

Modified Thromboelastography and its Variables: Platelets, Fibrinogen and Erythrocytes.

Kesav Jagadeesan¹, J. Kabalimurthy², K. Ramadevi³, N. B. Venkataraman⁴

¹ PhD. Research Scholar, Annamalai University and Consultant Surgeon,
K. J. Hospital, Chennai, Tamilnadu, India

²Professor of Surgery, RMMCH, Annamalai University.

³Director, Professor & Head of Institute of Biochemistry, Madras Medical College, Chennai, Tamilnadu, India

⁴Interventional Consultant Cardiologist, Vasantham Hospital, Nagercoil, Tamilnadu, India

Abstract

Thromboelastography (TEG) has been reported to be useful to detect impaired platelet function in patients undergoing cardiac surgery with cardiopulmonary bypass (CPB), to predict excessive microvascular bleeding (EMB), guide platelet transfusion and administration of desmopressin acetate (DDAVP). As the conventional TEG has a test completion time (TCT) of greater than 30 mins we had earlier reported a modified TEG (TEG-rTT) which was activated by 0.025% of recombinant tissue thromboplastin (rTT) to shorten the TCT to 7 mins. However, TEG-rTT is known to be affected by decrease in concentrations of platelets and coagulation factors including fibrinogen. Therefore in this work we examined the *in vitro* effects of varying reductions of platelets and fibrinogen and their interactions, as well as decreases of red blood cells concentrations on TEG-rTT. The results showed that the elastic shear modulus (G) increased progressively with increase in platelet concentration (10,000 – 350,000/ μ L) with TEG-rTT. While the onset of clotting or reaction time (R) was unaffected, the effect of platelet concentration on G value was blunted progressively with decreasing fibrinogen concentration particularly at concentrations less than 200mg/dl. Also G value increased with decrease in RBC concentration. One possible explanation for the increased G values obtained in the TEG-rTT is based on the presence of tissue factor receptors on platelets. TEG is a low shear stress system where there is increased platelet-platelet interaction when compared to platelet aggregation studies which is a high shear system. The binding of von Willebrand factor (vWF) to the glycoprotein Ib receptors on platelets is increased in a high shear system leading to lower G values than TEG-rTT. To the best of our knowledge we are the first to report the effect of RBC on TEG. The findings of this study demonstrated that severe reductions in platelet, fibrinogen and RBC concentrations which occur in patients undergoing CPB will have an impact on G value measured by TEG-rTT.

Keywords: TEG-rTT, Platelet function, Fibrinogen, RBC, Tissue factor

Introduction

Cardiopulmonary bypass (CPB) induces several complex disturbances in the coagulation and fibrinolytic systems [1,2]. Among the major haemostatic abnormalities with CPB are thrombocytopenia, transient platelet dysfunction and prolonged prothrombin time (PT) largely as a result of coagulation factor deficiencies due to hemodilution and hypothermia, particularly with prolonged CPB time and

accelerated intravascular coagulation [3,4]. Multiple qualitative defects including decreased aggregation and adhesion, reduced binding of fibrinogen and depletion of α granules have been reported to result from CPB [5,6]. Routine coagulation tests are unable to assess such major haemostatic disturbance arising due to CPB like alteration of interaction between the coagulation cascade and platelet surface caused by altered platelet function. During CPB, coagulation function and heparin activity are monitored routinely using activated clotting times (ACT) [7]. Although rapid and easy to use, ACT can assess coagulation only upto the time of initial fibrin formation and does not reveal information about platelet-fibrin interactions, clot retraction or clot lysis, all such aspects of coagulation which are affected by CPB.

