as may be found associated with hypotonic solutions may, by disrupting the membrane and exposing inner leaflet provide negatively charged PS lipids acts to trigger blood coagulation.

2.1.5.2 Role of Leukocytes in Coagulation

Despite laboratory evidence of leukocytosis in association with *in vivo* thrombosis, it has taken long for blood coagulation to be regarded as part of body's inflammatory process, with a contribution from leukocytes (Charles T. Esmon, 2003; Levi & van der Poll, 2010). Microscopic

examinations of thrombus have always revealed aggregates of leukocytes intertwined with fibrin mesh and platelets. From the available evidence currently, participation of leukocytes in blood clotting occurs at three levels: stimulus altered membrane characteristics, leukocyte secretion products and interaction with platelets and endothelium.

Experimental observations have revealed that stimuli altered leukocyte membranes lead to increased surface expression of TF several fold (Imamura, Kaneda, & Nakamura, 2002), more predominant in monocytes (Mészáros et al., 1994) in response to inflammatory mediators such as lipopolysacharides, immune complexes and cytokines (Bouchard & Tracy, 2001, 2003) which are also elevated at sites of thrombus (Fuchs, Brill, & Wagner, 2012). And there is compelling evidence that leukocytes migrates to sites of injury earlier than platelets and the source of TF in platelets is derived from leukocytes via microparticles (Bouchard & Tracy, 2003).Concomitantly stimulated leukocytes increase their expression of adhesion molecules such as p-selectin GP ligand-1(PSGPL-1) that helps their interaction with platelets and endothelium (Lindmark, Tenno, & Siegbahn, 2000; Merten & Thiagarajan, 2000). Therefore, leukocytes form mixed aggregates with platelets and thus influence the structure of thrombi.

In addition to expression of adhesion molecules, secretory products of leukocytes are powerful initiators and stabilizers of blood clotting. Notable among these is neutrophils extracellular traps (NETs) known to trap other cellular elements such as RBC and platelets (Fuchs et al., 2012).These NETs, consisting of chromatin material mixed with selected cytoplasmic proteins on contact with clotting factors induce intense activation of clotting factors (Brinkmann & Zychlinsky, 2012)in addition to providing polyanionic nucleosomes that bind TFPI thus

protecting the factors from inhibition (N. Wohner, 2008). Alongside NETS release are free radical reactive oxygen species that activate coagulation factors and cause membrane lipid peroxidation (Zawrotniak & Rapala-Kozik, 2013), and shedding of thrombogenic microparticles (Darbousset et al., 2012). In addition, secreted proteases such as elastase, cathepsin G, and matrix metalloproteinases enhance the von Willebrand factor-dependent platelet adhesion (Borissoff & ten Cate, 2011), (S. Jain et al., 2012; S. K. Jain, Palmer, & Chen, 1999). These facts therefore demonstrate that findings of leukocytosis in thrombotic states, or incorporation of leukocytes into thombus is not accidental but are an integral part of haemostasis.

2.1.5.3 Cell membrane derived micro vesicles or microparticles

Microparticles are small 0.1 to 100µM sized membrane-derived phospholipid vesicles shed from stimulated cells present in blood in physiological state and their concentration may vary under pathological conditions. Although various names such as exosomes (EXS) and microparticles (MPS) have been used to designate these extracellular vesicles (EVs), strictly speaking micro vesicles or micro particles are derived from plasma membrane and exosomes refer to intracellular origin. These vesicles generally originate from various types of cells such as red blood cells, platelets, leukocytes, endothelial cells and also tumour cells, but most, 70-80% are of platelet origin (Owens & Mackman, 2011).

Although EXs and MPs possibly arise from separate mechanisms, they share numerous similarities and participate in haemostasis as well as other numerous biological processes including intercellular communication (Tissot et al., 2013).Triggers for MP formation are chemical (chemokines, thrombin and cholesterol enrichment) and physical (shear stress,

hypoxia), (Zhou et al., 2014). The process of formation from cells of origin involves stimuli mediated activation, apoptosis or necrosis. The consequences of these processes are: cytoskeletal disruption, membrane blebbing and eventual pinching off (fig 2-8). Key in this process is rise in intracellular Ca++.

Rise in intracellular calcium inhibits floppase but activates flippase and scramblase leading to PS exteriorization. Also Ca++ activates enzymes calpain and gelsolin which hydrolyzes contractile proteins actin binding proteins. Specifically there is cleavage of actin capping proteins, the consequence of which is lack of cytoskeletal support with unrestricted water gradient directed inwards. Thus osmotic forces facilitate water entry and create an area of increased hydrostatic pressure. The net result is membrane budding and microparticle formation rich in PS and PE as well as bioactive cytoplasmic proteins. Thus, their composition mirror cells of origin, but with a high concentration of substituents (Morel, Jesel, Freyssinet, & Toti, 2011).

Procoagulant functions of microparticles relate to their membrane surface composition and core content which are: lipid raft membrane domains rich in cholesterol and negatively charged phosphadylserine on external membrane leaflet facilitating contact coagulation activation; high tissue factor expression participating in the extrinsic coagulation initiation. Additionally, high concentration of membrane proteins such as GP1, p-selectins and integrins have been implicated in platelet adhesion and aggregation reactions (Morel et al., 2011).

By blebbing or pinching off from other cells, it carries concentrated procoagulant factors, mainly TF and PS facilitating thrombus formation from site of origin. It possible that crystalloid

solutions, due to ionic and osmotic forces may determine the membrane hydrostatic and enzymatic activity leading to microparticle formation and thus influence whole blood coagulation reactions. But this has not been examined systematically.

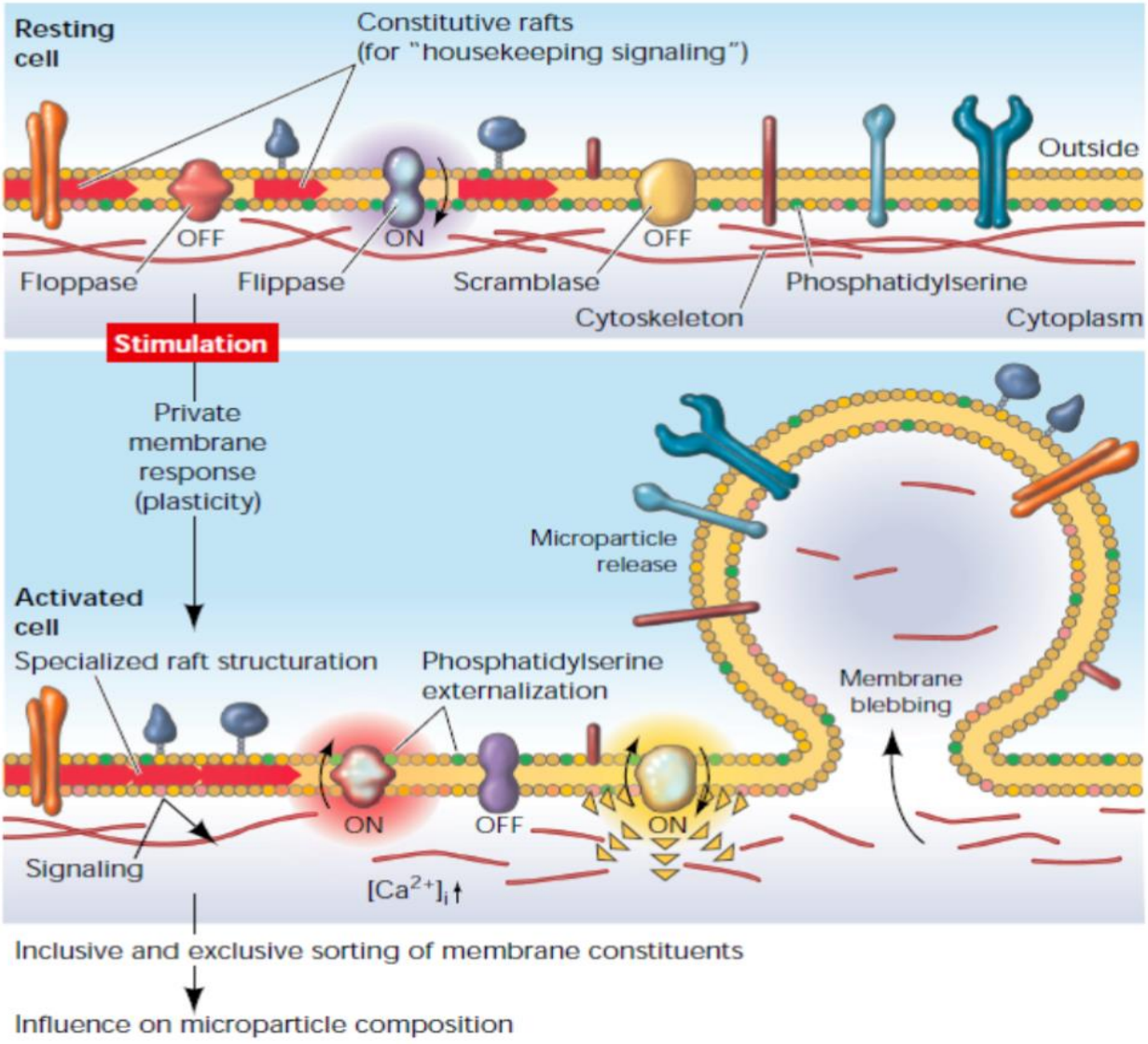

Figure 2-7 Schematic illustration of Process of microparticle formation from cell membranes (Hugel, 2005)

2.1.6 Mechanical Properties of Blood Clots

In haemostasis, clots must be stiff enough to stop haemorrhage yet reasonably elastic to withstand shear forces, a property called viscoelasticity. A clot formed from purely platelets is weak and withstand circulatory forces poorly. Much of the clot elasticity arises from fibrin fibers while stiffness is a function of ligation or cross linking conferred by FXIII. The origin of clot strength is still unknown but depending on clot structure and biochemical properties, it is thought to be interaction of fibrin meshwork with platelet retraction (Guthold et al., 2007; John W. Weisel, 2004).

During fibrin polymerization there is finite transition from fluid to solid states termed the gel point. This is the point when there is sudden development of clot stiffness. Thereafter, stiffness increases as a function over time, correlating with other measures such as turbidity or light scattering (Hantgan & Hermans, 1979; John W. Weisel, 2004; John W. Weisel & Litvinov, 2013). The mechanical strength, arising from gel point is a function of, and interaction between fibrin fibres and platelets (John W. Weisel, 2004).

Structural origin of clot stiffness have been correlated with network features, particularly fiber lengths, fiber diameters and branching densities as seen on electron microscopy (Ryan et al., 1999). Clot rigidity correlates with increasing fiber and branch point density (John W. Weisel, 2004), network structure as a function of fibrinogen concentration, fibrin fiber cross linking and other determinants of clot structure such as FXIIIa and calcium (Ryan et al., 1999).

Factors that modify fiber thickness also modify mechanical properties. For example, lowering concentration of thrombin enzyme reduces rate of fibrinopeptide release, resulting in slower production of fibrin monomers. Consequently, protofibrils grow longer and aggregate laterally producing long thick fibers. In contrast, higher thrombin concentrations cleave fibrinopeptides at a faster rate leading to faster protofibril polymerization but slower lateral aggregation. The end result is short oligomers that join before lengthwise or lateral association hence thin but highly branched fibers (J. W. Weisel & Nagaswami, 1992)(Ryan et al., 1999). Similarly, decreased fibre size is observed with high fibrinogen concentrations owing to higher monomer formation not matched with lateral assembly. Also, increased fibre sizes are associated with elevated Ca++ concentrations owing to enhanced fibrinopeptide release, facilitated lateral assembly from decrease in repulsive charges as calcium binds to sialic acid on fibrin/fibrinogen and stabilization from cross linking (Ryan et al., 1999).

2.1.6.1 Correlates of fibrin structure and gel point

At the point of clotting time, coinciding with abrupt transition from fluid sol state to solid or gel state is correlated with many biochemical and physical events. The gel point corresponds to clotting time ~4.7 minutes on minimally altered whole blood (Saulius Butenas, Bouchard, Brummel-Ziedins, Parhami-Seren, & Mann, 2005). At this point, FXa generated by extrinsic pathway is on the decline being approximately 25-30% of the total remaining while prothrombinase initiated by intrinsic pathway is on the steep rise at 80% maximal capacity. This also corresponds to transition from thrombin generation initiation to propagation phases when thrombin amounts are 10 nM or 4% of total amount, the rest of 96% of thrombin being generated after gel point (Saulius Butenas et al., 2005), (Kathleen E. Brummel et al., 2002). At gel point,

thrombin is at 4% of its potential, and only less than 0.2% is required to activate its substrates (FV, FVIII, FXIII, osteonectin. Most thrombin (96%) is generated after clotting time. This coincides with appearance of thrombin β-chain that has a lag time of 3.6 minutes and achieves maximum levels after 20 minutes (Kathleen E. Brummel et al., 2002). From these observations, very little thrombin is required to participate in positive feedback reactions thus suggesting that most thrombin generated after gel point could be involved in non-clot functions. At this thrombin amount only osteonectin, FVIII, FV and FPA have been activated during the initiation phase, whereas most FPB and FIIβ chain are generated in the subsequent propagation phase that follows(Kathleen E. Brummel et al., 2002).From these observations it may be deduced that factors reducing amount of thrombin may not interfere with clotting or gel point, but on mechanical properties.

With the visualization of clot formation most fibrinogen has disappeared from the liquid phase and only 50% FPA has been cleaved. This correlates with maximum FpA concentration while FpB is just beginning to rise. At this point, fibrinogen/fibrin is almost (>95%) out-of solution indicating polymerization. Cross linking involving FpB occurs much later after gel point(K. E. Brummel, Butenas, & Mann, 1999). At gel point, there is abundance of fibrin intermediates consisting of dimers, trimers, but little lateral aggregation which occurs many minutes after gel point. The gel point occurs when fibrin monomers predominantly form protofibrils, but before full fibrinopeptide release and subsequent fibre network formation (Lorand, 2005).

At the incipient gel point, fibrinogen is 80% depleted while FpA is 40% of its maximum value. Release of fibrinopeptides continues to rise well after gel point. Fibrinopeptide release achieves maximum value well after gel point and thereafter decreases as they get incorporated into

protofibrils. FXIII activation is coincident with FpA release, and attendant γ-γ cross links are formed prior to and coincident with clotting. Significant FpB release occurs subsequent to FpA release, but after clotting and γ-γ cross linking is virtually complete. Thus fibrin protofibrils at gel point contains intact Bβ chains, therefore FpB release is not a requisite for γ-γ cross linking (K. E. Brummel et al., 1999). Therefore, measurement of fibrinogen or FPA levels may not correspond to gel point or clotting time.

2.1.6.2 Characteristics or Properties of Fibrin Clot

The conversion of fibrinogen to fibrin gels is accompanied by the development of certain characteristics, whose change with time often indicating clotting time. One of the elements is sudden characteristic increase in rigidity that continues to rise with time after clotting time (Ferry & Morrison, 1947). Other measures include abrupt changes in friability, syneresis, tensile strength, viscosity, elasticity, opacity and compaction. These can be used to classify clots into two extreme types-fine or coarse (Ferry & Morrison, 1947).These types correlate with clotting time and can be modified by ionic strength as well as thrombin and fibrinogen concentrations.

In as much the fine and coarse clots have been described, the description was in relation to reactions of pure fibrinogen and thrombin. Whether these properties may be found in plasma and least in whole blood is yet to be seen. The presence of other proteins in plasma having procoagulant and anticoagulant effects may confound the picture. Further, ionic and osmotic effects of crystalloids may interact with cellular elements in whole blood with consequent influence on clot properties. These can only be elucidated by empirical inquiry whose results are presented in this report.

2.2. Laboratory Evaluation of Coagulation

Although it is widely acknowledged that haemostatic functions of blood to seal vascular breakages can only be performed owing to its elasticity and rigidity when it clots, controversy still remains on how to reliably detect gel point or clotting. In essence discussions of methods are based on application of the principles of detection of gel point, or point of change of state from liquid to solid, knowledge which was derived from polymer science or Newtonian fluids. Of note is that blood is neither a pure Newtonian fluid obeying linearity nor a pure polymer as it clots with suspended cellular elements. Complication arises since there is mutual interaction between non-protein plasma constituents and its cellular elements, such as the influence of osmolality/tonicity on blood cells, effects of cellular lytic products on coagulation factors and other cells, and physical characteristics after gelation. In an attempt to estimate *in vivo* situations using *in vitro* tests, various methodologies and techniques have been adapted over the years.

2.2.1. Plasma Clotting Time Based Tests

These are also called kinematic or clotting time based coagulation tests. They were developed as screening tests for coagulation factor deficiencies, or to monitor therapeutic effects of anticoagulation medication.

2.2.1.1. Standard Plasma Assays or Routine tests

These assays are based on the fact that since the coagulation factor pathways are sensitive to and can be initiated by specific reagents, so, in the presence of saturating levels of trigger, a defect can be inferred. They were designed to estimate factor deficiencies or monitor effects of anticoagulants. For reliability and reproducibility, the tests are performed on plasma. These assays determine time needed for platelet poor citrated plasma to clot after adding of excess

thromboplastin (tissue factor) for extrinsic pathway or contact activator for intrinsic pathway and calcium (Rochon & Shore-Lesserson, 2006). Therefore, the results and interpretations are dependent on type and source of reagent. They are commonly used in clinical laboratories.

The prothrombin time (PT) test was described and standardised by Armand Quick in 1935 to measure prothrombin (FII) but is also sensitive to the extrinsic pathway coagulation factors (FVII, FX, FV, FII and fibrinogen). It is a one stage test measuring time required for clot formation after addition to plasma of tissue thromboplastin consisting of tissue factor (TF), phospholipid and calcium. Thromboplastin was originally derived from animal organ extracts, particularly rabbit, but owing species specificity, most laboratories now use recombinant TF repilidated. Owing to variability in response, the reagents have to be standardized with International Standardization Index (ISI) sample close to 1, and World Health Organisation (WHO) recommended reporting of results as international normalized ratio (INR) since 1983 (WHO,1983).

Activated partial thromboplastin time (aPTT) test in seconds, employs surface activators such as kaolin and is sensitive to the intrinsic or contact factor pathway (FXII, FXI, FIX and FVIII). The term 'activated partial thromboplastin time (aPTT)' was coined in the original description of test in 1953 in which only the phospholipid concentration of the test was controlled, as opposed to surface activators which accelerated clotting but did not correct the prolonged clotting times of haemophiliac plasma. Essentially, the term means phospholipid is present but no tissue factor. The test is also known as kaolin cephalin clotting time (KCCT) or partial thromboplastin time with kaolin (PTTK)when kaolin is used as activator and cephalin as source of phospholipids.

Cephalin is a phospholipid substitute that replaces platelet phospholipids in the test. The role of kaolin is to act as a surface activator by binding directly to FXII resulting in FXIIa initiating intrinsic coagulation. Kaolin opacity makes the optical detection of end point difficult in automated systems and therefore alternatives employed include micronized silica, celite or ellagic acid.

There are many problems associated with current aPTT testing. It is not yet possible to standardize the aPTT and introduce common units. The triggering of contact activation with a variety of materials such as kaolin, silica or ellagic acidin the aPTT has been reported to lack reproducibility and reliability which originates from a poor onset of clotting and unpredictable patient response to surface activation. The lack of standardized aPTT materials and measurement methods and the large variety of commercially available reagent types has been highlighted. Overall, aPTT reagents demonstrate varying responsiveness to factor deficiencies and lupus anticoagulant. The reference interval for healthy controls must thus be determined locally. To reduce the variability in aPTT results, pre-incubation of a plasma sample with surface activator and phospholipids is recommended as the standard procedure. This, in turn, introduces multiple pre-analytical steps that complicate the assay and prolongs the total test time. This also leads to more complex automation to be performed such as the requirement of pumps for mixing. Furthermore, most commercially available aPTT devices allow for clotting time monitoring in plasma but not in whole blood samples. These limitations cause the test to be less accessible as it requires time-consuming sample preparation, trained personnel and the use of laboratory-based equipment such as centrifuges.

While there is widespread use of the aPTT internationally, the reagents and instruments used in the determination of the aPTT have changed significantly over the last 25 years. A distinct disadvantage of the aPTT is the loss of reproducibility across tests from different manufacturers as well as lot-to-lot variation in test kits from the same manufacturer. The aPTT is also well-known for its variability in responsiveness as a result of differences in reagent composition such as phospholipid or activator type, and methodological differences such as the type of instrument/coagulometer used. Nevertheless aPTT is still the gold standard screening test for factor deficiencies in the intrinsic coagulation pathway.

Thrombin time test (TT) - is employed to test the activity of thrombin in the presence of administered inhibitors such as heparin and other antithrombins. The test involves addition of excess bovine or human thrombin, which overcomes inhibitors or anticoagulants, reflecting conversion of fibrinogen to fibrin. Since it uses active α-thrombin, recalcification is not necessary.

2.2.1.2. Detection of clotting end point or gel point

Although PT and aPTT coagulation tests are differentiated by the addition of activators added to plasma, they are essentially clot based coagulation assays that rely on the measurement of time to onset of detectable clot formation as an end point. These are based on the principle that the clot is formed when fibrin (ogen) in solution rapidly transform into a solid state, called sol-gel transformation, a point which is still controversial. The point of contention, contributing to difficulty in comparability of test results, revolves around debate as to which physicochemical characteristic best describes the gel point.

A variety of methods exist to detect the clotting point. Most methods are based on changes of physical property of the clot. The simplest and gold standard is a visual inspection of gel formation. This can be detected manually by tilting test tube filled with blood, back and forth till it stops moving. Though this method is still the WHO gold standard for calibration for coagulation equipments, it does suffer many limitations including: being manual it is operator dependent thus could lead to different clotting time reporting by different observers in the same samples, it does not lend itself to automation as well as inability to test many samples at a go.

2.2.1.3 Mechanical clot detection

With demand for automation in testing many blood samples, some instruments have adopted detection of change of clot rigidity. This mechanical clot detection involves monitoring the movement of a steel ball within the test solution. As clot formation occurs, the movement of the ball changes, which is detected by the sensor. There are two variations of the method: a change in the movement of the steel ball may be detected when there is increased viscosity of the test solution or by a break in contact with the magnetic sensors when the steel ball becomes incorporated into a fibrin clot as the cuvette rotates. The formation of an insoluble fibrin network in the mixture serves to complete an electrical circuit in the clot detection system (Deepak, 2013; Bai, 2008).Mechanical clot detection using a steel rod as opposed to a steel ball is also a common method of detection.

A major benefit of mechanical clot detection is that there is no interference due to physical characteristics such as lipemia or haemolysis. Some also analyse whole blood, removing need for centrifugation. However, it is impossible to observe graphics of clot formation or may present problems of endpoint detection in some samples with low fibrinogen levels.

In the modified form, electromechanical method, where clotting factors and inorganic ions at clotting time concentrate and contribute to impedance and therefore stop movement of a probe. However, it is not suitable in presence of ionic crystalloids during haemodilution and is prone to external noise from vibrations.

2.2.1.4 Optical Detection

Due to limitations of mechanical methods of clot detection, some instruments employ photo-optical clot detection methods, also known as turbidometry. These rely on detection of change in optical density (OD) of a test sample as it clots. As the plasma sample clots, it becomes more optically dense and the amount of light falling on a photo-sensitive detector decreases (i.e. transmitted light decreases).The drop or change in light is determined as the endpoint (Deepak, 2013).In a study comparing photo–optical and mechanical PT clot detection systems, excellent agreement was found for both methods (Deepak2013).However, photo-optical methods are prone to interference by chromogenic substances within the test sample, therefore difficult to reliably mark gel point. The same difficulties also limit chromogenic and fluorescent methods.

Techniques such as immunologic or nephelerometric methods are suitable for individual factor assays but are of little value for clotting time detection in clinical samples.

Clotting time tests essentially measure the onset of clot formation which results from the formation of fibrin fibers in the blood sample. However, such assays are inherently imprecise due to the highly variable nature of the clot formation process and the sample matrix.

Routine coagulation tests have a number of limitations namely; elimination of contribution of platelets and other cellular elements in haemostasis. Also, application of excess coagulation factors to activate clot formation is not physiological. Whereas circulating TF concentrations are below 40pM, routine clotting assays involve addition of over 200 nM TF that are obviously unphysiological (Saulius Butenas et al., 2009). Further, they stop at the time point of clot formation therefore neither informative about clot dissolution (hyperfibrinolysis) nor clot strength. In essence, the tests are terminated when thrombin is still less than 10nMol, and before fibrin polymers are ligated by FXIIIa. They therefore are not able to estimate thrombus stability. Overall these tests were not only developed for monitoring haemophilia and anticoagulation therapy, but are also considered artificial since they are best performed on non-cellular blood components therefore eliminating important haemostatic interactions. A major analytical limitation of clot based assays is that lack of clot formation may be due to deficiency of thrombin generation or inhibition of its activity or lack of fibrin polymerization independent of thrombin(Daniel Bolliger et al., 2010). Therefore, in such a case, it is often impossible to deduce which arm of coagulation cascade is affected. Nevertheless, they are still useful as screening tests to guide more specific and complicated coagulation assays.

In the new modified routine test, Clot waveform analysis, has been developed to overcome some of the limitations of PT or aPTT (Toh & Giles, 2002).In addition, more useful information can be obtained by calculating first and second derivatives. It is gaining clinical applications in investigation of DIC, haemophilia and monitoring anticoagulant therapy independent of thrombin (Shima, Thachil, Nair, Srivastava, & Scientific and Standardization Committee, 2013). However, universal uptake still remains a challenge. This is due to the fact that there is not much

experience with this assay since the parameters seem quite unknown, and there is not much literature with regard to clinical validation (Lancé, 2015).

2.2.1.5 Laboratory Assays of Fibrinogen

Fibrinogen is the substrate for thrombin enzyme, that when activated gels out of solution to form a clot. Therefore, its determination is performed as a routine screening test in cases of prolonged clotting time. Various methods exist to aid in its determination and results are reported in mg/dL. These methods generally fall under functional or clotting method such as Clauss clottable assays or PT fibrinogen based on prothrombin time, immunological detection of fibrinogen antigen independent of functions and gravimetric based on clot weight. Of these, Clauss method is the oldest and routinely used in clinical laboratories (Clauss, 1957). It is the gold standard or reference quantitative method for determination of functional or clottable fibrinogen in plasma. The Clauss method of fibrinogen determination utilizes supra-physiological/excess amounts of thrombin and depends on identification of clotting time as an end point which is converted to fibrinogen levels by comparison to normogram. These thrombin concentrations by-passes reactions cascades in prothrombin activation to thrombin that is designed to surmount any inhibitor present in mixture.

Clauss method is based on the principle that, in the presence of excess thrombin, soluble fibrinogen is converted to insoluble fibrin clot and clotting time (in sec) is inversely proportional to fibrinogen concentration in plasma. Using standard dilutions, result for amount of fibrinogen in test is read off a table (mg/dL) by comparison with control plasma results. The use of a high concentration of thrombin (typically 100 U/ml) ensures that the clotting times are independent of thrombin concentration over a wide range of fibrinogen levels, and to minimise the effect of

'inhibitory substances' within the plasma e.g. heparin, elevated levels of FDPs. The method assumes a linear correlation between clotting times in the region of 10-50s. However, care has to be taken in interpretation of results from fibrinogen values out of the linearity range, either too low or too high concentration ranges. This method was originally manual, but has now been automated.

In the PT derived fibrinogen method, fibrinogen level is derived from PT performed in a range of plasma dilutions, the results read off a calibration curve. The derived fibrinogen is a simple and in-expensive test and is widely used. However, the test can give misleading results in some disorders and is not recommended for routine laboratory use.

Both the Clauss and PT derived fibrinogen assays rely on clot formation as an end point. The assumption is that excess thrombin will always clot fibrinogen, and therefore a prolonged clotting time correlates to low fibrinogen substrate. However, this is not always the case in test plasma samples, since there may be strong thrombin inhibitors or lack of fibrin polymerization post thrombin. If such a case is suspected, then other specific methods that do not depend on clot formation need to be employed (Miesbach, Schenk, Alesci, & Lindhoff-Last, 2010).

Other methods of fibrinogen determination which are independent of clot formation include: immunological assays, gravimetric measurements or heat precipitation assays which measure protein concentration rather than functional activity. However, they are of little value in coagulation studies. Furthermore, they are unsuitable for haemodilutional studies since assays

involve buffers that may introduce confounders when crystalloids solutions are under consideration.

2.2.2 Thrombin Generation

Thrombin is the key coagulation enzyme, whose activity determines whether blood in fluid would form clots, or remain as liquid. Therefore, in non-clottable mixtures which is often assumed to arise from thrombin inhibited activity or attenuation of its generation, though not always the case.

Determination of Thrombin, like other coagulation factors, cannot be investigated quantitatively by chemical means. The concentration or activity must be evaluated in relative to reactions that occur. Like most enzymes its activity is demonstrable by effects on substrate conversion to products, the methods which vary in type of substrate, or techniques of product analysis.

Physiological actions of thrombin are demonstrable by its ability to coagulate fibrinogen, which has led to a number of definitions and adoption of different activity units. However, use of clot end point in determining thrombin activity may be problematic in clotting assays since inhibitors of prothrombin-thrombin conversion or thrombin may also affect fibrin monomer polymerization or gel formation, in which thrombin plays no role. Non suitability of fibrinogen clotting as an indicator for thrombin activity arose out of the observations that the subsequent fibrin monomers produced from thrombin activity consists of three reversible steps. Thrombin (T) is involved is in only the first step which is a limited proteolysis to release fibrinopeptides (FpA and FpB) from fibrinogen to produce fibrin monomer (Scheraga, 2004).Subsequent interaction of fibrin monomers that ends up in a clot is a biophyiscal process that occurs in four overlapping stages,

independent of enzymatic activity. Substances that inhibit fibrin dimer formation, protofibril linear growth, lateral aggregation and fibre cross linking may give the impression of prolonged clotting time or no clot at all thus leading to false conclusion of disordered thrombin generation/activity which may not be true. In this respect many substrates indicative of thrombin activity independent of fibrin clot formation has replaced fibrinogen.

Thus the two processes, fibrinogen cleavage and fibrin monomer, can be influenced independently. Also, the relationship between thrombin concentration and clotting time is rectangular hyperbolar and not linear, an inverse relationship. Furthermore, description of activity during calibration only applied to pure thrombin and fibrinogen, and presence of inhibitors for either factor could confound the activity. With observations that showed that thrombin had esteratic activity independent of clotting activity, a non-clottable thrombin substrate such as p-tosyl l-arginine methyl ester (TAMe) replaced fibrinogen. The thrombin activity was monitored by color change. However, TAMe was found to accelerate fibrinogen precipitation independent of thrombin, thus unsuitable as an indicator of thrombin activity in plasma (Abilgard, 1964).Although Sheraga followed hydrolysis by potentiometric method, use of TAMe substrate for thrombin was found non- specific since other enzymes also hydrolyze TAMe (Scheraga and Laskowski, 1957). TAMe was thereafter replaced by other synthetic substrates.

Because thrombin is a serine proteinase specific for serine peptide bonds, a variety of synthetic substrates have been prepared to match that specificity. Thrombin cleavage of fibrinogen occurs only at Arg residues. However, the cleavage site is not specific thus resulting in many products.

Also, Thrombin from any mammalian species will clot the fibrinogen of any other mammalian species. The next step was to add a coloured chemical group, such as *p*-nitroaniline, to aid in the quick measurement of the enzymatic activity using spectrophotometry in the visible light range. This allows determination of its products in plasma. Thus thrombin (human and bovine) will catalyze the hydrolysis of several peptide *p*-nitroanilides such TAMe.

In addition to chromogenic substrates developed on the basis of short peptides terminating with para-nitroaniline (*p*-nitroaniline), conceptually analogous assays have been developed using different chemical characteristics. Another approach utilizes peptide thioesters in which hydrolysis generate a colour associated with the liberated sulfhydryl group. Several other reagents have been developed that generate colour after hydrolytic cleavage, but these reagents are more commonly used in research than in clinical coagulation laboratories (Budzynski, 2001)

The problem of chromogenic substrates was that they do not have exclusive specificity for a single enzyme; these compounds will react with several enzymes. For example, chromogenic substrates for plasmin have the following cross- reactivities: with plasmin, thrombin, Factor Xa, activated protein C, and tissue plasminogen activator (tPA). Furthermore, a reaction with a chromogenic substrate is sensitive to changes of temperature and pH. Consequently, these 2parameters must be maintained at a constant level during the reaction. Hydrolysis of chromogenic substrates by proteinases is affected by the composition of the buffer used to maintain pH as well as by reagents added to the analysed specimen such as inorganic salts, polyethylene glycol, or serum albumin. Therefore, it is important to maintain the same

experimental conditions and the same amounts of the added reagents to obtain reproducible results in the laboratory (Budzynski, 2001).

The most important initial experimental limitation of chromogenic assays was that it could be done only in defibrinated platelet poor plasma (PPP). Thus at least two important components of blood coagulation (i.e. platelets and fibrinogen/fibrin) were absent (Kumar, Béguin, & Hemker, 1995). These limitations were overcome by fluorogenic substrates which have replaced earlier chromogenic substrate methods (H. Coenraad Hemker, 2008). Fluorogenic substrates have been synthesized using coumarin derivatives linked to a peptide by an amide bond. Enzymatic hydrolysis liberates free coumarin derivatives, which are fluorescent. Measurement of fluorescence is much more sensitive than visible colorimetry, but the former is also more susceptible to disturbances by fluorescence quenching agents (H. Coenraad Hemker, 2008)

2.2.2.1. Thrombin generation Tests (TGT)

Initial methods of determination of thrombin activity in plasma were manual sub-sampling by tube-tilt method which was calibrated against parallel samples of purified thrombin and fibrinogen of known activity. In fact, the WHO standards still use this as the reference method. The problem is that it is a static measure which gives no clue in the evolution of thrombin amounts from the zymogen over time, from initial small amounts, peak to termination of activity.

First description of the continuous determination of thrombin activity in plasma was done independently by two groups (Macfarlane & Biggs, 1953)(Pitney & Dacie, 1953). Subsequently, Hemker and group pioneered modifications of the work that initially used fibrinogen as substrate and manual sub-sampling. These techniques involved stopped assays in which several test

samples were incubated with triggers and after a fixed period each sample was stopped with an inhibitor. The results were plotted to give activity over time (Scopes, 2002). However, these had limitations, mainly the inhibitors denatured the enzyme and therefore the progression of events post inhibition could not be estimated; and also the curves drawn assumed linear relationship, a phenomenon rarely encountered in biological reactions.

With advances in analysis of enzyme activity in general, continuous assays were incorporated in the determination of thrombin generation. With the availability of synthetic chromogenic or fluorogenic substrates, thrombin activity could be followed spectrophotometrically by continuously monitoring absorbance or fluorescence of a sample over time, and any deviation from linearity could be observed (Hussein & Al-Hassan, 1987). Thereafter, computer automation was an added innovation (H. C. Hemker, Willems, & Béguin, 1986). Currently, the method, Thrombogram-Thrombinoscope Calibrated Automated Thrombogram (CAT) performed on a fluorometer gives continuous recording of thrombin generation in plasma over time. The method runs test sample in parallel to a known thrombin calibrator. The parameters for analysis are lag time (time to onset of 5 nM thrombin), peak thrombin level, and endogenous thrombin potential (ETP) which represents the area under the curve.

Although originally performed on plasma, it is now technically possible to perform thrombin generation (TG) in whole blood(Marisa Ninivaggi et al., 2012; Tappenden, Gallimore, Evans, Mackie, & Jones, 2007).Many scientific articles concerning Thrombin generation methods have been published (Baglin, 2005; Castoldi & Rosing, 2011; Alisa S. Wolberg, 2007). TG determination is gaining clinical application in the investigation of congenital and acquired

bleeding problems as well as thrombotic conditions (K. E. Brummel-Ziedins, Pouliot, & Mann, 2004; V. Chantarangkul, Tripodi, Clerici, Negri, & Mannucci, 1998; Dargaud, Trzeciak, Bordet, Ninet, & Negrier, 2006; H. Coenraad Hemker, Al Dieri, De Smedt, & Béguin, 2006a; Hézard, Bouaziz-Borgi, Remy, & Nguyen, 2006).

Although Thrombogram-Thrombinoscope had suffered from lack of standardization, especially with regard to pre-analytical variables and experimental conditions, many have been addressed (Gerotziafas et al., 2005). The optimal conditions found were thromboplastin dilution of 1: 1000, platelet count higher than 50x 10^9/L, and synthetic phospholipids concentration of 4μL.

One of the limitation of continuous thrombin generation assays using exogenous synthetic thrombin substrates, is modification of TF initiated TG curves (S. Butenas & Mann, 2007),. Specifically, lag phase is prolonged and peak thrombin level is higher suggesting competitive inhibition of thrombin positive feedback reactions (H. C. Hemker & De Smedt, 2007) but the practical significance for each substrate remains to be explored. Nevertheless, caution should be exercised in interpretation of TG curves especially in clinical specimens.

Most of the assays mentioned are indirect, in that they determine end product of thrombin. They thus do not give information on differentiation of prothrombin activation products. Immunological methods are sensitive in detecting non clotting products such as meizothrombin from active α-thrombin(Shuman & Majerus, 1976). In this respect, KG Mann and group pioneered ELISA determination of the evolution of thrombin-antithrombin (TAT) complex over time that led to a description of the major phases of thrombin generation as initiation phase (time

to 5nM thrombin), propagation phase (explosive or thrombin burst) and peak height and termination phases (Butenas and Mann, 2001) that are similar to the CAT™ assay system (Figure 2-9) .Measurement of TAT combined with F1.2 determinations as products of prothrombin activation (K. Brummel-Ziedins, Whelihan, Ziedins, & Mann, 2006; Chandler & Velan, 2003) are claimed to give a better estimate of prothrombin conversion to thrombin. However, they neither predict thrombin clotting activity nor its esteratic activity. Alternatively, although determination of FpA and FpB are evidence of thrombin amydolytic action on fibrinogen, their determination are however too laborious for routine clinical use.

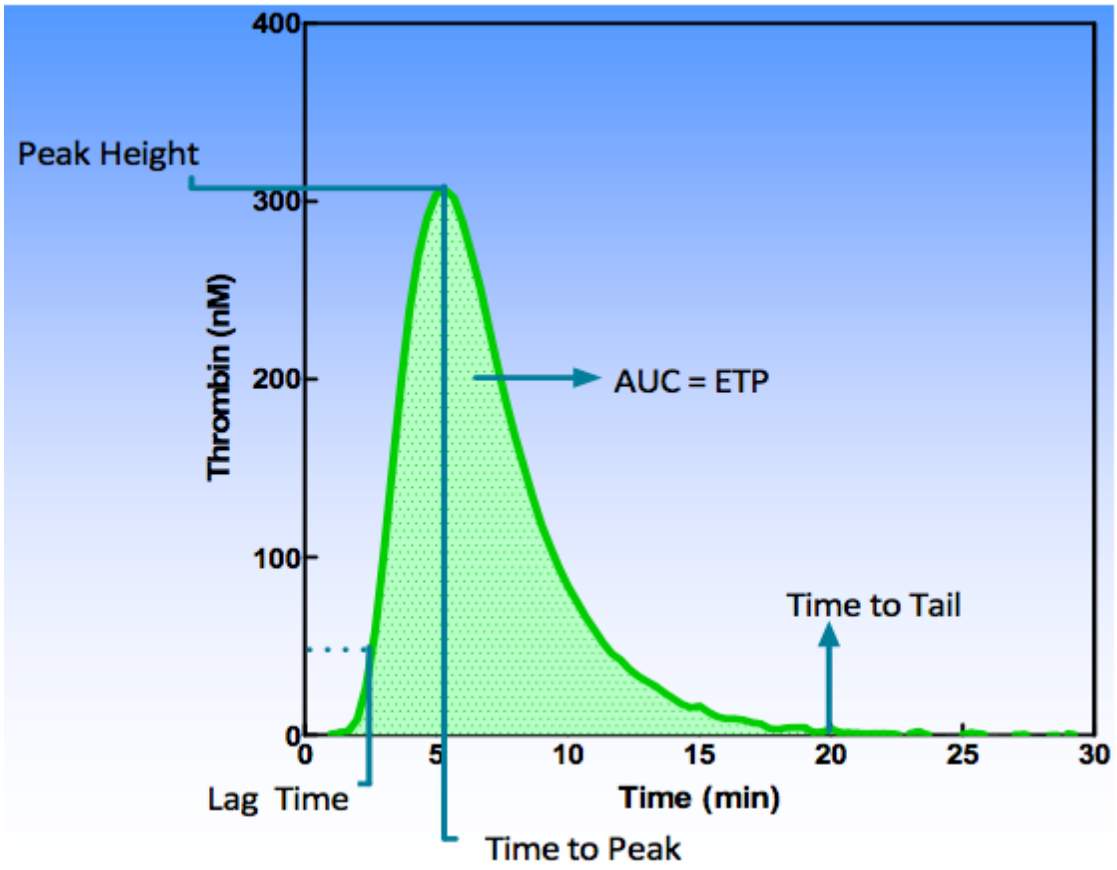

Figure 2-8 Typical Thrombin generation curve and parameters as displayed by CAT™software (Source-Thrombinoscope data on file). AUC- area under curve, ETP-endogenous thrombin potential, lag time represent time to predetermined thrombin level, usually 5nM;

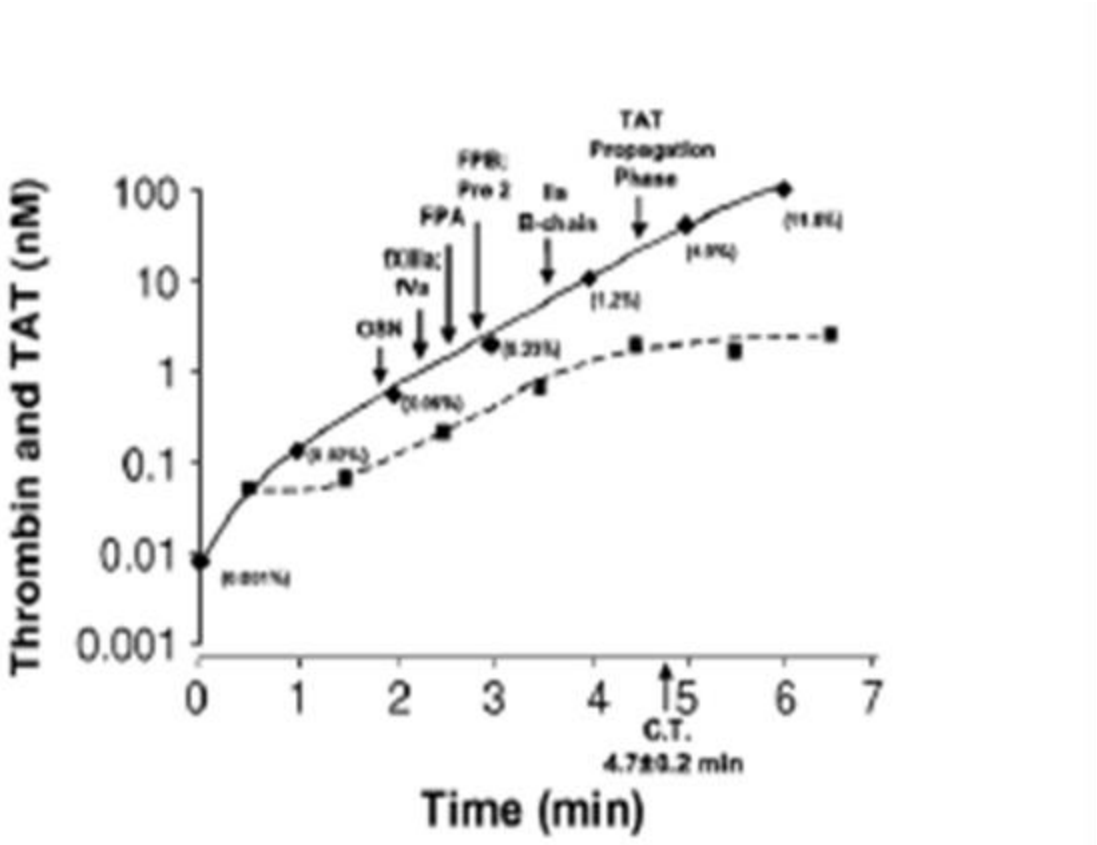

Figure 2-9 Thrombin activities during lag phase (Brummel, 2002).
osn-osteonectin, FVa-Fva, FVIIIa-FVIIIa, FPA-fibrinopeptide A, FPBfibrinopeptide B, pre 2-pre peptide 2 of thrombin, Ia β chain-fibrinogen β chain, TAT-thrombin antithrombin complex, C.T-clotting time shown by arrow≈4.7 minutes

Overall, a lot of information has been derived from analysis of thrombin generation. In particular, clotting time in routine, standard plasma fibrin-based clotting tests correspond to transition between lag phase and propagation (Kathleen E. Brummel et al., 2002; Kenneth G. Mann et al., 2006) when amount of thrombin is only less than 5% of total generated (fig 2-10). Most of thrombin (>95% of total) is generated after clotting point. Therefore determination of thrombin generation in clotting plasma has been claimed to give more information than PT or aPTT.

2.2.3 Viscoelastic Coagulation Methods

These are based on the principle that as blood clots the resulting fibrin formation and cross linking alters its viscosity and elasticity as a function of fibrin formation, rate of fibrin build up and tensile strength of the clot. During the coagulation process, blood changes its physical properties from the status of a pregel viscoelastic fluid to a viscoelastic solid. The turning point between the two physical conditions has been previously defined as the gel point (GP)(Evans et al., 2008). Before reaching the GP, blood reacts to changes in shear rates (SR) as a non-Newtonian fluid, exerting its viscous properties; after the GP, the forming clot under flow conditions manifests both the properties of a fluid (viscosity) and of a solid (elasticity), and these mixed characteristics take the name of viscoelastic properties of the clot. Many operational definitions of a blood 'Gel Point', 'Clotting Time' (CT) or 'clot formation time' criterion are based on the attainment of an *arbitrary* level of dynamic rigidity during coagulation, at a particular frequency of oscillation (Evans, 2006). These criteria are applicable to whole human blood clots, and other biogels such as collagen and gelatin.

In whole blood, the major determinant of gel point is thrombin generation, and associated platelet reactions that leads to clot network formation that contributes to clot stiffness or rigidity (Ranucci, Laddomada, Ranucci, & Baryshnikova, 2014). These principles have been applied in design of rheological coagulometers such as Thromboelastography (TEG) or thromboelastometry (ROTEM).

2.2.3.1 Thromboelastography (TEG)

In its most basic form the TEG consists of an inner cylinder (referred to as the 'Pin') suspended on a torsion wire and an outer cylinder (referred to as the 'cup') in the form of a cuvette, which

performs unsteady oscillation. A sample (typically 0.36ml) of blood is placed within the cup which moves back and forth, typically every 10 seconds, through an angle of 4 degrees 45 minutes. The eventual formation of a fibrin network (or clot) between the surfaces of the pin and cup are deemed to result in a coupling of the motion of the latter to the former. The resulting pin movement is recorded by an electromagnetic transducer and is plotted as a displacement (in *millimetres*) in both the clockwise and counter clockwise directions as separate curves on a chart-type record called a 'thromboelastogram'. TEG measurements will inevitably involve both elastic and viscous contributions from the evolving clot, and it is well established that liquids with a sufficiently high shear viscosity will generate substantial thromboelastograph readings, despite the absence of fluid elasticity. TEG parameters are typically (fig 2-12)-lag time, R (min), coagulation time, k (min), alpha angle, α-angle (^{0}C), maximum amplitude, MA (mm), lysis time at 30 and 60 minutes after MA (Donahue & Otto, 2005; Luddington, 2005).

The 'lag time', R, is the time that elapses between the start of TEG data collection to a pin movement greater than 2 mm on the chart recording defined as the reaction time. It is the interval necessary to initiate fibrin network formation. Various other TEG parameters which are used to characterise the course of coagulation, include (i) the 'k value' - the time taken from the beginning of clot formation until the TEG amplitude of the TEG output trace reaches an arbitrary value 20mm; (ii) the'alpha (α) angle' between the line in the middle of the thromboelastogram and the line tangential to the developing "body" of the TEG trace; (iii) the 'maximal amplitude'(MA) - the greatest amplitude of the thromboelastogram; (iv) the amplitude of the thromboelastogram 60 minutes after MA and; (v) the 'clot lysis index' - the amplitude at 60 minutes, expressed as a percentage of MA. Aspects of the relationships between

thromboelastogram parameters and linear viscoelastic parameters derived from rheometrical studies of coagulation are arbitrary, and therefore tests are best performed before and after intervention. In addition to its original use in providing a global coagulation profile, other uses of the TEG have included measurements of the 'Shear Elastic Modulus Strength' (Glidden, Malaska, & Herring, 2000), and estimation of generation of thrombin(Rivard et al., 2005), though less specific and suffers from lack of validation.

Thromboelastography can also be used to estimate functional fibrinogen levels in a method known as Modified Thromboelastography (Katori, Szlam, Levy, & Tanaka, 2004; Katori, Tanaka, Szlam, & Levy, 2005)(Virginia A. Bowbrick, Mikhailidis, & Stansby, 2003; Craft et al., 2004). In this method, platelet aggregation inhibitors such as cytochalasin D is added (to eliminate contribution from platelets) to a sample and TEG test run. The result of clot strength or maximum amplitude is compared to a control without added platelet inhibitor. The difference reflects contribution of platelets to clot strength. However, contributions of other cellular elements still remain which are not sensitive to platelet inhibitors. Furthermore, since no saturating thrombin is added, it may be difficult to interpret flat TEG trace, which could arise from either thrombin deficiency or excessive fibrinolysis (Harr et al., 2013; Kalina et al., 2008). In the case of a flat TEG trace, parallel samples are run containing protamine and fibrinolytic inhibitors. However, the task would require multiple assays that are neither economical nor clinically useful.

A variety of Thromboelastography machine designs are now available differing in computer software and transduction systems which include: Thrombelastograph™ 5000 (Haemoscope,

Niles, Skokie, USA) (fig 2-11), ROTEG ™ (Pentapharm, GmbH, Munich, Germany)-uses optical transduction, ROTEM™ (Pentapharm, GmbH, and Munich, Germany) - modified ROTEG with ball bearings(Franz, 2009; Ganter & Hofer, 2008; Luddington, 2005).

The major advantages of the Thromboelastography over conventional coagulation tests is testing clot formation within the whole blood hence estimating the interaction of coagulation factors in blood with cellular elements as well as information on clot strength and dissolution all from one sample concurrently. TEG can be performed on plasma, platelet concentrates, citrated and recalcified samples. Thromboelastography distinctively offers benefit over clotting time based coagulation tests because it begins to gather information where these methods stop. The time course of monitored parameters-strength and integrity of clot may be useful clinically in effective haemostasis (John W. Weisel, 2004).

Another major advantage of Thromboelastography/metry over conventional routine coagulation tests is that it can be performed on whole blood or plasma, whether citrated or not to estimate blood clot lysis in a sample. It is the most sensitive real time analyzer of ongoing blood clot lysis. The principle relies on displaying time dependent blood clot strength regression after maximum amplitude has been attained. It is sensitive to endogenous fibrinolytic activity, exogenous fibrinolytic agents as well as testing the effects of antifibrinolytic agents. In addition, the sensitivity and reliability in hypercoagulability and haemorrhage exceeds routine/ standard coagulation tests (Essell, Martin, Salinas, Thompson, & Smith, 1993; Howland, Schweizer, & Gould, 1974; Wong, Liu, & Glassenberg, 1995; Zuckerman, Cohen, Vagher, Woodward, & Caprini, 1981).

Despite the many benefits of TEG measurements, limitations are still experienced. A major concern regarding TEG or ROTEM is that they provide a measure of the Viscoelastic properties that is expressed in arbitrary units (mm), based on the transmission of the movement of blood to a pin. In these tests, the shear rate, SR ,is fixed at a very low value (0.5 sec ^{1}) that is not found in any part of the natural circulation (Ranucci et al., 2014). Furthermore, a principal drawback of the TEG is that the strain amplitude experienced by the sample is uncontrolled and changes significantly during the course of coagulation (Evans et al., 2008). This feature of the instrument's operation results in TEG measurements transgressing the non-linear viscoelastic regime during coagulation monitoring with the result that the TEG's operation can substantially modify clot structure during the measuring (Evans, 2006). All these drawbacks have been acknowledged and applications in testing are driven by its richness of information and sensitivity. In acknowledging some of these drawbacks, standardization problems were addressed in one International Society for Thrombosis and Haemostasis (ISTH) consensus statement (Chitlur et al., 2011a).

Other methods that are based on principle of viscometry and rheometry are cone-on plate- (Ranucci et al., 2014), modified platelet contractile force (Marcus E. Carr, 2003).However, both viscometry and rheometry methods are research tools that look at blood like any other polymer gel. In essence they are of limited clinical applications since calibration and validation data in clinical settings are largely unavailable (Falvo et al., 2010).

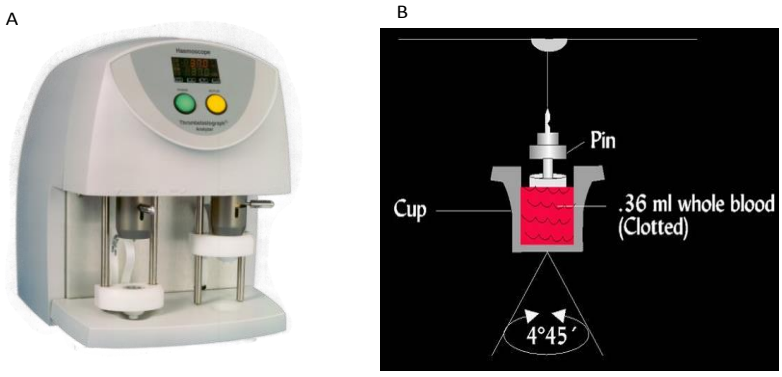

Figure 2-10 Thromboelastograph machine and principles of operation
A-TEG machine, B-0.36 ml of blood placed in cup which rotates arounf pin at $4^0$45' angle

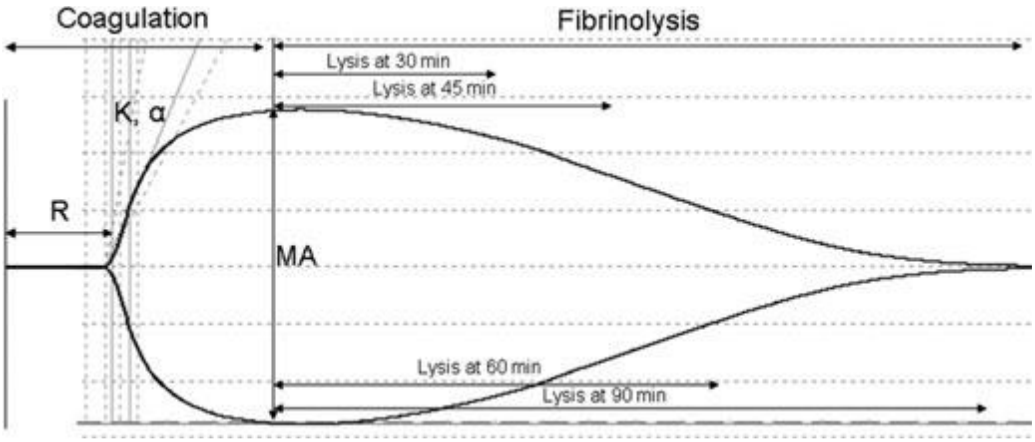

Figure 2-11 Typical TEG tracing. R-time to onset of clot formation (mins), K-predetermined value representing clot formation upto 20 mm amplitude, α-angle in degrees, MA-maximum amplitude (mm), lysis at 30, lysis 45& lysis 60-time to decrease amplitude 30 minutes , 45 minutes and 60 minutes after maximum amplitude respectively. Adapted from ref (Chitlur et al., 2011b)

2.2.4 Assessment of Platelet Functions

Blood platelets are now recognised to play many biological roles apart from haemostasis. They are involved in inflammation, host defence, tumour growth and atherosclerosis. Notwithstanding these multiplicities of roles, most well tested and known laboratory evaluation methods have majorly been described in relation to participation in primary haemostasis. Therefore this review will concentrate on platelet haemostatic function tests accordingly. The different laboratory methods under consideration are based on assessment of aspects of platelet reactions-vs adhesion and shape change, aggregation, on analysis of physical properties of clot, and measurement of compounds released under exogenous stimulation as surrogate markers for secretion. Laboratory methods evaluating platelet functions assess platelet reactions in isolation or as combined tests-adherence, shape change; granule content release/secretion, activation/receptor expression. Of these, assessment of aggregation is the most preferred method in clinical use.

2.2.4.1. Bleeding time

Historically, older methods to estimate *in vivo* platelet participation in haemostasis were platelet count and Ivy or Duke (1910) bleeding time. The method involves making a standardized puncture on an arm and recording time to haemostatic plug formation that leads to wound occlusion. For many years it was the main screening tests for platelet function disorders. However, it is associated with many confounders and thus not reflective of platelet function per se(Rodgers, 1990).Moreover, apart from lack of specificity, reproducibility between laboratories is an issue. It has been abandoned in many institutions.

2.2.4.2. Swirling

This is a non-invasive method for testing the quality of platelet rich plasma (PRP) for transfusion. Platelet swirling is caused by light diffraction due to the alignment of normal discoid

shaped platelets. When these discoid platelets align, light that is diffracted creates a cloudy or swirling like appearance. It is performed holding a bag of PRP horizontally, approximately 12-30 inches in front of 50 to 100 watt light source. The bag is moved gently with the fingers in motion in front of light source. In the thin areas of PRP bag, an appearance of cloudy or swirly appearance will be observed. The results are recorded as absent or negative, positive or extensive, moderate or intermediate swirl (Bertolini & Murphy, 1996; Mathai et al., 2006).

Swirling is a qualitative attempt to check for the presence of discoid-shaped platelets through visual inspection of the platelet bag by tapping or gently rotating the unit in front of a light source. The test is based on the principle that viable inactivated platelets are discoid in appearance, while activated platelets are generally spheroid. The discoid-shaped, non-activated platelets scatter the incident light in different directions resulting in a moving opalescence or swirling. By contrast, activated, spheroid platelets affected by storage lesions (such as microparticle formation, P-selectin expression, morphologic changes or loss of discoid shape) lose the ability to scatter light in different directions. In effect the swirling effect is lost and the platelets have a dull unchanging appearance on visual inspection. It is an old method for testing platelet concentrate viability (Mathai et al., 2006).

Although swirling was initially touted as a simple, non-invasive method for assessing platelet viability due to its correlation with pH values the subjective nature of the technique, its lack of sensitivity, and ongoing discussion between *in vitro* assessments and correlation with platelet function and transfusion efficacy have raised serious questions about the role of swirling as an indicator of platelet quality (Bertolini & Murphy, 1996; Mathai et al., 2006).Moreover, for

quality control, determination of pH, morphology, size and shape, and response to aggregating agents may need to be performed. However, these assays are time consuming and involve elaborate techniques thus unsuitable neither for routine use nor for many samples. Furthermore, information provided makes no reference to platelet interactions with and participation in clot formation especially with regard to crystalloid haemodilution.

2.2.4.3Hypotonic Shock Response (HSR) and Extent of Shape Change (ESC)

The HSR measures the ability of platelets to recover their volume after being exposed to a hypotonic environment. Exposure of platelets to hypotonic environment results in rapid initial swelling, followed by a gradual decline as the platelets resume their baseline size. With maintenance of normal membrane integrity and energy metabolism, platelets are able to extrude the water to regain normal volume. In the test, the addition of water to the cuvette containing PRP causes the platelets to swell because of the influx of water. Platelet swelling results in a decrease in the refractive index of the platelets and an increase in transmitted light as measured on a spectrophotometer (Lindahl & Ramström, 2009a).

In ESC assay, platelets are stimulated by agonists such as ADP, and proportions of platelet with discoid morphology that change to spherical shapes are followed. This transformation is associated with a decrease in transmitted light. The extent of the decrease in transmitting light has been shown to be directly related to the percentage of discoid platelets in the platelet-rich plasma (Holme, Moroff, & Murphy, 1998; Holme & Murphy, 1978; Lindahl & Ramström, 2009a). Visualization of morphological change of platelets from discoid to spherical with extrusion of pseudopods on activation can be detected by electron microscopy or flow cytometry

(Kuwahara et al., 2002). Also, it can be estimated by an initial decrease in light transmittance and the disappearance of oscillations of stirred platelet suspensions in the platelet aggregometer. However, it's not a suitable parameter in haemodilutional studies due to: premature *in vitro* activation and shape change arising from venepuncture; contact activation and exposure to varying temperatures. Furthermore, a reliable method differentiating in *vivo* from *in vitro* shape change remains largely unavailable. Furthermore, investigation of platelet activity in the absence of intact endothelium is a challenge.

Although ESC and HSR have been considered the 'gold standard' for in vitro estimation of platelet viability, lack of stable controls has precluded further standardization of these biologic assays even within a single laboratory. Therefore, the use of more quantitative methods such as aggregation have been advocated and adopted.

2.2.4 .4 Platelet Aggregometry

The original optical method for determination of platelet aggregation was a modified spectrophotometer that measures light transmission or turbidity of aggregating platelet rich plasma (PRP) compared to platelet poor plasma (PPP) as control (G. V. Born & Cross, 1963; G. V. Born, Dearnley, Foulks, & Sharp, 1978; G. V. R. Born & Cross, 1964). The principle relies on the fact that un-aggregated platelets suspended in plasma are turbid and therefore absorb light. However, as platelets are subjected to activators, they change shape, aggregate and fall to the bottom of cuvette despite continuous stirring thus allow more light to pass through the sample. In this method, optical density (OD) of light source through stirred platelet sample is followed over time. The readings are set such that platelet poor plasma (PPP) to represent 100% light transmittance (LT) compared to platelet rich plasma (PRP) representing 0% light transmittance

as control (fig 2-13). The OD of test sample is determined and plotted along the sample calibration(Harrison, 2009).

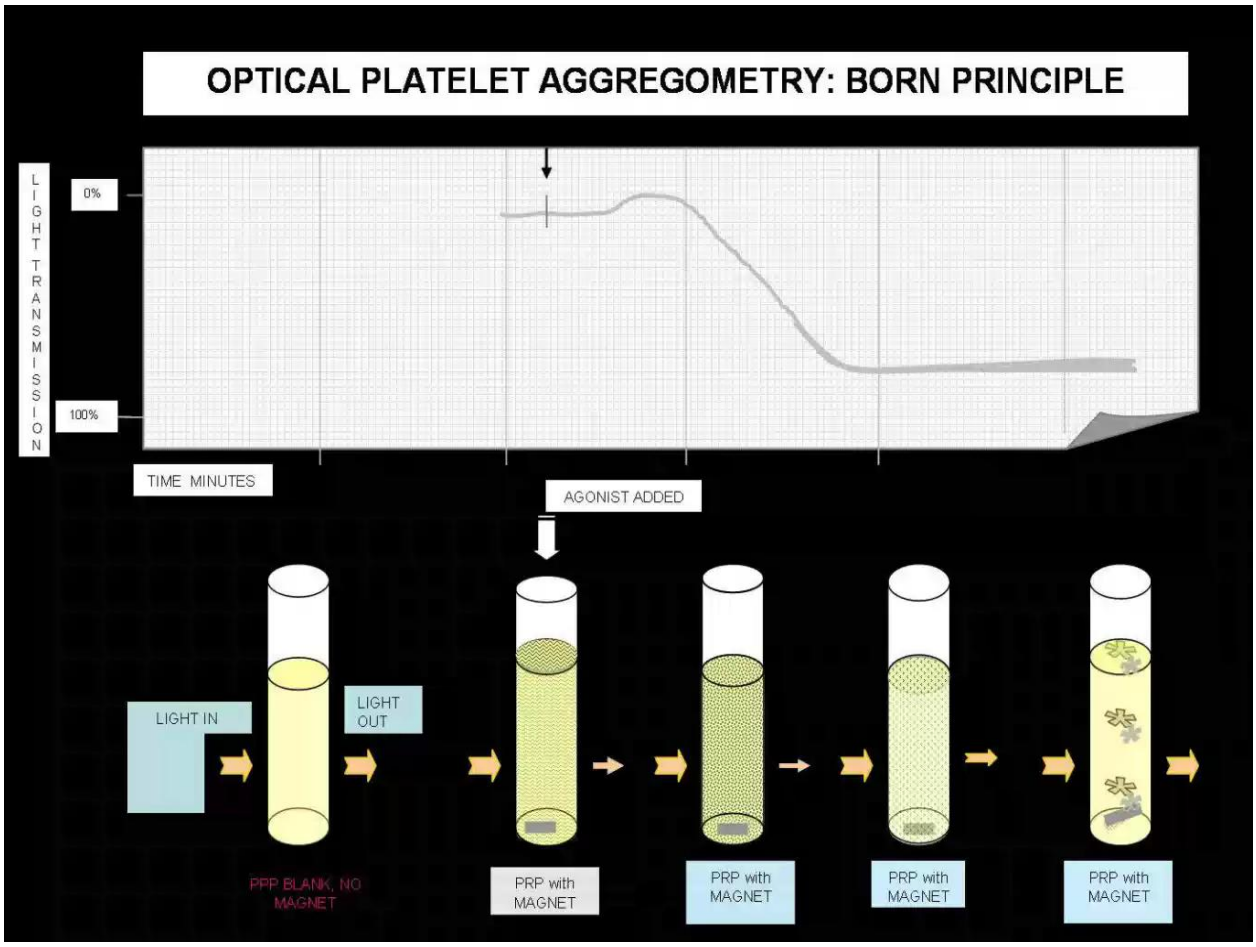

Figure 2-12 Principles of Born platelet aggregometry: Inset (top of figure) typical photometric trace recording light transmittance from 0%(PRP without agonist) to 100% (full aggregation). Below of figure shows steps of platelet changes in cuvette (Helena, data on file).

Platelet aggregation studies can be performed using platelet rich plasma (PRP), washed platelets, or whole blood, though PRP is the most common sample. Usually, analysis is done in the presence of primary agonists such as ADP, Arachidonic acid (AA), collagen and thrombin which react with their corresponding receptors on platelets. Additionally, secondary agonists such as

adrenaline, which become highly effective in the presence of low levels of primary agonists, are employed when investigating antiplatelet drugs such as aspirin. An antibiotic agonist ristocetin is applied to further define von Willebrand Factor (vWF) effect on aggregating platelets, (Paniccia, Priora, Alessandrello Liotta, & Abbate, 2015).

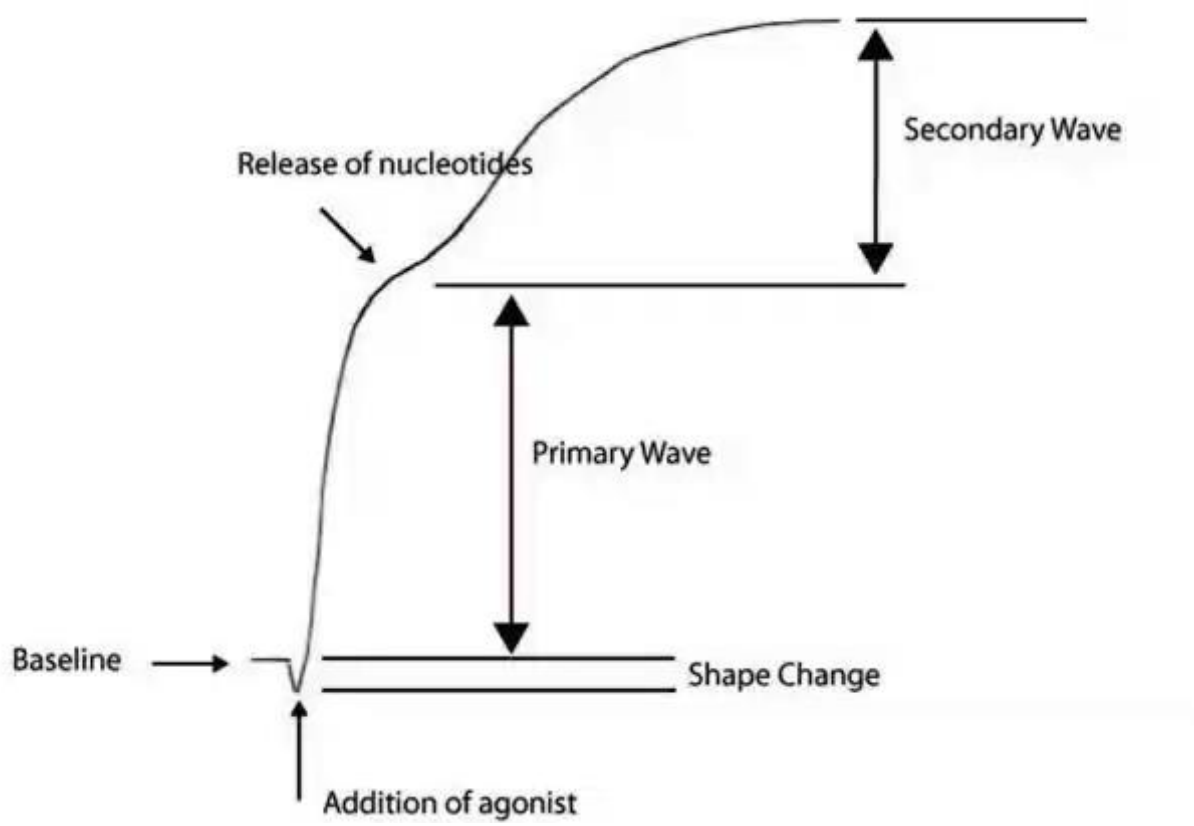

Figure 2-13 typical annonated platelet aggregation curve showing events observed with some agonists

A Biphasic 1^0 and 2^0 wave requires intact prostaglandin pathways (Chronolog data on file)

Analysis of platelet sample OD curve provides much information of platelet events during aggregation (Figure 2-14). For example, platelet shape change can be estimated by an aggregometer (Holme et al., 1998; Holme & Murphy, 1978). Briefly, transmission of the light decreases when platelets transform from discoid to a more spherical shape as confirmed on

microscopy. The longer the formation of filopods lasts, the longer is the duration of shape change. Immediately following addition of agonist, there is decrease in OD corresponding to platelet shape change commonly associated with ADP, AA and collagen. This is expressed as a percentage LT below baseline (D) and duration of shape change in seconds (T). Other parameters include lag time to onset of aggregation (sec), maximal aggregation velocity (%/min) and maximal aggregation as % LT obtained (Figure 2-13).

The curves of platelet aggregation are agonist specific. For example, ADP at low doses and adrenaline-induced stimulation produces biphasic curves. The initial primary aggregation curve, in ADP and adrenaline agonists, correspond to release of stored agents, and secondary aggregation curve after another short delay correspond to synthesis and release of new agonists (Figure 2-14).Eventually, the aggregation stabilises at its lowest giving maximum aggregation (Harrison, 2009; Paniccia et al., 2015).

However, results from some studies indicate that although shape change always precedes aggregation, it is not necessary and does not initiate platelet aggregation (Maurer-Spurej & Devine, 2001). Therefore, its presence should not be taken as an indication of aggregation.

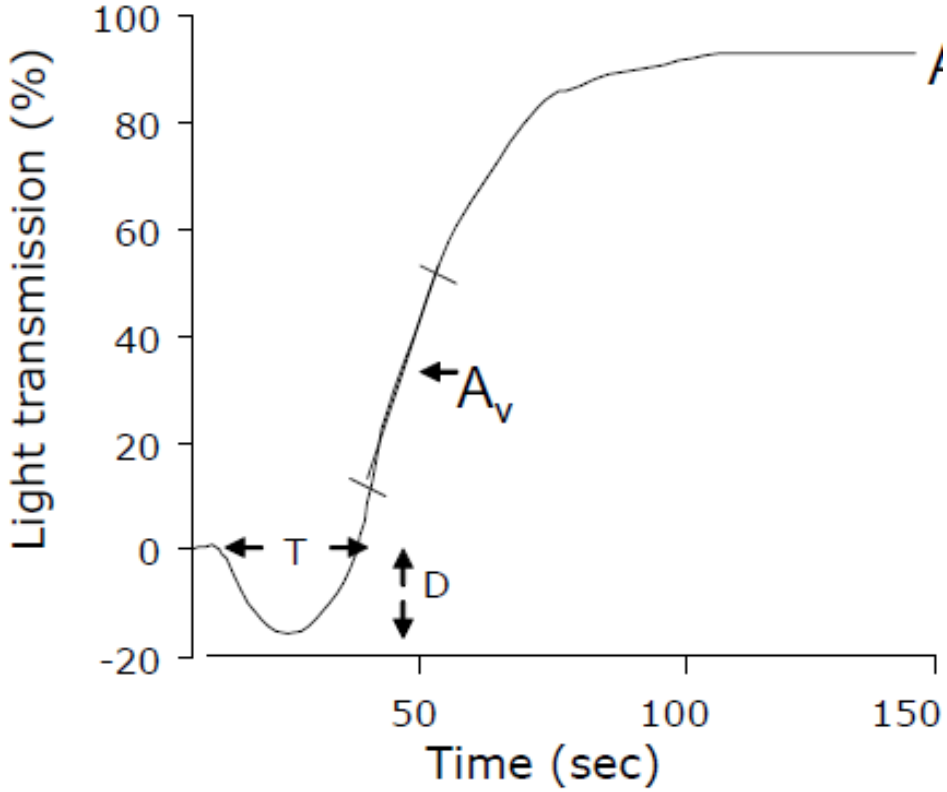

Figure 2-14 Platelet aggregation curve showing shape change: A-maximal aggregation (%), Av-maximal aggregation velocity (%/min), D-maximal decrease in light transmission during shape change (%) and T-duration of shape change (sec). Curve adapted from ("Laboratory analyses for evaluation of platelet disorders and platelet concentrates - laborato.pdf," n.d.)

Although Born LTA has become the gold standard for laboratory comparison of methods of platelet aggregation in investigation of both congenital and acquired platelet defects, however, it has limitations. Sensitivity is affected by level of fibrinogen concentration, since fibrinogen/fibrin acts as a bridge between platelet-platelet interactions (Landolfi et al., 1995). A major weakness is that the method neither detects granule secretion nor adherence which has to be inferred. Pertinent to this is that in an aggregometer, platelet clumping occurs under low, non-laminar shear conditions, experimental conditions that do not adequately simulate the flow-dependent recruitment and adhesion over thrombogenic surfaces *in vivo*. Furthermore, defective

aggregation may arise from inadequate amounts of agonists used, as well as drugs and other substances in blood such as lipemia. Moreover, analysis should be performed within two hours of collection, and care taken to exclude any platelet activation during processing.

Prenalytical factors that may affect quality of results include: Clean venipunture as hemolysis may release ADP with attendant premature activation of platelets, lipemia may obscure OD during PLT aggregation, PRP contact with glass and thus prematurely activated platelets, samples has to be capped to prevent loss of CO_2 – change sample pH (optimal pH for PRP should be maintained within the range 7.7–8.0), samples storage at room temperature – prevent cold inhibition of platelet aggregation, and perform testing within 3 hours of sample collection to avoid platelet destruction and activation. A major weakness of the method is that it is neither sensitive in detection of micro-aggregates nor platelet hyperfunction (Harrison, 2009).

Due to the labour intensive nature of the method, more recent innovations to overcome these limitations have been introduced. Whole blood impedance aggregometers that detect platelet adherence to surfaces under flow and Lumia aggregometers that detect release reactions based on fluorescence substrates. These are designed to utilize small blood samples, provide faster turn-around times as well as simultaneous measurement of platelet aggregation together with ATP secretion. Other newer methods include Clot Signature Analyser (CSA), Cone and Platelet Analyser (CPA) and Platelet Function Analyser (PFA-100) all which can detect platelet adhesion, activation and aggregation under shear conditions, though, have a number of limitations. Nevertheless, LTA is still the preferred method since it evaluates the final event in platelet reactions-platelet aggregation to one another and to fibrin (Gurney, Lip, & Blann,

2002)(Harrison, 2009; Zhou et al., 2014).Therefore, it is commonly applied in many studies in estimating platelet functions during crystalloid haemodilution.

2.2.4 .5 Platelet Activation

Various approaches have been adopted investigating the consequences of platelet activation.

These include determination of increased expression of surface receptors or assays of secretory products. Whereas determination of membrane glycoproteins by flow cytometry as markers of activation is widely practiced, it suffers from the following drawbacks: P-selectin is non-specific as it has been found to be dependent on the anticoagulant and time of sample collection. Moreover, its value has been questioned as it may be shed or re-internalised after activation and possibility of recycling (Gurney et al., 2002). Because of these limitations, other markers of platelet activation and are analysed alongside p-selectin. Glycoprotein IIb/IIIa is the most abundant adhesion receptor on platelet surface and binds a variety of ligands such as vWF, fibrinogen as well as fibrin, vitronectin and fibronectin; it is analysed using PAC-1 monoclonal antibody. Glycoprotein V expression is restricted to platelets and megakaryocytes but is not well studied; Glycocalicin, proteolytic fragment of gpIb.

Although the platelet activation products can be measured by ELISA, it is still unclear which of the soluble membrane products truly reflects activation rather than some other aspects of platelet physiology. Moreover, determinations of specific alpha granule secretory products in plasma have the following limitations:-levels are increased with lysis, inadvertent venepuncture, and lack of correlation with other platelet function tests; Platelet factor 4 (PF4)- is a heparin binding protein and therefore plasma levels may be up regulated up to 20 times by the presence of anticoagulant. Also, beta thromboglobulin makes up 10% of alpha granules but shares 50% homology with PF4. Other dense granule products such as serotonin, non metabolic pool of ADP

and ATP, calcium and pyrophosphate are not exclusive to platelets and hence not specific. Determinations of metabolic products such as PGI_2 are not specific since they are derived from many sources apart from platelets which include smooth muscles, neutrophils and endothelium. Additionally, these activation products participate in events preceding activation, when technically platelets are not yet active (Lindahl & Ramström, 2009b), (Kamath, Blann, & Lip, 2001). A major weakness of these assays for soluble plasma markers of activation is lack of sensitivity and specificity owing to lack of comparative data. Furthermore, these markers are post-event - giving data about events that have already happened apart from having methodology and specificity issues.

2.2.4 .6 Flow cytometry

The technique relies on incubation and labelling activated platelets with fluorescence material or monoclonal antibodies. The antibodies with tagged fluorescent dyes bind specifically to individual platelet proteins, granules and lipid membranes. Detection is by passing light beams across the platelet samples and recording scatter. It is useful for investigating platelet activation markers such as p-selectins; glycoproteins and platelet shape change whose expression increase on activation. Use has been made in detection and quantification of surface receptors and antigens or expression of new epitopes as well as their ligands. Additionally, application has been made in determining platelet-leukocyte aggregates as well as microvesicle detection and characterization. It has high sensitivity in detecting activation as low as 0.8% of platelets (Lindahl & Ramström, 2009a).

However, a major limitation of flow cytometry is that it is expensive and time consuming, continuous measurement is not possible and disaggregation may occur during haemodilution

procedures if samples are not fixed. Furthermore, platelets may become activated during preparation and storage of platelet concentrates.

2.2.4 .7 Platelet adhesion

A unique feature is requirement for flowing blood and ability to withstand shear induced stress exerted by haemodynamics of flow. The classical adhesion test is to count platelets before and after passage of heparinised blood through a column filled with glass beads coated with vWF and collagen. The principle relies on immobilization of vWF on collagen surfaces and passing a stream of blood over it. The tests measure time to occlusion of blood flow and many commercial instruments are available (Lindahl & Ramström, 2009a). However, platelet adhesion at low shear rates may occur independent of vWF, including fibronectin, fibrinogen and thrombospondin. Thus, the lack of adhesion on VWF surfaces underestimates this function. Moreover, at low agonist levels, it may not be observable (Kuwahara et al., 2002). Furthermore, adhesion also includes aggregation thus not specific (Paniccia et al., 2015). A great limitation is lack of wide availability of the instruments as they are still in the phase of clinical trials.

2.2.4 .8 Measurement of platelet clotting and clot elasticity-clot viscoelasticity

These measure combined participation of platelets in whole blood clot formation as a consequence of interaction with fibrin (ogen) and other blood cells. During blood coagulation, the blood viscosity increases as fibrin network forms, and the elasticity of the clot depends on platelet contractile force among other factors such as haematocrit, fibrinogen concentration and thrombin generation. The change in blood clot viscoelasticity can be monitored by Thrombelastography (TEG)/Thrombelastometry (ROTEM) or free oscillation rheometry (FOR). In order to distinguish contribution of platelets from fibrinogen, platelet inhibitors such as cytochalacin D or immunological inhibitors are included in assays (Virginia A. Bowbrick et al.,

2003). Algorithms are used to calculate platelet contribution to elasticity modulus from the frequency and damping data. A commercial TEG modified algorithm of platelet mapping has been described (Bochsen, Wiinberg, Kjelgaard-Hansen, Steinbrüchel, & Johansson, 2007; Virginia A. Bowbrick et al., 2003; Cattano et al., 2013; Weitzel et al., 2012). Notwithstanding the labour intensive nature of TEG platelet mapping for assessment of platelet function, comparatively, it suffers from low sensitivity and predictability in relation to other platelet tests such as aggregometry and flow cytometry (Virginia A. Bowbrick et al., 2003).

2.2.4 .9 Measurement of Platelet clot retraction

After aggregation, platelets develop force that reduces clot volume that occurs only during clot formation. Clot retraction is typically assessed in vitro by measuring volume of serum extruded from the clot or decrease in size of clot mass referred to as compaction, methods which are difficult to perform and less accurate. More accurate methods include Hemodyne (Hemodyne, Inc., Richmond, and VA), Sonoclot (Sienco, Morrison, CO) and Thrombelastography. Thrombelastography estimate of clot retraction relies on the principle that reduction of amplitude after reaching MA is an indication of clot retraction. However, in a study (Katori et al., 2005)it was demonstrated that amplitude reduction occurred both in samples displaying clot retraction as a function of platelet count as well as those that were spiked with fibrinolytic agent. Therefore, it may be concluded that TEG amplitude reduction is not specific to either platelet clot retraction or low dose fibrinolysis.

2.2.5 Other Laboratory Methods in Evaluation of Coagulation Reactions Not in Clinical Use

2.2.5 .1 Electron Microscopy

Microscopic examination of blood clots offers physical proof of fibrin formation and thus the gel point that can be related to other measured clotting parameters. They give a deeper insight into the nature of the morphological modifications of fibrin and platelets and interactions, and to confirm suspected abnormalities (Pretorius, 2013; Pretorius et al., 2007). Scanning electron microscopy (SEM) - provides greater surface detail of the intermediate steps in fibrin synthesis and cross linking. They correlate structural features with clot strength and clotting time (Kawasaki, Katori, Kodaka, Miyao, & Tanaka, 2004; Mardel et al., 1998). Scanning electron microscopic examination of fibrin clots have ascertained that the gel network (the diameter of the polymer chain and the typical mesh size of the gel network) and various properties of clots (mechanical strength, syneresis, opacity, and clot retraction) are associated with the structural characteristics of fibrin networks. A key point is that the analysis of network structure, which is represented by fractal dimension, mesh size and orientation ordering, may be useful for understanding the physiological phenomena as well as the physical properties of clots formed under various physiological conditions (Evans, 2006). Though useful in research settings, its clinical application is limited since it is technically laborious and sample preparation may introduce some artefacts, especially with negative staining.

2.2.5.2 Detection of procoagulant microparticles or micro vesicles

The commonest method to measure and characterize microparticles is by flow cytometry. It has high sensitivity and simplicity in measurement of cellular surface antigen levels useful in classification in cellular source of origin such as TF, receptors such as p-selectin and glycoproteins (GP), and lipid asymmetry (PS exposure). However, flow cytometry has detection

limit of 100nM owing to limited forward scatter sensitivity (Zhou et al., 2014).Other methods such as electron microscopy, atomic force microscopy, laser-induced nano-tracking, and dynamic light scattering are useful adjuncts, but cannot provide biological properties of MPs (Owens & Mackman, 2011). Due to laborious nature of the technique and lack of correlation with actual blood clotting, it is not a method for coagulation testing. It is an adjunct but cannot supplant known coagulation tests.

2.2.5.3 Assessment of Fibrinolysis

Laboratory evaluation of fibrinolysis is challenging using plasma clot based routine coagulation tests, as they cannot distinguish fibrin dissolution from failure of its formation in non clottable samples. Biochemical tests designed to quantify fibrin degradation products (FDP) or D-dimers are specific but are not sensitive to ongoing or active fibrinolysis (Cesarman-Maus & Hajjar, 2005; Elnager et al., 2014). These latex immunological test assays cannot distinguish present ongoing clot formation with dissolution. Likewise plasma assays of fibrinolytic enzymes such as tissue plasminogen activator (t-PA) or plasminogen only give estimate of fibrinolytic potential in patient samples but not active fibrinolysis (Sidelmann, Booth, Hoffmann, Nesheim, & Rosén, 2000). Furthermore, these assays are time consuming as well as technically difficult to perform.