Address for Correspondence

Dr. Kesav Jagadeesan, Department of Surgery, K. J. Hospitals, 182, PH Road, Chennai - 600084, Tamilnadu, India Mobile: +91 996277795
E-mail: drkesav@yahoo.com

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TEG is a point-of-care (POC) method which enables a complete evaluation of the process of clot initiation and structural characteristics of the formed clot and its stability [8]. Some researchers have undertaken work on the efficacy of monitoring coagulation with TEG and reported it to be a useful tool in the management of haemostasis during major surgical procedures and in critically ill patients in the intensive care unit. TEG is a low shear stress flow system which involves limited transport of platelets leading to an increase in platelet concentration at the cup wall (platelet diffusivity) and therefore a low incidence of platelet collisions. This is likely to limit its sensitivity to detect abnormalities of vWF with its platelet receptors and platelet prostaglandin metabolism.

Among the TEG variables the reaction time (R) value is the time from the start of the assay to the beginning of clot formation. It is measured from the start of the tracing to the point where the curve is 1mm wide and is similar to whole blood clotting. Prolongation of R value is associated with clotting factor deficiencies or inhibitors and anticoagulants such as heparin. The R value may be prolonged in thrombocytopenia as platelets provide the phospholipid surface for coagulation reactions. The maximum amplitude (MA) is the width of the curve at its widest point and represents the maximum clot elastic shear modulus (G) attainable. It is a function of platelet count and fibrinogen level which are the structural components of the clot. Thrombocytopenia, hypofibrinogenemia and very long clotting times associated with severe factor deficiencies or heparin therapy will also reduce the MA. The elastic shear modulus (G) which is a measure of clot strength is calculated by substituting the MA value in the formula $G = [(5000 \times MA) + (96 - MA)]$. The G value increases exponentially in proportion to the amplitude of the thromboelastograph tracing and is affected by changes in platelet and fibrinogen levels and their interactions at low concentrations. To the best of our knowledge the effect of RBC on TEG has not been examined by researchers till now.

Researchers have previously used different types of activators to shorten the TCT of TEG [9,10]. Our earlier findings showed that employing rTT as an activator at a low concentration of 0.25% was optimum for shortening the TCT of TEG upto 7mins which was well below the TCT of conventional TEG (30 mins) [11]. However there are some reports that suggest even with modified TEG the effects of reduction in platelets and fibrinogen are not eliminated [12,13]. Hence this study was designed to explore the potential usefulness of the modified TEG (TEG-rTT) as a POC test for CPB associated platelet dysfunction by simulating *in vitro* blood alterations commonly encountered in patients undergoing cardiac surgery. In this work we examined the effects of varying concentrations of platelets,

fibrinogen and RBC on TEG variables R and G as well as the interactive effects of low concentrations of platelets and fibrinogen.

Materials & Methods

The study protocol was approved by the Institutional Ethical Committee of K.J Research Foundation, Chennai, India and written informed consent was obtained from all volunteers. Blood was collected from four healthy adult volunteers in the age group of 30 to 45 years in each study group. Subjects with known history of bleeding abnormalities, receiving any medications for the past 2 weeks including nonsteroidal anti-inflammatory drugs (NSAIDs), aspirin, aspirin like effect drug and oral contraceptives were excluded from the study.

Based on our previous work [11] 0.025% rTT which was the lowest concentration that yielded maximum G in normal citrated platelet rich plasma (C-PRP) at a concentration of 250,000/ μ l was selected as the amount for activating TEG in this study. Citrated platelet free plasma (C-PFP) was prepared by centrifugation of C-PRP at 12,000 g for 3 minutes using an Eppendorf microcentrifuge. C-PRP with different platelet concentrations was obtained by mixing different volumes of the initial C-PRP and C-PFP. The effects of fibrinogen and platelet concentrations were examined by centrifugation of ACD-PRP at 12,000g for 3 minutes and resuspending the platelet pellets immediately in mixtures of either C-PFP and afibrinogenemic plasma (George King Bio-Medical, Overland Park, KS) or C-PFP to which different amounts of human fibrinogen (Sigma Chemical Co, St Louis, MO) has been added. Fibrinogen concentration in the initial plasma and final plasma mixtures was measured by Clauss assay [14] using an automated clot timer (STA, Diagnostica, Asinieres, France).