Global tests of fibrinolysis such as euglobulin lysis test (A. A. Smith, Jacobson, Miller, Hathaway, & Manco-Johnson, 2003)or thrombelastography/metry (Kupesiz et al., 2010; Nielsen, Cohen, & Cohen, 2006)have been developed. Detection of clot dissolution in euglobulin lysis test was initially by naked eye, but is now currently followed spectrophometrically to track down changes in absorbance(A. A. Smith et al., 2003). Although this has been the standard fibrinolytic test, its major drawback is separation of cellular elements from plasma rendering their contribution to fibrinolysis activation or inhibition unavailable. Thromboelastography has the

advantage of displaying blood clot lysis in real time. However, it is often difficult to distinguish clot lysis from clot retraction by percent lysis time as both presents with reduction in amplitude (Katori et al., 2005).

2.2.6 Effects of Pre-Analytical Variables on Coagulation Tests

Reliability of coagulation assays depends on control of a number of pre-analytical factors, which otherwise contribute to test results variability. These factors, which may shorten or prolong clotting time include: application of tourniquet for venous stasis, size of needle for blood collection, type of sample container, anticoagulant employed, storage temperature and duration, centrifugation speeds and pipette dimensions in blood sampling techniques (Lippi & Guidi, 2004; Lippi, Montagnana, Salvagno, & Guidi, 2006; Lippi, Salvagno, Montagnana, & Guidi, 2005).

Application of tourniquet to assist in identification of veins before venepuncture, though generally regarded in activating clotting, is controversial as it is not universally supported by empirical data. In a study to investigate effect of application of standard 60mm Hg venous stasis for 1 and 3 minutes on routine coagulation tests revealed that although levels of haematocrit, fibrinogen, FVII,FVIII and FXII increased, the effect on PT or aPTT were either prolonged or not significant (Lippi & Guidi, 2004; Lippi et al., 2006, 2005). This is in keeping with other studies that have investigated the influence of tourniquet application in surgery, which apparently also shows an increase in fibrinogen (Rosenson, Staffileno, & Tangney, 1998)and activation of fibrinolytic system as well (Aglietti et al., 2000). Though the amount of occluding pressure, size of tourniquet and duration are rarely mentioned in comparative studies,

nevertheless, the current consensus is to apply 60mmHg pressure for 1minute and release pressure as soon as the needle is properly placed (Lippi et al., 2012).

Unless coagulation tests are performed immediately, blood is usually collected in test tubes with inhibitors to facilitate for plasma separation and storage before performance of tests.

It is common practice for haematology blood count samples to be collected in EDTA containers, which though is considered unsuitable for coagulation studies. However, in a study comparing EDTA and citrate samples, good comparability and correlation was found for PT determination in Owrens reagent performed in either EDTA or citrated samples in patients taking oral anticoagulants (Horsti, 2001). In contrast, another study found test results were different in EDTA compared to citrate (Crista, 2009).However, EDTA samples bind ca++ with high affinity and require higher amounts of calcium for recalcification than citrate (Macey et al., 2002) as well as undesirable high levels of potassium (Bowen & Remaley, 2014).

Similarly, older methods that compared oxalate and citrate anticoagulation for coagulation testing gave similar results of PT citrate at equal concentrations (Lovelock & Porterfield, 1952; Quick & Stefanini, 1948). However, oxalate was found to adsorb heparin and therefore was unsuitable for clinical monitoring of heparin effects (Anon). Furthermore, FV is unstable in oxalate in addition to slow complex formation with calcium therefore considered inadequate and unsuitable for precise coagulation testing (Narayanan, 2000). In this respect, most modern methods do not use oxalate as an anticoagulant for clinical laboratory coagulation testing.

Citric acid and its salts (citrates) inhibit coagulation by binding (chelating) calcium and thus removing a critical component of the coagulation cascade. The use of tri-sodium citrate as an anticoagulant dates back to a time before the First World War when it was developed as an additive for donor blood. Its use still persists as an additive in blood specimen collection containers, blood bank collection bags and in renal replacement therapies. The anticoagulant properties of this additive are based on its ability to complex with calcium ions in the blood and thus render them unavailable for participation at various key points of the coagulation pathway. The concentration of tri-sodium citrate, for this purpose, has evolved considerably over the past nine decades in view of its influence on comparability of coagulation test results.

Based on studies by Seegers (W. H. Seegers, McCLAUGHRY, & Fahey, 1950) who reported that prothrombin specimens kept over timein trisodium citrate anticoagulants ranging from 5% to 30% concentrations underwent autocatalysis to active thrombin dose-dependently without addition of either calcium or thromboplastins, lower concentration have been adopted. Currently, most specimen tubes contain either 3.2% or 3.8% (109 or 129 mmol/L respectively) trisodium citrate. The clinical Laboratory standard (CLSI) recommends superiority of 3.2% as opposed to 3.8% which is in general agreement with clinical studies (Adcock, Kressin, & Marlar, 1997).

Citrate anticoagulation is dose dependent. Higher citrate concentrations chelate calcium and render blood incoagulable with fixed amounts of recalcification used (V. Chantarangkul et al., 1998; Veena Chantarangkul, Clerici, Bressi, Giesen, & Tripodi, 2003). A study by Calatzis (Calatzis, Toepfer, Schramm, Spannagl, & Schiffl, 2001) revealed that citrate affects coagulation factor enzyme reaction kinetics independent of calcium chelation or its recalcification but had no

influence on fibrin polymerization. This was evidenced by dose dependent increase in Kaolin clotting time but which was not evident in Ecarin clotting time and TEG clot formation time as well as clot firmness. This explains why coagulation proceeds on recalcification despite persistence of citrate as a molecule.

The addition of aqueous calcium chloride solution ('reagent calcium') for recalcification and initiation of coagulation reactions in the laboratory is a critical step. If there is an excessive amount of citrate in the plasma specimen, some of the reagent calcium will be 'quenched' – a potential source of error. The lower concentration of citrate additive (0.105 – 0.109 mol/L) lessens the likelihood of residual citrate in the plasma specimen. Levels of residual citrate can also be elevated in under-filled specimen tubes and in specimens from polycythaemic patients (a significant issue with haematocrits >0.6L/L). Levels of residual citrate will be higher where specimen tubes containing 0.129 mol/L citrate are used in these situations. A volumetric ratio of 9 parts of blood to 1 part of the liquid citrate additive is used – this is critical (Potgieter, Pool, Prinsloo, Botha, & Olorunju, 2010).

The amount of liquid citrate additive can be adjusted to compensate for abnormally elevated haematocrit values by a certain formula (Marlar, Potts, & Marlar, 2006). This formula was developed for high haematocrits above 0.5. In anaemic subjects, Siegel (1998) found that although results were significantly different when adjusted formula was used, however there was no correlation with haematocrit. They therefore advised against adjusting for anaemic samples.

The choices of tubes and inhibitors have a bearing on coagulation results. It has long been known that glass test tubes stimulate contact activation than propylene or siliconized tubes. These were

born out of studies that demonstrated that clotting time was increased in the order glass>collodion>paraffin> synthetic plastic, whether without anticoagulants, citrated and recalcified, normal or haemophilic plasma (Lozner, Taylor, & MacDonald, 1942). Since then, a number of studies have examined coagulation tests results performed in glass compared to plastic.

In a study comparing coagulation tests performed in 3.2% citrate either in glass or plastic tubes, it was found that PT and APT clotting times were statistically prolonged in glass than plastic. However, these differences were not clinically significant (Gosselin, Janatpour, Larkin, Lee, & Owings, 2004). In another study, TEG parameters in samples collected in glass tubes with 3.2% sodium citrate showed linear decrease with time of storage as opposed to plastic tubes(Stover & Stammers, 1995). These data suggest plastic tubes may be preferable to glass. These claims have been supported by data from a number of studies finding plastic containers to be favourable to glass (Gosselin et al., 2004; Kratz, Stanganelli, & Cott, 2006; Yavaş, Ayaz, Köse, Ulus, & Ulus, 2012). From a logistical point of view, since glass may break during handling, plastic tubes may be preferable. Therefore, most coagulation test tubes are currently made of polypropylene to prevent contact activation before assays are done.

The prevalence of haemolysed blood samples in clinical coagulation laboratory specimens is estimated at 3.3%(Lippi et al., 2006)and presents analytical challenge especially if photo-optometric methods are employed, and is the most frequent cause for specimen rejection. Clinical observations that haemolytic conditions are associated with hypercoagulability (Ataga, 2009), (Gladwin & Kato, 2008; Rother, Bell, Hillmen, & Gladwin, 2005) are supported by

empirical evidence that products of cell lyses induce blood clotting (Reiter et al., 2002). In clinical situations, apart from *in vivo* haemolytic diseases, blood cell lyses usually arises from specimen collection manoeuvres such as multiple punctures, mechanical destruction from narrow lumen needles, or vigorous mixing in collection tubes. In studies evaluating effects of haemolytic samples on routine coagulation tests, Lippi (Lippi et al., 2006)found direct increase in PT and D-dimers with percent lyses, in contrast to APTT and fibrinogen which was inversely related. In a related study, Laga (Laga, Cheves, & Sweeney, 2006)found that RBC lysis significantly affected extrinsic coagulation activation pathway as evidenced by strong correlation with FVII, FX and F 1.2 but not FXII or FVIII. However, there was poor correlation between coagulation tests with extent of haemolysis or supernatant haemoglobin in the same study. But these were stroma free haemoglobin. The differences in the two cited studies can be found in methodology of inducing haemolysis. Lippi study used mechanical freeze-thaw while Laga and group used mechanical homogenizer techniques. Reconciliation of the two study findings could arise on the extent of release of procoagulant microparticles as well as negatively charged phospholipid membrane vesicle and ferric iron and not haemolysis per se. These findings have implications in interpretation and conclusions drawn from haemodilution studies with hypotonic solutions incubated over a period of time.

2.2.7 Effects of Experimental Conditions on Coagulation Tests

One area in coagulation research which has concerned standardization bodies is in the use of exogenous coagulation triggers, especially in citrated samples. Apart from calcium recalcification, this majorly concerns source, concentration and activity of thromboplastins. Of note are publications concerning standardization of reagents so as to achieve comparative results, especially INR or ISI (International Sensitivity Index).

A key variable in blood coagulation testing is levels of ionised calcium. Calcium or coagulation factor IV is an important coagulation factor for both the intrinsic and extrinsic coagulation pathways. The ionized calcium form is responsible for salt bridge formation between negatively charged phospholipids and coagulation factors with Gla domains such as FIXa and FVIIIa (intrinsic Tenase) and TF-FVIIa (extrinsic Tenase) and FXa-FVa (prothrombinase) (Calatzis et al., 2001). It does not appear essential for thrombin amydolytic activities since active forms such as α-thrombin are able to cleave fibrinogen without recalcification (Calatzis et al., 2001), or fibrin polymerization.

Calcium is present in blood plasma in three fractions which are in equilibrium with one another, i.e., the ionized and complex bound calcium, which together comprise the diffusible and ultra-filtrable fraction, and the non-diffusible calcium which is bound to the plasma proteins. The ionized calcium is considered to be the physiologically active fraction, and must be maintained between 1.0 and 1.3 mmol/l in the test plasma (Calatzis et al., 2001). The practice of calcium chelation therefore aims at lowering the active, ionized calcium fraction. This results in a decrease of the ionized calcium fraction to between 0.3 to 0.35 mmol and an increase of the complex bound calcium in the blood to establish adequate anticoagulation. Therefore, the practice of collection of blood samples in tubes containing calcium chelators, citrate, oxalate or EDTA, prevents inadvertent clot formation before analysis during storage, transport or when dealing with large samples.

It is an acknowledged fact that recalcification may vary since amount of anticoagulants may not be exactly matched. Controversy still remains what volume, concentration and form of calcium to be added. Most coagulation standards have recommended calcium chloride in 0.2M concentration for coagulation testing, but little data is available regarding lower or higher concentrations. Although an alternative source of calcium such as calcium gluconate is clinically infused in cases of massive transfusion, it provides one third ionised calcium per 10 mg dose(Jeffreys, 2001), but there are no data on its use for recalcification of citrated specimens in coagulation studies. Perhaps this is due to relatively low solubility and ionisation as well as lower amounts of ionised calcium released per unit weight compared to calcium chloride.

In routine coagulation testing, addition of equivolumic amounts of calcium chloride further compound haemodilutional effects, a fact rarely acknowledged. Whereas dose response curves indicate that clotting time goes through a minimum as calcium concentration increases (Conio, Dondero, Troglia, Trefiletti, & Patrone, 1975; Conio, Trefiletti, Troglia, & Patrone, 1976), (Lovelock & Porterfield, 1952), and findings that plasma calcium levels beyond 0.56 mM produce no added increase in clotting time (Calatzis et al., 2001; James & Roche, 2004), addition of fixed volume and concentration of calcium chloride warrants further scrutiny. Furthermore, although citrate chelation has high affinity for calcium and magnesium, it also chelates other divalent ions such as Mn++ and Fe++. At the moment, restitution of these ions has not been described, and it is not known whether the issue should be of analytic importance though some studies suggest iron could have an influence on coagulation reactions/ thrombus stability (Lipinski, Pretorius, Oberholzer, & Van Der Spuy, 2012)(Praticó et al., 1999).

Controversy involving standardization of thromboplastins is still not yet resolved. This is because initial sources from animal (human or rabbit brains) were not comparable owing to variability in tests results. Rabbit brain gives different test results from human tissue extracts. This may be explained by differential concentration of TF in different tissues (Astrup, 1965) (Drake, Morrissey, & Erdington, 1989). A recent study found that there was a difference in sensitivity obtained not only with thromboplastin from animal sources, but also with recombinant ones (Saulius Butenas, 2013).

Clotting time is dependent on TF concentration (Saulius Butenas et al., 2005, 2009), which shows that increase of TF shortens clotting time non-linearly, possibly by log relationship. TF is an integral membrane protein whose expression increases with strength of stimulation (Campbell, Brummel-Ziedins, Butenas, & Mann, 2010).Membrane bound TF form acts as a trigger for extrinsic pathway as well as a receptor for circulating FVII (Orfeo et al., 2005a). However, coagulation tests performed in plasma assume it is active in the aqueous soluble phase, which is far from the true situation since its membrane is fixed. Moreover, the commonly employed 200 pM of TF concentration in routine clotting tests is far more than what has been observed *in vivo* (Saulius Butenas et al., 2005). Therefore, commonly employed concentrations look arbitrary rather than physiological. It is imperative therefore that the clotting tests results reflect the concentration of TF in the analyte.

In view of variability in potency of thromboplastins from animal sources, they are being replaced by recombinant factors. This brings another area of contention since recombinant forms produced from *E.coli* synthesis may not reflect the human physiological form. Furthermore, in a study comparing different commercial recombinant thromboplastins, (S. A. Smith & Morrissey,

2004) found each type and lot produced different PT test results and varied sensitivities in the same plasma and coagulometer. This strongly suggests that variations in coagulation test results may arise not only from factor deficiencies but also in sensitivity to type and source of thromboplastins reagent employed.

Although phospholipids are well regarded as necessary for initiation of coagulation justifying relipidation during coagulation testing, the optimal concentration and composition is still a matter of active research. Different models have been put forward to explain the role of phospholipids in the kinetics of blood coagulation (H. C. Hemker, Dieijen, Rosing, Tans, & Zwaal, 1980). The significance of phospholipid composition on clotting test results was best illustrated in findings that each composition of thromboplastin lot and the type of coagulometer used to measure clotting time had a bearing on clotting index sensitivity (Kitchen & Preston, 1999). Subsequently, recent research has demonstrated that clotting time sensitivity was independent of molar ratio of phospholipid to TF (S. A. Smith & Morrissey, 2004), but more on proportional ratio between PS to PC, the effect of PE being synergistic with PS (S. A. Smith, Comp, & Morrissey, 2006; S. A. Smith & Morrissey, 2004) (S. A. Smith & Morrissey, 2004;S. A. Smith et al., 2006). It is becoming clear that optimal results are obtained when PS to PC is 20:80 (H. C. Hemker et al., 1983, 1980).Thus variations in clotting results may arise from different sensitivity of factor deficiencies to phospholipid composition in the analyte reagents. However, phospholipid composition has more impact on clotting sensitivity than TF concentration and this is exagerated when ionic strength of medium is increased (S. A. Smith & Morrissey, 2004; S. A. Smith et al., 2006).

2.3 Effects of Crystalloid Solutions on Coagulation System

Many publications which have addressed the effects of haemodilution on coagulation have compared crystalloids with colloids without giving operational definitions of crystalloids and thus assuming that crystalloids are made of uniform solutes. By definition, crystalloids are solutions of small inorganic and organic salts in water. The most distinguishing criteria from colloids is molecular size of not more than 30kDA and ability to permeate biological membranes with ease (Severs, Hoorn, & Rookmaaker, 2014). From the initial isotonic saline (0.9% NaCl) solutions a lot of modifications have been made such that even NaCl is available in different strengths with addition of other inorganic and organic molecules such as lactate, acetate, gluconate in various combinations (Smorenberg et al., 2013) (Table 2-1). The list also includes organic molecules such as mannitol, dextrose which though are not are not resuscitation fluids, are infused for other therapeutic applications.

Table 2-1 Composition of commonly used crystalloid solutions

Crystalloid solution	Osmolality M0sm/kg	pH	Na^+ (mmol/l)	K^+ (mmol/l)	Ca^{2+} (mmol/l)	Mg^{2+} (mmol/l)	HC_{O3} (mmol/l)	Lactate (mmol/l)	Other buffer (mmol/l)	Cl^- (mmol/l)
0.9 % Saline	308	5.5	154							154
Ringer's	309		147	4	5					156
Ringer's lactate	273	6.5	130	5.4	2.7			29		109
Ringer's lactate	273	6.5	130	5.4	2.7				29[a]	
Hartmann's Modified Balanced Fluids	280	6.5	131	5.4	2				29[a]	112
	290		145	4	2.5	1			24[a]	127
	294	5.5	140	5		1.5			50[b]	98
	299	5.5	140	10	5	3		8	47[a]	103
1.4% Na bicarbonate	333	>7.0	167				167			
4.2 Na bicarbonate	1,000	>7.0	500				500			
8.4% Na bicarbonate	2,000	>7.0	1,000				1,000			
3% saline	1,026		513							513
7% saline	2,394		1,197							1,197

a-lactate; b-acetate; c-gluconate (Smorenberg et al., 2013)

Traumatic injuries are often accompanied by blood loss and the main aim of crystalloid fluid infusion is restoration of blood volume even though solute contents and concentrations vary according to formulation. However, 25-30% of severe trauma cases develop coagulopathy.

Coagulopathy defined as 1 ½ times prolongation of plasma clotting time tests (MacLeod, 2008; MacLeod et al., 2003; Maegele et al., 2007) is associated with high mortality. While acknowledging the multiple risk factors associated with trauma coagulopathy, most authors have previously assumed it is caused by dilution due to intravenous crystalloids, and therefore 'dilutional coagulopathy models'(Hirshberg et al., 2003; Wafaisade et al., 2010). The justification for the models is that, by diluting the blood clotting factors by infused crystalloids, the clot forming ability would intuitively be impaired. However, this 'dilutional coagulopathy' concept is questionable as it does not take into account the crystalloid formulations and is supported by little empirical evidence. Furthermore, comparability of study results varies according to methods of coagulation testing and which blood component specimens have been analysed.

2.3.1 Routine Tests in Crystalloid Haemodilution and Coagulation

Tocantins *et al* (Tocantins et al., 1951a) demonstrated for the first time that addition of 0.85% NaCl to plasma accelerated blood clot formation. The mechanism(s) underlying this seminal observation has remained a matter of debate among researchers to date, with some opining that it could have been an experimental artefact or error, or a subject of imprecise coagulation methods employed then. Nevertheless, only a few investigators have arrived at the same conclusion using normal saline (0.9% NaCl) as a comparator but different results have been obtained when NaCl

concentration was changed or different types of solutes bringing into focus the effects of fluid osmolality and chemical composition.

Studies on the effects of Crystalloid osmolality on coagulation have recorded mixed results. Most of these studies only compared 7.5% NaCl (Hypertonic saline, HTS) with isotonic saline. For example Reed *et al* (Reed et al., 1991a)reported that hypertonic NaCl not only prolongs clotting time by virtue of its concentration but also on serial dilution. Although the study provided strong indication that increased saline concentrations induce hypocoagulability *in vitro*, it was not specific considering the inherent ionic strength or just concentration effects. Moreover, the conclusion that the effects were due to increase in Na+ ions on coagulation factors as propounded by Reed lacked a comparator and ignored the contribution of chloride anions. Nevertheless, this was the first study that indicated that change from normal to hypertonic saline concentrations was associated with impairment of prothrombin time (PT), activated partial prothrombin time (APTT) and thrombin time (TT).

In a related study, Wilder *et a l*(Wilder, Reid, & Bakaltcheva, 2002b) using routine coagulation tests compared the *in vitro* effects of a number of solutions at 2400mOsm concentrations at uniform 20% plasma dilutions. They found that although all solutions had similar osmolality, the effect of HTS profoundly prolonged time to clot formation more than other organic solutions (dextrose, mannitol and sorbitol) at identical concentration. This study introduced another dimension to concentration effects-predominance of ionic strength over osmolality. However, only one concentration (2400mOsm) was tested, therefore further references as to the contribution of ionic strength from the cited work required further validation. Although the

results demonstrated isotonic saline caused hypercoagulability as opposed to HTS which caused hypocoagulability, the effects of intermediate concentration at which transition occurred has neither been determined nor well documented. Furthermore, the mechanisms for differences in behaviour of solutes could not be accounted for.

The data reported by Wilder (Wilder et al., 2002b)were in great contrast to a previous study that had found increased clotting time of pure fibrinogen by thrombin compared to sorbitol which had no effects (Einarsson, 1975). These laboratory effects of glucose were also not in keeping with clinical observations of hyperglycemia and hypercoagulability in association with diabetes mellitus (Dunn, Ariëns, & Grant, 2005; Jörneskog et al., 1996; Pieters et al., 2008).

The relationship between diabetes mellitus and thrombosis is well established (M. E. Carr, 2001; Grant, 2007; Kluft & Jespersen, 2002). In particular, blood from uncontrolled diabetic patients has shortened clotting time (Egeberg, 1963) and increased clot strength (Blagosklonnaia, Krasil'nikova, Zaĭed, & Babenko, 1990; Yürekli, Ozcebe, Kirazli, & Gürlek, 2006a) and altered clot morphology (Jörneskog et al., 1996; Pieters et al., 2008) resistant to fibrinolysis (Dunn etal., 2005). These effects have long been associated with hyperglycaemia (Ceriello, 1993). However, a direct link between hyperglycaemia and coagulation is currently a subject of debate as evidenced by numerous observations which report that correction of hyperglycaemia does not normalize coagulation abnormalities (Knöbl et al., 1994). This calls into question the role of sugars in fibrin structure in particular and clotting in general. It must be appreciated that the clotting time tests are conducted on plasma and not whole blood while *in vivo* coagulation involves whole blood.

Mannitol is a congener of glucose both having molecular weight of 182 but differing in orientation of hydroxyl molecules at carbon atom in position 2. Due to this unique structure, it is impermeable to cell membranes and therefore clinically employed in reduction of brain swelling. Although for a long time it was considered innocuous with regard to blood coagulation, some case reports have described clinical coagulopathy in association with mannitol infusion (Bullock, Hanemann, Murray, & Teasdale, 1990; Sansing et al., 2003). The mechanisms for these clinical observations still remain unclear even though haemodilution with hypertonic mixture of glucose, mannitol and glycine did not show evidence of change from undiluted control on PT and aPTT (Wilder et al., 2002b).

2.3.2 Crystalloid Solutions on Thrombin Generation

Monkhouse (Monkhouse, 1959) while performing crystalloid haemodilution with isotonic fluids noted that the samples which accelerated plasma clotting as evidenced by shorter clotting time also had reduced antithrombin levels as well. He postulated that this allowed the procoagulant factors to act unopposed which was the beginning of the concept that the crystalloid haemodilution enhancement of coagulation was related to reduction in the natural anticoagulant, antithrombin III. Many other authors have also reported association of crystalloid haemodilution induced hypercoagulability and reduced plasma antithrombin levels confirming previous findings and also that hypercoagulability was attenuated when the antithrombin was restored to pre-dilutional level (Nielsen, Lyerly, & Gurley, 2004; T. G. Ruttmann, Jamest, & Lombard, 2001; T. G. Ruttmann, Lemmens, Malott, & Brock-Utne, 2006; Szlam et al., 2008). However, in one study although all parameters indicated hypercoagulability, the fall of antithrombin did not correlate to extent of dilution (K. F. J. Ng, Lam, & Chan, 2002) casting doubt on explanation

based on antithrombin levels. It is imperative to note that these experiments were performed on serial dilutions with isotonic saline and direct measurements of thrombin in the same haemodiluted plasma were not measured concurrently therefore putting into question the generalizability of the postulate irrespective of crystalloid composition. Probably, reduced antithrombin activity could arise from its sensitivity to particular solutes or ionic milieu and not dilution *per se*.

In clinical studies in which haemodilution was maintained by isotonic saline accompanied by direct measurements of thrombin and antithrombin levels, it was found that thrombin lag time remained unchanged despite progressive reduction in thrombin peak levels that correlated with changes in antithrombin levels (Daniel Bolliger et al., 2010; D. Bolliger, Szlam, Levy, Molinaro, & Tanaka, 2010; Sniecinski et al., 2008). These findings correlated with an *in vitro* study in which plasma was diluted with normal saline which also showed progressive reduction of thrombin peak levels without changes in thrombin lag time with 5pM TF trigger concentration (De Smedt, Wagenvoord, & Coen Hemker, 2009a). However, in the same haemodiluted plasma samples triggered with either 1pM TF or kaolin, the thrombin lag time became progressively lengthened with dilution. These study results suggested that the relationship between plasma crystalloid dilution and thrombin generation is complex which cannot be explained on the basis of isolated antithrombin level measurement.

Since no ideal resuscitation crystalloid fluid has not been described there is still ongoing debate regarding the effects of crystalloid fluid formulation on coagulation. In a study carried out to investigate the effects of crystalloid composition on thrombin generation, it was found that the

changes were dependent on extent of dilution and solute concentration (K. Brummel-Ziedins et al., 2006). However, there was discordance between thrombin antithrombin (TAT) and fibrinopeptide (FPA) levels. Whereas, at each dilution, the order of decrease in TAT was normal saline (NS)>Ringer's lactate (RL)> hypertonic saline (HTS), for FPA it was LR>NS>HTS. The changes of FPA corresponded to osteonectin levels but not thrombin mass.

These results were a great paradigm shift demonstrating that thrombin generation is dependent not only on dilution but also on crystalloid composition. However, the cited study only considered surrogate markers of thrombin (TAT and FPA) and direct measurement of thrombin in haemodiluted plasma with different solute types and concentration remained largely unexplored.

2.3.3 Use of Thromboelastography in Evaluation of Effects of Crystalloid Solutions on Whole Blood Coagulation

Many studies on the effects of crystalloid solutions on human coagulation have been done diluting plasma without taking into account that when blood clots, it occurs as whole blood without separation into individual components. Perhaps, this was due to difficulties in methods of analysis that has been solved by Thromboelastography.

It had initially thought that crystalloids have no inherent effects on coagulation apart from plasma haemodilution of coagulation factors which led to use of normal saline as a comparator. However, Ruttmann (1996) using thrombelastography demonstrated for the first time that haemodilution with isotonic saline induces a hypercoagulable state indicating its intrinsic properties on whole blood coagulation. The same author later on used various isotonic fluids and arrived at the same conclusion and commented that crystalloid haemodilution is procoagulant

irrespective of pH, osmolality or type of solutes (Thomas G. Ruttmann, Montoya-Pelaez, & James, 2007). The proposition was not supported by empirical evidence, and could only be true for isotonic solutions.

For a long time it has been thought that calcium in some balanced salt solutions such as 'Ringer's lactate' (RL) or 'Hartmanns' solutions may lead to clot activation when blood transfusion are administered through the same filters concurrently. However, this concern does not appear significant since in a study that compared the rate of clot formation in transfusion filters did not find any difference between normal saline and RL (Cull, Lally, & Murphy, 1991). Similarly, no significant differences on coagulation have been demonstrated between them in plasma coagulation tests (Timothy John Coats & Heron, 2004; Ekseth, Abildgaard, Vegfors, Berg-Johnsen, & Engdahl, 2002). Whereas RL contains calcium, a known coagulation factor which is absent in normal saline, the presence of calcium at this concentration plays very little role on coagulation effects (T. J. Coats, Brazil, & Heron, 2006). This probably casts doubt on the role of calcium in coagulation at concentrations normally found in many resuscitation fluids.

In comparative studies using Thrombelastography, it was demonstrated that RL is more hypercoagulable than NS (Boldt, Haisch, Suttner, Kumle, & Schellhase, 2002; Anthony M. Roche, James, Bennett-Guerrero, & Mythen, 2006a). RL is hypotonic compared to NS, has Na and Cl lower (130 and 109 mMol/l compared to 154 and 154 mMol), respectively for the two solutions. Although the osmolality for NS and RL are 295 and 280 mOsm respectively, the marked difference in composition is chloride ions. The difference in Na+ is only 9 mMol, while it is 35 mMol for chloride ions. These data suggest that increase in solute concentration

especially ionised ones may impair whole blood clotting. The greater chloride concentration may account for the differences in parameters but controlled laboratory investigations have not been done.

Magnesium is a divalent cation present in some resuscitation fluids such as ‘Hartmans’ solution that is expected to compete with calcium in coagulation reactions, and probably procoagulant owing to increased thrombin generation (Ravn, Lassen, Bergenhem, & Kristensen, 2001). Clinical experience in which magnesium sulphate was infused for other indications showed that clotting time as well as clot formation time were increased with concomitant decrease in alpha angle and clot firmness in keeping with hypocoagulability (Ames, McDonnell, & Potter, 1999)(Thomas G. Ruttmann et al., 2007; Na, Chung, Hwang, & Do, 2012; Na, Shin, Kang, Hwang, & Do, 2014) in contrast to another study which showed hypercoagulability (Choi, Lee, & Park, 2005). But these were *in vivo* studies where multiple factors may be at play, especially release of vasopressin from hypothalamus and osmotic activity on endothelium. The tests that demonstrated hypercoagulability were done in very sick liver transplant patients and therefore not a true reflection of its physiological effects on clotting factors.

Unlike *in vivo* studies, *in vitro* haemodilution with magnesium solutions have consistently demonstrated hypocoagulability (Jankun et al.., 2013) in keeping with its infusion in healthy volunteers. However, due to inconsistency of study findings and in view of high concentrations administered in volunteers, it is still doubtful whether the effects were due to hyperosmolality, or sulphate anion which is a kosmotrope on the Hofmeister series. Furthermore, no investigations have been done with magnesium chloride solutions

Studies on the effects of Crystalloid osmolality on coagulation have recorded mixed results. Whereas Tan *et al* (Tan, Tan, Ng, & Loh, 2002) found 7.5% HTS impaired all TEG parameters at dilution of 10% and above in contrast to isotonic saline (Ruttmann, 2003; Ruttmann et al., 2007, 2006, 2002, 2001, 1996). These studies provided strong indication that increased saline concentrations induce hypocoagulability in vitro, and demonstrated clearly that coagulation outcomes may not simply be accounted for on the basis of haemodilution of clotting factors. However, the reasons to account for the difference in NaCl concentration on coagulation and the mechanisms remain unknown to date.

In a study comparing effects of different concentrations of saline with hypertonic mannitol *in vitro*, it was found that the two molecules produced different results. While hypertonic mannitol was associated with increased ROTEM clotting time as well as clot formation time with concurrent reduction in MCF and alpha angle, changes produced by iso-osmolar NaCl concentration were much less. In the same study isotonic saline was associated with hypercoagulability (Luostarinen, Niiya, Schramko, Rosenberg, & Niemi, 2011). This reinforced previous finding that haemodilution with mannitol or its combination with Ringers acetate or hydroxyethyl starch inhibited whole blood coagulation (Lindroos, Schramko, Tanskanen, & Niemi, 2010). The mechanism for discordance in effects between NaCl and mannitol has not been established.

Even though diabetes mellitus is considered a hypercoagulable state, thromboelastographic evaluation of type II diabetes failed to show enhanced whole blood coagulability (Yürekli et al., 2006a). Acute complications of diabetes are always associated with many electrolyte imbalances

which could confound the picture and mask coagulation effects of glucose. Furthermore, many metabolic products in association with long term diabetic complications are often at play and it is difficult to distinguish coagulation effects of diabetes in this scenario. However, unlike mannitol, no study has been conducted *in vitro* to define the effects of dextrose on coagulation *per s*e using thromboelastography.

With the findings that antithrombin levels were reduced proportional to extent of dilution, many authors have used this to explain the haemodilution induced hypercoagulability (Nielsen et al., 2004; T. G. Ruttmann et al., 2001, 2006; Szlam et al., 2008). However, when either thrombin or fibrinogen concentrates were added to haemodiluted blood samples, TEG parameters were not restored to predilution levels (Schols, Heemskerk, & van Pampus, 2010). This suggested the inadequacy of antithrombin hypothesis in explaining hypercoagulability, and probably another factor was at play. However, this has not been identified to date

2.3.4 Effects of Crystalloid Solutions on Platelet Aggregation

Most coagulation screening tests only consider adequacy of platelet counts without confirming their clot forming ability even in cases of clinical bleeding. There is accumulating evidence that platelet functions are decreased following trauma with normal platelet count (Saillant & Sims, 2014; Solomon et al., 2011; Stansbury et al., 2013)(White, 2013; Kutcher et al., 2012). The role of crystalloid solutions in causing platelet dysfunctions remains a laboratory phenomenon since the existing methods depend on triggering aggregation with agonists whose concentration and physiological release are still under debate.

In a study comparing the effects of hypertonic saline (7.5%) (HTS) to isotonic saline on platelet aggregation, it was found that maximal platelet aggregation as well as shape change were markedly inhibited by HTS relative to control (Reed, Johnston, Chen, & Fischer, 1991b). The authors attributed this to increase in medium ionic strength and Na+ ions. However, this was contradicted by demonstration that addition of ouabain to hypertonic solution was associated with procoagulant responses that correlated with increase in cellular volume (Tomasiak, Stelmach, Rusak, Ciborowski, & Radziwon, 2007). This was against expectation since oubain would naturally augment increase in intracellular Na+ and thus lead to attenuation of aggregation as predicted by *Reed at al*. In a related study with different alternative crystalloid solutions whose concentrations were kept at 2400 mOsm similar to the previous study, it was found that all hypertonic solutions (NaCl, glucose, sorbitol, glycine and mannitol) inhibited platelet aggregation and shape change irrespective of solute type (Wilder et al., 2002b). This therefore illustrated the deficiency of the theory of increase in ionic strength in explaining platelet aggregation responses. Probably the platelet responses are molecular specific depending on which receptors or signal pathways are affected.

Many others have investigated the influence of molecular composition of crystalloid solutions on platelet reactions with mixed results. For example, increase in mannitol concentration was found to inhibit platelet aggregation in a similar degree to free radical scavengers such as desferrioxamine, catalase and deoxyribose (Praticó et al., 1999). This finding was an added twist to determinants of platelet aggregation apart from the well cited classification as ionic or non-ionic crystalloids. However, another group found that haemodilution with mannitol dose dependently increased platelet binding to fibrinogen as well as p-selectin expression suggesting

pro-aggregant action (Keating, Sobel, & Schneider, 2003). In the same study, iso-osmotic concentrations of mannitol and dextrose mimicked results in patients with uncontrolled diabetes mellitus suggesting that increased osmolarity and hyperglycemia were associated with increased platelet activation. Similar results of increase in platelet fibrinogen binding and p-selectin expression were obtained with diverse molecules different radio-active contrast media (Hay & Bull, 1995, 1996a; Laffan, Dawson, & Gooding, 1997) in contrast to another one where platelet activation was attenuated by contrast agents (Ji, Ghaly, Hjemdahl, Tornvall, & Li, 2005). It is still questionable whether the mechanism of platelet activation could be ascribed to contrast agent induced degranulation or some other means given that these contrasting results were obtained with different methods-flow cytometry in whole blood as opposed to light transmission aggregometry in platelet rich plasma.

2.3.5 Clinical Significance of Crystalloid Haemodilution on Coagulation

Enhanced coagulation following crystalloid haemodilution has been demonstrated by employing different coagulation techniques. For example, coagulation analysis in whole blood using Biobridge method (Heather et al., 1980), thromboelastography (T. G. Ruttmann, 2003; T. G. Ruttmann et al., 1996) and routine coagulation tests on plasma (Tocantins, 1951) all concluded crystalloid haemodilution induces a hypercoagulable state. However, clinical relevance, the mechanism responsible for the observations or whether the same results would be replicated with crystalloid made from other solutes of varying concentrations has been a matter of debate within the scientific community. In one journal commentary, some authors questioned the authenticity of observations opining that crystalloid enhancement of blood clotting may have been an *in vitro* artefact arising from sedimentation of red cells in diluted equipment probes (T. G. Ruttmann, 2003). This reflects that the observation is far from being an established fact.

The clinical relevance of crystalloid haemodilution on blood coagulation has only found one study associating it with demonstrated *in vivo* thrombosis. In a clinical trial involving a cohort of patients scheduled for abdominal surgery (cholecystectomy) evaluating the association between haemodilution and thrombosis (Janvrin, Davies, & Greenhalgh, 1981) where patients were into two groups: Group A called dry were not given any form of fluid drip; while group B, identified as wet received intravenous isotonic crystalloids. Surprisingly, the wet group were found to have twice the incidence of deep venous thrombosis (DVT) compared to the dry group in the post operative period. From this, it was concluded that the drip caused thrombosis against the prevailing conventional thought that DVT was caused by dehydration- induced haemoconcentration (Janvrin, Davies, & Greenhalgh, 1980). This study discounted surgical stress as the cause since both patients groups underwent similar surgical procedures, except postoperative fluid management. The findings by Janvrin *et al*., (1980) was a paradigm shift which was corroborated many years later by findings of hypercoagulability, as determined by TEG, in patients undergoing vascular surgery and infused with intravenous fluids (T. G. Ruttmann et al., 2002).

The data of hypercoagulability were in strong contrast to findings of coagulopathy or hypocoagulability with consequent haemorrhage post fluid resuscitation in trauma patients(MacLeod, 2008; MacLeod et al., 2003). Perhaps the difference in coagulation outcome between the two studies can be accounted for by early but marked inflammatory response arising from uncontrolled tissue injury in trauma patients compared to controlled situations in surgery. It

is still unclear why crystalloid haemodilution would lead to hypercoagulability *in vitro* and *in vivo* despite observed coagulopathy in trauma patients

2.4 Other Factors that Influence Blood Clotting

Despite knowledge that clotting factors together with blood cells are sensitive to medium in which they function, contribution of other blood components such as electrolytes and plasma proteins are usually not taken into discussion of the clotting process. These probably account for indirect modulators of the clotting process giving rise to different coagulation test results.

2.4.1 Interaction of Fibrin (Gen) with Plasma Proteins

2.4.1.1 Fibronectin

Plasma fibronectin is usually low but increases with platelet activation. Its presence does not influence clotting time, an indication it has no effect on either free fibrinogen or fibrin monomer intermediates prior to gel point. However, after gel point, fibrin fibres thickness and networks are increased indicating its incorporation in the gel different from activity of FXIIIa (Nair & Dhall, 1991; Okada, Blombäck, Chang, & Horowitz, 1985; A. S. Wolberg, 2010).

2.4.1.2 Thrombospondin

This is a protein released from α -granules of activated platelets at sites of thrombus formation. It binds to fibrinogen at the α-C domains to stabilize binding to platelet GpIIb-IIIa. Increase in its concentration shortens clotting lag time as well as thinner but more numerous fibrin networks (Ruggeri & Mendolicchio, 2007)

2.4.1.3 Von Willebrand factor

Both vWF and fibronectin are substrates for FXIIIa which incorporates and cross links them to fibrin α-chain. The net result is decreased rate of fibrin polymerization as it stabilizes the interaction under slow clotting conditions (Hada, Kaminski, Bockenstedt, & McDonagh, 1986). Therefore, vWF plays two distinct roles in clot formation. First, it promotes platelet binding to sub-endothelial surfaces and to other platelets (platelet aggregation). Secondly, it also functions as a transporter molecule for factor VIII, protecting it from proteolysis, thus preserving its catalytic role in the coagulation cascade (Pepin, 1995)("The role of von Willebrand factor in coagulation," n.d.).

2.4.1.4 Albumin

Depending on concentrations and preparations, albumin either shortens the onset of time to fibrin formation and enhances fibre thickness (Nair & Dhall, 1991; A. S. Wolberg, 2010), or decreases fibrin fibre thickness independent of and synergistic with fibrinogen. Thin but branched fibers have been demonstrated in the presence of albumin, as well as a decrease in density. These effects are observable post gel point indicating inhibition of protofibril lateral aggregation (Galanakis, Lane, & Simon, 1987; Nair & Dhall, 1991).These changes can be attributed to electrostatic hindrance repulsion between negatively charged albumin and fibrinogen/fibrin inhibiting lateral aggregation during fiber formation. Generally, albumin also affects many coagulation factors by inhibiting their oligomerization, thus decreasing activity (Simhadri et al., 2014).

2.4.1.5 Immunoglobulins

Data on effects of immunoglobulins on coagulation available suggests inhibition of fibrin protofibril lateral aggregation with, however, paradoxical increased mechanical rigidity (Gabriel, Smith, Folds, Davis, & Cancelosi, 1983). Therefore, in the presence of increased plasma immunoglobulins, clotting time may not be affected but influence on haemostatic resistance to vascular stress.

2.4.2 Interactions of fibrin with platelet and leukocyte integrins

Cellular elements interact and modulate fibrin fibres by a variety of mechanisms; mainly by direct interactions between cellular receptors and the fibrin network, activities in cellular releasates and pro- and anti-coagulant activities of the cells themselves. These findings suggest that the types of cells present during coagulation (TF-bearing cells, platelets) determine the aspects of fibrin structure and stability (A. S. Wolberg, 2010).

Fibrinogen and fibrin can bind to cellular elements via integrin receptors. The integrin receptors are $\alpha_M\beta_{2\ in}$ leukocytes, αIIb β3 (GpIIbIIIa) in leukocytes, as well as non-integrin receptors VE-cadherin and ICAM-1. It has been shown that platelet aIIbb3 interactions with the fibrin network promote the formation of a dense fibrin network surrounding the platelet aggregate. Moreover, this network is highly resistant to tissue plasminogen activator-mediated fibrinolysis (Collet et al., 2001).

Proteins and inorganic molecules secreted from quiescent cells or released during cellular activation modulate fibrin clot quality *in vitro* and may influence fibrin stability *in vivo*. Platelets in particular contain several proteins and molecules that influence fibrin quality. For instance,

platelet transglutaminase activity released during platelet activation increases the fibrinolytic resistance of platelet-rich clots. Platelet factor IV reduces fibrin network porosity and decreases clot elastic and loss moduli. Polyphosphate, a polymer of inorganic phosphate released from platelet dense granules during platelet activation prolongs clot lysis in a calcium dependent manner. In addition to direct effects on fibrin network formation and structure, cells may enhance local fibrinolytic activity by expressing urokinase plasminogen activator, urokinase plasminogen activator receptor, or tissue plasminogen activator (S. A. Smith & Morrissey, 2008).

Cells may also suppress endogenous fibrinolytic activities through secretion of plasminogen activator inhibitor and/or TAFI. Given the prominent role of thrombin generation on fibrin structure and stability observations that cells expressing a high level of TF activity promote a rapid onset of fibrin production and dictates the quality of the fibrin network (A. S. Wolberg, 2010) directly support the hypothesis of cell based coagulation model (M. Hoffman & Monroe, 2001; Maureane Hoffman, 2003a).

2.5 Summary of Literature review

Blood coagulation is a complex interaction of cells, proteins and electrolytes and no single paradigm can accurately describe the phenomenon. The coagulation tests in clinical use are based on diverse scientific principles and therefore description of effects of exogenous substances on the clotting process needs to take this into account when comparing results.

From the literature review, it is apparent that the effects of crystalloids on coagulation are a function of solute content, concentration and extent of dilution. However, the overall mechanisms of effects of individual solute contents and concentrations are not well understood

neither are they well documented. Furthermore, lack of uniformity in conclusions arises from use of different blood specimens employed in the various coagulating tests. There is therefore need to perform a comprehensive study investigating the possible crystalloid concentrations and solute contents on blood component specimens with a view to harmonising the coagulation test results that can inform clinical practice.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

The study was laboratory based analytical, experimental, comparative study of the effects of various crystalloid solutions on blood coagulation *in vitro*. The intervention was haemodilution of blood specimens with index crystalloid solutions serving as the test, and the corresponding undiluted sample acting as the control. The experiments were divided into groups as per the objectives into:

i. Routine blood coagulation testing
ii. Thrombin generation
iii. Thromboelastography
iv. Platelet aggregation

3.2 Study Sites

The studies were conducted in the following sites: Routine coagulation and platelet aggregation tests were carried in haemostasis laboratory in conjunction with regional blood donor and transfusion services of Kenyatta National Hospital; Thrombin generation tests were carried in Cardiovascular Research Institute of Maastricht (CARIM), University of Maastricht, Netherlands; Thromboelastography was carried out in the department of Anaesthesia Laboratory, University of Cape Town (for pilot study), and Kenyatta University Department of Medical Physiology

3.3 Study Population

Blood samples for the study were sought from healthy human volunteers who had presented for blood donation at the Blood Transfusion Unit of Kenyatta National Hospital. These were for the routine coagulation tests, Thromboelastography for citrated whole blood samples, and platelet aggregation. Subjects from Groote Schur General Hospital of Cape Town University provided blood samples for Thromboelastography in native, non-citrated whole blood.

3.3.1 Inclusion Criteria

Males or females who had provided voluntary informed consent for study of at least 18 years and not more than 30 years, body mass index between 18 and 28.

3.3.2 Exclusion Criteria

History or confirmed diagnosis of bleeding disorder, systemic illness such as hypertension or diabetes mellitus, current usage of medications acting on the coagulation system or platelets, history of trauma in the preceding two weeks, having received intravenous fluids in the preceding two weeks, females who were pregnant, breast feeding or on hormonal contraception, known diagnosis of malignancy or HIV, and individuals on antiretroviral drugs.

3.3.3. Sample Size Determination

The sample size was determined in consideration of the need to demonstrate at least a seventy percent deficit in coagulation between solutions of different solute types and concentration at a power of 80% (1-β) with a significance level of 5% (α=0.05), as guided by the formula (Dawson and Trapp, 2004) and previous studies (Lindroos, 2010) substituting in the formula:

$$n=\frac{(z_{\alpha/2}+z_{\beta/2})^2 * 2\delta^2}{d^2}$$

Where

n is the sample size

$z_{\alpha/2}$ is the critical value of the normal distribution at α/2 (e.g. for a confidence level of 95%, α is 0.05 and the critical value is 1.96)

$Z_{\frac{\beta}{2}}$ is the critical value of the Normal distribution at β (e.g. for a power of 80%, β is 0.2 and the critical value is 0.84)

$\delta^2$is the population variance – which is 8 (Lindroos, 2010) from TEG stuies.

d^2 is the difference to be detected set as per previous similar studies (Lindroos, 2010) at 10mm of TEG maximum amplitude. Therefore, substituting in the formula:-

$$n=\frac{(1.96+0.84)^2 * 2 * (8)^2}{10^2}$$

$$n=\frac{(7.84) \quad * 2 * (64)}{100}$$

$$n=10.04$$

A minimum sample of 10 subjects for each experiment was therefore determined appropriate.

3.3.4 Allocation of study subjects to experimental groups

In the study, 50 normal young adults were recruited after they met inclusion criteria and signed informed consent. Actual experiments were conducted on samples from 38 (20 males, 18

females) of these adults, age range 20 to 25 years and a mean haemoglobin level of 11g/dl. The distributions per study for the various arms are outlined in the Table 3-1 below:

Table 3-1: Distribution of subjects per study

Study subjects	Routine	TEG	Platelet Aggregation
Number Consented	20	16	14
Destroyed samples	2	2	2
Inadequate samples	1	0	0
Machine malfunction	0	3	2
Actual number whose samples were tested	17	11	10

Thrombin generation study was conducted using pooled samples from Sanguine, Synapse BV, Cardiovascular Institute of Maastricht, Netherlands.

3.3.5 Sampling techniques

The recruitment of study subjects was convenient consecutive as they presented to the blood donor unit. The subjects who declined to give consent were replaced by picking the next subject until the desired sample size was achieved.

3.4 Materials

3.4.1 Crystalloid solutions

Sodium chloride (NaCl), sodium gluconate, chorine chloride, mannitol, and dextrose solutions, in defined concentrations - 300, 600, 900, 1200, 1500, 1800 milli-osmoles/litre confirmed by freezing point depression on Osmometer (Gonotec, USA) – were used.

3.4.2 Coagulation reagents

Thromboplastin, Kaolin, CaCl2 and bovine thrombin were from Stago (Asnier, France) for use in routine coagulation tests.

For thrombin generation, the following materials were used: platelet free plasma (Sanquin, The Netherlands), recombinant tissue factor (Innovin) (Dade-Behring;USA), phospholipid (PPL) solutions (Synapse, Maastricht, the Netherlands) - DOPS, DOPC and DOPE (20%-60%-20%), Calcium chloride ($CaCl_2.2H_2O$), potassium chloride (KCl), tri-sodium citrate dehydrate ($C_6H_5Na_3O_7.2H_2O$), sodium hydrogen carbonate ($NaHCO_3$) and d-glucose water free ($C6H_{12}O_6$) (all from Merck, Germany), thrombin substrate Z-Gly-Gly-Arg-AMC (Bachem, Switzerland), bovine serum albumin (BSA) and D-mannitol (Sigma-Aldrich Chemie GmbH, Germany), Thrombin Calibrator (alpha-2-M- thrombin complex) (Synapse, Maastricht, The Netherlands), and Kaolin light.

For platelet aggregation, platelet agonists - ADP and Arachidonic acid (AA) (Helena Biosciences, UK) reconstituted with double distilled water to a final concentration of 200µM and 500µM respectively were used.

3.5 Methods of Blood coagulation testing

3.5.1 Blood Sample Collection

Blood was collected once from each of the healthy volunteers for *in vitro* studies. From each healthy volunteer 12 ml of blood was collected from freely flowing ante cubital vein using G18 scalp vein set applying minimal stasis, and collected into 3.2% citrated Tubes (Becton

Dickinson, NJ, USA) in a ratio of 1 part citrate and 9 parts blood. The first 4 ml was discarded to avoid contamination with tissue factor.

3.5.2. Routine Coagulation Tests

Blood was collected in citrated tubes from healthy human subjects who met inclusion criteria. Platelet poor plasma (PPP) was obtained by centrifuging blood at 1600-2000 x g for 15 mins at room temperature. Aliquots of 0.4mls platelet poor plasma (PPP) were then mixed with 0.1mls of the index crystalloid solutions defined above. The blood samples in polypropylene tubes was inverted three to four times to ensure uniform mixing.

Routine clotting time were determined by Coagulometer (Dahde-Behring, NJ, USA) using the standard laboratory coagulation methods (Andrew *et al*, 1998; Robert *et al*; 2001). The coagulometer uses automatic pippeting system and provides print out of test result readings for PT, aPTT, INR and fibrinogen levels. The coagulometer was calibrated using reagents provided by the manufacturer performed weekly as part of quality control.

Briefly, prothrombin time (PT) was performed by placing aliquotes of 0.1ml plasma in a test tube in a water bath at 37 ^{0}C and 0.1 ml thromboplastin added. After 3 minutes of incubation, 0.1 ml of pre-warmed $CaCl_2$at a concentration of0.025 mols/L was added to mixture to start coagulation reaction.

The Activated Partial Thromboplastin Time (aPTT) was performed by first mixing equal volumes of phospholipid reagent (0.1 ml) and kaolin suspension (0.1 ml) in a test tube and left to warm in a water bath at 37 ^{0}C. Then 0.1 ml of plasma was placed in a test tube to which was added 0.2 ml of kaolin-phospholipid mixture and incubated for exactly 10 minutes. The reaction was started by adding 0.1 ml of pre-warmed $CaCl_2$at a concentration of 0.025mols/L. The

clotting times were detected automatically by the machine using mechanical electro-magnetic detection of rotating steel ball method.

The fibrinogen concentration was determined by adding excess bovine thrombin into plasma. The clotting time is compared automatically to a standard curve by the machine from inbuilt algorithm to give reading of fibrinogen in sample (mg/L).

The test results were recorded: PT (sec) (normal laboratory range 11 to 16 secs), aPTT (sec) (normal laboratory range 27 to 40 secs), INR (ratio) (range 1.0 to 1.3) calculated from PT, and fibrinogen (mg/dl). The performance of PT has been reported as having 88% specificity and 88% sensitivity, and APTT giving 50% sensitivity and 100% specificity for factor deficiencies (Daniel Bolliger et al., 2010)

3.5.3 Thrombin Generation

The crystalloid solutions tested were mannitol (2.5%, 5%, 10%, 15% and 20% w/v concentrations), NaCl (0.9%, 2.5%, 4%, 5%, 7.5%, 10%, and 20% w/v) and Ringer's Solution (RS). These were representative of neutral non ionic and ionic crystalloids respectively.

Frozen plasma was obtained from Sanguin (Synapse, University of Maastricht, Netherlands) kept at -80^{0}C. Samples were warmed to 37^{0}C and vortexed before use. Aliquotes of plasma were pippeted into ependorf tubes and diluted with index crystalloid solutions in 1: 5 ratio (20% dilution). Thrombin generation (TG) was carried out using Thrombinoscope™ calibrated automated thrombogram (CAT) (Synapse BV, Maastricht, Netherlands) in triplicate as described by Hemker *et al* (H. Coenraad Hemker et al., 2003; H. Coenraad Hemker, Al Dieri, De Smedt, & Béguin, 2006b). Thrombin generation was monitored by following the fluorescence changes accompanying splitting of fluorogenic substrate by the generated thrombin in the test.

Calibration was obtained by running a parallel sample with known thrombin-like of the thrombin calibrator (α-2-M-Thrombin complex).

Briefly, in a 96-well plate, 20 µL of calibrator (600 nM alpha-2-M- thrombin complex) was added to three wells and 20 µL of trigger solution (0.5 to 5 pM tissue factor (TF) and 24 µM phospholipids (in 5 mg/ml BSA) was added to another three wells; 80µL of plasma samples diluted with index crystalloids was added to all six wells.

Extrinsic activation was achieved by innovin (TF) in concentrations of 0.5pM, 1pM and 5pM in triplicates. In parallel, intrinsic activation was achieved using 100nM kaolin.

The 96-wells plate was incubated on a plate heater for 10 minutes at 37 °C. In the meantime, the FluCa solution (0.1 M $CaCl_2$ and 2.5 mM of Z-Gly-Gly-Arg-AMC in 60 mg/ml BSA) was prepared and incubated in a water bath at 37 °C. When the experiment was started, the dispenser squirted 20 µL of FluCa solution in each well at zero time. Fluorescence was measured at 390 nm and 460 nm excitation every 20 seconds over a period of 60 minutes. The signal from the two wells (calibrator and sample) was simultaneously measured. From the calibrator well, the calibration constant was obtained at every level of fluorescence. With these values, the molar amount of thrombin present at any moment in the coagulating sample was calculated. Data were exported from the CAT which generated thrombin generation curves from the data obtained. The experiments were repeated using defibrinated plasma.

The software enabled the estimate of the following parameters: (a) the Lag Time of thrombin generation (minutes), (b) the time to reach the maximum concentration of thrombin (time to Peak) (minutes), (c) the maximum concentration of thrombin (Peak) (millimoles), (d) the total duration of thrombin generation activity (Start Tail), and (e) the total amount of thrombin

activity assessed as the area under the curve recorded as the endogenous thrombin potential (ETP).

3.5.4 Thromboelastography

Fresh whole blood was collected into citrated tubes and kept at room temperature. Aliquots were taken and diluted with index crystalloid solutions in 1: 5 ratio (0.1ml crystalloid solution and 0.4 ml whole blood) (20% blood volume dilution).

Thromboelastography was determined by Thromboelastograph™ (TEG) 5000 (Haemoscope, Skokie, Il, USA) on whole blood samples after dilution with the crystalloid and recalcification according to the manufacturer's instructions(Donahue & Otto, 2005; Glidden et al., 2000; Zambruni, Thalheimer, Leandro, Perry, & Burroughs, 2004). TEG machine calibration was performed using reagents provided by the manufacturer.

Briefly, 340µL of whole blood mixed with crystalloid solutions under tests was pipetted into pre-warmed TEG cuvettes (at 37^0C) containing 20µL of calcium chloride. Immediately after transferring the blood samples to TEG cups,the machine was started and allowed to run for 60 minutes. TEG parameters R time (mins), k (mins), α-angle (degrees) and MA (mm) were recorded in a spreadsheet.

In separate experiments, TEG testing was done with modification as follows:

(a) Native whole blood collected innon-citrated polypropylene tubes and analysed immediately within 4 minutes without recalcification. The aim was to determine whole blood coagulation in absence of citration.

(b) Citrated blood samples haemodiluted with highest concentration of crystalloid solution that showed hypocoagulability pippeted into TEG cups spiked with either (i) thrombin

(10 IU) or (ii) fibrinogen (1mg). This was to estimate which of the coagulation factor concentrate could restore the hypocoagulability.

3.5.5 Platelet Aggregation

Platelet aggregation was performed on blood obtained from healthy subjects who met the inclusion criteria after overnight fast. Twenty ml of venous blood was collected atraumatically with minimal occlusion in citrated tubes (BD) (1 part 3.2% sodium citrate to 9 parts blood) and kept at room temperature. Platelet rich plasma (PRP) was obtained by centrifugation at 150-200g for 10 -15 minutes. The PRP was carefully removed to avoid contamination with RBC, buffy coat and placed in stoppered plastic tube at room temperature until tested. The remaining blood was centrifuged at 2000g for 20 minutes to obtain platelet poor plasma (PPP). Standardization of PRP was done by diluting with PPP to achieve platelet counts between 200 to 400 platelet counts X 10^9/L. The aggregometer was switched on about 30 minutes before the tests to be performed to allow the heating block to heat to 37^0C. The stirring speed was set at 900rpm.

Duplicates of PRP (400µL) were mixed with each crystalloid solution (100µL) and incubated for 15 mins at 37^0C in a water bath. From each PRP (diluted) and PPP (undiluted acting as control)tubes, 450µL was pippeted and transferred into Aggram cuvettes and incubated for 3 minutes and thereafter inserted into appropriate channels of the platelet aggregator heating block. After 1 minute the stirrer was inserted into the plasma. Calibration was set at 0% light transmission aggregometry (LTA) for PRP and 100% LTA for PPP. The samples were allowed to warm to 37^0C for 2 minutes before addition of agonists. To each PRP containing cuvette, 50µL of either AA or ADP agonist were added and channel activated and monitored for 10 mins at a constant stirring speed of 1000rpm at 37^0C.

Controls were run in parallel and consisted of PRP without the crystalloid diluents. Inhibition of platelet aggregation was expressed as a percentage decrease in the area under the curve compared to control. The analysis of a platelet aggregation curve was noted by the presence of a primary and secondary wave. The platelet aggregometry parameters recorded were: lag time (sec), max aggregation (%), shape change (% LTA below baseline after initiation of reaction) and velocity of aggregation (%/sec).

3.6 Data Handling and statistics

Data was entered into paper based proforma and transferred to a spreadsheet database. Conventional measures of central tendency and spread were used to describe the data. Variables of interest were tested for normality using both Kolmogorov-Smirnoff and Shapiro-Wilk tests, and hypothesis of normality was done if either test was significant. Data was analysed using STATA (Intercooled STATA version 11.0, Texas, USA). Analysis of variance (ANOVA) and Kruskal Wallis equality of population tests was applied to determine differences in continuous data as guided by the distribution of data. Statistical significance was set at 5%. Results are presented in tables and graphs as appropriate.

3.7. Ethical Considerations

An approval was sought and obtained from the Institutional Human Research and Ethics Committee of Kenyatta National Hospital and University of Nairobi (Appendix 2), and University of Cape Town (Appendix 3). Study subjects were recruited as per criteria in Appendix 1. The study was conducted as per ethical guidelines on biomedical research defined by International Commission of Harmonization on Good Clinical Practice. A written consent

was obtained from all study subjects recruited into the study (Appendix 1). Confidentiality was maintained by coding of identity information of the study subjects and no identifiable individual data was recorded.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Routine Coagulation Results

PT, INR, Fibrinogen and APTT data were normally distributed, with Shapiro Wilk test consistently revealing a $p < 0.05$ for trend analysis. There were significant changes in routine coagulation tests with increasing NaCl concentrations.

4.1.1. Effects of NaCl Concentration on Prothrombin Time

The data were not normally distributed, and therefore the appropriate measure of central tendency was medians. The undiluted control plasma had a median PT of 16.1 secs (IQR 15.3-17.1; N=17).

Prothrombin time increased linearly with increase in NaCl concentration ($p<0.001$: chi square test for trend) (Figure 4-1).

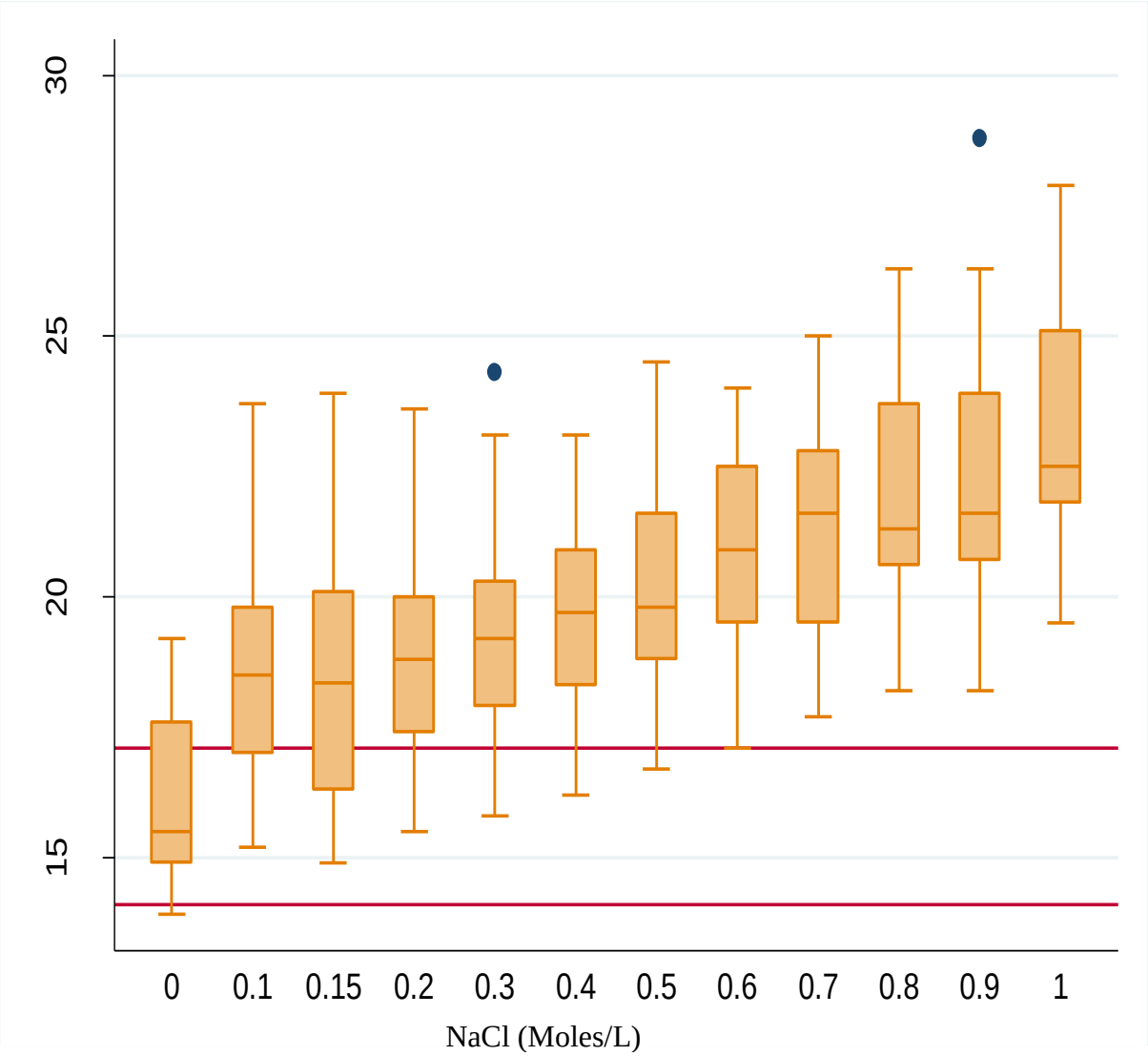

Fig 4-1 Changes in PT with increase in NaCl concentration
Data are for medians with 25% and 75% interquartile ranges, red line across represents normal laboratory ranges (13-15 secs) (N=17). * represents outliers

4.1.2. Effects of NaCl Concentration on INR

The median INR before dilution was 1.2 (IQR 0.99-1.32; N=14).The INR increased with increase in NaCl concentration ($p<0.003$: chi square test for trend from undiluted control). The INR mirrored PT changes. All the median INR were above the upper laboratory reference range (Figure 4-2).

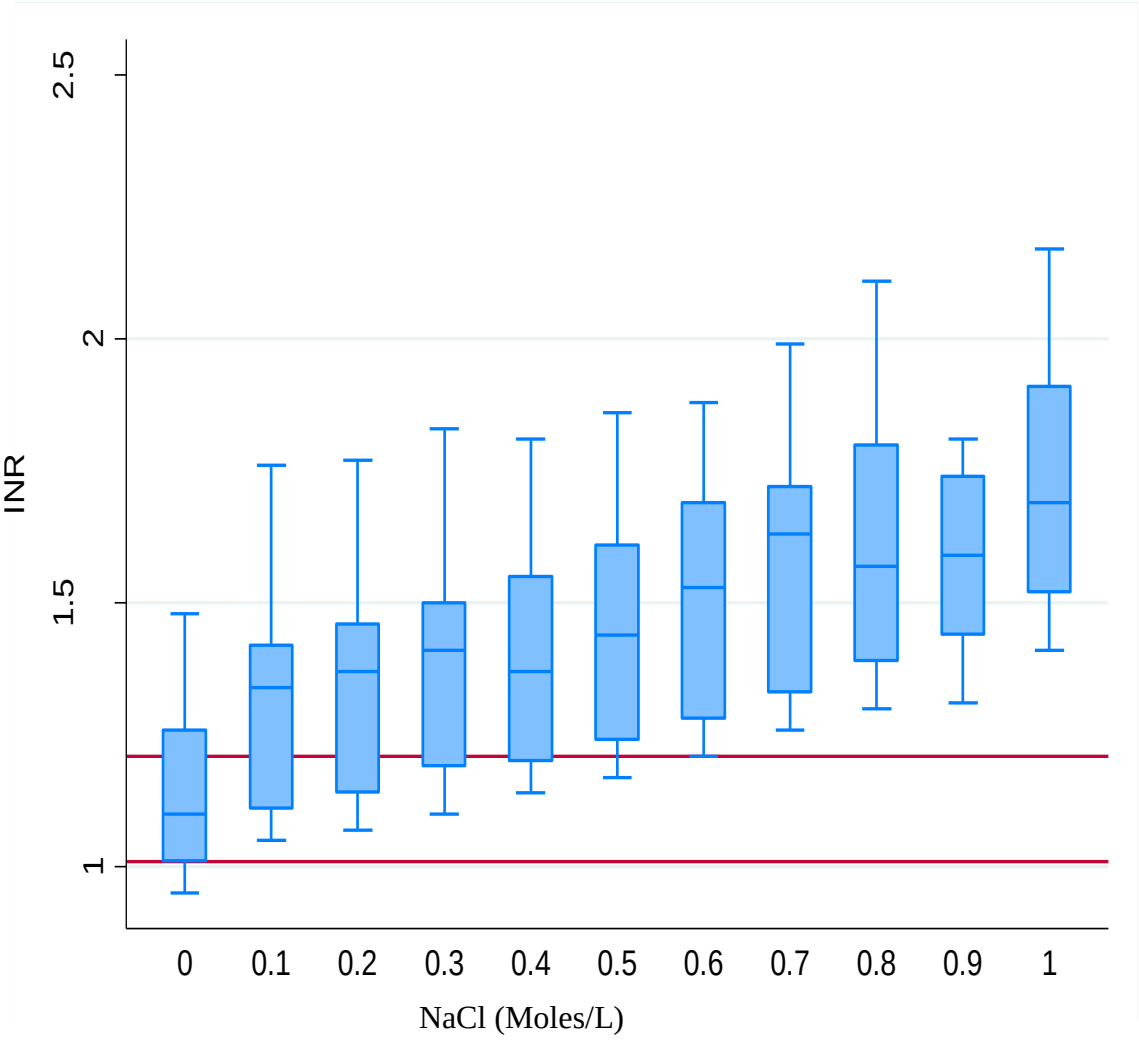

Figure 4-2 Effects of NaCl concentration on plasma INR
Data represents medians and 25th and 75th interquartile range (N=17). The red line across represents normal laboratory reference ranges (1.0-1.2). Note INR is a ratio without units.

4.1.3. Effects of NaCl concentration on aPTT

The median APTT for undiluted control was 33.98 secs (IQR 31.94-36.02; N=14). aPTT significantly increased with increase in NaCl concentration ($p<0.001$: chi square test for trend) (Figure 4-3).

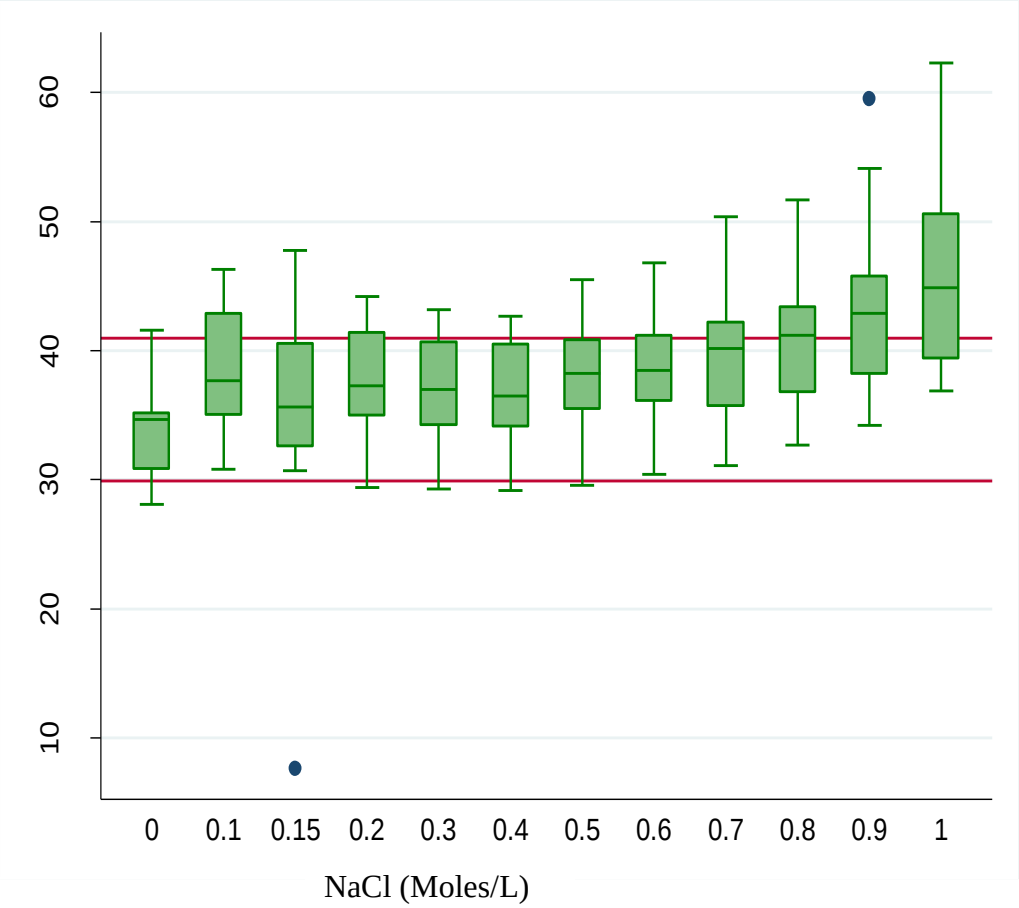

Figure 4-3 Effects of NaCl on aPTT
Results are for medians and 25th and 75th interquartile range (N=17). Red lines across represent normal laboratory reference aPTT range (27-37 secs).

4.1.4. Effects of NaCl Concentration on Fibrinogen Levels

The median fibrinogen for undiluted control was 283.9 mg/dL (IQR 249.9-317.8; N=12). Fibrinogen levels decreased with increase in NaCl concentration ($p<0.001$: chi square for trend) (Figure 4-4).

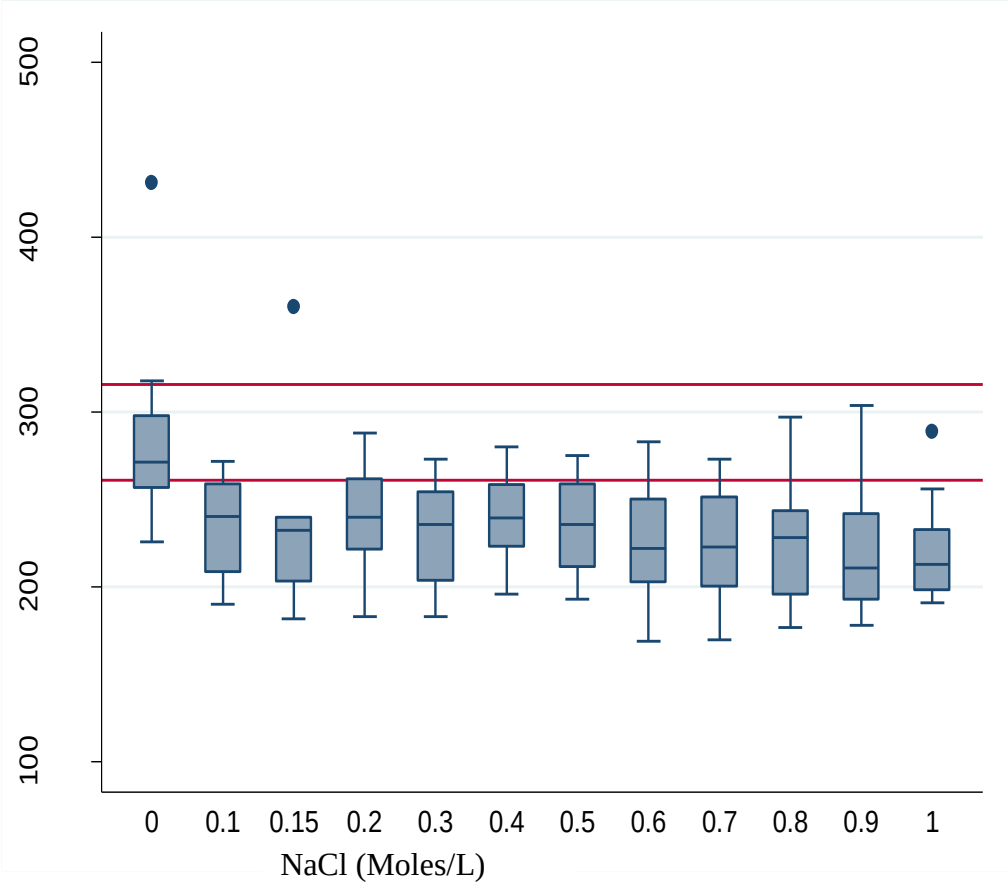

Figure 4-4 Changes of fibrinogen concentration with increase in NaCl concentration
Data plots for results are for medians with 25th and 75th interquartile ranges (N=11). Red lines across represent normal laboratory reference range (250-320 mg/dL of fibrinogen).

4.1.5 Effects of other Crystalloid Solutions on aPTT

The data were normally distributed, therefore mean as a measure of central tendency was appropriate. The mean aPTT for undiluted control was 31.24 secs (SE 2.84; 95% CI 25.62-36.85; N=17). Only choline chloride at 1800 mOsm/L was associated with a significant increase in aPTT than control (mean 78.42 secs; SE 3.75; 95% CI 71.2-85.83; P<0.01). All the other solutions did not show significant difference compared with the undiluted control. Even though dextrose 50% suggested higher value than control with a tendency towards significance (mean 41.81; SE 4.7; 95% CI 32.53-51.10; P=0.06), it was not significant.

(Figure 4-5).

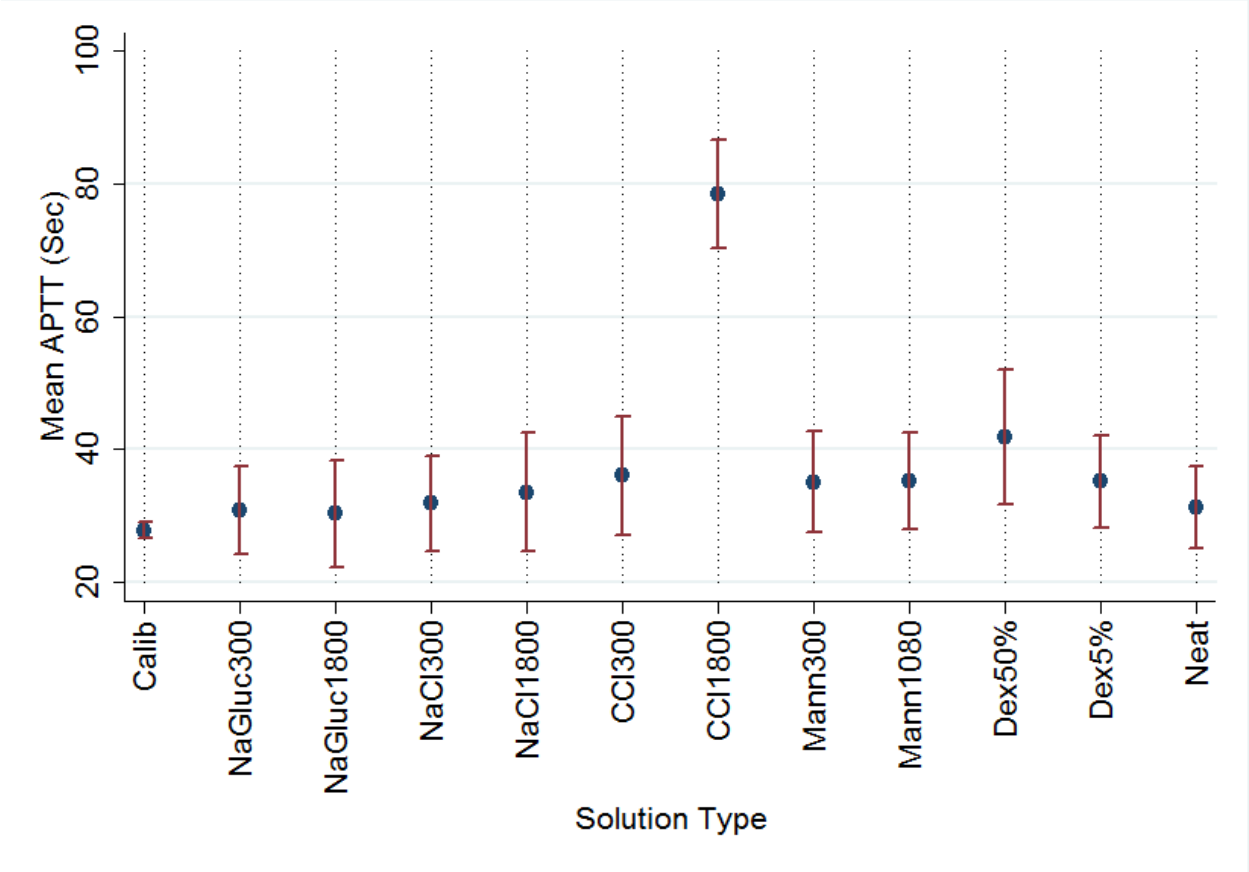

Figure 4-5 Effects of other crystalloid solutions on intrinsic coagulation activation

Data were plotted for error bars of mean of 17 experiments and 95% CI. Calib-machine calibrator standard, Neat-undiluted control, NaGluc-sodium gluconate, NaCl-sodium chloride, CCl-choline chloride, Mann-mannitol, Dex-dextrose.

4.1.6 Effects of other Crystalloids on PT

The data was normally distributed therefore mean was used as a measure of central tendency. The mean PT for the undiluted control was 13.8 secs (SE 0.33; 95% CI 13.17 to 14.49). All the solutions, except Mannitol 300mOsm/L and Dextrose 5%, were associated with a significant longer clotting time compared to the control ($P<0.05$, $N=17$) (Figure 4-6).

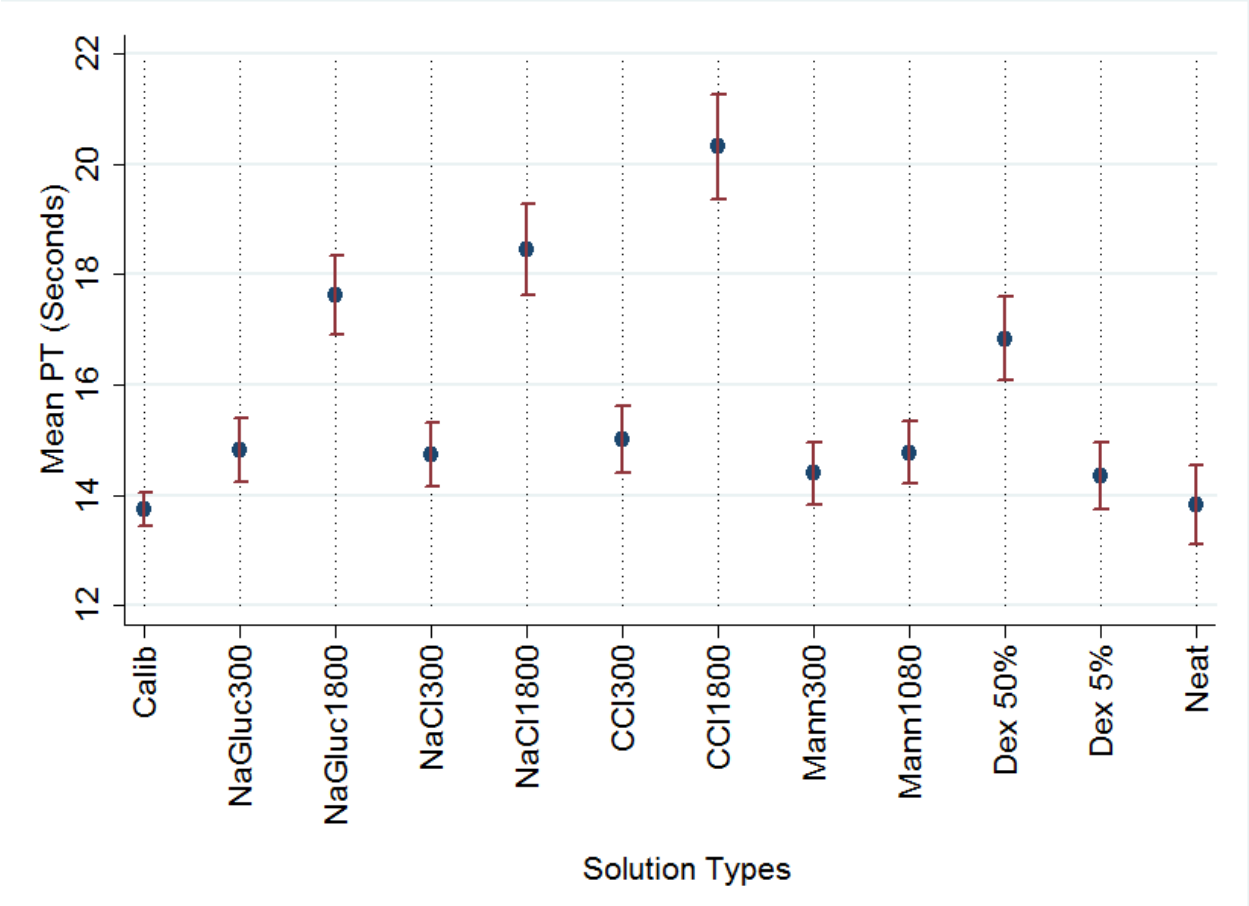

Figure 4-6 Effects of crystalloid solutions on extrinsic coagulation activation

Data are Plots are for means as error bars and 95% CI. Calib-machine calibrator standard, Neat-undiluted control, NaGluc-sodium gluconate, NaCl-sodium chloride, CCl-choline chloride, Mann-mannitol, Dex-dextrose.

In consideration of defined normal laboratory ranges, while PT values were out of range when plasma samples were diluted with NaCl concentration of 0.2 Moles/L and above (Figure 4-1), the corresponding INR was out of defined range only when diluted with NaCl concentration of 0.5 Moles/L and above (Figure 4-2). Clottable fibrinogen levels were out of range for any NaCl concentration (Figure 4-4). The aPTT values largely remained within normal reference range for all the NaCl concentrations ranges used in the study (Figure 4-3)

This study found direct linear relationship between NaCl concentrations and plasma clotting time within concentration range from 0.1 to 1M. This is consistent with previous studies (Reed, 1992; Wilder, 2002) which found plasma clotting time was prolonged in the presence of hypertonic 7.5% NaCl compared to isotonic concentrations. However the investigators used different methods since they compared only two concentrations (7.5% vs 0.9% NaCl) rather than full range which does not address effects of intermediate concentrations as one progresses from isotonic to hypertonic concentrations. Their results were therefore uninformative whether relationship is linear or exponential. Furthermore, their tests were performed on reference plasma (Reed, 1992) instead of freshly collected samples and the control was phosphate buffered saline thus assuming uniform composition of procoagulants and anticoagulants in plasma. Involvement of phosphate may introduce an additional confounder. Additionally, lack of undiluted control precluded generation of baseline coagulation characteristics before crystalloid haemodilution.