The effect of RBC concentration on TEG variables was examined by adding different amounts of packed RBC (prepared by differential centrifugation of ACD-WB from which PRP had been removed at 450 g for 10 minutes at room temperature) to C-PRP containing a standard platelet concentration of 250,000/ μ l. Briefly the procedure consisted of mixing aliquots of packed RBC prepared from ACD-blood by differential centrifugation with aliquots of C-PRP to yield the desired RBC-PCV (% packed cell volume). The PCV and platelet concentration in the mixtures were determined by electronic cell counting (Sysmex Haematology Analyzer). Wright stained smears of reconstituted blood mixtures were examined for platelet clumping. Visual examination of plasma from reconstituted blood mixtures showed no haemolysis. TEG-rTT was prepared on aliquots of the mixtures using 320 μ l of reconstituted blood, 20 μ l acid soluble bovine collagen (Sigma Chemical Co, 100 μ g/ml) 10 μ l rTT and 10 μ l of 0.6M CaCl₂ as reported earlier.

Statistical Evaluation

All results were expressed as mean \pm standard deviation and compared by analysis of variance provided by the computerised software package InStat, version 3.0. Values with $p < 0.05$ were considered significant.

Results

Effect of Platelets

The results of experiments designed to assess the effect of varying platelet concentrations in C-PRP on TEG-rTT are shown in Figure 1. G is used as actual measure of clot firmness (shear elastic modulus strength) and is measured in dyne/cm^2 . The G value increases exponentially in proportion to the amplitude of the tracing. There was a continuous linear increase in G with increasing platelet concentration upto 150,000/ μL beyond which the curve plateaued and showed a much slower rate of increase upto a maximum of 350,000/ μL studied, which was consistent among the four donor platelet preparations. The maximum G value obtained at each platelet concentration among the different donor platelet preparations was not related to the plasma fibrinogen concentration of that donor sample. There was no effect of platelet concentration on R values.

Effect of Fibrinogen

Figure 2 shows the results of experiments designed to examine the interactive effects of varying concentrations of fibrinogen and platelets on G value. Abnormally low fibrinogen concentrations ($< 100\text{mg/dl}$) and platelet count between 50,000 and 150,000/ μL had no significant effect on G measured by TEG-rTT. With increasing platelets concentrations above 150,000/ μL the effect of fibrinogen at

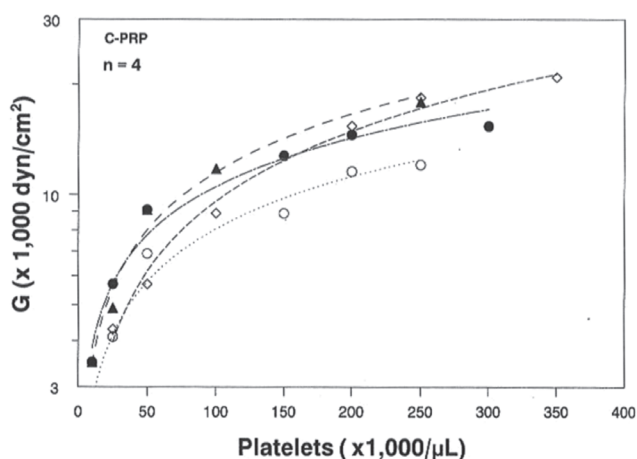


Figure 1: Effect of platelet concentration on shear elastic modulus (G) value of TEG-rTT

lower concentrations on G values was minimal. However, with increasing fibrinogen concentration throughout the normal range and moderately elevated fibrinogen concentration (600mg/dl), the effect of increasing platelet concentrations on G was progressively magnified. R values progressively decreased with increasing fibrinogen concentration independent of platelet concentration (data not shown).