The present data alongside previous work revealing anticoagulant effects of increasing saline concentration can be interpreted on the basis of increasing ionic strength of NaCl (Scheraga, 2004). This is consistent with previous data that demonstrated that apart from prolongation of clotting time in experiments utilizing pure thrombin and fibrinogen (Seegers and Smith, 1942; Ferry and Morrison, 1947; Ferry and Shulman, 1949; Latalo, 1962; Waugh, 1953; Conio, 1976), other properties of fibrin were altered by increasing ionic strength (Waugh and Patch, 1953; Ferry and Morrison, 1947; Latalo, 1962). The process was reversible on diluting to lower concentrations implying the process is temporary arrest of clotting reactions. This was most likely post thrombin cleavage of fibrinogen to fibrin monomers, but before fibrin monomer polymerization. However, the postulate is speculative since no published results have been done

evaluating effects of NaCl concentration on thrombin generation in plasma. Also, their results had limited applicability since plasma clotting involves complicated reactions between pro-coagulants and natural anticoagulants which are absent in simplified purified factor simulations.

The findings in this study that there was negative correlation of fibrinogen levels with increasing saline concentration were unexpected and new (Figure 4-4). Since the clotting still occurred though after a prolonged period, the results indicate that thrombin in hypertonic medium was still active and able to cleave fibrinogen. This therefore strongly suggests that the explanation for prolonged clotting time was not interference with thrombin activity but most likely retardation of fibrin monomer polymerization. This line of thinking is strongly supported by studies that found evidence of maintained thrombin activity in presence of hypertonic saline (Grant, 1985; Froelich, 2006) despite increasing clotting time (Reed, 1991; Wilder, 2002), and despite lack of fibrinogen clotting (Landis et al, 1981). Thus lack of clotting could only arise from impaired biophysical polymerization of fibrin monomers. Therefore, in this study, decrease in clottable fibrinogen levels with increasing NaCl concentrations at uniform dilution is most likely due to inhibition of fibrin polymerization which is a biophysical process. However, this hypothesis can only be tested if concurrent tests of thrombin generation are carried in the same haemodiluted plasma samples.

Reduction of clottable fibrinogen as determined by Claus method in the presence of increasing ionic strength in this study (Figure 4-4) despite uniform dilution can present potential clinical implications especially if other alternative methods such as immunological techniques report no changes in fibrinogen levels. This becomes pertinent since clinicians may be confused as to

which test is best suited for optimal therapeutic fibrinogen factor replacement. However, in clinical practice the issue is of little significance since saline concentrations above 1000 mOsm/L (3%) have limited clinical applications except in treatment of elevated intracranial pressure and/or as in small volume resuscitation (Reed,1992). Therefore for most fluid resuscitations where the most preferred crystalloids are isotonic to plasma, the problem of reduced fibrinogen with hypertonic saline is unlikely to be encountered.

The order of increase in clotting time with ionized solutes was: Sodium gluconate < sodium chloride< Choline chloride (Figure 4-6). In contrast, effects of choline chloride were more pronounced at all concentrations in comparison to other solutions on both PT and aPTT (Figure 4-5& 4-6). The present study results are in contrast to enhancement of coagulation with serial dilution of plasma with isotonic saline (Tocantins et al., 1951a). Instead, the results show that plasma clotting time is dependent on crystalloid solutions solute type and its concentration, an example of Debye-Huckel theory of ionic strength (Cace, 1997). Neutral and organic solute ions despite increase in concentration maintained clotting time close to undiluted control. This pattern is in keeping with Hofmeister series of lyotropic effects of salts ("how_hofmeister_ion_interactions.pdf," n.d.). From the results, it is evident that effects of charged anions were greater than cations, and organic less than inorganic. This pattern is in keeping with observations where anions at high concentrations such as sulphates, phosphates and citrates promoted prothrombin autocatalysis to thrombin in contrast to chlorides salts of sodium, potassium, ammonium or magnesium (W. H. Seegers et al., 1950; Walter H. Seegers & Smith, 1942). Thus, the salt's anion is the most important factor in determining the time to clot end point.

Overall, it is evident that chloride containing salts dose-dependently prolonged clotting time in both systems (Figure 4-5 and 4-6), an effect attenuated by sodium. The effect of ionic strength does not appear significant unless in chloride salts, since effects of choline chloride were greater than those of equiosmolar concentrations of sodium gluconate (Figure 4-5& Figure 4-6). Nevertheless, it is not possible using routine clotting tests to differentiate whether impairment was on thrombin generation or fibrin polymerization.

The experimental results in this study of prolongation of clotting time in the presence of chloride are in agreement with the hypothesis of chloride's lyotropic effect (Baldwin, 1996). Chloride anion being a chaotrope has less hydration energies therefore allowing proteins to remain in solution or have salting-in effects resulting in delayed precipitation of fibrin. Although choline and chloride are both chaotropes, effects of anions usually predominate. Nonetheless, the pronounced clotting time prolongation in the presence of increasing concentrations of choline chloride could be attributed to synergistic effects of choline and chloride.

The current experiment demonstrates clearly that increase in choline chloride increases clotting time out of proportion to osmotic concentration. Comparison with other similarly concentrated crystalloid further confirms its unique molecular properties on the clotting process. Although it has been taken that prolonged clotting time in the presence of increasing NaCl is due to ionic strength (Ferry & Morrison, 1947; Latallo, Fletcher, Alkjaersig, & Sherry, 1962; Walter H. Seegers & Smith, 1942), this study reveals that this can only be true when chloride ions are in the mixture. This is in agreement with

experiments carried out examining different aspects of clots derived from clotting of pure fibrinogen by thrombin in which ionic strength was controlled/maintained with varying NaCl concentrations where increase in clotting time also corresponded with changes in other properties (Ferry & Morrison, 1947; Latallo et al., 1962; Shulman, 1953; Waugh & Patch, 1953) whether salt solutions were added before or after thrombin suggesting they were independent of thrombin. Also, other evidence include impairment of fibrin monomer aggregation in the presence of high ionic strength contributed by NaCl (Hantgan & Hermans, 1979), decrease in fibrin fibres diameter with increase in ionic strength which correlated with exponential increase in clotting time (Conio et al., 1975, 1976). Cl- anion was the most important specific variable regulating fibrin fibre morphology (E. Di Stasio et al., 1998; Enrico Di Stasio, 2004; Vindigni & Di Cera, 1996). These findings therefore establish that the contribution of ionic strength in impairment of coagulation is a specific function of cl- ions.

Haemodilution with concentrated chloride containing solutions or large volumes of saline are the main cause of hyperchloremia (McFarlane & Lee, 1994). Hyperchloremia has been associated with more blood loss requiring more blood products, and had deranged coagulation parameters than those with normochloremia in humans (Boldt et al., 2002; Scheingraber, Rehm, Sehmisch, & Finsterer, 1999), and in laboratory animals (Kiraly et al., 2006). These results suggest coagulation effects attributable to strong ionic strength are likely due to chloride ions even when other factors are controlled.

The contribution of chloride in impairing blood clotting has been suggested in clinical studies comparing isotonic saline (high Cl- 154 mMol) versus plasmalyte (balanced salt solution low in

chloride at 100 mMol). Therefore, a crystalloid fluid high in chloride or dilution that increases chloride load will show prolonged clotting time.

Choline is a bulky molecule and weakly hydrated hence its participation on the coagulation reactions is very minimal. But contribution of choline cannot be ruled out since routine coagulation tests involved thromboplastin relipidation. Choline could still play a role in reducing effective optimal ratio of PS to PC (20:80) thus contributing to prolonged clotting time and exaggerating the effect of ionic strength in enhancing clotting factor deficiency sensitivity to thromboplastins (S. A. Smith & Morrissey, 2008).

Solutions containing gluconate were associated with short clotting time despite increase in its concentration in this study. The anion gluconate though classified as Kosmotrope is big a molecule that does not participate in coagulation reactions therefore haemostatically inert (Baldwin 1996). However, no comparative study has been done on evaluation of effects of gluconate in relation to other anions using routine tests.

The effects of glucose alone, and in combination with mannitol on plasma clotting has been studied (Wilder et al., 2002b). In an *in vitro* study, clotting time in both PT and aPTT were not significantly prolonged with dextrose haemodilution ranging from 5%, 10% to 20% plasma volume replacement. Additionally, another in another study (Bakaltcheva, O'Sullivan, Hmel, & Ogbu, 2007) it was found that coagulation was not affected when plasma was incubated with dextrose, mannitol, trehalose, or sorbitol for up to 5 days. In contrast, others had found that found that clotting time of pure fibrinogen by thrombin was longer in the presence of 3%

dextrose solutions compared to no change in sorbitol and control without additive (Einarsson, 1975). Clotting times were restored when glucose was dialyzed from mixture with fibrinogen or when the amount of thrombin was doubled. Although the same compounds had earlier been found to have minimal effects on clotting of pure fibrinogen by thrombin at high concentrations, physical properties of formed fibrin clots were different (Ferry and Shulman, 1949; (Shulman, 1953). These studies suggest osmolality of hydroxyl compounds-as represented by mannitol and dextrose have minimal effect on plasma coagulation factor activity, and lack of activity is conferred by charge neutrality.

The minimal change in clotting time despite high concentration in organic solutes such as mannitol, dextrose and anion gluconate is this study is in agreement with results obtained by Wilder et al (Wielders, Béguin, Hemker, & Lindhout, 2004), where effects of hypertonic organic solutes (glucose, glycerol, sorbitol and mannitol) were much less than hypertonic NaCl on PT, aPTT or TT. The current study results therefore further reinforces the notion of predominance of ionic strength over concentration on coagulation parameters, according to their position on the Hofmeister series (Baldwin, 1996). The difference in effects of crystalloid solutions can be accounted for on the basis of physicochemical characteristics based on salting in or out of proteins, and ionic strength as a marker for solute charge activity (Baldwin 1996). Since mannitol or dextrose molecules have high hydration energies thus binding water with greater affinity, they therefore tend to promote salting- out or precipitation of proteins. The fibrin monomers that arise from thrombin cleavage in their presence tend to have low solubility or fractional increase in the remaining little ‘free water’ and therefore tend to gel out of solution.

It is speculated that dextrose affects fibrin polymerization, but not thrombin activity or proteolysis. This is reinforced by findings in an experiment in which fibrinogen was clotted with thrombin in absence and presence of 10mg/dL of added glucose, where glucose only shifted the onset of light scattering intensity but not the slope. Analysis of line weaver Burk plot indicated that the data points for thrombin-fibrinogen mixtures in absence and with added glucose were coincident indicating no inhibition of thrombin enzyme (Kubota et al., 2004). The effects were more on gel structure and not on dynamics of gel point. From this study in comparison to aforesaid cited results, it may be inferred that increase in clotting time in the presence of glucose arises due to inhibition of fibrin monomer polymerization, and not on thrombin.

It must be appreciated that a typical PT assay employs approximately 200 nM TF, which will produce a clot in 11–15 s. Since at the point of visible clot only 10–30 nM thrombin has been generated , at the high TF concentrations used, robust generation of factor Xa by factor VIIa–TF renders unimportant the contribution of the factor VIIIa–factor IXa complex in clot end point assays (S. Butenas & Mann, 2007). Therefore, with saturating TF levels, routine coagulation test may not be sensitive to slight to modest changes in thrombin generation. This is one of the limitations when no difference is reported for incremental doses of crystalloid solutions.

Another major limitation of routine coagulation assays is that it uses clot formation as an end point to estimate deficiency of coagulation factors. This is one event in a series of coagulation factor reactions. Therefore, any reference as to impairment of thrombin generation or on fibrin polymerization must remain speculative.

4.2 Effects of Crystalloids on Thrombin Generation

4.2.1 Effects of Mannitol Haemodilution on Intrinsic Activation with Kaolin

Thrombin generation with kaolin activation in the intrinsic system remained unchanged despite increase in Mannitol concentrations, up to 20% w/v (Fig 4-7). Lag time, time to peak, peak thrombin and ETP remained qualitatively unchanged from the undiluted control.

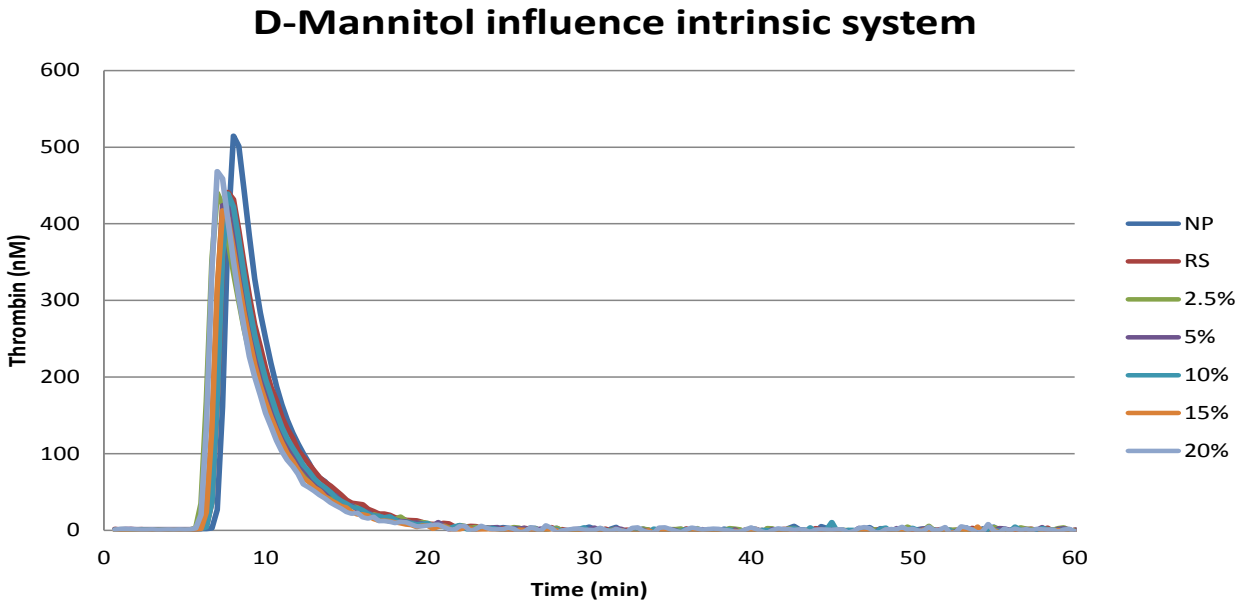

Figure 4-7 Influence of different concentrations of D-mannitol on the thrombin generation

Haemodilution was 1 part crystalloid and 4 parts plasma. Intrinsic coagulation activation was triggered by kaolin. NP-normal plasma; RS-Ringer's lactate; mannitol concentrations ranged from 2.5%, 5%.10%, 15% and 20% w/v as indicated.

4.2.2 Effects of Plasma Dilution with Different NaCl Solution Concentrations on Intrinsic Activation with Kaolin

Thrombin generation curves in different NaCl concentrations were qualitatively similar, though lag time were progressively lengthened at each saline concentration, shifting the whole thrombin generation curve to the right. The corresponding ETP and peak thrombin levels were also progressively decreased with NaCl concentration, but to a lesser extent (Figure 4-8). Interestingly, thrombin generation was totally inhibited in 5% NaCl and other higher concentrations, occurring at a lower level than in extrinsic system (Figure 4-13). Comparison of effects of mannitol and NaCl concentrations shows that thrombin generation parameters lag time (Figure 4-9), ETP (Figure 4-10) and peak height (Figure 4-11) did not change in mannitol unlike in NaCl.

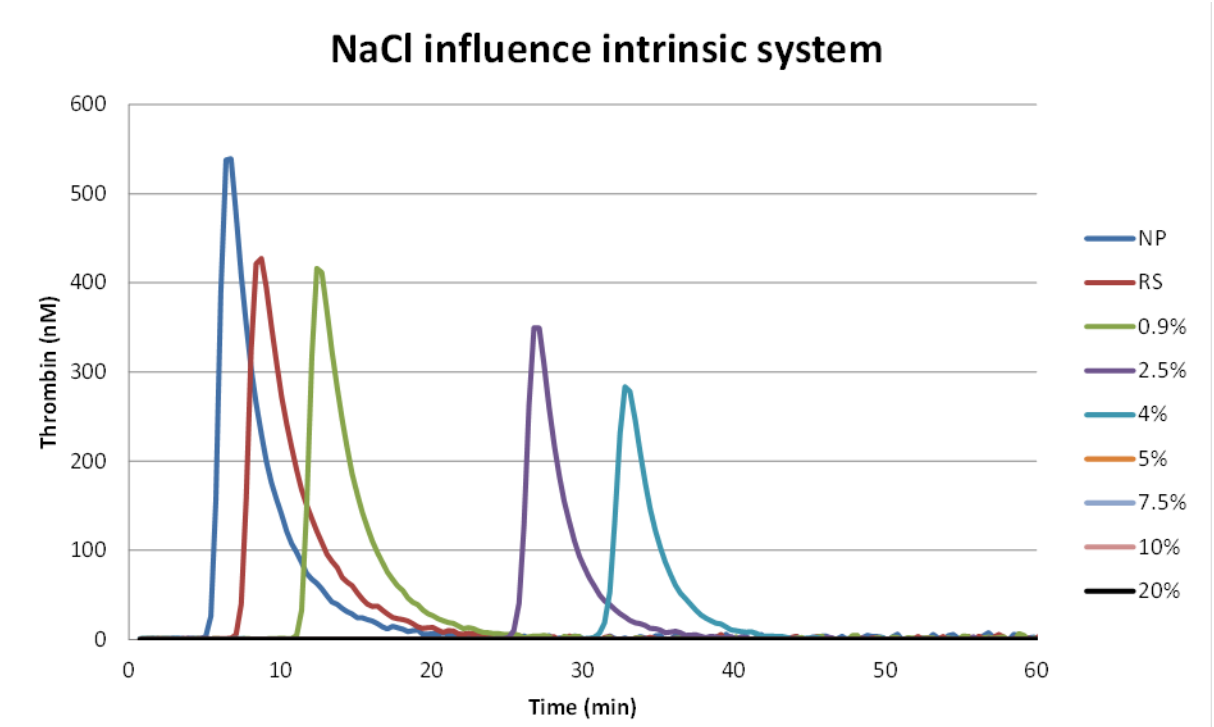

Figure 4-8 Influence of NaCl concentration on intrinsic coagulation system.

Plasma dilution was kept constant at 20%. Tests were carried out in triplicate; error bars are shown (standard deviation).

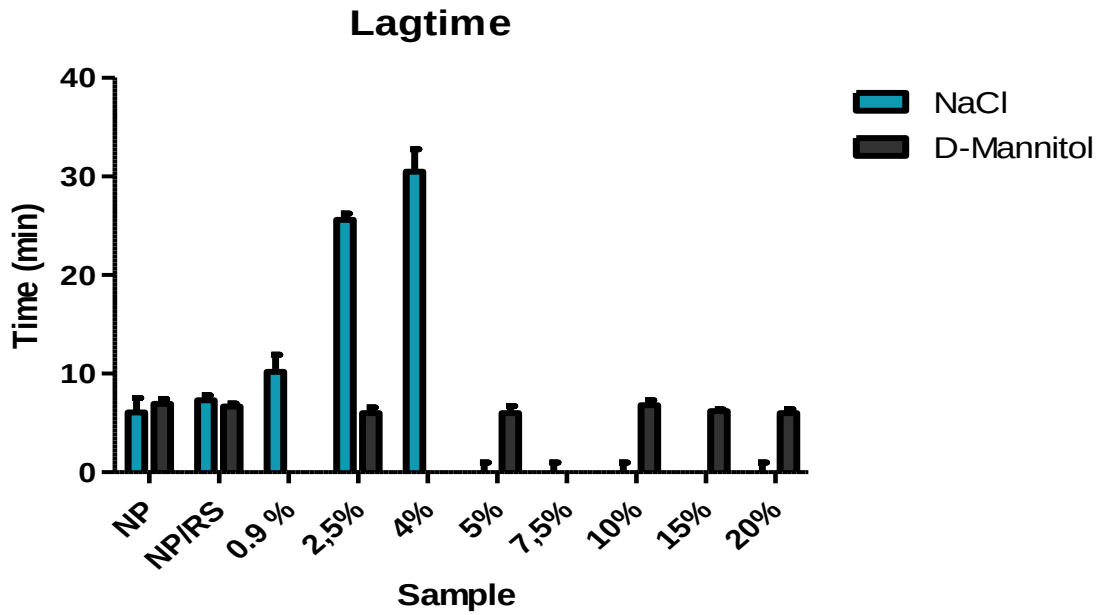

Figure 4-9 Comparison of thrombin lag time in intrinsic system activation in NaCl and mannitol at different concentrations

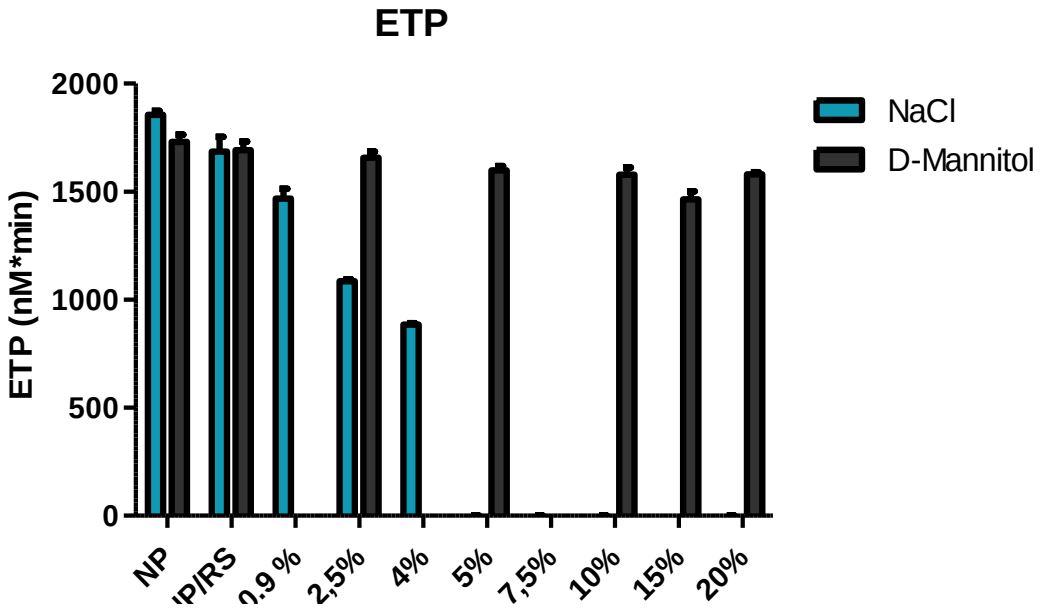

Figure 4-10 Comparison of thrombin ETP in intrinsic activation in NaCl and mannitol at different concentrations

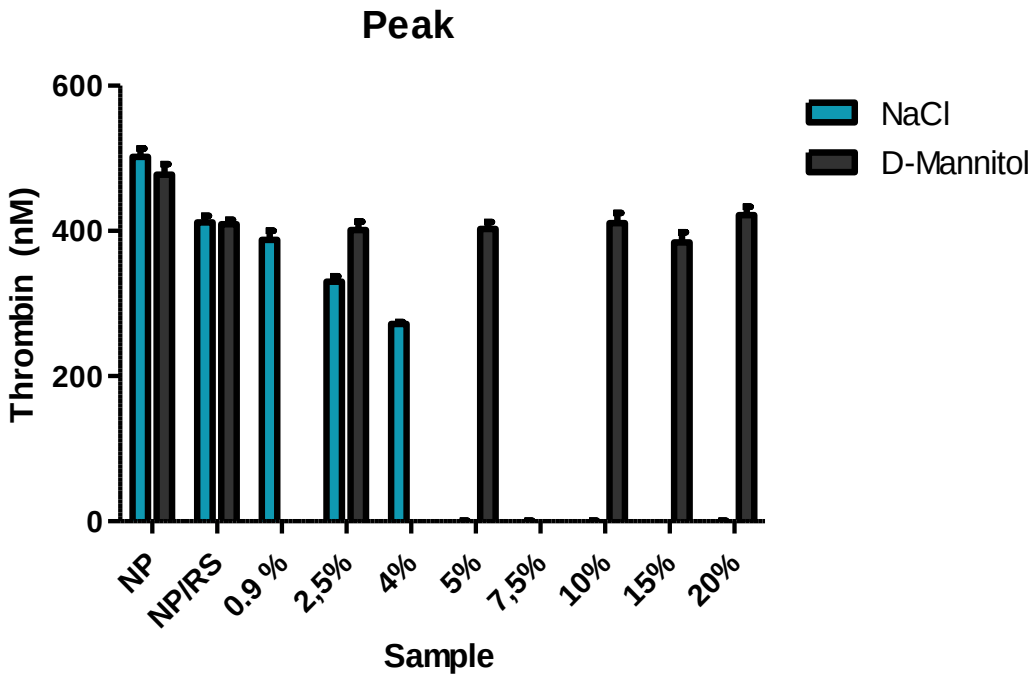

Figure 4-11 Comparison of peak thrombin levels in intrinsic activation with NaCl and mannitol at different concentrations

Tested samples are shown on x-axis. Plasma dilution was kept constant at 20%. Tests were carried out in triplicate; error bars are shown (standard deviation).

4.2.3. Effects of Mannitol on Extrinsic Activation with 1 pM TF.

Haemodilution with mannitol within the concentration range up to 20% (1000 mOsm/L) had minimal influence on thrombin generation parameters when 1 pM TF was used to trigger coagulation in the extrinsic coagulation (Figure 4-12).

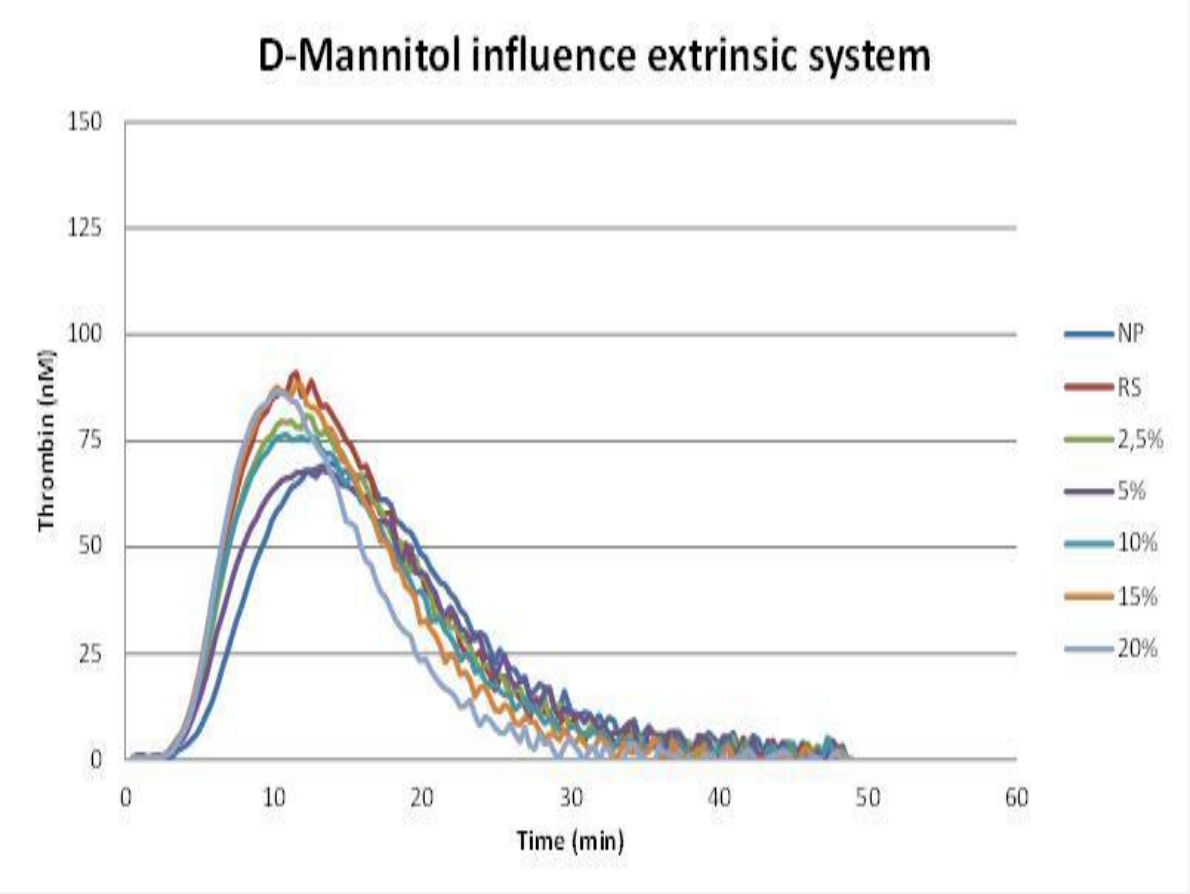

Figure 4-12 Effects of Mannitol concentration on extrinsic activation with 1 pM TF

NP-normal plasma, RS-Ringer's solution, mannitol concentrations (2.2%, 5%, 10%, 15%, 20%). Tested samples are shown on x-axis. Plasma dilution was kept constant at 20%. Tests were carried out in triplicate; error bars are shown (standard deviation).

4.2.4. Effects of NaCl Concentrations on Extrinsic Activation with 1 pM TF

When 1 pM TF was used to trigger plasma coagulation diluted with increasing concentration of NaCl, lag time changes were parabolic having minimum inflection point values between 2.5% to 4% NaCl (833 mOsm/L to 1333 mOsm/L). The corresponding ETP decreased with increase in NaCl concentration despite broadening of thrombin generation curves owing to longer time to termination. Overall thrombin generation was totally inhibited in 7.5% NaCl and higher concentrations in the extrinsic activation system with 1pM TF (Figure 4-13).

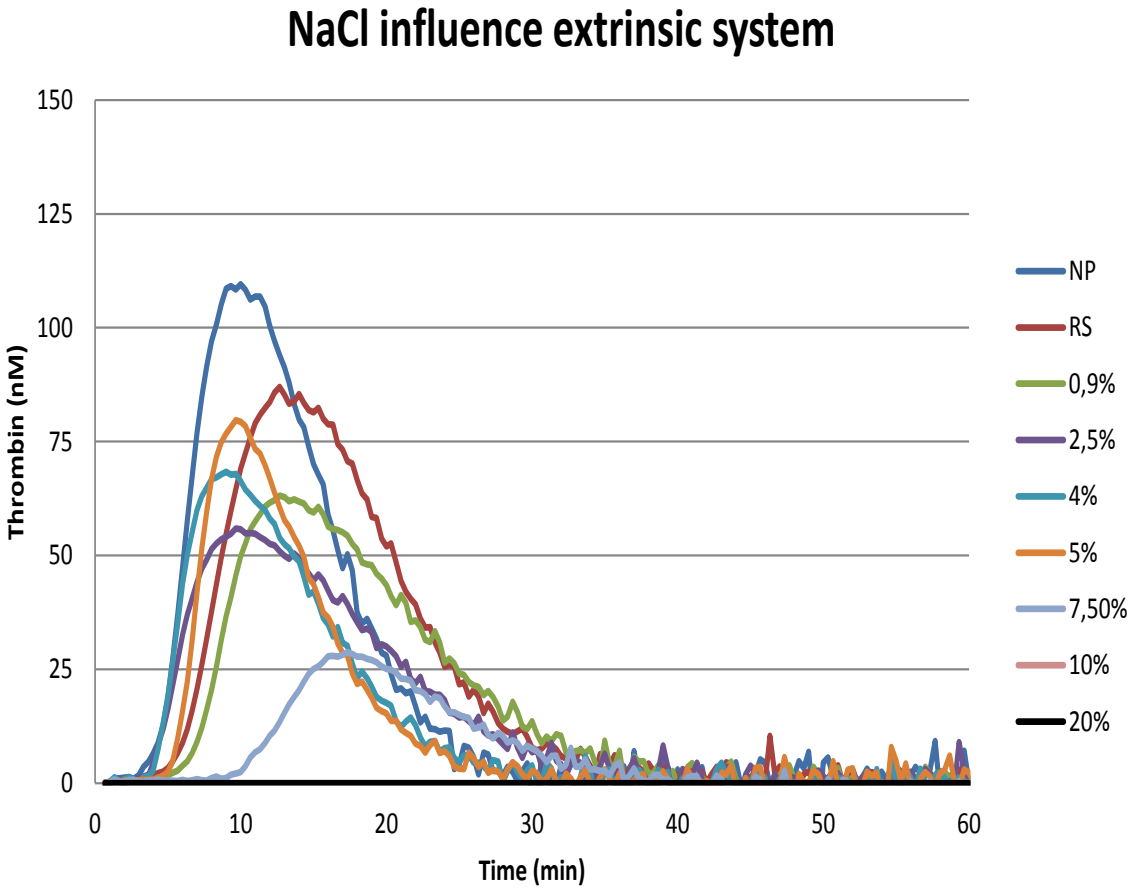

Figure 4-13 Influence of NaCl concentration on extrinsic activation with 1 pM TF
NP -normal plasma, RS-Ringer's solution and NaCl concentrations (0.9%, 2.5%, 4%, 5%, 7.5%, 10%, 20%).

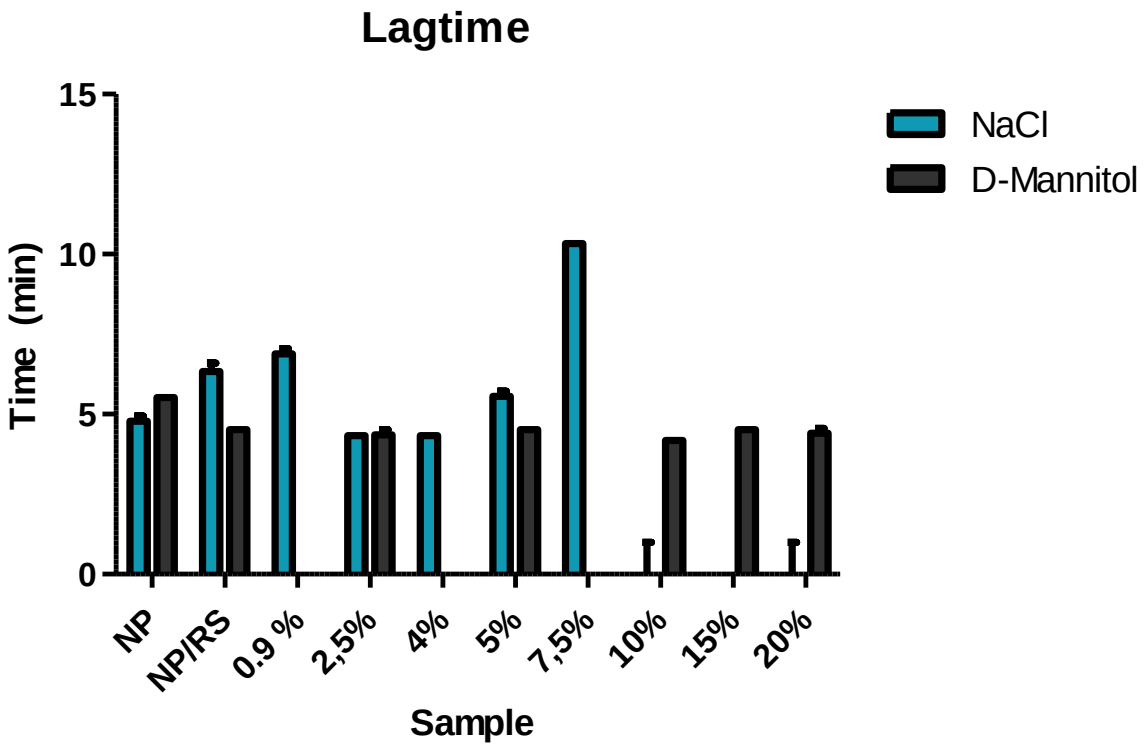

Figure 4-14 Comparison of thrombin lag time in different concentrations of NaCl and mannitol in extrinsic activation with 1 pM TF

Tested samples are shown on x-axis. Plasma dilution was kept constant at 20%. Tests were carried out in triplicate; error bars are shown (standard deviation).

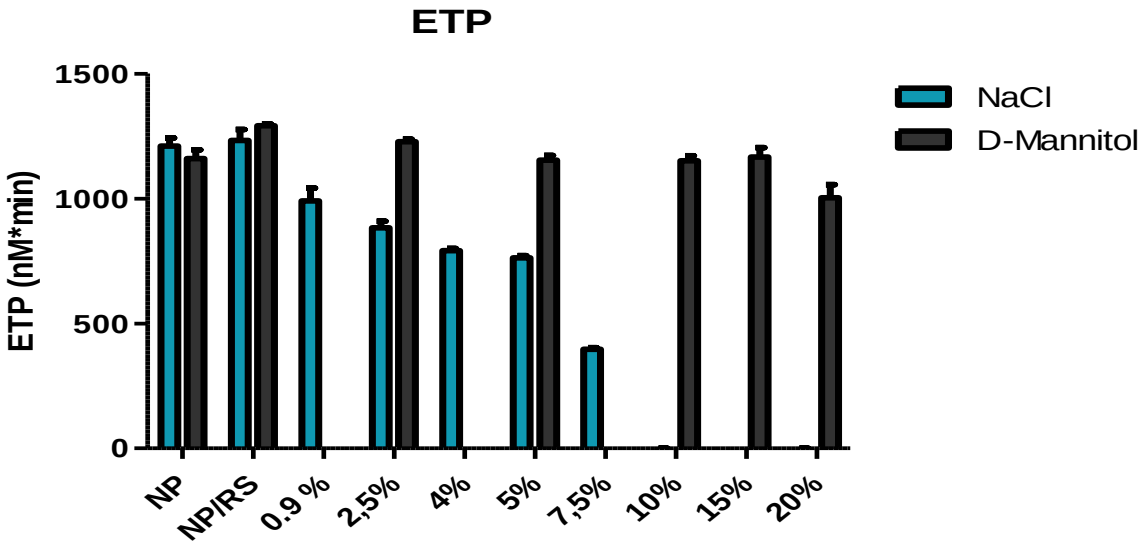

Figure 4-15 Comparison of ETP in extrinsic system activation with 1 pM TF in different concentrations of NaCl and mannitol

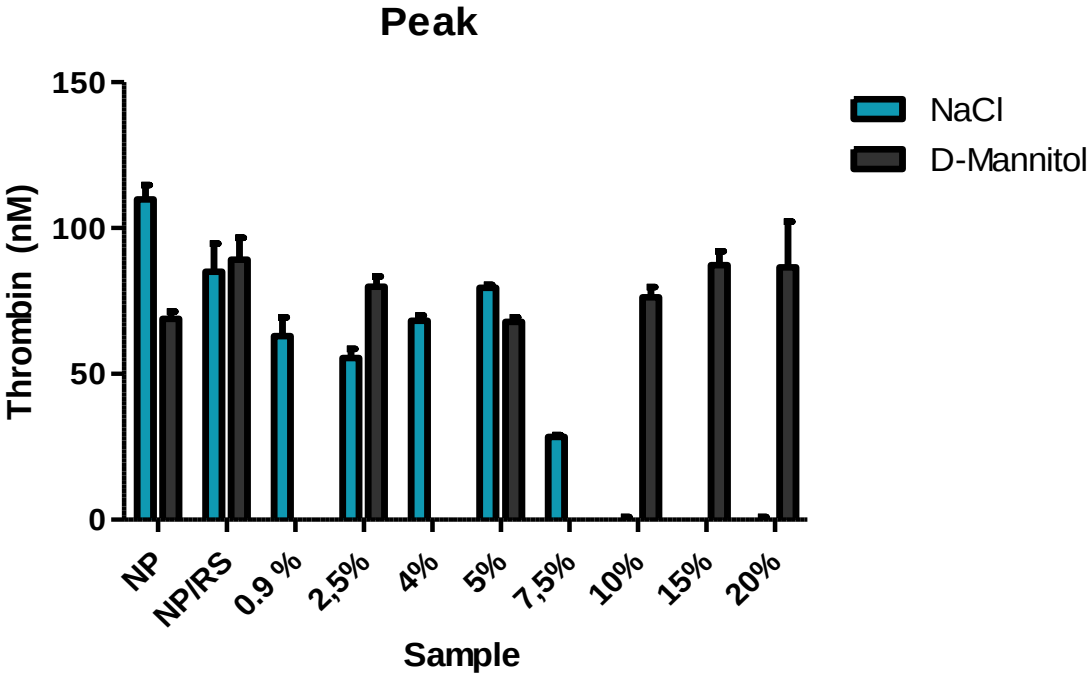

Figure 4-16 Comparison of peak thrombin in extrinsic activation with 1 pM TF in different concentrations of NaCl and mannitol

Again, in extrinsic TF activation comparison of Thrombin generation (TG) parameters showed lag time (Figure 4-14), ETP (Figure 4-15) and peak height (Figure 4-16) remained unchanged in mannitol unlike in NaCl solutions**.**

4.2.5 Effect of low TF Concentration (0.5 pM) Compared to Kaolin on TG Curves of Plasma Diluted with 5% NaCl

In plasma diluted with 5% NaCl, there were differences in extrinsic and intrinsic activation. In extrinsic system activation with 0.5 pMTF, TG lag time was indistinguishable from control, though slope was slightly steeper as well as peak levels (Figure 4-17). In the kaolin activated samples, 5% NaCl diluted samples showed no evidence of thrombin generation, in contrast to undiluted control (Figure 4-18). This clearly demonstrates that in plasma diluted with 5% NaCl, even small amounts of TF are able to trigger thrombin generation which was not seen with kaolin.