Effect of Erythrocytes

The results of experiments designed to assess in detail the effects of RBC on G and R of TEG-rTT, are presented in Figure 3. The G values progressively increased with decreasing RBC concentration throughout the range examined (50-0% PCV). However at higher haematocrit values (30-50% PCV) a modest decrease in platelet concentration was observed in the reconstituted C-WB, presumably due to a dilution effect induced by plasma carry-over with the addition of packed RBC during blood reconstitution, which may have contributed to the decrease in G values. However, R values remained unchanged and independent of RBC concentration in these experiments. The addition of collagen had no significant effect on R and G values with variable hematocrit values.

Discussion

Several tests have been clinically investigated with regard to their potential usefulness in detecting blood loss in CPB patients. The template bleeding time (BT) which is an *in vivo* test should be practically suitable as it is sensitive to abnormalities affecting the formation of the initial platelet plug following small vessel injury (primary hemostasis) and generally not affected by even severe abnormalities of blood coagulation. Indeed, in several studies the

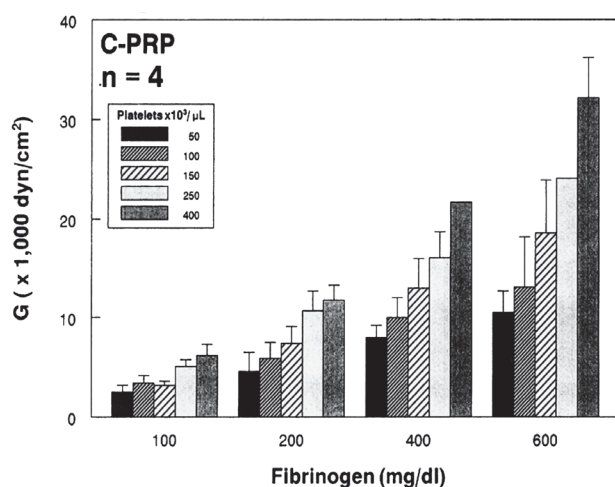


Figure 2: Effect of fibrinogen concentration on G value of TEG-rTT at different platelet concentrations. At each fibrinogen concentration, the effect of five different platelet concentrations was studied.

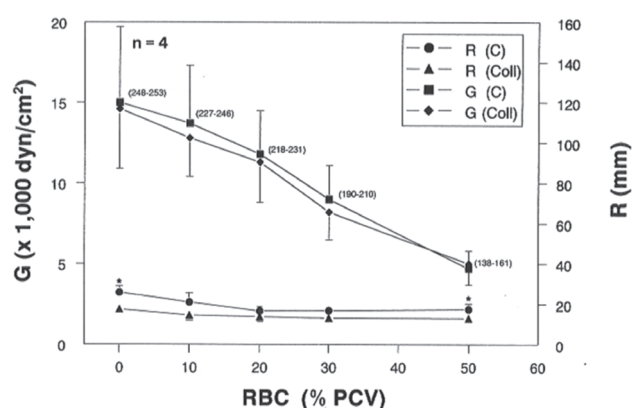


Figure 3: Effect of RBC on G and R values of TEG-rTT with and without the addition of collagen. The numbers in parenthesis indicate the range of platelet concentration in the reconstituted blood mixture.

postoperative template BT was shown to be predictive of post-operative blood loss [4,15-17] and response to platelet transfusion and infusion of DDAVP in CPB patients. In one study, [18] where no correlation between postoperative bleeding time and blood loss was found, the older Duke ear lobe bleeding time test was used and was thought to be less sensitive and reproducible than the more standardized template bleeding time. However BT, a seemingly simple test is time consuming and is affected by numerous analytical variables. Hence it must be performed by highly skilled and experienced individuals. It is also very difficult to perform this test in patients undergoing surgery. There was one report that postoperative whole blood impedance aggregometry did not correlate with postoperative blood loss. A more recently developed POC test, the HemoSTATUS® is thought to measure predominant changes in platelet activating factor induced availability of platelet procoagulant activity [19]. This test was found to be useful in predicting postoperative blood loss, platelet transfusion requirements and response to DDAVP [20, 21]. However these findings were only partially confirmed by some [22,23] and not confirmed by others [24].