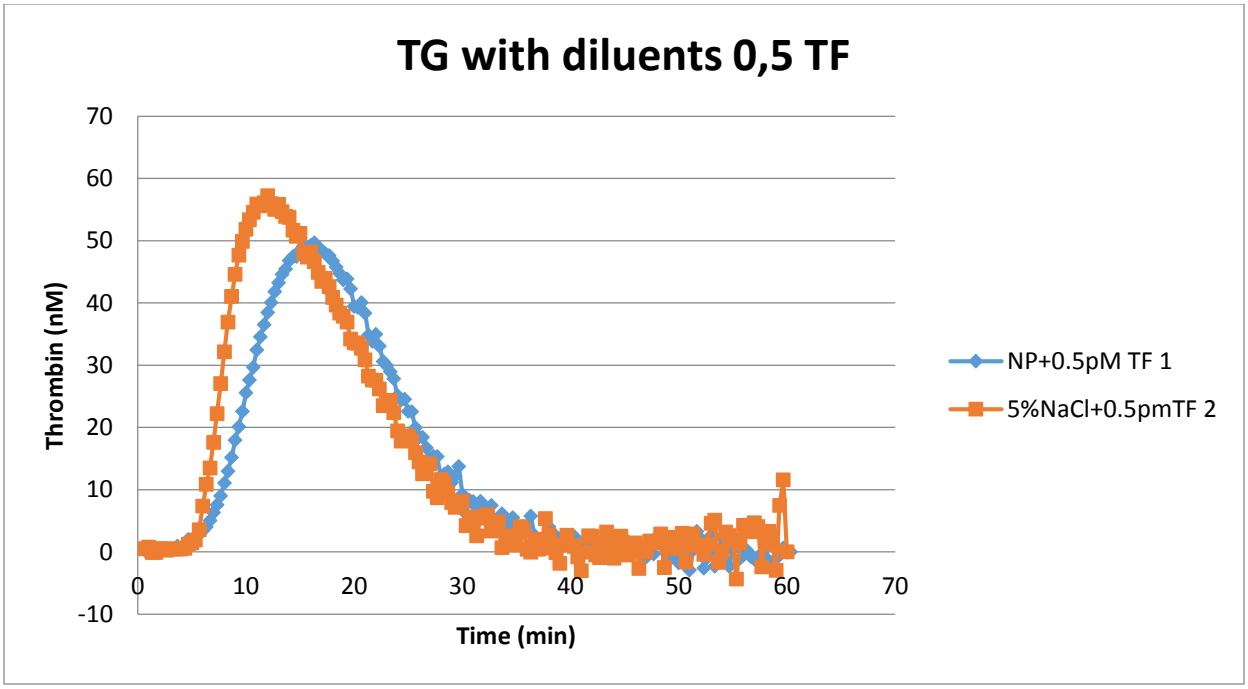

Figure 4-17 Effects of 0.5 pM TF on plasma diluted with 5% NaCl compared to undiluted control

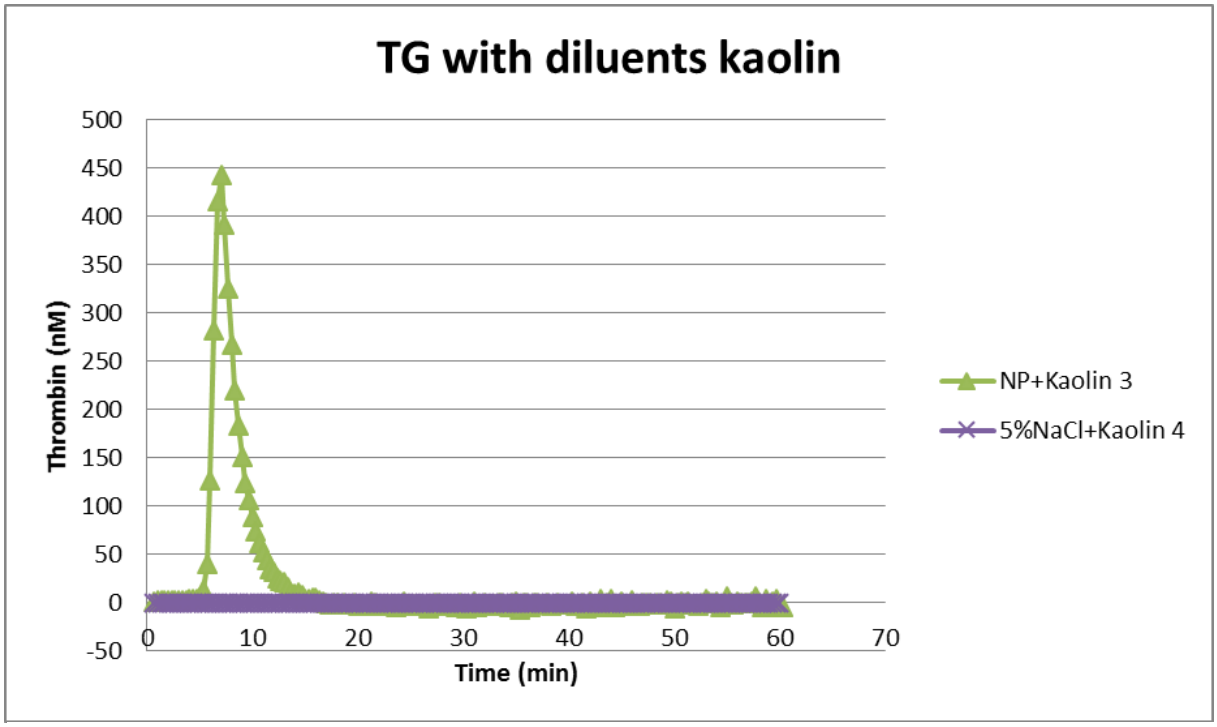

Figure 4-18 Effect of 5% NaCl on intrinsic coagulation activation with kaolin NaCl-sodium chloride, NP-normal plasma, pM-picomolar, TF-Tissue factor

When 1 pM TF was used as a trigger, lag time remained fairly similar except that thrombin generation peak levels were reduced with concomitant broadening of the TG curves (Figure 4-19). Figure 0-19 Effects of 1 pM TF activation on thrombin generation in plasma diluted with different concentrations of NaCl or 20% mannitol compared to undiluted control.

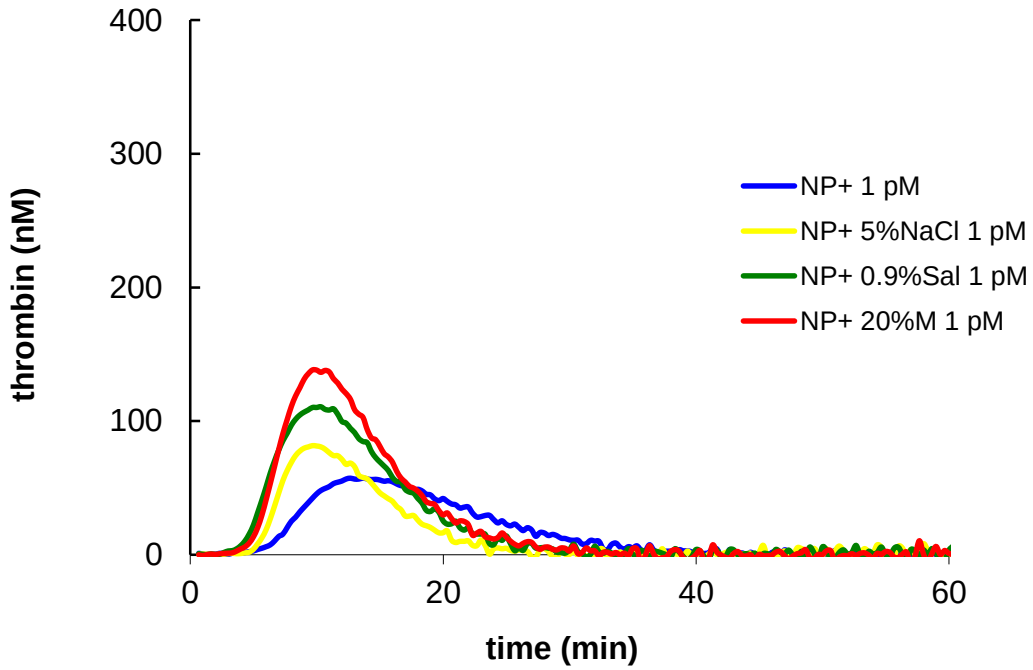

Figure 4-19 Effects of 1 pM TF activation on thrombin generation in plasma diluted with different concentrations of NaCl or 20% mannitol compared to undiluted control.

In plasma haemodilution with either 20% mannitol or 5% NaCl and triggered with 1pM TF, no differences in lag time were noticed. The undiluted control had a decreased slope and lower peak but slightly prolonged termination phase. In contrast, the propagation phases in 5% NaCl and mannitol were similar but steeper than undiluted control but TG peak was higher in 20% mannitol than 5% NaCl. The termination phases for 5% NaCl and 20% mannitol were parralel but less than undiluted control (Figure 4-19).

When 5pM TF was used to trigger extrinsic coagulation, TG curves in plasma haemodiluted with either 20% mannitol or 5% NaCl had similar lag time and propagation phases. However, peak thrombin was in the following order 20% mannitol>5% NaCl> undiluted control. Although peak thrombin in 5% NaCl was higher than undiluted control, its ETP was smaller owing to earlier termination phase (Figure 4-20).

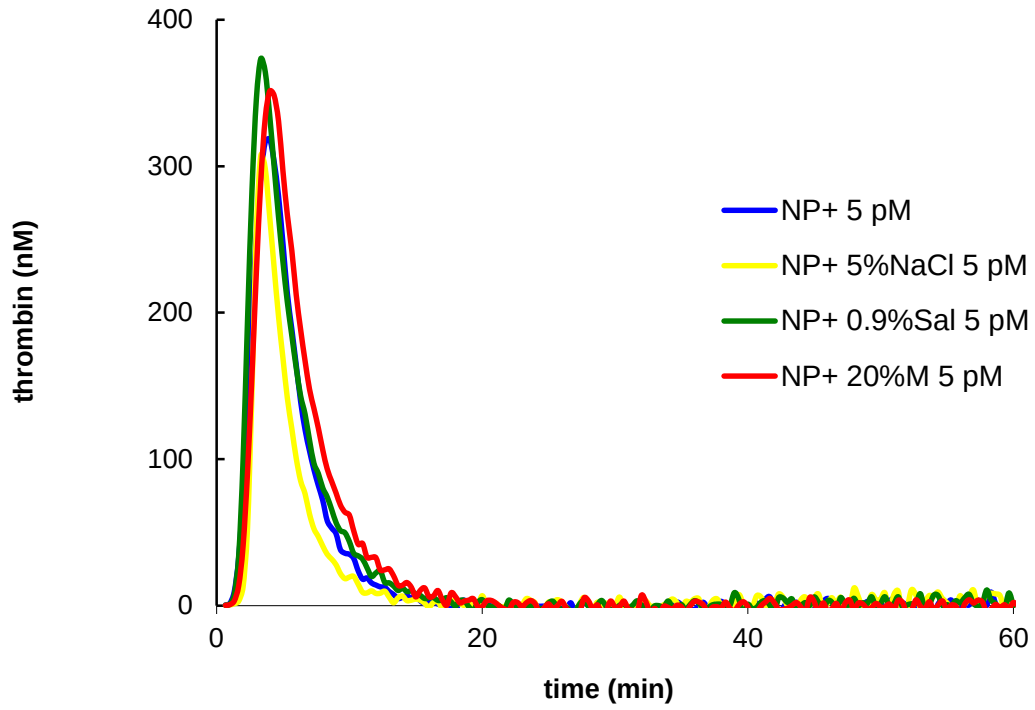

Figure 4-20: Effect of 5 pM TF on thrombin generation in plasma diluted with 5% NaCl, 0.9% NaCl or 20% mannitol compared to undiluted control

NP-normal plasma, M-mannitol, NaCl-sodium chloride, pM-picomolar, Sal-saline (0.9% NaCl)

When 1 pM TF concentration was compared to 5 pM TF, the effect of trigger concentration was quite evident. The TG curves for the different concentrations of crystalloid solutions were

qualitatively indistinguishable in 5 pMTF. With 1 pM TF, all the diluents produced greater thrombin than undiluted control, with variations. Although lag time were similar, the order of peak thrombin levels, from greatest to least were: 20% mannitol>0.9% NaCl> 5% NaCl> undiluted plasma. This demonstrates that without TF, differences in diluents concentrations are apparent. But as TF trigger concentrationwas increased, the differences became obliterated. This means that the inhibitory effects of NaCl concentrations on thrombin generation can be overcome by increasing TF suggesting their action are upstream in the coagulation cascade.

When the experiments were repeated in defibrinated plasma, using 1 pM (Figure 4-21) and 5 pM TF (Figure 4-22), the results were not different. This suggests that presence or absence of fibrinogen has no influence on inhibitory activity of NaCl concentrations. Although at 5pM TF 5%NaCl had a greater peak than undiluted control, its ETP was less than control undiluted, owing to earlier termination phase. This suggests enhanced synergistic inhibitory effects or destabilisation of thrombin dimer.

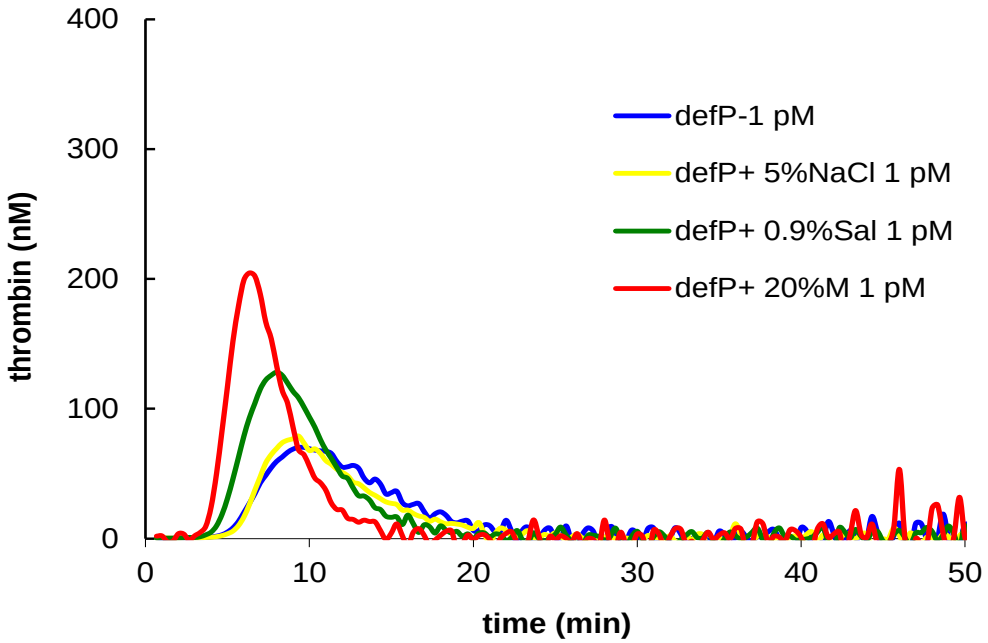

Figure 4-21 Effect of 1 pM TF on thrombin generation in defribinated plasma diluted with either 5% NaCl or 205% mannitol.

defP-defribrinated plasma, M-mannitol, NaCl-sodium chloride, pM-picomolar.

As TF trigger concentration was increased from 0.5 pM through 1pM to 5 pM, the most noticeable effect was shortening of lag time and increasing slope of thrombin generation during propagation phases. Defibrination did not alter TG curve changes produced by TF concentrations suggesting no role for fibrinogen on TG curves.

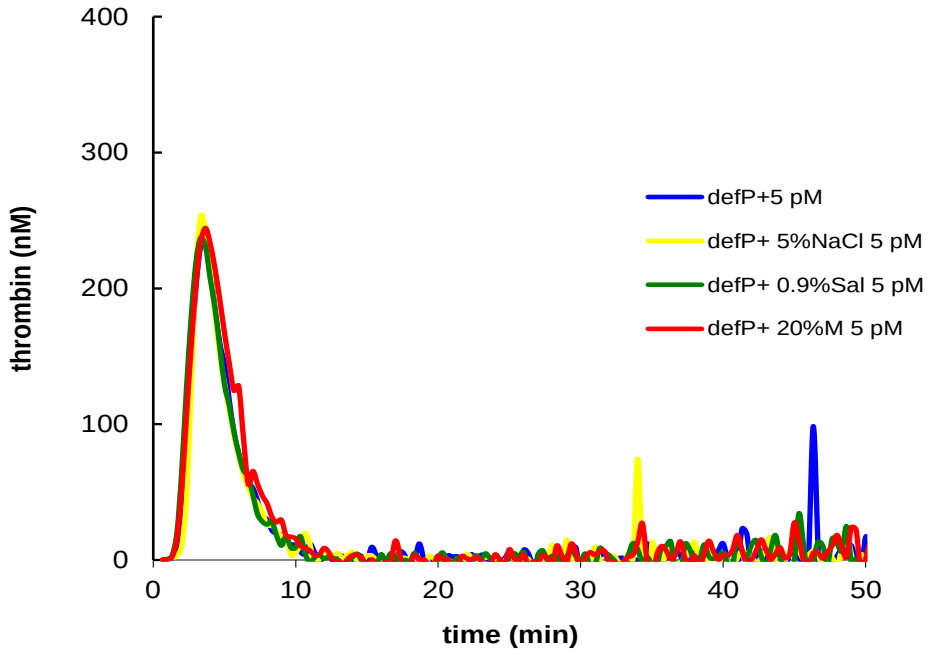

Figure 4-22 Effect of 5 pM TF on thrombin generation in defrinated plasma diluted with either 20% mannitol or 5% NaCl.

defP-defribrinated plasma, M-mannitol, NaCl-sodium chloride, pM-picomolar

In the previous study using routine coagulation tests (objective 1), it was found that increasing concentrations of ionic crystalloids prolonged time to plasma clot formation while neutral organic diluents did not. This is an effect attributed to ionic strength of ionic crystalloids. Since the mechanism of coagulation impairment was unclear, whether the inhibition was on thrombin or fibrin polymerization, this part was designed to test effects of diluent's ionic strength on thrombin generation. Solutions of NaCl and mannitol were used since they represent prototypical ionic versus neutral crystalloids in clinical use. Pooled normal plasma was diluted at 20% volume replacement with the diluents before being tested on fluorogenic CAT™ assay.

The findings in this study that increase in mannitol concentrations had no effects on the TG parameters either on intrinsic or extrinsic system can be explained by physicochemical properties of mannitol. Mannitol is a neutral, non-ionic organic molecule. The absence of charge on mannitol molecules precludes its interactions with charged protein coagulation factors hence lack of influence on TG parameters: lag time, propagation, peak, termination or ETP even when its concentration is increased. This is because, mannitol being a Kosmotrope stabilizes protein native structures in solution (Kunz, 2010), and therefore preserves enzymatic activities of coagulation factors, especially thrombin and FX, despite change in its concentration. This also explains observations obtained with mannitol on routine coagulation factors.

In contrast to mannitol, effects of NaCl were dependent on concentration, type of activator and amount of TF trigger (Figures 4-8, 4-13, 4-17, 4-19 and 4-20). The differences in TG pattern between mannitol (Figure 4-9, 4-10, and 4-11) and NaCl (4-14, 4-15, 4-16) demonstrates sensitivity of procoagulant factor activation on crystalloid ionic strength.

It is evident from this study that, in intrinsic activation, thrombin generation lag time increases with increasing NaCl concentration without affecting propagation phase, such that at 5% NaCl or higher concentration abolished thrombin generation. This pattern of thrombin generation is identical to one reported in the presence of ionic contrast media (Al Dieri, Béguin, & Hemker, 2003), an effect attributable to ionic strength. This pattern is also similar to thrombin generation in presence of decreasing FIX in synthetic stimulation experiments (van ’t Veer, Hackeng, Delahaye, Sixma, & Bouma, 1994)(Saulius Butenas, Orfeo, Gissel, Brummel, & Mann, 2004), in plasma (M. Ninivaggi et al., 2011). Identical TG patterns have also been observed with

decreasing concentrations of FIX on prothrombinase complex at low TF concentrations, situations mimicking decreased intrinsic activation (Béguin, Dol, & Hemker, 1991; Xi, Béguin, & Hemker, 1989b).

Generally, lag time in kaolin activation (intrinsic) takes longer than in the corresponding TF activation (extrinsic). This is due to slow rates of reactions in the assembly of intrinsic Tenase than in extrinsic system (Keularts, Zivelin, Seligsohn, Hemker, & Béguin, 2001; Louvain-Quintard, Bianchini, Calmel-Tareau, Tagzirt, & Bonniec, 2005; M. Ninivaggi et al., 2011; Wielders et al., 2004).

The the mechanism underlying progressive prolongation of lag phase resulting in delay in onset of propagation phase with each NaCl concentration in the intrinsic system could be attributed to interferences by ionic strength on kaolin activation in the upstream pathway of intrinsic reactants. Since Intrinsic Tenase (kaolin-FXII-FXI-FIX complex assembly) requires anionic membrane surface for optimal function, antagonistic effect of NaCl concentration can be considered as contributing to electrostatic repulsions. This has been confirmed in experiments demonstrating decreased FXII activation in presence of increasing ionic strength maintained by NaCl (Rosing, Tans, Govers-Riemslag, Zwaal, & Hemker, 1980; Rosing, Tans, & Griffin, 1985), also affecting rates of contact activation reactions (Tankersley, Alving, & Finlayson, 1983; Tankersley, Fournel, & Schroeder, 1980).

The findings in this study that show increasing concentration of NaCl but not mannitol progressively lengthens intrinsic TG lag phase is most likely an effect of ionic strength on FIX

and FX activation. This is supported by evidence from experiments in which activation rates and functions of FIX and FX were reduced with increasing ionic strength independent of salt type (McGee, Teuschler, & Liang, 1998, 1999). Furthermore, activation rates of FIX to FIXa and its interaction with FVIIIa has been found to decrease with increase in ionic strength contributed by NaCl (Lenting, Donath, van Mourik, & Mertens, 1994). Since FIX and FX enzyme substrate binding domains interact with negatively charged phospholipids via Ca++ bridges, increase in univalent anions abrogate the interactions and decrease activation efficiency. It may therefore be concluded that increasing NaCl concentration causes FIX inhibition impairing intrinsic tenase assembly and hence delay in intrinsic activation lag time. Therefore, TG patterns in intrinsic system demonstrating progressive prolongation of lag time without affecting propagation velocity (Figure4-8) can be interpreted in terms of inhibition of FIX and Kallikrein activation (Sugo, Kato, Iwanaga, Takada, & Sakakibara, 1985; Tankersley et al., 1983, 1980), but not FXII activation by kaolin (Kirby,1983). The putative mechanism underlying ionic strength inhibition of FIX is by acting on its Gla domain and inhibition of its dimerization that is essential for its activity (Simhadri et al., 2014).

It appears that increased ionic strength only delays initiation phase without interfering with propagation phases of thrombin generation (Figure 4-8). But it has been reported that once assembled, intrinsic tenase is 50 times more efficient than extrinsic tenase in generating FXa in the common pathway (S. Butenas & Mann, 2007; Kenneth G. Mann et al., 2003). Subsequently, FXa that arises from the tenase combines with FVa and forms prothrombinase that converts prothrombin into trace amounts of active thrombin in the initiation phase. The trace amounts of thrombin formed participates in positive feedback reactions leading to further activation of FV

and FVIII (Jesty & Beltrami, 2005) in the amplification phase that sets stage for thrombin burst (Kathleen E. Brummel et al., 2002).

Since the intrinsic activation propagation phase velocity was not affected in this study(Figure4-8), it may be concluded that, consistent with previous findings, increasing ionic strength neither interfere with positive feedback activation of cofactors FVIII or FV, nor binding of active forms of FVIIIa and FVa to their enzymes (Higgins & Mann, 1983). Moreover, this fact is reinforced by known facts that thrombin binding to its natural negative regulators Thrombomodulin (Baerga-Ortiz, Rezaie, & Komives, 2000; Dang et al., 1995; R. De Cristofaro, Picozzi, Morosetti, & Landolfi, 1996) and antithrombin (J. C. Fredenburgh et al., 1997; James C. Fredenburgh et al., 2004; Gopalakrishna & Rezaie, 2006), is decreased with increasing ionic strength of medium. These two facts explain the steep curve with kaolin activation at each NaCl concentration (Figure 4-8).

Caution must be exercised regarding interpretation of mechanism of delay in lag phase in intrinsic system in the presence of increasing NaCl concentration contributing ionic strength. Several studies (Béguin, 1987; Béguin et al., 1991; Béguin, Lindhout, & Hemker, 1988) have found that lag time increases partly due to inhibition of positive thrombin feedback activation of factors V, VIII, and X as demonstrated by amount of heparin anticoagulants added. It was later found that FVIIIa was the most important determinant of lag phase duration, as its addition to prothrombinase in the presence of phospholipids shortened onset of intrinsic activation (Béguin et al., 1991, 1988; Kumar et al., 1995; Lindhout, Govers-Riemslag, van de Waart, Hemker, & Rosing, 1982; Rosing et al., 1980). Therefore, lag time represents time necessary for trace

amounts of thrombin (<5% total thrombin) to activate FVIII to FVIIIa and FV to FVa. The thrombin burst (>95% total thrombin) is produced by thrombin initiated chain reaction during positive feedback mechanisms. Though ionic strength could delay thrombin activation of FVIII, but once activated FVIIIa activity is enhanced (Schmidt, Stewart, Mathur, Krishnaswamy, & Bajaj, 2005). However, others have found thrombin is more efficient at activating FV (Myles, Yun, Hall, & Leung, 2001; Segers et al., 2007), FVIII (Nogami et al., 2005), and FXI (Yun et al., 2003) in the presence of increasing ionic strength (Segers et al., 2007). These apparent contradictions must be viewed in light of their experimental set-up: simulation with purified factors and not in plasma.

Experimental evidence suggests that effects of NaCl concentration on α-thrombin may be minimal and therefore lengthening of lag time may arise from inhibition of prothrombinase rather than thrombin. This is born out of evidence from a study that activity of meizothrombin and thrombin that were derived from prothrombin were not affected by NaCl mediated increase in ionic strength. In contrast, full prothrombinase activity was inversely related to NaCl concentration when a different substrate was used to monitor activity (Tans et al., 1991). These data strongly reinforces the postulate that increase in ionic strength inhibits upstream factors-FXa, FIXa and FXIIa but not thrombin.

Further support for the claim that the effects on thrombin generation were upstream of coagulation factor but not on α-thrombin enzyme are derived from a study that investigated effects of group IA and IIA salts on α thrombin properties (Landis, Koehler, & Fenton, 1981). In the cited study, fibrinogen clotting time increased with ionic strength without affecting thrombin

esteratic activity monitored using a non-fibrinogen synthetic substrate. Also, there was no corresponding change on spectral absorption and spin resonance spectra indicating stability of thrombin. These facts indicate that increase in ionic strength has no gross disturbance on thrombin protein conformation and hence catalytic activity. Therefore, prolongation of lag phase and diminution of thrombin generation peaks without affecting propagation is independent of thrombin enzymic activity (Figure 4-8).

Effects observed with increasing NaCl concentration on TG in the extrinsic system activation were more complex. A distinctive characteristic difference of TG in two activation systems was that at each TF concentration, the lag phases did not vary much from one another (Figure 4-17, 4-19, and 4-20) unlike in intrinsic where NaCl concentration had a profound effect (4-8 and 4-18). This can be explained by the fact that changes in ionic strength have limited effect in TF-FVIIa binding kinetics (Krishnaswamy, 1992, 2013). Also, in an experiment comparing FXa activation at different ionic strengths, McGEE et al (1998) found that FXa generation was one and a half times faster in extrinsic than in intrinsic systems. Since lag time depends on amount of trigger, it may be concluded that ionic strength, as contributed by NaCl has minimal role on extrinsic activation, a position supported by data in this study.

Two observations can be derived from the TG curves obtained with different TF concentrations on haemodilution with NaCl: (1) at 1 pM TF, effects produced by NaCl on TG curves were biphasic (Figure 4-13&4-19), and (2) when TF was increased from 1 pM to 5pM, lag time became shorter and differences between individual curves were indistinguishable (Figure 4-19& 4-20). At a given TF concentration, each increment of NaCl concentrationfrom 0.9% upto 4%,

there was progressive steepening but narrowing of TG curves despite unchanged lag time and peak height. However, from 5% NaCl and higher concentrations, TG curves started having longer lag phase with shorter termination phase. With 7.5% NaCl TG curves resembled those obtained in the presence of inhibitors –longer lag phase, decreased propagation phase slope and broadened curve (De Smedt, Wagenvoord, & Coen Hemker, 2009b). This phenomenon could be due to either faster inactivation of thrombin or to reduced formation, or both. Since effects of NaCl concentrations were more pronounced in narrowing the TG curves at each TF trigger concentration, it can be deduced that this is by suppressing intrinsic Tenase in the prothrombinase complex. The concentration that does this was from 5% NaCl with complete annihilation at 7.5% and higher. Therefore, the current result demonstrating increasing ionic strength causing narrowing of TG curves or early termination suggests that the mechanism of inhibition of thrombin generation is by suppressing second wave of prothrombinase contributed by late onset intrinsic activation (De Smedt et al., 2009b).

It is well known that in the extrinsic system activation of thrombin, the duration of initiation phase is dependent on the balance between TF and TFPI concentrations (Butenas, 2001). Thus while high TF causes brisk onset or shortening of lag phase, increase in TFPI causes prolongation of lag time duration (Hockin, Jones, Everse, & Mann, 2002). Therefore, shortening of TG lag time as TF concentration was increased from 1 pM to 5 pM was most likely due to TF overcoming the inhibitory effects of TFPI.

Build up of FXa in intrinsic activation occurs much later than in extrinsic system (S. Butenas & Mann, 2007). Therefore, activation with low concentration TF produces initially brisk but

reduced FXa over time, which is predominantly intrinsic. But as TF-FVIIa gets neutralized by TFPI, followed by a later but more sustained FXa through FIXa in the Josso_Loop that produces a second wave of prothrombinase (Hockin et al., 2002; K. G. Mann et al., 2003; Kenneth G. Mann et al., 2006, 2003) responsible for broadening of TG curves. But once trace amounts of thrombin is formed, its participation in explosive positive feedback generation during propagation phase is un-hindered. This is responsible for the biphasic curves mentioned above.

At low TF concentration TG were broader but were progressively narrowed with increased TF concentration (Figure 4-13, 44-17, 4-19, and 4-20). This is because as TF trigger concentration is increased from 1pM to 5 pM, TG generation changes from intrinsic to predominantly extrinsic. In Low TF concentration (1 pM) thrombin generation is mostly by Josso-loop, where TF-FVII directly trigger initial transient trace amounts of prothrombinase followed later by indirect activation of FIXa-FVIIIa producing a sustained second wave of prothrombinase (De Smedt et al., 2009b; Hockin et al., 2002; K. G. Mann et al., 2003, 1988; Kenneth G. Mann, 2003).

With 5 pM TF (Figure 4-20), TG curves had a shorter lag time, higher peak as well as earlier termination, patterns which were indistinguishable from one another (undiluted, 20% mannitol, 0.9% NaCl and 5% NaCl). At this trigger concentration TG generation is mainly extrinsic with little contribution from Josso-Loop (De Smedt et al., 2009b). This therefore explains narrowing of TG curves owing to absence of second wave of prothrombinase. So, with high TF concentration, reaction rates are much faster and TG is predominantly extrinsic.

It is quite evident from this study, which is in agreement with other studies, that the pattern of thrombin generation is dependent on amount and type of trigger used as well as crystalloid ionic strength. It is generally considered that TF is the 'physiological' coagulation initiator. However, this must be considered with a lot of caution since there is no "physiological" concentration of tissue factor. This is because, *in vivo*, it is not a soluble reactant but a component of membranes that are large compared to other molecules. Reaction velocities are therefore determined by diffusion rather than by chemical interaction (H. Coenraad Hemker et al., 2006b).

Apart from influence on termination phase, increment in NaCl concentration upto 5% was accompanied by steepening of propagation phase at 1 pM (Figure 4-13, 4-19 and 4-21). Analysis reveals that the sigmoid TG curve twists to the left. This reflects enhanced thrombin activity arising from being either unchained from negative propagation phase regulators (Baerga-Ortiz et al., 2000; R. De Cristofaro et al., 1996; J. C. Fredenburgh et al., 1997; Segers et al., 2007) favouring positive feedback reactions and/or allosteric potentiation (Dang et al., 1995; Orthner & Kosow, 1980; Wells & Di Cera, 1992a).

The explanation for enhanced thrombin activity (steeper propagation phase slope) in the presence of increasing ionic strength (upto 4% NaCl) arises from three mechanisms, all interrelated. In the first place, high concentrations of NaCl provides Na+ ions which then occupies Na+ binding sites on thrombin (Enrico Di Cera, 2003; Huntington, 2008; Pineda et al., 2004). Once bound, Na+ initiates allosteric modification on thrombin that increases activity of its active site for fluorogenic substrates, as well as enhanced exosite 2 favouring activation of FV and FVIII to FVa and FVIIIa respectively (Segers et al., 2007). At the same time, the ionic strength induced

structural modification on thrombin decreasing its binding to negative allosteric inhibitors: Thrombomodulin (Baerga-Ortiz et al., 2000) and antithrombin (J. C. Fredenburgh et al., 1997). This allows unopposed thrombin amydolytic activity as evidenced by enhanced cleavage of synthetic substrate.

Thrombin is an allosteric enzyme with several binding sites influencing its activity. Its activity depends on salt concentration of the medium. Orthner and Kosow (Orthner & Kosow, 1980) first brought to our attention that amidolytic activity of thrombin was enhanced in the presence of monovalent cations, especially Na+ containing salts. This was determined in purified α-thrombin activity on small peptides substrates. They further demonstrated enhanced activity on physiological substrate fibrinogen. The same results were replicated by Wells (Wells & Di Cera, 1992a). In their experiments, fibrinogen clotting time in the presence of increasing cations concentration was related in u-shaped pattern passing through a minimum value, then increasing with ionic strength. For Na+ containing diluents, clotting time was least in 5.0 mMol; while in Choline chloride it was least in 10 mMol. However in 100 mMol of either diluents in their study, clotting time was 2.3 fold increased in NaCl in contrast to Choline chloride where it increased 7.6 fold. This significant large difference in clotting time despite similar ionic strength suggests positive allosterism of thrombin activity in presence of sodium.

In other experiments, it has been shown that activity of α-thrombin is increased in the presence of NaCl as opposed to other diluents as evidenced by increase in release of fibrin peptide A and B from fibrinogen (Bah et al., 2006; Dang et al., 1995; Pineda et al., 2004; Shainoff et al., 2002; Vindigni & Di Cera, 1996). Molecular binding sites for Na+ on thrombin have been identified

(Bah et al., 2006; Pineda et al., 2004). This explains the previous observations of salt dependence activity of thrombin allosterism. Thus thrombin activity is slow and aniticoagulant at low Na+ concentration, and fast and procoagulant towards or around 200 mMol Na+ (Vindigni & Di Cera, 1996).

Although binding of Na+ to FX and FIX has been demonstrated, catalytic activities of both intrinsic and extrinsic as well as prothrombinase for their physiological substrates appear independent of it. It appears Na+ is necessary for initial activation for FVa, FVIIIa and FXIa preceding thrombin burst, during transition from initiation to propagation phases of TG (Kenneth G. Mann et al., 2003; Page & Di Cera, 2006).

Identification of sodium allosteric effects on thrombin different from clot inhibiting effects has been made possible when other synthetic substrates are substituted for fibrinogen as markers of activity. Reduction in fibrinogen clotting with increase in ionic strength maintained by NaCl has been a recurring theme (Landis et al., 1981). This is due to the fact that although thrombin activity in releasing fibrinopeptides may be enhanced, subsequent biophysical fibrin polymerization is independent of thrombin but is sensitive to negatively charged anions, particularly chloride (E. Di Stasio et al., 1998). Therefore, experiments that use fibrinogen clotting as an indicator for thrombin activity in the presence of increasing NaCl concentrations are fraught with limitation of accuracy, since thrombin release of fibrinopeptides may still occur but may not proceed to eventual polymerization. These facts explain the discrepancy between the results derived from routine clotting tests compared to thrombin generation in this study.

In a recent observational study which sought to document complications associated with infusion of hypertonic saline, it was reported that the rates of hypernatremia (Na+>155 mMol) were three times, while DVT rates were one and half times higher in the group that received 3% saline compared to normal saline (Froelich, Ni, Wess, Ougorets, & Härtl, 2009). Additionally, rates of DVT were twice likely in those patients with hypernatremia than those with normal plasma Na+ levels. These clinical observations corroborate findings of thromboses in association with hypernatremia described in some case reports (Kamijo, Soma, Hamanaka, Nagai, & Kurihara, 2003; Vikrant, Pandey, Raina, & Sharma, 2007). Although these were observational and were not powered to detect an association between plasma Na+ and coagulation outcome, nonetheless, procoagulant role of Na+ is highly suggestive.

Specific effects of chloride on thrombin activity have not been succinctly described. However, decrease in activity after 4% NaCl suggests threshold inactivation by increase in ionic strength, most likely an effect of Cl- ions. Generally, increases in chaotropicity of Hofmeister salt series have been associated with reduction in other enzyme activity (Cacace, Landau, & Ramsden, 1997). This is by destabilizing the native enzyme protein conformation. Additionally, the concentration-dependent 'bell shaped' effects of NaCl on TG parameters can be explained on the basis of competition between electro selectivity of ions of the Hofmeister series on protein interactions (Kunz, 2010, Chapter 1)(Zoldák, Sprinzl, & Sedlák, 2004). The reduced or abolishment of thrombin generation at higher NaCl (>5% NaCl) may be ascribed to ionic strength effects in destabilising thrombin protein dimer conformation essential for its activity (Simhadri et al., 2014), most likely an effect of chloride ions (E. Di Stasio et al., 1998).

4.3 Effects of Crystalloid Solutions on Coagulation as Assessed by TEG

This section determined whether inclusion of whole blood, instead of the usual plasma, in coagulation tests would yield additional information not evident from plasma based assays. The data shows non normal distribution, and therefore median was the measure of central tendency.

4.3.1 Effects of NaCl on TEG

This part of experiments sought to investigate whether the observed effects of increase in saline on whole blood coagulation was limited to general concentration or due to influence of ionic strength. The effects of increasing NaCl concentration on TEG parameters shows that changes in TEG parameters are parabolic with an axis around 0.3M (600 mOsm) (Figure 4-23, 4-25and 4-26). These are in contrast to linear relationship obtained with routine coagulation tests on plasma (Figure 4-1 to 4-4).

A scatter plot of NaCl concentration against R values was parabolic, inflection point around 0.3 M NaCl (Figure 4-23). From 0.7 M NaCl concentrations and above, there were wide variations in R values, although the mechanisms are not clear. In fact in most TEG traces above 1M NaCl, it was difficult to differentiate the R values from a flat trace since the machine only gives a value if the amplitude is 2mm or more. The R values at higher concentrations may be considered unreliable. All in all, the traces shows that the clotting time was modulated by the blood cells in the specimens, reflecting different haematocrits, platelet counts or the leukocytes interacting with ions in solution.

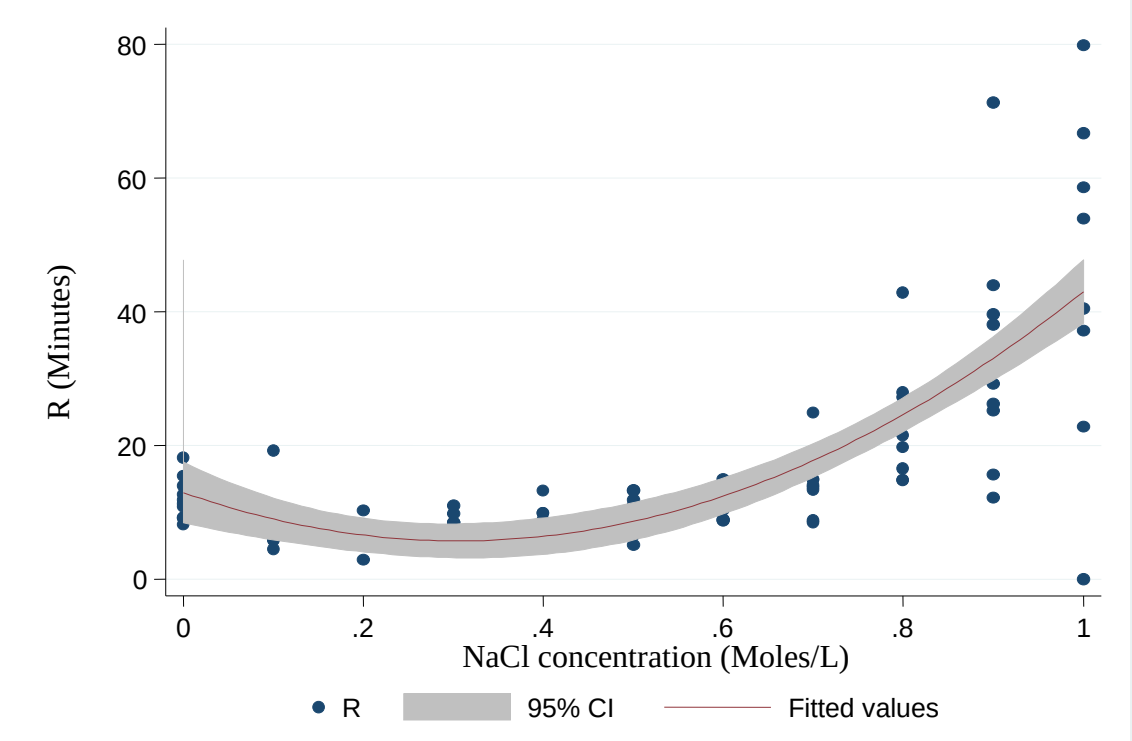

Figure 4-23 Effect of NaCl concentration on TEG R

R values for citrate anticoagulated whole blood diluted (20% v/v) with different concentrations of NaCl. Data scatter plots for raw values, and best fit regression line ($p<0.005$). Y-axis R in minutes, and X-axis concentration in Mols/L. Point of inflection for R was 0.3 Mols/L

The scatter plot of K-values showed positive linear relationship with increase in NaCl concentrations. However, there were wide variations at concentrations from 0.7m NaCl, maybe giving the impression of linear instead of curvilinear relationship (Figure 4-24). It is of note that K-values represent time elapsed from 2 mm to 20 mm. It is possible that, since higher concentrations were associated with hypocoagulability may account for this linear pattern.

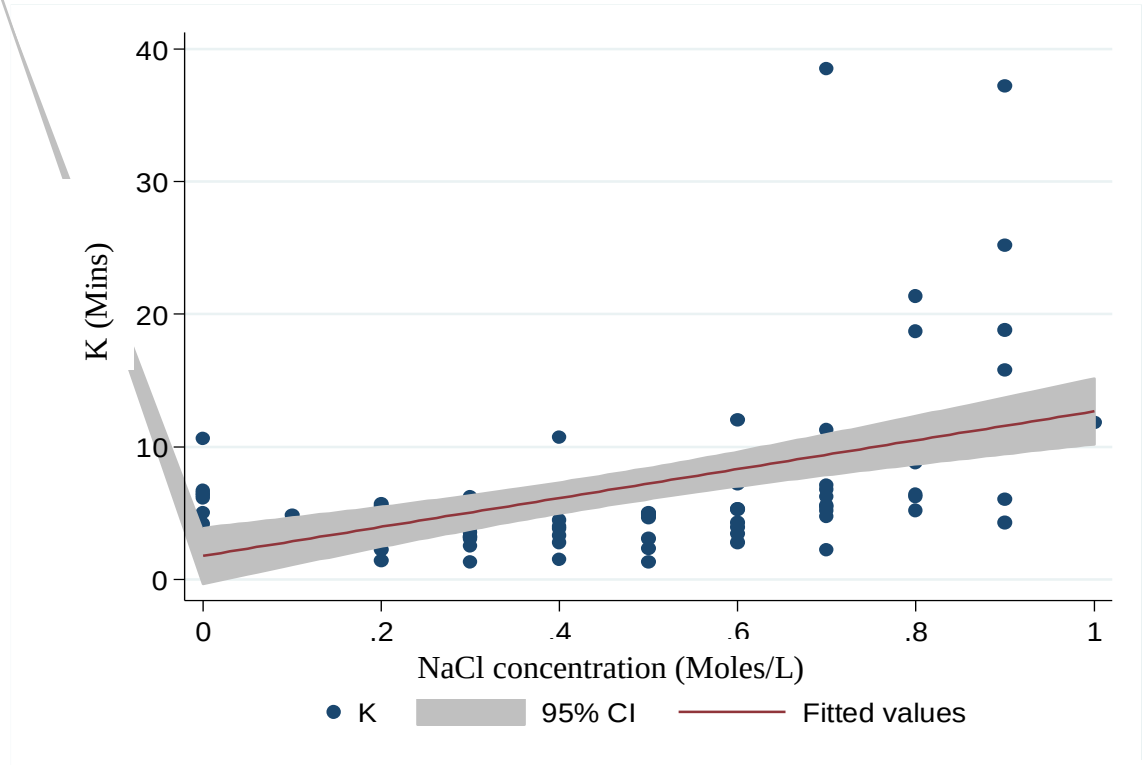

Figure 4-24 Effect of NaCl concentration on TEG K values

Scatter plot for K values in minutes (Y-axis) against NaCl concentration (X-axis) in Moles/L. Citrated whole blood was diluted with different concentrations of NaCl.

The relationship between TEG α-angle values and NaCl concentrations were curvilinear with a plateau inflection point around 0.3 M. NaCl concentrations above 1 M were associated with flat traces consistent with profound hypocoagulability (Figure 4-25). This reflects reduced fibrin polymerization build up, progressive inhibition of platelet aggregation or fibrin-platelet interactions. The scatter for α-values at high NaCl concentrations did not have wide variations unlike in contrast to K-values (Figure 4-24).

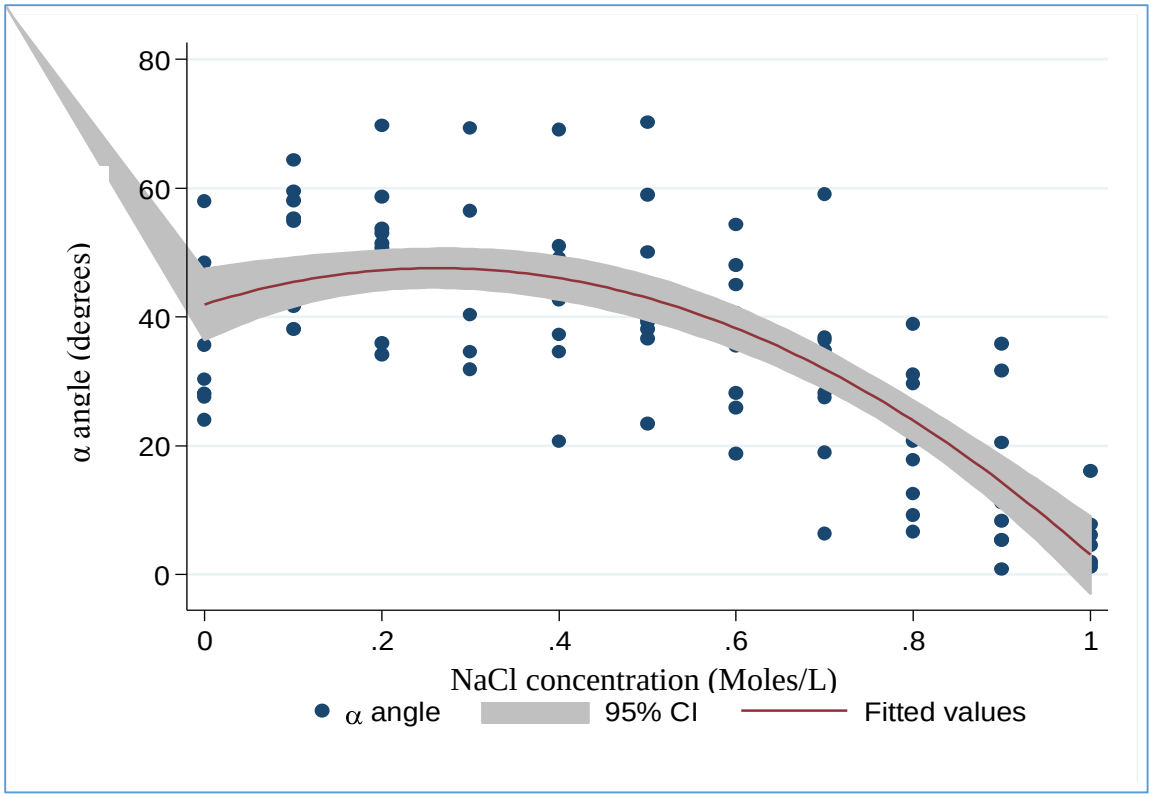

Figure 4-25 Effect of NaCl concentration on TEG alpha angle

Scatter plot for α-angle in degrees (Y-axis) against NaCl concentration in Moles (x-axis). Citrated whole blood was diluted with different concentrations of NaCl.

The clot strength, as indicated by the Maximum amplitude (MA) progressively decreased with increase in NaCl concentrations (Figure 4-26). This was qualitatively similar to changes in α-angle (Figure 4-25), with an inflection point around 0.3 M (Figure 4-26). Since MA is indicative of platelet functions, this indicates that there was progressive reduction of platelet functions with increase in NaCl concentrations. The changes were significant, best fit regression line ($P<0.005$).

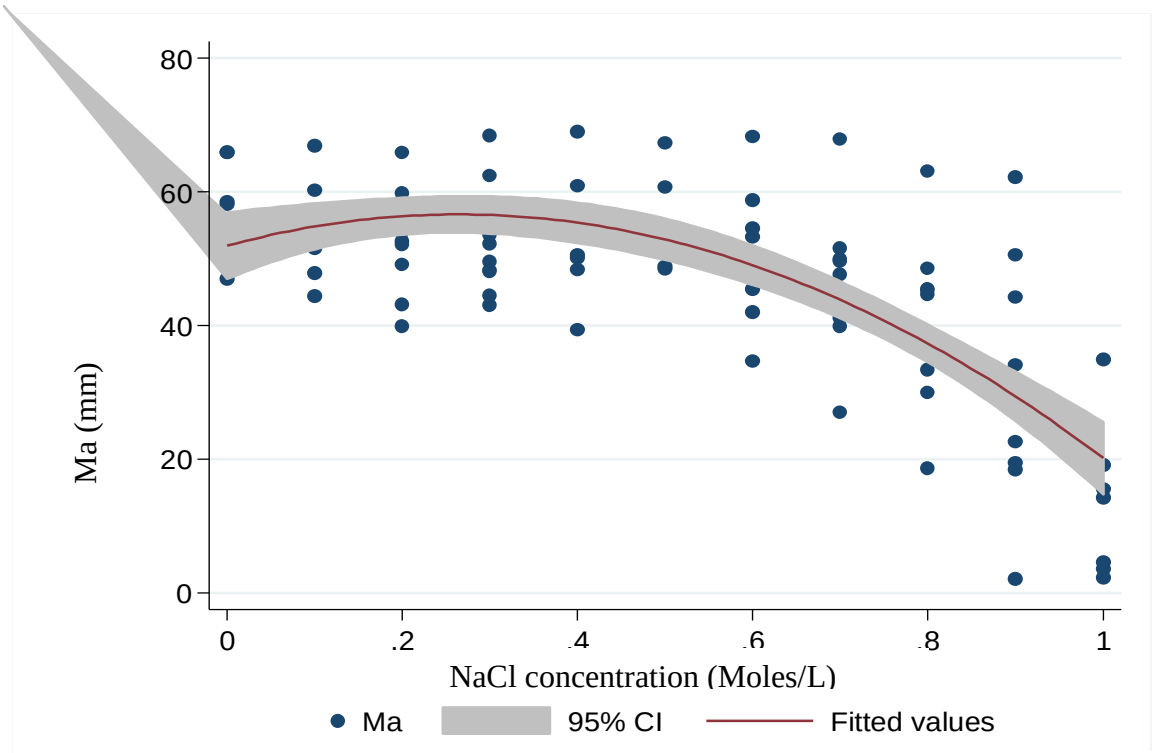

Figure 4-26 Effect of NaCl concentration on TEG Maximum amplitude

Citrate anticoagulate whole blood was diluted with different concentrations of NaCl. Scatter plots of Ma in mm (y-axis) against NaCl concentration (x-axis) in Mols

4.2.2 Effects of Other Ionic Crystalloid Solutions on Citrate Anticoagulated Whole Blood Coagulation as Assessed by TEG

The pattern of relationship between other ionic crystalloids and TEG parameters was parabolic and were qualitatively similar. From 900 mOsm/L crystalloid concentrations, there was divergence in sensitivity in coagulation impairment in the order: choline chloride>NaCl>sodium gluconate. The changes were significant ($p<<0.01$) (Figure 4-27).

The median R value for undiluted control was 11.8 minutes (IQR10.5, 13.5). The trend of R values for each increase in crystalloid solution concentration was significant ($p<0.005$).

Although the difference between the various types of crystalloids at each concentration was not significant (P=0.84) this was explicit at lower concentrations of 300 mOsm to 600 mOsm. The R values in 1200 mOsm to 1800 mOsm increased exponentially with crystalloid concentration. At these high ionic crystalloid concentrations, the difference between Choline chloride and sodium gluconate were significant (P<00.1). Overall, at each NaCl concentration the pattern was Choline chloride>NaCl>sodium chloride (Figure 4-27).

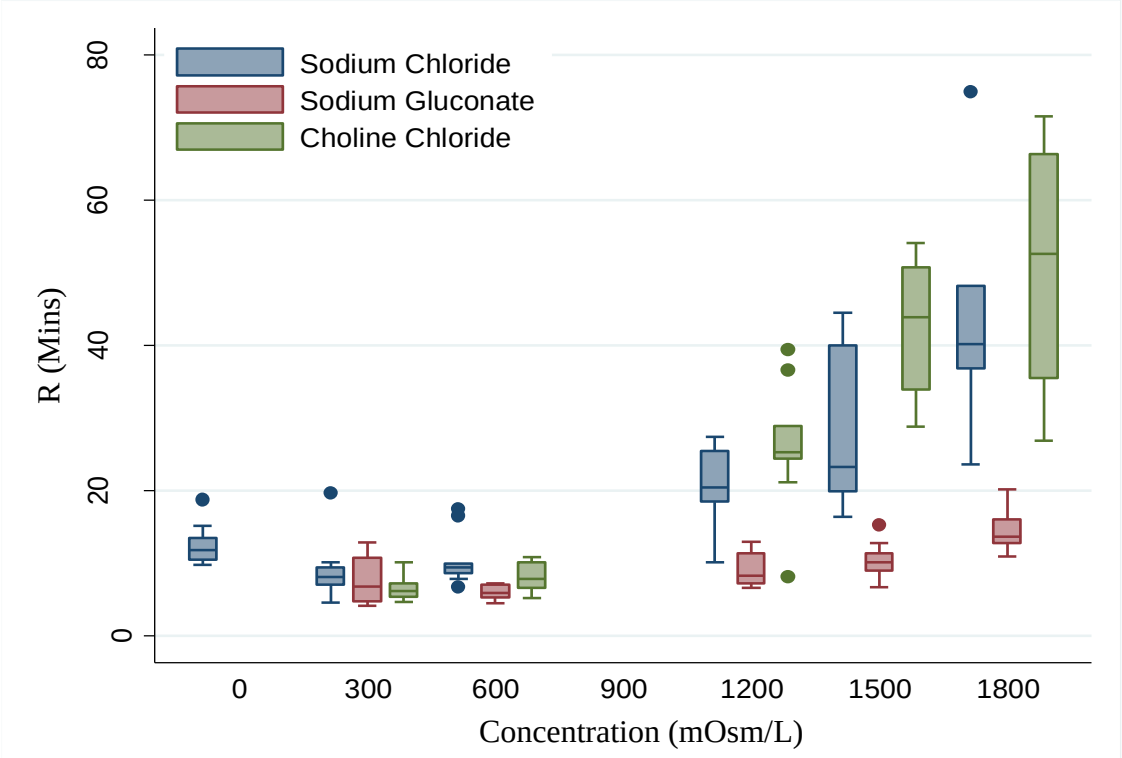

Figure 4-27 Effect of different concentrations of different crystalloid fluid types on TEG R values.
Citrate anticoagulated whole blood was diluted with crystalloid solutions (20%v/v). Box plot with whiskers of median R is in minutes; concentration in mOsm/L; * represent outliers.

The median K value for undiluted control was 5.9 minutes (IQR4.1,6.5). The trend of change in K values for each crystalloid solution with each concentration was significant (p<0.005). However, the difference between the various crystalloid solutions at each concentration was not significant (Figure 4-28).

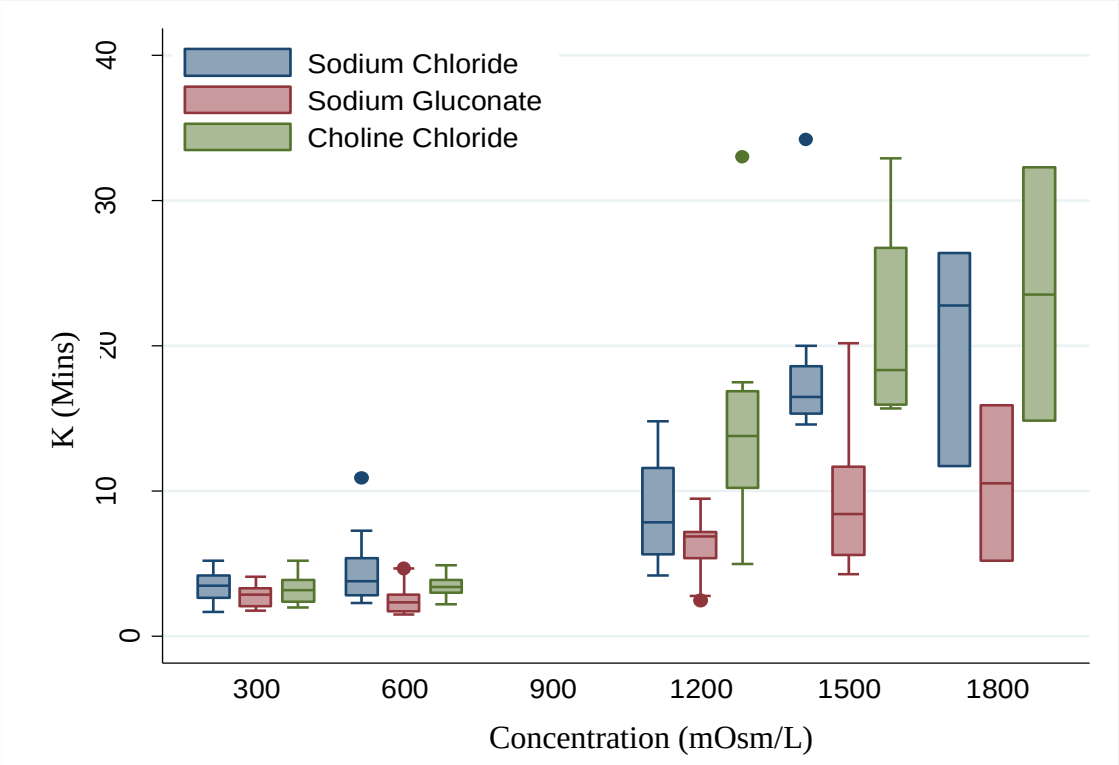

Figure 4-28: K values of different fluid types at different concentrations.

Citrate anticoagulated whole blood was diluted with crystalloid solutions (20%v/v). Box plot of K values in minutes, concentration in mOsm/L, * outliers. (N=11).

The median α-angle for undiluted control was 31.05 degrees (IQR 24.7,41.4; N=11). Increase in crystalloid solutions significantly decreased α-angle ($p<0.001$), although there was no differences between solutions at each concentrations (Figure 4-29).

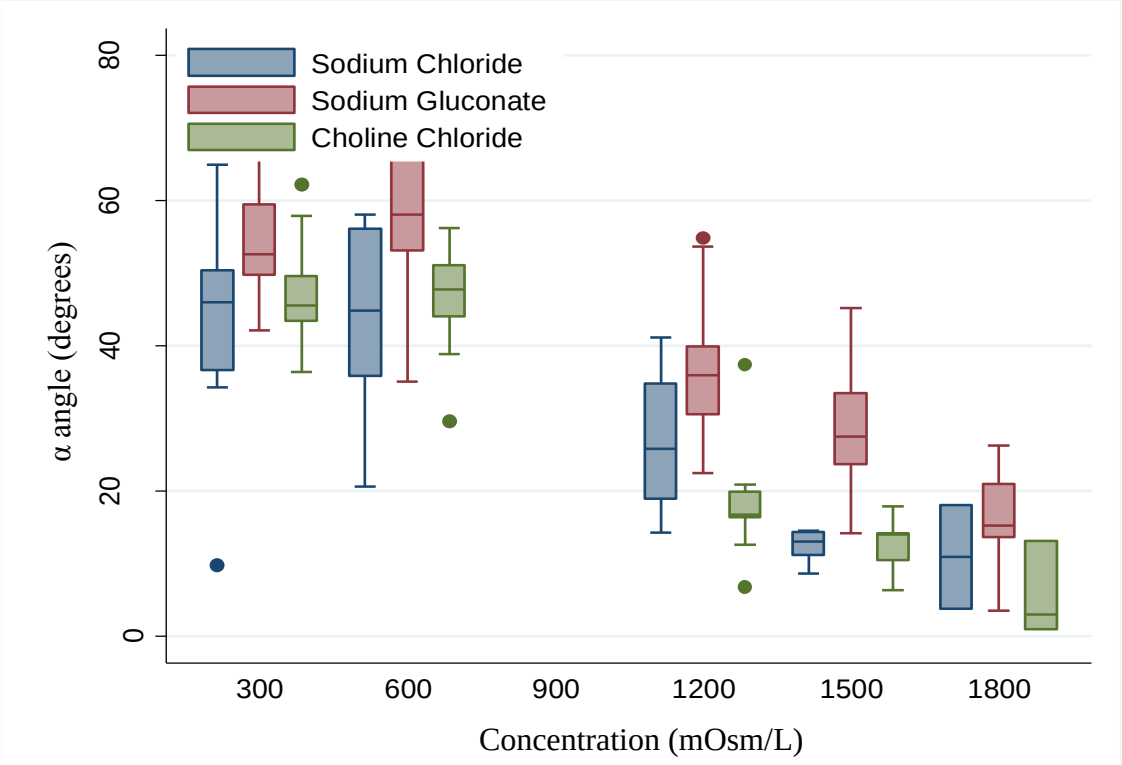

Figure 4-29: Effects of different fluid types at different concentrations on α-angle (degrees).

Citrate anticoagulated whole blood was diluted with crystalloid solutions (20%v/v). Box plot of α-angle in degrees, concentration in mOsm/L, * outliers (N=11)

The median Ma for the undiluted control was 53.55 mm (IQR50.2-57.2; N=11). The Ma decreased with increase in crystalloid solution concentration ($p<0.002$). There was no statistical difference between the different crystalloid solutions at each concentration (Figure 4-30).

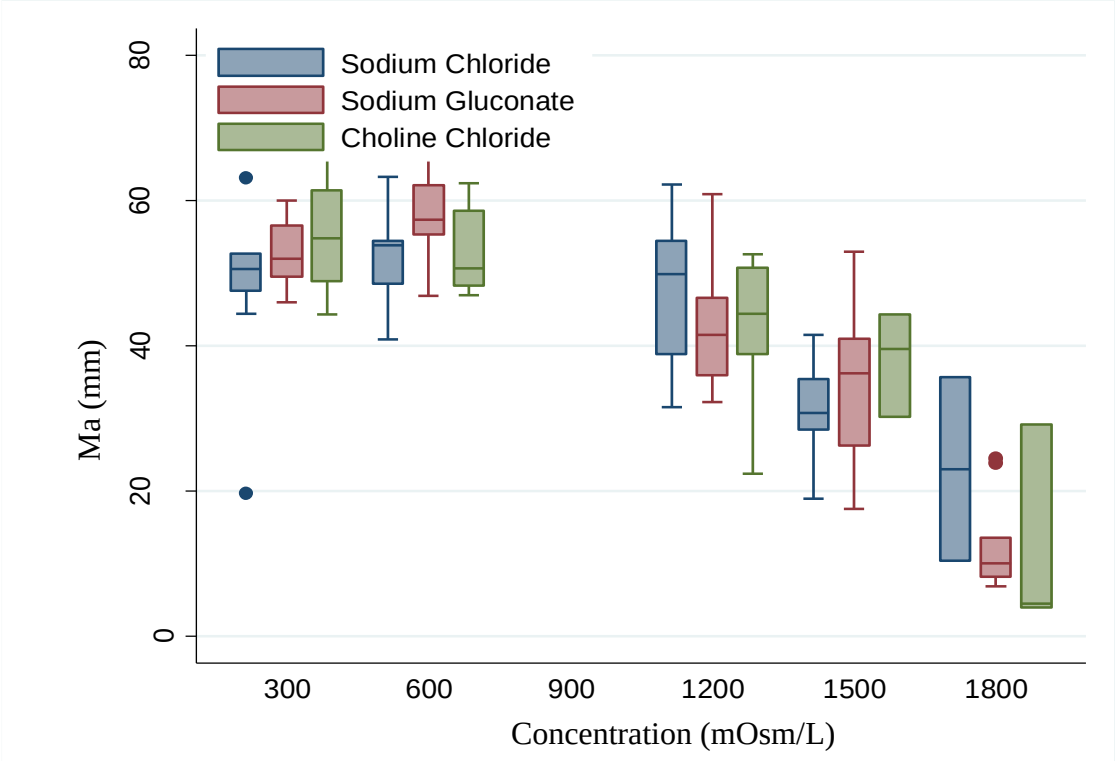

Figure 4-30: Effects of different fluid types at different concentrations on TEG MA.

Citrate anticoagulated whole blood was diluted with crystalloid solutions (20%v/v) Box plot of ma in mm, concentration in mOsm/L, *outliers, (N=11) * Kruskall Wallis equality of populations test used.

4.2.3 Effects of Neutral Organic Non-ionic Crystalloid-Dextrose and Mannitol on Whole Blood Coagulation as Determined by TEG.

The solutions tested were those normally used at therapeutic concentrations- isotonic and then at hypertonic.

(a) Effects of non-ionic crystalloid solutions compared to normal saline in citrated whole blood

Although mannitol and dextrose produced shorter time to onset of clot formation than saline, rate of clot formation were faster (greater alpha) but weaker clot strength (lower Ma) than control undiluted. At hypertonic concentrations, effects of 1000 mOsm/L saline were not different from

control in contrast to similar concentration of mannitol (20%) that significantly reduced clot strength. Generally, effects of mannitol and dextrose concentrations were more evident on clot strength than time to onset of clot formation out of proportion observed with NaCl (Table 4-1).

Table 4-1: Median values of organic non-ionic crystalloid solutions on citrate anticoagulated whole blood coagulation

Solution	R(mins)	K	alpha	MA
Neat	12.90 (IQR 11.00, 17.40)	5.10 (IQR 3.50, 7.00)	32.40 (IQR 27.70, 46.90)	55.80 (IQR 54.30, 57.00)
300 mOsm Mannitol	8.5 (IQR 4.20, 11.70)	4.00 (IQR 3.20, 5.60)	43.60 (IQR 35.90, 57.90)	48.70 (IQR 41.50, 52.30)
300 mOsm NaCl	10.70 (IQR 7.70, 13.00)	3.75 (IQR 3.00, 5.20)	44.85 (IQR 34.70, 50.00)	53.4 (IQR 47.4, 56.60)
5% Dextrose (300 mOsm)	9.80 (6.10, 10.80)	3.70 (IQR 3.20,5.800	43.95 (IQR 33.70, 51.60)	53.20 (IQR 45.90, 57.00)
20% Mannitol (1000 mOsm)	9.55 (IQR 4.20, 11.50)	29.65 (IQR 3.80, 55.50)	13.40(IQR 9.90, 17.70)	14.40 (IQR 8.60, 18.6)
1000 mOsm NaCl	12.65 (IQR 10.20, 16.60)	4.9 (IQR 3.80, 5.90)	38.00 (IQR 33.20, 44.20)	51.6 (IQR 49.2, 55.4)
50% Dextrose (>2500 mOsm)	57.95 (IQR 42.30, 70.00)	-	1.35 (IQR 0.95, 9.10)	2.10 (IQR 2.05, 3.95)

Data are for median values and 25th and 75th interquartile ranges. Citrate anticoagulated whole blood was diluted with the various non-ionic crystalloid solutions at 20% (v/v)

(b) Effects of mannitol solutions on native non-citrated whole blood as assessed by TEG

The objective of this experiment was to investigate whether absence of citrate anticoagulation has an influence on whole blood coagulation. It can be seen that changes induced by normal saline compared to control undiluted whole blood samples were not significant. In contrast, effects of mannitol were dose-dependent significant reduction of clot strength as evidenced by decrease in alpha angle and Ma indicative of hypocoagulability (Table 4-2).

Table 4-2 Mean TEG parameters of effects of mannitol or normal saline on non-citrated whole blood coagulation.

Solution	R (mins)	K (mins)	α angle(degrees)	MA (mm)
Control(undiluted)	10.1(±2.1)	3.1(±1.05)	51.8(±10.5)	60.4(±6.86)
0.9% saline(20 %v/v dilution)	15.6(±21.4)	2.6(±0.77)	53.1(±11.8)	57.4(±6.89)
5% mannitol(20%v/v dilution)	12.6(±4.5)	4.8 (±4.33)	43.9 (±13.35)*	50.3 (±8.35)*
20% mannitol(20% v/v dilution)	18.2 (±20.6)	12.4 (±2.93)*	13.6 (±5.95)*	17.4 (±9.26)*
20% mannitol(5%v/v dilution)	9.9(±2.8)	4.1(±2.49)	45.5 (±13.75)	57 (±7.73)

* Significant ($p<0.005$). Values are for means ±SD. Citrate anticoagulated whole blood was diluted with mannitol or NaCl and different concentrations.

Haemodilution as a cause of hypocoagulability can be discounted since at similar haemodilution, coagulation results varied by diluents type and concentration. The predominant effects of mannitol on alpha angle (α) and clot strength (Ma) test results suggests that, mannitol as a molecule impairs platelet aggregation, fibrin polymerization or both.

In this study, TEG results done on whole blood showing parabolic relationship between coagulation parameters and saline concentration (Figure 4-23, 4-25 and 4-26) were in sharp contrast to positive linear relationship observed with plasma based routine coagulation tests (Figure 4-1 and 4-2). A possible source of difference in the two samples is contribution of cellular elements in whole blood specimens. A plausible mechanism to explain the discrepancy between the two observations is ‘cell based coagulation model’ (M. Hoffman & Monroe, 2001; Maureane Hoffman, 2003a, 2003b). In this new concept, contribution of cellular elements,

RBC, WBC and platelets, in blood clot formation are recognized. Unlike the plasma based coagulation cascade, blood coagulation reactions occur on the surface of cellular elements, especially platelets and RBC. The reactions are initiated by TF from TF bearing cells, mostly monocytes and leukocytes.

In blood, coagulation cofactors TF, FV, FVIII are membrane bound (Kenneth G. Mann, 2003; Kenneth G. Mann et al., 2003). Membrane binding increases their catalytic efficiency for FVII, FX and FII, in the formation of tenase and prothrombinase, by four to six orders of magnitude as well as protection from inhibition by Antithrombin and TFPI (Orfeo, Butenas, Brummel-Ziedins, & Mann, 2005b). Furthermore, membrane surfaces stabilize FIX and FX and protect them from inhibitory effect of increasing ionic strength and increase probability of substrate binding as well as protection from TFPI, unlike when they are freely suspended in solution (McGee et al., 1998, 1999)(Kuharsky & Fogelson, 2001). A consequence of this interaction is augmented tenase and prothrombinase complex assembly in blood clot formation. In addition to providing membrane surface for coagulation factor reactions, neutrophils protects factor V from inactivation by APC as well as release of reactive oxygen species that enhance thrombin generation and platelet aggregation thus modulating blood clotting (Altieri, 1995; Bouchard & Tracy, 2001, 2003). However, these observations remain to be validated in the presence of varying diluents' concentration. With regard to saline concentrations, inhibition of platelet-leukocyte aggregation has been reported at supratherapeutic doses (Huang et al., 2010). This partly explains TEG parameters (α-angle and Ma) obtained with 1200 mOsm and higher.

An alternative explanation for the behaviour in whole blood may rest on crystalloid electrolyte constitution**.** A consequence of haemodilution with increasing saline concentrations is increase of sodium concentrations, which may reach hypernatremic proportions depending on the initial sodium levels and diluents concentration. Although hypernatremia may cause hypertonicity, it has been associated with thrombosis of dural sinus (Kamijo et al., 2003) and DVT (Vikrant et al., 2007). Continuous infusion of hypertonic saline (CHS) have been associated with hypernatremia (Na>155 or 160) which correlated with thrombosis (Froelich et al., 2009) and increase in thrombin activity (high FPA) (Grant, Tate, Hughes, Davies, & Prentice, 1985)(Froelich et al., 2009). These results suggested enhanced thrombin activity in presence of increasing sodium concentrations reinforcing a strong association between saline haemodilution and DVT (Heather et al., 1980; Janvrin et al., 1981).

Another dimension in support of cellular procoagulant behaviour could be due to Hofmeister ions on biological membranes. According to this theory, membranes swell in the presence of increasing ionic salt concentrations (Kunz, 2009; Petrache, Zemb, Belloni, & Parsegian, 2006), and possibly by inhibiting plasma membranes enzymes that maintain membrane phospholipid asymmetry. As a consequence, procoagulant effects arise from: exposure of Phosphatidyl serine (PS) from inner cytoplasmic side to the outer membrane, an increase in surface area for coagulation factor interactions and structural support for membrane dependent coagulation factor reactions (Kalafatis, Swords, Rand, & Mann, 1994; Lentz, 2003). Based on the above mentioned facts, observed differences between ionized, charged diluents as opposed to neutral, uncharged diluents in this study may be hypothesized as follows; Charged/ionic crystalloids augment

anionic milieu supportive of coagulation factor reactions on surface of cellular elements up-to a certain concentration. In contrast, mannitol and dextrose being electrically neutral do not affect membrane dynamics and therefore lack cellular procoagulant mechanisms.

The concentration-dependent 'bell shaped' effects of NaCl and other diluents on TEG parameters can be explained on the basis of competition between electro selectivity of ions of the Hofmeister series on protein interactions (Kunz, 2009; Zoldák et al., 2004). Thus, hypocoagulability induced by diluents with concentrations from 1200 mOsm/L and above could most likely arise from chaotropic effects on coagulation proteins and membrane lipid perturbations. This would need to be verified in further studies.

Another possibility to explain discrepancy between plasma based coagulation tests and whole blood TEG results is the contribution of microparticles generated from either monocytes or erythrocytes. These have been found to transfer procoagulant initiation factors to platelet (Saulius Butenas et al., 2009; Rauch et al., 2000). The high speed centrifugation at 2000g to obtain plasma may have precluded microparticles that are normally separated at centrifugation speed of 800-100 g. Perhaps absence of procoagulant microparticles from plasma explains lack of crystalloid procoagulant activities in plasma.

Apart from facilitating dissemination of TF from initiator cells, they also provide lipid raft rich in negatively charged PS on the outer membranes. This together with surface anchoring of prothrombinase amplifies thrombin generation. However, due to electrostatic hindrances, it has been found that their shedding are inhibited by extracellular K+ or high ionic strength, but

unaffected by low ionic strength or in the presence of non-ionic solutions such as sucrose (Pizzirani et al., 2007). Therefore, high ionic strength may have inhibited production of procoagulant microparticles, thus explaining the bell shaped TEG parameters (R, K, alpha and Ma) the presence of ionic NaCl, choline chloride and sodium gluconate as compared to either mannitol or dextrose. But for this postulate to be accepted there would need to be unambiguous demonstration of microparticle shedding and procoagulant functions as a function of changing external osmolality or ionic strength.

Haemodilution with glucose solutions was to create *in vitro* condition simulating hyperglycaemia that is associated with hypercoagulability in diabetes mellitus. The results indicate that 5% dextrose (iso-osmolar with plasma) may be hypercoagulable but not significant (Table 4-1). However, hyperosmolar 50% dextrose induced whole blood hypocoagulability which tended towards significance (Table 4-1), against expectation. This finding in the current study was in contrast to those in other studies of hypercoagulability only in extrinsic activation but not in either intrinsic or routine coagulation tests in clinical studies in type 2 diabetes mellitus (Yürekli, Ozcebe, Kirazli, & Gürlek, 2006b) or hypercoagulability in diabetic patients undergoing pancreatic-kidney transplants (Burke et al., 2004) probably reflecting activity of supraphysiological amounts of TF used as a trigger. Moreover, the differences may arise not from direct dextrose effects, but from glucose metabolic end products and/or hyperosmolar changes. Furthermore, other studies suggest no direct roles for glucose (Grant, 2007) but leukocyte counts (Rauch et al., 2000). Thus leukocyte co-activation with platelets in presence of glucose may be the sentinel event in thrombogenesis. This probably acts through generation of neutrophil extracellular traps, a concept which has been described in relation to other hypertonic

solutions but not investigated in this study. From this study, direct link between glucose and whole blood coagulation as determined by TEG remains unequivocal *in vitro*.

In the current study, it is demonstrated using Thrombelastography, that mannitol dose dependently impair coagulation of whole blood *in vitro.* The effect decreases blood clot strength, as evidenced by reduction in MA and alpha angle with minimal effect on clot activation. Findings of reduced Ma as well as alpha in the current study (Table 4-1&4-2) are consistent with those observed in presence of platelet aggregation inhibitors as assessed by TEG (Bochsen et al., 2007; Chakroun et al., 2006; Gottumukkala, Sharma, & Philip, 1999; Lang et al., 2004; Scharbert, Auer, & Kozek-Langenecker, 2009) and ROTEM™ (Lindroos et al., 2010; Luostarinen et al., 2011). The findings in the current study and the previous ones done demonstrated greater effects on clot strength in mannitol group compared to undiluted control suggesting impairment was more on platelets than on fibrin polymerization. These results correlates with tests performed on Light Transmission aggregometry (Beilin, Arnold, & Hossain, 2006; Katori et al., 2004, 2005). The dose-dependent antiplatelet effects of mannitol using optical aggregation tests are confirmed in the next section of this study. The effect may be mediated by increased hypertonicity and hydroxyl group of mannitol on platelet functions, and unlikely due to interference with either thrombin generation or fibrin polymerization.

Failure to demonstrate effects of mannitol on routine coagulation tests suggests its action is on cellular elements in whole blood rather than plasmatic components in blood. This postulate is reinforced by findings in the previous section in this study that at 20% plasma dilution, mannitol in the concentration up-to 20% w/v (1000 mOsm/L) does not alter thrombin generation either in the extrinsic (Figure 4-12) or intrinsic activation (Figure 4-7).

TEG results obtained from haemodilution with saline and mannitol on citrated samples (Table 4-1) compared to neat, non-citrated samples (Table 4-2) reveals procoagulant effects in citrated samples. The result displayed by the table clearly demonstrates an effect of pre-analytical citrate anticoagulation and consequent recalcification as variables on coagulation test results. Several authors have pointed artificial effect of citrate on TEG parameters. A trend towards hypercoagulability has also been documented in other studies comparing citrate storage (Zambruni et al., 2004) compared freshly collected and tested clinical blood samples (Gilman, Koch, Santrach, Schears, & Karon, 2013; Mancuso et al., 2003; Wasowicz et al., 2008). Citrate does not inhibit contact pathway activation of FIX (Roche, 2003) (Anthony M. Roche et al., 2006a, 2006a; Anthony M. Roche, James, Bennett-Guerrero, & Mythen, 2006b) as evidenced by shorter TEG initiation phase in citrate (Fenger-Eriksen, Ingerslev, Tønnesen, & Sørensen, 2009) that is enhanced by duration of storage (V. A. Bowbrick, Mikhailidis, & Stansby, 2000). Interestingly, Mann (2007) demonstrated that thrombin generation dynamics lag time and peak were artificially altered in citrated plasma with subsequent recalcification which become more significant with longer incubation period. Therefore, although citrate chelation inhibits blood clotting, thrombin generation still proceeds on recalcification despite citration. This demonstrates prothrombin- thrombin conversion is less affected by citrate molecules, although independent contribution of sodium in sodium citrate cannot be ruled out (W. H. Seegers et al., 1950), and thrombin activity increases with duration of storage and concentration of citrate. Auto-activation of prothrothrombin to thrombin is facilitated by increasing citrate concentrations, and citrate chelation slows but does not stop the process on the upstream calcium dependent factors.

An argument may be made that hypercoagulability with recalcification could arise from calcium ions. However, this is countered by findings that plasma calcium levels above 0.56 mM/L do not produce added changes on clotting time (Ataullakhanov, Pohilko, Sinauridze, & Volkova, 1994; Calatzis et al., 2001; James & Roche, 2004), unless levels fall below the threshold. In contrast, recalcification produces only modest changes on clot firmness as assessed by TEG even at very low calcium levels (Calatzis et al., 2001; James & Roche, 2004). Therefore, when a threshold of plasma calcium is reached, no added advantage is achieved with increase in ionized calcium concentrations, instead there is paradoxical increase in clotting time (Conio et al., 1975, 1976). This may be taken as an increase of chloride ions overriding gains made by calcium ions.

The conventional practice of recalcification of citrated plasma samples assumes that calcium is the only participant and thus its restoration will normalise clot formation. This line of thinking assumes that citrate is innocuous and thus plays no role on the coagulation factor interactions. However, this is not the case as illustrated by hypercoagulability of haemodiluted neat fresh samples compared to undiluted controls, an observation not seen with citrated samples (A. M. Roche, James, Grocott, & Mythen, 2003). The citrated undiluted samples although showing hypercoagulability compared to neat non-citrated, the difference on haemodilution is lost. This strongly suggests procoagulant effect of citrate independent from recalcification. It may therefore be surmised that the subtle procoagulant effects on haemodilution become less evident in the background of citration.

The putative effect of sodium citrate on blood coagulation may arise from two aspects: (1) presence of sodium that allosterically enhance thrombin activity (Di Cera, 1995); (2) citrate

being a kosmotrope on the Hofmeister lyotropic series (Cacace et al., 1997) stabilises coagulation factor proteins with consequent salting out or promotion of clotting. This may explain the TEG results in this study showing hypocoagulability in neat (Table 4-2) compared to citrated samples (Table 4-1). This demonstrates that results obtained from citrated samples may be hypercoagulable, and therefore not true reflection of *in vivo* state.

4.2.4 Thrombo-Elastograph Parameters after Intervention with Fibrin and Thrombin

The data from previous section demonstrated that more than 3 fold increase in crystalloid concentration induced hypocoagulability irrespective of solute type (Table 4-3). Therefore in an attempt to explore which coagulation factor could be useful in reversing this pattern, TEG cups were spiked with coagulation factor concentrates before adding whole blood haemodiluted with index hyperosmolar solutions.

Table 4-3: TEG parameters obtained with hypertonic crystalloid solutions before factor supplementation

Crystalloid solution	R (min)	K (min)	A-angle (degrees)	MA (mm)
Neat/Undiluted	8.75	3.55	47.3	58.5
300mosm NaCl	8.15(IQR7.1,9.5)	3.5(IQR2.6,4.2)	46(IQR36.6,50.4)	50.6(IQR47.5,52.7)
1800mosm NaCl	40.2 (IQR36.8,48.2)	22.8 (IQR11.7,26.4)	10.95 (IQR3.8,18.1)	23.05 (IQR10.4,35.7)
1800mosm Na. Gluc.	13.7 (IQR12.8,16.1)	10.6 (IQR5.2,15.9)	15.3 (IQR13.7,21)	10.1 (IQR8.1,13.6)
1800mosm ChCl	52.7 (IQR35.5,66.35)	23.6 (IQR14.8,32.3)	3 (IQR.9,13.2)	4.5 (IQR4,29.2)

Data are for medians (IQR). Citrate anticoagulated whole blood was diluted with various crystalloid solutions at the highest concentrations tested.

4.2.4. 1 Effects of Thrombin Supplementation on TEG parameters when diluted with hypertonic crystalloid solutions

Thrombin supplementation/spiking had the effect of predominantly shortening R and K time than on alpha and Ma. However, improvement in alpha and Ma in ionic crystalloids were greater than those in non-ionic crystalloids (mannitol or dextrose). Interestingly, Ma in sodium gluconate decreased instead of improving which was odd considering shortened R and K (Table 4-4).

Table 4-4 Effect of thrombin supplementation on TEG parameters obtained with hypertonic crystalloid solutions

Solution	R (mins)	K (mins)	A-angle (degrees)	MA(mm)
300mosm NaCl + Thrombin	0.5	9.1	39.35	21.9
1800mosm NaCl + Thrombin	0.65	13.15	39.7	30.4
1800mosm Na. Gluc. + Thrombin	0.65		30.15	3.5
1800mosm ChCl + Thrombin	2.65	7.2	23.95	18.5
20% mannitol + Thrombin	1.5		18.55	9
50% Dextrose + Thrombin	2.65		10.8	2.75

Data represent mean of two replicates. Thrombin 10 iu was placed in TEG cups into which citrate anticoagulated whole blood diluted with the various hypertonic crystalloid solutions were added.

The results demonstrate that the hypocoagulability induced by hyperosmolar solutions, thrombin spiking preferentially shortened R and K time without significant changes in alpha angle and maximum amplitude or clot strength (Table 4-4). This is comparable to a study by Szlam (2008) who spiked normal pooled plasma with increasing concentrations of thrombin *in vitro* (at

1,2,5,10 nM final concentration) and found progressive decrease in R time together with simultaneous increase in alpha angle and MA. The changes in R time were significant while those in alpha and Ma were not significant. It may therefore be inferred that changes induced by thrombin supplementation are more significant on coagulation initiation rather than on clot strength. Our study results thus reveals that thrombin spiking only restores impaired kinetic reactions (shortened R and K) but not clot strength, which is a marker of fibrin polymerization. The current study results are consistent with what is known about thrombin activity in forming fibrin monomers, which spontaneously polymerize as a biophysical process independent of thrombin. Therefore, in presence of high ionic strength fibrin lateral aggregation is still affected despite spiking with thrombin. This has clinical implications in increased haemorrhage associated with hypertonic saline that may not be responsive to thrombin factor concentrates, since haemostatic functions are dependent on fibrin mechanical strength not amenable to thrombin correction.

4.2.4 2 Effects of Fibrinogen supplementation on TEG obtained with hypertonic crystalloid solutions.

Fibrinogen supplementation increased α-angle and Ma out of proportion to changes in R and K values (Table 4-5). However, the changes were suboptimal compared to those produced by thrombin (Table 4-4).

Table 4-5 Mean TEG parameters obtained with fibrinogen supplementation.

Solution spiked with fibrinogen	R(mins)	K(mins)	α-angle (degrees)	MA (mm)
300mOsm NaCl + Fibrinogen	5.05	2	59.3	68.75
1800mOsm NaCl + Fibrinogen	15.3	6.8	29.8	53.5
1800mOsm Na. Gluc. + Fibrinogen	23.25	13	10.15	21.85
1800mOsm ChCl + Fibrinogen	20.05	7.15	36.7	45.15
20% Mannitol + Fibrinogen	6.1	3.2	33.85	30.5
50% Dextrose + Fibrinogen	7.1		27.05	7.75

Data represents mean of two replicates. TEG cups were spiked with 10 mg of fibrinogen into which citrate anticoagulated whole blood diluted with the various hypertonic solutions were added.

Data represents mean of two replicates. TEG cups were spiked with 10 mg of fibrinogen into which citrate anticoagulated whole blood diluted with the various hypertonic solutions were added.

The current study results demonstrating significant improvement in rate of clot build up (alpha angle) and clot strength (maximum amplitude) with minimal changes on R and K in hypertonic crystalloids spiked with fibrinogen is consistent with previous studies where supplementation with fibrinogen resulted in significant increase in alpha angle and MCF without affecting CT (Haas et al., 2008) (De Lorenzo et al., 2006).

Lack of complete restoration of TEG parameters impaired by hypertonic solutions on spiking with either thrombin or fibrinogen indicates another factor is at play. This is most likely platelet.

4.4 Effects of Crystalloids on Platelet Aggregation

This section was a follow up on the results obtained with Thrombelastography that indicated that maximum amplitude was reduced with increase in crystalloid concentration (Figure 4-25, 4-26 and 4-30). This was indicative of reduced platelet aggregation. It was therefore imperative to specifically investigate using a method which is the gold standard for platelet aggregation.

4.4.1 Effects of Crystalloid Solutions on Platelet Aggregation Induced By ADP

Stimulation with ADP agonist produced crystalloid solution concentration-dependent inhibition of maximal aggregation, which was statistically significant ($P<0.05$ for trend line). The changes on maximal aggregation were more marked though inconsistent in crystalloid concentrations of 1500 mOsm and 1800 mOsm. Overall, the differences between solute types at all concentrations for each type were not significant. Platelet aggregation was inhibited by crystalloid concentration irrespective of solute type.

Analysis of corresponding shape change showed that while platelet change significantly increased in solutions at 300 mOsm compared to undiluted control ($p<0.001$), it was totally abolished in crystalloid concentration of 1000 mOsm and higher. When taken together, maximal aggregation patterns did not correspond to shape change in each crystalloid concentration when agonist was ADP (Figure 4-31).

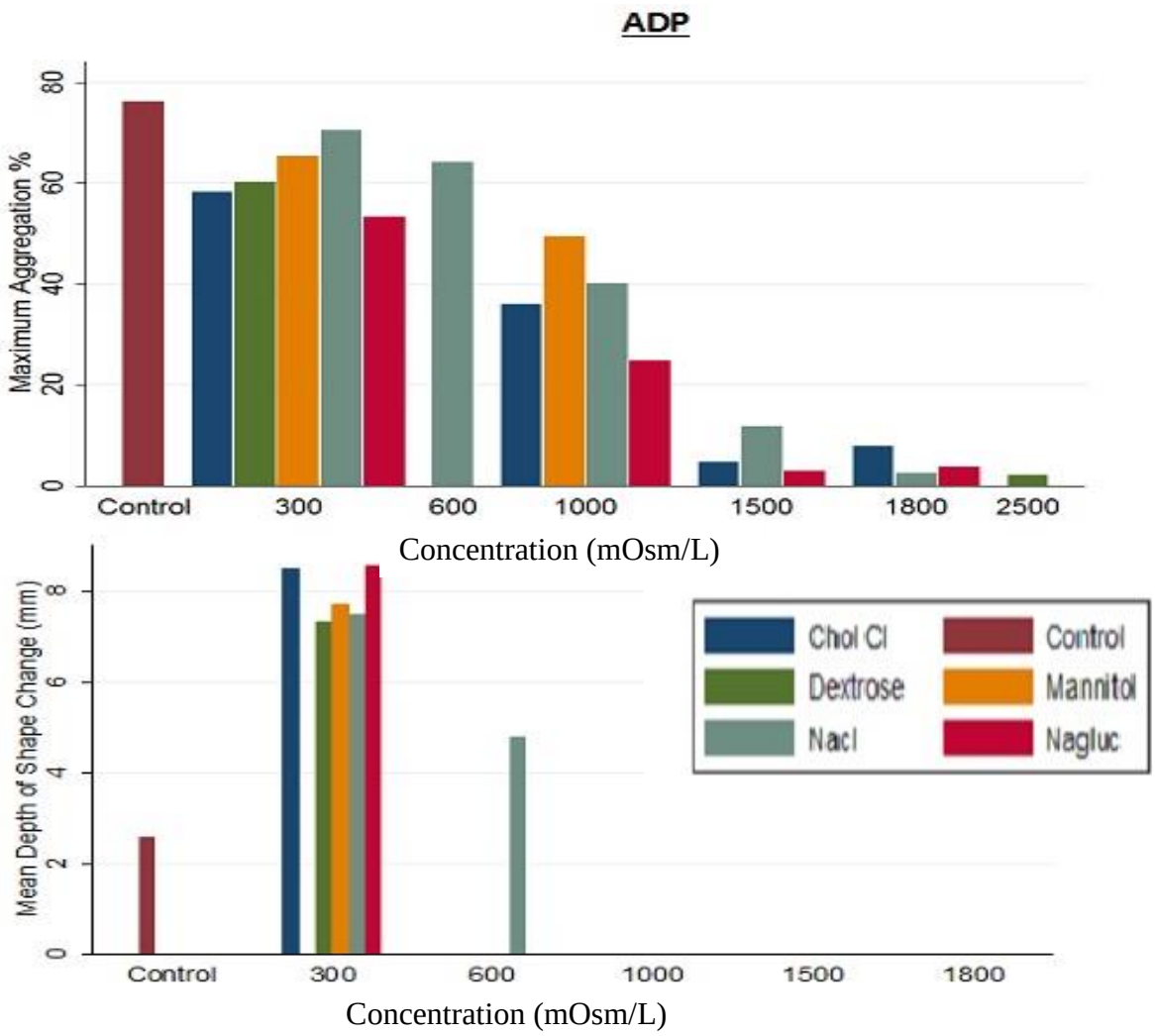

Figure 4-31 Effect of crystalloid solutions on platelet aggregation induced by ADP.

Platelet rich plasma was diluted with the various crystalloid solutions and aggregation induced by ADP agonist. ADP-adenosine diphosphate, top panel-maximum aggregation (%), lower panel-shape change (%)

4.4.2 Effects of Crystalloid Solutions on Platelet Aggregation Induced by Arachidonic Acid (AA)

When platelet aggregation was induced by AA, there was reduction in maximal aggregation at all concentrations from control. However, the aggregation levels remained fairly unchanged up to crystalloid concentration of 1000 mOsm. Platelet aggregation inhibition was only significant in 1500 and 1800 mOsm ($p<0.05$). The corresponding platelet shape change was increased in crystalloid solutions with concentration of 300 mOsm compared to control undiluted. However,

in contrast to ADP, platelets in 1000 mOsm still had some evidence of shape change, though greatly diminished. Again, the differences between diluents' solute types were insignificant.

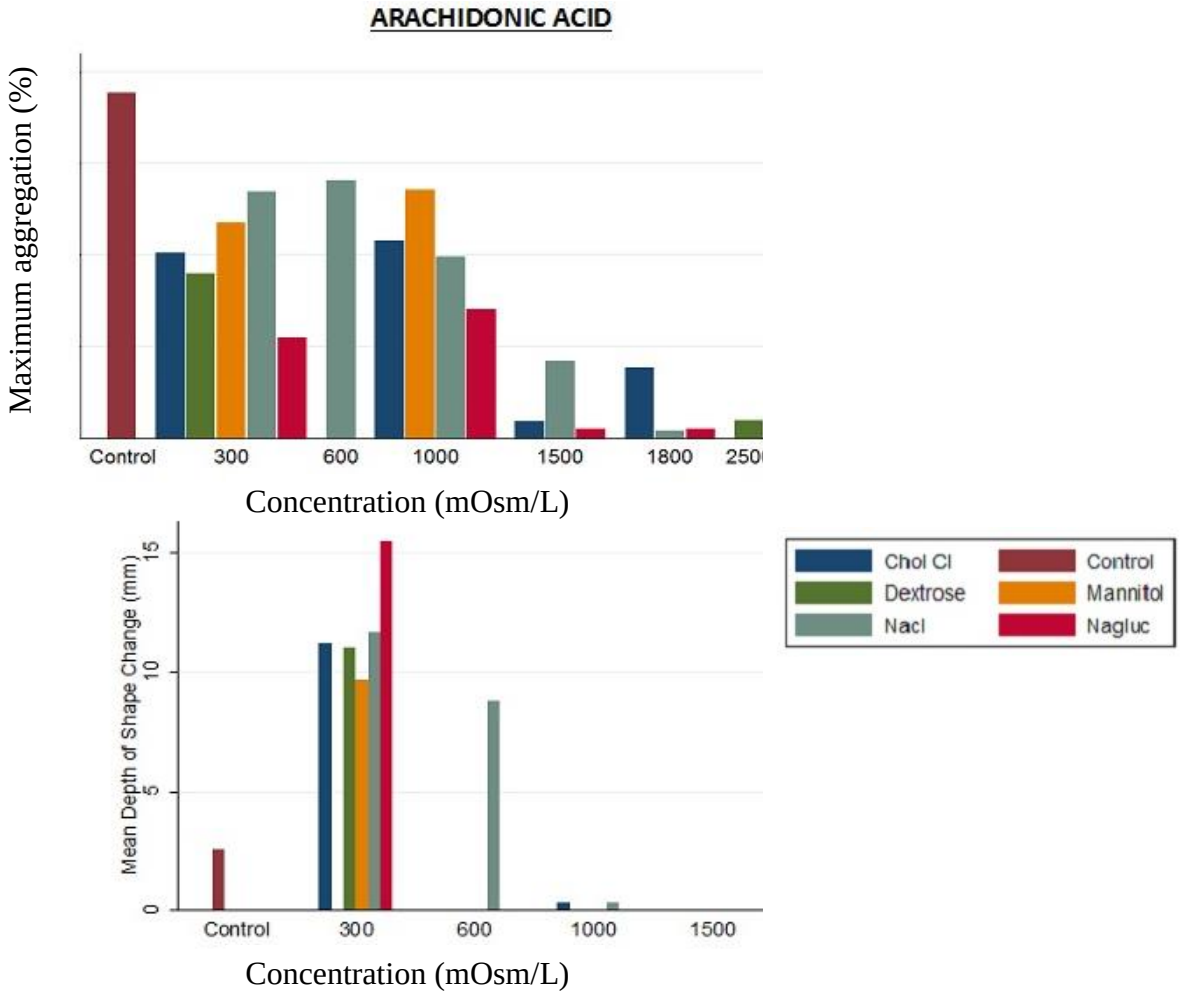

Figure 4-32: Effect of crystalloid solutions on platelet aggregation induced by arachidonic acid (AA).

Platelet rich plasma anticoagulated with citrate was diluted with various crystalloid solutions and aggregation induced by arachidonic acid. AA-arachidonic acid, top panel maximum aggregation (%), lower panel-shape change (%).

When results obtained with the two agonists were juxtaposed against one another, stimulation with AA tended to lag behind ADP at each concentration (fig 4-32). This may reflect differences between agonist potencies-AA are less potent than ADP (Cattaneo et al., 2013; Harrison, 2009)

Results of NaCl concentration dependent inhibition of platelet aggregation in this study are in agreement with previous studies. For example, Reed (Reed et al., 1991a) performed serial dilution of PRP with 7.5% NaCl (~2500 mOsm/L)(HTS) and found progressive deterioration in platelet aggregation when 10% or more of normal plasma was replaced by HTS in the presence of ADP or collagen agonists, whereas there was no change with normal saline at similar dilutions. A similar study (Wilder et al., 2002a) also reported impairment of platelet aggregation in response to thrombin agonist with 2400 mOsm/L saline at 20% volume replacement compared to normal saline control. However, these previous studies were done using only two fixed crystalloid diluents concentrations: 300 and 2400 mOsm. This therefore did not answer the question of crystalloid-concentration or dose-response relationship, i.e progression of platelet aggregation with diluents concentration. This study has bridged this gap by showing that there is a dose – response relationship between diluents concentration and platelet maximum aggregation, though qualitatively varying in agonist used.

In a study by Bradley et al (2001), the researchers used increasing concentrations of Saline (290, 320 and 350 mOsm) and induced platelet aggregation using ADP, Epinephrine and Collagen. They found that platelet aggregation inhibition differed according to the type of agonist and saline osmolality. Platelet aggregation remained unchanged in 290 mOsm saline to all agonists tested. However, while in 320 mOsm saline platelet aggregation inhibition was small and only significant for collagen and epinephrine but not to ADP, in 350 mOsm aggregation inhibition was large and observed for all the three agonists tested. With respect to NaCl concentration, this study results are essentially in agreement with those reported by Bradley with ADP and AA agonists, except that in this study others such as thrombin, collagen and epinephrine were not

tested. In this respect, extrapolation of results may be difficult since agonists have different signal transduction pathways with different sensitivities.

In vitro investigations of effects of glucose on platelet aggregation have yielded different results. For example while this study results are consistent with results published by others (Wilder et al., 2002b) who reported 2400 mOsm/L dextrose at 20% volume dilution significantly inhibited platelet aggregation as well as shape change when thrombin was used as an agonist, which was in contrast to other findings of enhanced platelet aggregation in presence of incremental concentrations of glucose when ADP or collagens were used as an agonist (Keating et al., 2003; Sudic et al., 2006; Tang et al., 2011)(De La Cruz et al., 2004). Variations in study results may arise from differences in duration of incubation. Whereas in the current study mixture of platelets with dextrose were incubated for ten minutes, in the above cited studies incubation times ranged from 60 to 90 minutes. The longer incubation time allowed for glucose metabolism with subsequent downstream signalling mechanism, and production of reactive oxygen species or free radicals which are known platelet pro-aggregators (Tang et al., 2011)

The platelet effects of glucose have been correlated to oxidative metabolic products of glucose (Chaudhry, Sagone, Metz, & Balcerzak, 1973; De La Cruz et al., 2004; Sudic et al., 2006). Additionally, in another study, full aggregatory influence was developed in whole blood in the presence of leukocytes (Sudic et al., 2006) and erythrocytes (S. Jain et al., 2012; S. K. Jain et al., 1999). These findings suggest hyperglycemia - induced platelet pro-aggregation requires extended period for metabolic products elaboration in concert with contribution from other formed cellular elements. This was unlikely in this study since incubation period of less than 10

minutes was too short a time and contribution from RBC and leukocytes were omitted since PRP samples analysed had no other cellular elements. Nevertheless, it is possible that inhibitory response observed in this study was due to lack of proaggregatory effects of free radical ordinarily contributed by leukocytes and RBC. Overall, increased platelet activation in presence of glucose is best demonstrated in whole blood when RBC and leukocytes are taken into account.

Since incubation period in our study was too short for metabolic processes to manifest, the effects observed could be attributed solely to acute hypertonic stress on platelets. This is supported by observation that addition of isoosmotic concentrations of glucose and mannitol produced similar response in this study compared to another study (Keating et al., 2003). These results suggest osmolality has a stronger influence, in the short term, than crystalloid molecular type on platelet reactivity.

Although Mannitol and dextrose have similar molecular weights of 182, differing only on the orientation at carbon 2, mannitol is impermeable to biological membranes while dextrose penetrates with ease. Despite this fact, there were no differences between mannitol and dextrose on platelet aggregation in the current study. Mannitol inhibited platelet aggregation dose dependently in the presence of either ADP or arachidonic acid. Previous studies of effects of mannitol on platelet aggregation have revealed responses to be agonist specific. For example, while Clark (2011) reported inhibition of epinephrine mediated aggregation as opposed to no effect with collagen, others (Koerner, Sahlmen, Zimmermann, Cardoso, & Kubanek, 1994) reported both ADP and collagen inhibition and dilution dependent inhibition with thrombin (Wilder et al., 2002a).

Antiplatelet effects of mannitol are a consistent finding in many research reports although opinions are still divided on its mechanism of action. A recurring proposition is mannitol free radical scavenging function during platelet activation. Data in support for this line of claim arises from similarity in activity with known free radical scavengers like superoxide dismutase and catalase which inhibit platelet aggregation, an effect mimicked in presence of mannitol (Handin, Karabin, & Boxer, 1977; Praticó et al., 1999). However, though free radical scavenging property may be a factor, it is possibly overridden by hyperosmolality since platelet inhibition was observed in presence of incremental doses of heterogeneous molecules other than mannitol in this study. Moreover, hyperosmolality induced antiplatelet effect has also been observed with Non-ionic radiographic contrast (Hay & Bull, 1995, 1996b) and therefore not limited to mannitol alone.

Various mechanisms have been advanced to account for effects of various crystalloid diluents on platelet aggregation. Overall, these theories revolve around specific effects of diluents on membrane transport and hence osmotic changes, regulation of intracellular Ca++ and pH, and intracellular signal transduction and metabolic pathways.

The claim that extracellular fluid composition influences platelet membrane solute transport system with possible consequences on aggregation is born by evidence that thrombin activated platelet aggregation is augmented by external Na+ medium (Agam, Argaman, & Livne, 1989) and is accompanied by rise in intracellular Ca++ and pH (Oscar A. Gende, 2003, 2003, 2004, 2005), or blockage of Na+-Ca++ exchanger by Ouabain (Tomasiak et al., 2007). These results

suggested role of Na+ cation exchanger in procoagulant response. However, other experiments have shown that this is not specific for Na+, as inhibition of Na+/H+ (Agam et al., 1989) or Na+/Cl- (Spalding et al., 1998) exchangers have failed to demonstrate similar procoagulant responses. Instead, the only consistent findings was correlation of platelet shape/ volume change with fluid osmolalirity and aggregation (Tomasiak et al., 2007) discounting roles of specific solutes on membrane transporters and subsequent influence on intracellular events in platelet aggregation as suggested by some authors (O. Gende, 2003; O. A. Gende, 2003; Oscar A. Gende, 2003, 2003, 2004, 2005). Indeed, in this study it was found that lower crystalloid concentrations caused increment in platelet shape change, which also correlated with aggregation. But at higher crystalloid concentrations shape change was abolished and associated with diminished or no aggregation. Therefore, data from this study is in support of Tomasiaks (2007) osmolality postulate, rather than specific solute constituents.

Although chloride ions have been suggested to play a role in promoting platelet shape change and procoagulant reactions (Harper & Poole, 2013) (Vaitkevicius, Turner, Spalding, & Lockette, 2002), there was no difference between chloride containing solutions and others in this study. Perhaps lack of definitive action in this study could be related to type and dose of agonist used compared to the latter cited works. Nevertheless contribution of chloride anion appears small relative to osmolality changes.

A common mechanism to account for antiplatelet effects of crystalloid solutions in this study appears to be diluents induced extracellular hypertonicity and therefore suppression of platelet reactions (Pollard, Tack-Goldman, Pazoles, Creutz, & Shulman, 1977; Rizoli, Rotstein, Parodo,

Phillips, & Kapus, 2000). Evidence for this claim is derived from experiments in which platelets exposed to increasing hypertonic stress were smaller and failed to develop filopodia in response to known agonists like ADP or thrombin (Armitage & Hunt, 1982a; Armitage, Parmar, & Hunt, 1985)(Law, 1983). Failure to undergo shape change when stimulated by agonists also corresponded with decreased aggregation (Law, 1983)(Armitage & Hunt, 1982b; Armitage et al., 1985; Kahn & Meryman, 1973). Hyperosmolality has also been associated with reduction in intracellular ca++levels (Oscar A. Gende, 2003, 2004, 2005) and serotonin exocytosis (Lundberg, Meryman, & Estwick, 1972; Pollard et al., 1977). Thus exposure of platelets to hyperosmolality with consequent suppression of exocytosis of procoagulant chemicals leads to aggregation inhibition. This is due to the fact that it precludes flip-flop exposure of inner membrane containing receptors that participate in platelet reactions-adhesion, activation, secretion and aggregation (R. F. A. Zwaal et al., 2005; R. F. Zwaal & Schroit, 1997).

Platelet procoagulant changes are often accompanied by shape change from discoid to spherical shapes (Pretorius et al., 2007). These are Specific to areas on platelet membrane where blebbing or vesiculation occurs (Dai & Sheetz, 1999). Membrane vesiculation provide micro domains rich in cholesterol lipid rafts that facilitate assembly of prothrombinase complexes and interaction of platelet cytoskeleton with fibrin that bring about aggregation (Del Conde, Shrimpton, Thiagarajan, & López, 2005; Hoylaerts, 2003; Sims, Wiedmer, Esmon, Weiss, & Shattil, 1989; Tuszynski et al., 1984). In the presence of external hypertonicity, procoagulant granule secretion driven by cytoskeletal actin remodelling is inhibited (Rizoli et al., 2000), P- selectin expression is reduced (Huang et al., 2010), attenuation of membrane adherence mechanisms (Thiel et al., 2001) and attenuation of intracellular ca++ mobilization irrespective of crystalloid solute type

(Oscar A. Gende, 2005) with subsequent inhibition of aggregation. These events abrogate the crucial platelet-leukocyte interaction necessary for full response. These facts are consistent with the current study findings.

Other possible mechanisms for hyperosmolality induced suppression of platelet aggregation arising from inhibition of shape change are as follows: reduction in intracellular Ca++ levels leads to stabilisation of cytoskeletal structures that prevents membrane bleb formation (microvesiculation) as well as exteriorization of PS by flip-flop from cytoplasmic surface. In this way platelet activation via P-selectin receptor expression is prevented. Without PS exposure and P-selectin expression, platelet aggregation cannot occur. In addition, it has also been found that increase in NaCl concentration decreases platelet GP1 expression and subsequent binding characteristics (Bevers et al., 2005). Therefore increase in ionic strength in this study may be a contributing factor for decreased platelet aggregation.

The initial decrease in light transmission in optical aggregometry has widely been interpreted to correspond to platelet shape change (Fantl, 1968; Livne, Grinstein, & Rothstein, 1987) which corresponds to cytoskeletal rearrangements (G. V. Born & Cross, 1963; G. V. Born et al., 1978; G. V. R. Born & Cross, 1964; Fantl, 1968) although opinions are still divided whether it precedes full aggregation (Maurer-Spurej & Devine, 2001). In this study it was found that platelet shape change as determined by optical methods is dependent on crystalloid concentration. This is consistent with findings of Pollard (Pollard et al., 1977) where in addition, serotonin secretion and osmotic lysis were also reduced by increase in NaCl concentration. Increase in external osmolality, by preventing platelet shape change, the consequence is to

decelerate platelet secretion and assembly of membrane dependent factors (tenase and prothrominase- FXa, FIXa, FXa, FVa and FVIIIa). The end result is altered platelet-fibrin interaction and thus reduction of aggregation (Heemskerk, Bevers, & Lindhout, 2002).

Whether the initial decrease in light transmittance is due to platelet shape change or microaggregate formation is difficult to make a distinction in this study owing to the method used. Elucidation can only be done by microscopic studies, either confocal or scanning electron microscopy (G. V. Born et al., 1978; Kraus, Strasser, & Eckstein, 2010; Maurer-Spurej & Devine, 2001), techniques which were not used to assess platelets in this study. Nevertheless there is no reason to doubt validity of initial decrease in photometric transmittance, since platelets only interact with fibrin upon activation (Hantgan, Taylor, & Lewis, 1985). Therefore, observations of lack of aggregation at high crystalloid concentrations in are in keeping with attenuation of activation, in which shape change is just one of manifestations.

In the current study, the effects of crystalloid concentration on platelet reactions followed photo-optometrically were the result of externally induced osmotic effects, more of a biophysical than a chemical process. This is consistent with other findings of dose- dependent osmolality reduction of platelet aggregation (Fantl, 1968) and platelet volume reduction (Livne et al., 1987) in response to osmotic stress. In these previous studies, platelet shape changes independent of crystalloid solution composition suggested simple osmotic behaviour, and that mechanical force or cytoskeleton does seem to play a crucial role in determining platelet reactions responses to osmotic gradients. Platelets thus act as osmosensors irrespective of crystalloids solute composition.

When TEG and platelet aggregation results in this study are compared, it demonstrates that human platelets are sensitive to external osmolality more than RBC. This is evidenced by linear dose-dependent inhibition of platelet aggregation (Figure4-31& 4-32) in contrast to parabolic relationship of coagulation in whole blood as observed in TEG studies (Figure 4-25&4-26). The explanation for the difference can be inferred from observations that found that platelets were sensitive to osmotic stress than RBC (Livne et al., 1987). This can be accounted for by presence of a different type of water channel, aquaporin AQP6, present in platelets but not in RBC (Lee et al., 2012). Hence, hypertonicity prevented platelet aggregation at a lower concentration than RBC. Therefore platelets are better osmosensors than RBC.

The photo-optical methodology of assessment of platelet function employed in this study has a number of limitations. In essence, only one of the platelet responses was assessed i.e change in light transmittance as a surrogate measure of both platelet shape change and aggregation. Other platelet responses to stimulation, such as adhesion, microscopic examination of shape change, secretion or release reactions and markers of membrane activation/receptor expression were not determined owing to limitations inherent in methodological issues. There is also problem in interpretation of aggregation results- reduction in aggregation curves may arise from impairment of fibrin polymerization or platelet filopodia formation, which cannot be differentiated by this method. Further, the use of PRP precludes contribution of leukocytes whose participation *in vivo* platelet aggregation is well recognized. Nevertheless, LTA method employed herein is a well recognized screening tests for evaluation of platelet function before performing some of the more specific sophisticated tests.

Clinical applicability may be a challenge, since *in vitro* studies use agonists whose physiological concentrations are not known. More fundamentally, it assumes events are static. *In vivo*, hypertonicity triggers release of vWF from endothelial cells, together which may compensate for platelet hypo responsiveness under flow conditions.

The mechanisms underlying differences in coagulation parameters between platelet aggregation in PRP compared to whole blood TEG warrant further investigation. Also investigations of hypertonicity-induced antiplatelet effects arising from this study would help in understanding platelet responses under altered plasma electrolyte states as may be found following volume resuscitation associated with coagulopathy.

4.5. General Discussion of previous Theories relating to crystalloid solutions and coagulation and in light of new findings in this study

Many theories have been advanced to explain the effects of crystalloid haemodilution on human blood coagulation. However, they did not take into account either the crystalloid solute content or concentration. Therefore a discussion of these postulates in the perspective of findings in the current study casts doubt on general applicability.

4.5.1. Haemodilutional Coagulopathy

For a long time it has been taken that coagulopathy that develops in trauma patients is related to dilution since they also receive intravenous fluids, which naturally reduces proportion of clotting factor functions. However, the data in this study and other publications do not support the literal mechanistic ‘haemodilutional coagulopathy’, but rather demonstrate that test results are dependent on solute content and concentration.

4.5.2. Haemodilution Enhances Coagulation

The data in this study, congruent with the latter, shows that coagulation outcomes are dependent on solute type and its concentration whether tests are done on plasma or whole blood, routine kinematic tests or TEG, citrated or native samples. These points out that test results are a function of experimental conditions and techniques employed in investigating coagulation.

Tocantin first brought to our attention that, against expectation, moderate crystalloid haemodilution (upto 40% v/v plasma dilution) would lead to shortening to time to clot formation(Tocantins et al., 1951a). This was also true in haemophilic plasma. Other workers have demonstrated the same findings using TEG in whole blood (Janvrin et al., 1981; K. F. J. Ng et al., 2002; K. F. Ng & Lo, 1996; T. G. Ruttmann et al., 1996). Analysis of their data shows that these findings were obtained with isotonic saline. However, when the same experiments were repeated by other workers using solutions of different solutes at different concentrations, findings have ranged from no change (Wilder et al., 2002a) to hypocoagulability (Reed et al., 1991a; Tan et al., 2002).

4.5.2.1 Crystalloid dilution induced reduction in antithrombin and procoagulant effects-a caveat

The findings in this study therefore casts doubt on the above mentioned postulate, and instead suggest individual diluents' components influence coagulation factor reactions. In the present study, haemodilution was kept uniform at 20% v/v. If reduction of antithrombin was the predominant factor, then all samples tested in the current study would have resulted in similar hypercoagulability which was not the case. Instead, the current study results cast doubt on applicability of the theory of 'reduction of antithrombin enhances blood coagulation'.

One of the well described theory accounting for haemodilution induced enhanced hypercoagulability (Janvrin et al., 1981; T. G. Ruttmann et al., 1996; Tocantins, Carroll, & Holburn, 1951b) is related to reduction in plasma antithrombin levels following crystalloid haemodilution (Monkhouse, 1959; Nielsen et al., 2006; Thomas G. Ruttmann et al., 2007; Tocantins et al., 1951a). According to this theory, haemodilution intuitively suppresses antithrombin participation in thrombin negative feedback reactions thus leaving procoagulant thrombin functions unopposed. Most studies citing this line of evidence were done using isotonic or buffered saline solutions after moderate haemodilution. However, in studies in which extent of dilution were kept constant while varying diluents' solute concentration, results obtained with TEG have ranged from hypercoagulability (Luostarinen et al., 2011; T. G. Ruttmann et al., 1996; Tan et al., 2002) to hypocoagulability. Therefore, even without the current study results, there is evidence of lack of consistency.

The mechanistic theory of antithrombin reduction on haemodilution leading to procoagulant effect (K. F. J. Ng et al., 2002; K. F. Ng & Lo, 1996; T. G. Ruttmann et al., 1996) focused on measurement of antithrombin before and after intervention, and addition of exogenous antithrombin attenuated the procoagulant state (Ruttmann, 2001). However, in another study, direct measurement of thrombin and TEG parameters lacked correspondence to antithrombin levels (Szlam, 2008. Furthermore, 20% saline dilution was still hypercoagulable compared to control in antithrombin deficient plasma samples (Nielsen et al., 2006). This suggests that even in the absence of antithrombin, saline still has an effect.

Since at uniform dilution AT would be expected to be equal, heterogeneity of findings therefore suggests mass reduction of antithrombin levels may not be the critical factor influencing

coagulation outcome in the face of changing diluents' concentration. With the current study findings in the perspective of previous studies, the concept of haemodilution induced hypercoagulability based on reduction of only antithrombin would require revision.

4.5.3. Haemodilution and Calcium

The coagulation test results in the current study were dependent on solute type and concentration leading to the conclusion that changes in calcium levels played no or minimal role in the results. This is because in the current study, all samples were diluted to 20% and therefore, accordingly plasma calcium levels would be expected to be reduced by similar magnitude in all blood samples.

Besides haemodilution, some researchers have suggested that reduction in plasma calcium could be responsible for observed coagulation differences between saline based and balanced salt crystalloid solutions (Martin et al., 2002). However, this is unlikely to be the case in this study since 20% saline haemodilution does not reduce calcium levels to below the threshold to support coagulation (Nielsen et al., 2006). This position is supported by experimental findings that hypocoagulability is only evident when calcium levels fall below 0.56 mmol/L (Calatzis et al., 2001; James & Roche, 2004). This fact is reinforced by studies that conducted haemodilution at 20% and 40% and found that calcium levels were all above 0.9 mmol, but saline based caused more coagulation derangements than balanced salt solutions (Roche, 2006). Even when calcium supplementation was done to saline based crystalloid solutions, the differences still persisted (Anthony M. Roche et al., 2006b). This suggested that, since calcium has a ceiling effect (Calatzis et al., 2001; James & Roche, 2004), coagulation outcome results may also be influenced by other solutes apart from calcium.

The effects of crystalloid haemodilution on coagulation obtained in this study does not support hypothesis that haemodilution induced hypocoagulability arises from reduced calcium, or influence of recalcification on the clotting process. Evidence refuting this hypothesis include data from a study (Darlington et al., 2011) derived from *in vitro* haemodilution with a number of solutions such as isotonic saline, Ringers lactate, albumin and starches in saline. In the cited study, while calcium levels linearly decreased with haemodilution, PT, aPTT, TEG (R, K, alpha and Ma) were curvilinear and showed hypercoagulability. Changes due to hypocoagulability began on extreme haemodilution from 50% and above. It is to be noted that calcium levels at 20% dilution changed from normal of 1.3 mMol to 0.9 mMol. At this level, coagulation was normal and consistent with other studies (A. M. Roche et al., 2003; Anthony M. Roche et al., 2006a, 2006b) (Calatzis et al., 2001).

4.5.4 Haemodilution and Chloride Ions

In this study, increases in ionic strength corresponded with prolongation of time to clot formation in routine coagulation tests. Further, crystalloid solutions with containing chloride ions had the most effect. These strongly suggest specific effect of chlorides on the coagulation reactions. Therefore conclusion of chloride chaotropic activities is consistent with previous studies.

Since it is well established that calcium levels increase exponentially with recalcification of citrated haemodiluted samples (Nielsen et al., 2006), another ion that also increases is chloride. The increase in chloride is twice the calcium levels when calcium chloride is added. Logically, increase in calcium chloride beyond the coagulation threshold, would disproportionately increase chloride beyond its normal plasma levels. At these supraphysiological levels chloride may begin

exerting its chaotropic effects. However, further empirical studies are warranted to conclusively confirm this position.

4.5.5 Haemodilution and Citrate Anticoagulation

All the routine coagulation tests in this study were conducted in plasma anticoagulated with citrate. While solutions of NaCl demonstrated linear increase in clotting time with increase concentration, clotting time remained unchanged in either mannitol or dextrose despite increase in concentration. Thrombelastographic evaluation of non-citrated blood samples showed hypocoagulabity compared to citrated samples. These illustrate the influence of citrate on coagulation, which unfortunately is not evident in routine coagulation tests. Citrate is a Kosmotrope that promotes blood coagulation, although a weak one. This puts into question the physiological relevance of coagulation tests performed on citrated blood samples. However, use of corn trypsin anticoagulation in coagulation studies that bypasses citration (K. G. Mann, Whelihan, Butenas, & Orfeo, 2007) has not become standard practice.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This study aimed to investigate the effects of crystalloid solutions on the human coagulation system in vitro. Various well validated coagulation tests were employed. Solutions of different solute types at different concentrations were tested. The results indicate the following novel findings:

i. Increasing concentrations of NaCl displayed positive linear relationship with kinetic clotting tests. In comparison with other solutes, ionic strength was the most important determinant, and presence of chloride ions prolonged clotting time more than other ions. In general, the pattern of solutes on the clotting time fits within the Hofmeister series: mannitol, dextrose and gluconate classified as kosmotropes do not influence clotting time even at high concentrations; chloride being a strong chaotrope and therefore in keeping with its lyotropic effects increases clotting time dose-dependently; Although Na+ and choline cations are classified as kosmotropes and chaotropes respectively, they have weak effects and therefore effects are overridden by corresponding anions.

ii. The effect of NaCl concentration on thrombin generation was parabolic, greatest effect observed around 3% w/v. This is consistent with Na+ allosteric effect on thrombin activity. However, thrombin generation was reduced in the intrinsic activation earlier than extrinsic system, owing to possible suppression of second wave of prothrominase on intrinsic tenase assembly. Suppression of thrombin generation by NaCl concentrations of 5% or higher, but which are corrected by

higher TF concentrations, strongly suggest inhibition of intrinsic Tenase (FXa) generation not affecting extrinsic (FVIIa) activation. Possible explanations include chloride electrostatic hindrance with negatively charged Gla domains of FXII, FXI and FIX. Mannitol is a kosmotrope which explains its minimal effects on the thrombin generations at the concentrations tested.

iii. Thrombelastography demonstrated whole blood coagulation was enhanced with increase in crystalloid concentration, reaching its peak around a concentration of 600 mOsm/L NaCl, and thereafter is impaired. The trend was replicated by all ionic crystalloid irrespective of solute type. All crystalloids dose-dependently reduced clot strength. For those samples where coagulation was impaired by high concentrations of crystalloids, supplementation with clotting factors revealed different sensitivities. While thrombin effects were consistently and mostly on R, fibrinogen supplementation were most and consistent on increasing MA but weak on R. However, effects of either factor supplementation were weak in 50% dextrose. A number of deductions can be made from these results, namely:

(a) Presence of cellular elements (RBC and WBC) further enhances coagulation which modulates role of crystalloid fluid osmolality, greatest at 600 mOsm/L but thereafter impaired

(b) The pattern of effects of crystalloids on whole blood coagulation as determined by TEG does not appear to follow Hofmeister series

(c) Neither thrombin nor fibrinogen concentrates alone fully restores impairment of coagulation resulting from high diluents concentrations

(d) Thrombelastography appears to be more informative than either routine coagulation tests or thrombin generation in investigation of effects of diluents on coagulation.

iv. Platelet aggregation revealed that crystalloids dose-dependently reduced platelet function irrespective of solute type. The pattern of effects cannot be predicted from Hofmeister series. The impairment was a function of osmolality rather than ionic strength; the effects of solute type were not significant. This demonstrates that in acute exposure to hypertonic solutions, platelets act as osmosensors.

5.2 Recommendations from this Study

5.2.1 Recommendations for clinical practice

i. To maintain integrity of human coagulation system, iso-osmolar crystalloid solutions should be recommended for resuscitation fluids.

ii. In analysis of human blood coagulation following crystalloid haemodilution, combination of coagulation tests should be employed, and tests performed in whole blood. In this respect, Thrombelastography should be a better option as it provides more information than the other tests performed on isolated blood components.

iii. Hypertonic solutions are to be avoided since they dose dependently inhibit platelet aggregation.

5.2.2 Recommendations for Policy

(i) Standards and regulatory authorities should limit iso-osmolar crystalloid solutions in resuscitation fluids to within the physiological range to avoid untoward effects on the human blood coagulation system.

(ii) Protocols guiding safety infusion of crystalloid solutions on the human coagulation system should include plasma electrolyte testing alongside the usual coagulation tests.

(iii) In correction of hypocoagulability induced by hypertonic crystalloid solutions, a combination of fibrinogen and thrombin factor concentrates as well as platelets should strongly be considered in trauma resuscitation guidelines.

5.3 Recommendations for Future Work

This study demonstrates our incomplete understanding of the effects of crystalloid solutions on coagulation and therefore, the following further research is recommended to determine:

(i) The effects of crystalloid solutions on blood clot morphology, and correlation with the clotting tests employed. This will help explain interaction of crystalloid solutes on blood clot structure in relation to resistance to fibrinolysis or bleeding.

(ii) The effects of the crystalloid solutions on plasma electrolyte changes and correlation to human coagulation, and whether the coagulation laboratory test results could be replicated *in vivo*.

(iii) Expression of procoagulant molecules such as tissue factor and phosphatidyle serine on cellular elements such as RBC, monocytes, leukocytes and platelets when haemodiluted by crystalloid solutions to further interrogate their relation to coagulation

(iv) Effect of crystalloid solutions on formation of nuclear extracellular traps (NETs) as contributors to hyper/hypocoagulability

(v) Effects of crystalloid solutions on the formation of procoagulant microparticles.

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APPENDICES

Appendix 1: Subject/ patient information and consent explanation form

5.1.1 Statement of consent

Title: The effect of crystalloid solutions on human coagulation system

Background information:
Crystalloids are solutions of small molecules commonly employed to restore and/or maintain blood volume following blood loss or shock. They are also used to dilute blood and clean apparatus during laboratory procedures. Examples include solutions of common salt (saline), glucose and others varying in concentrations and mixtures of constituents. Since they are introduced into the blood stream, the dilutional consequences may interfere with normal blood clotting mechanisms, either leading to delayed or accelerated clot formation. Though useful, little is known about the effect of the individual constituents or concentration on the blood clotting systems in humans.

5.1.2 Purpose of study
The major aim of this study is to find out how the crystalloid interact with the normal blood clotting system of humans when the nature of solutes and their concentrations are varied under laboratory conditions, and also what happens to the blood coagulation in cases of body salt and sugar derangements. The tests to be employed involve testing for time to clot formation, strength and morphology of clots formed. This will help our understanding of the interaction of the crystalloid solutions with the blood clotting system and thereby lead to better treatment strategies.

5.1.3 Your role as a study participant
You will only be required to consent and provide blood specimens voluntarily without coercion or intimidation. No further demands will be made. The provided blood samples will be processed as per the study protocol and discarded according to the public health regulations.

5.1.4 Rights as a participant
You can refuse to consent or withdraw from the study at any time without penalty or discrimination to you. You have a right to explanation about the study aims and what test will be done on the sample of blood provided. Confidentiality will be maintained at all times. You have a right to be treated fairly and with human dignity at all times, without tricks or deception.

5.1.5 Risks involved
No risks are known except pain at venipuncture

5.1.6 Benefits as a participant
You will be given a copy of the Haemoglobin test results on blood samples collected from you at no cost.

5.1.7 Consent declaration

I have been informed about the nature of the research and risks involved. I have had a chance to ask questions which have been answered to my satisfaction. If I have further clarifications about the study, am permitted to ask questions at any stage. I have been given no guarantee about the outcome. My participation is voluntary without any coercion whatsoever. I am at least eighteen years of age, having been born _____. I understand also that I may withdraw from the study at any time or refuse to answer a particular question without penalty.

By appending signature in this document is evidence of my willingness to participate and follow all instructions as prescribed during the study period.

Name of subject/Guardian: _____Signature:_____

Name of person obtaining consent

Name of witness: _____Signature:_____

Date: _____Time:_____

The above document is to be signed in duplicate, with one copy being kept by the researcher and the other by the subject.
Note:
If you have any questions about the study and your rights as human subject, do not hesitate to ask Principal researcher (Dr Ogweno), whose phone number is: 0725 715623 or 0733716152
Or
Lead Supervisor Prof NK Gikonyo -0722763186

Or

Chairman/ Secretary of KNH/UON Ethical research Committee Tel: +254 (20)726300-9

Appendix 2: KNH/UoN ERC Approval

Appendix 3: Graduate School Proposal Approval