TEG which has emerged as useful tool in the management of haemostasis during major surgical procedures has the drawback of a prolonged TCT. Our earlier findings showed that employing rTT as an activator at a concentration as low as 0.025% rTT was optimum for shortening the TCT of TEG (7 mins) which was well below the TCT of conventionally used inactivated TEG (>30 mins) [11]. The impact of varying platelet, fibrinogen and RBC concentrations and platelet-fibrinogen interactions on the R and G values of the 0.025% rTT activated TEG was probed in this investigation. Transient impairment of platelet function is one of the several important hemostatic abnormalities associated with cardiac surgery involving CPB which may contribute to CPB associated EMB.

Availability of TEG-rTT as a reliable POC test to detect CPB associated platelet dysfunction would be important in the early identification of EMB or patients likely to develop EMB during surgery. This would be highly beneficial for making judicious decisions about the need for transfusion of platelets and/or DDAVP to the patient. By this it is possible to avoid administration of these expensive and potentially risky agents to patients with surgical cause for bleeding requiring re-exploration, or having major coagulation defects requiring administration of plasma and/or cryoprecipitate.

Although, it has been suggested that TEG can help in limiting or directing the appropriate use of blood products during surgery, the contribution of platelets to the TEG profile has not been studied in detail. In TEG which is a low shear system the mechanism of platelet aggregation and adhesion is by fibrinogen GP IIb/IIIa receptor. In moderately high shear conditions the fibrinogen and von Williebrand factor via GPIIb/IIIa and GPIb receptors is mechanism for platelet aggregation and adhesion. Whereas in a very high shear system the aggregation mechanism is primarily through vWF via GPIb receptor [25,26]. Among the variables measured by TEG-rTT, MA is an overall estimate of platelet-fibrin interaction of high relevance and is superior over other hemostatic tests. It is postulated that MA is influenced by three different factors which include platelet function, platelet count and to a lesser extent fibrinogen concentration. Therefore reduction of MA may result from platelet dysfunction, from low platelet count or from low fibrinogen concentration. Few groups who studied the effect of platelet count on TEG parameters have reported that there was a linear correlation between platelet count and MA values [27,28]. The present study which was carried out to study the effect of platelet count demonstrated that there is a clear interaction between platelet count and G value which was in turn calculated from the MA value given by the TEG-rTT. This study therefore suggests that platelet count has to be considered while interpreting G of TEG-rTT in patients with complex haemostatic disorders. The G parameter of TEG-rTT may be very useful for assessing platelet transfusion requirements.

RBCs have a procoagulant and prothrombotic potential by influencing their effect on fibrin network formation, mechanical properties of clot, fibrinolytic resistance and clot retraction. RBCs can play a dual role by helping to form a platelet plug to stop bleeding at the same time producing thrombosis through various mechanisms. The main advantage of TEG compared to other conventional tests of hemostasis (BT, PT, aPTT, D-Dimer) is its ability to assess in *ex vivo* using a whole blood sample with many of the *in vivo* components that play a role in the formation of the hemostatic plug i.e., RBCs, white blood cells, platelets, clotting

factors, fibrinolytic proteins. Since RBCs are the major component of blood, the hematocrit (HCT) is a main factor that influences blood viscosity. High hematocrit levels cause high shear rates and therefore platelet-platelet and platelet-RBC collision increases thereby producing platelet aggregation [29]. Flow and/or rheological conditions of the blood affect coagulation and TEG evaluates the viscoelastic properties of the clot [30]. Therefore, the effect of HCT on TEG parameters was also evaluated in order to correctly interpret the TEG tracings in patients with high or low HCT values in conditions like blood loss anemia and disseminated intravascular coagulation. One group concluded in canine studies that HCT showed a strong correlation with some of the TEG parameters when platelets count was within the normal limits [31]. Our study showed that clot strength was weaker as the HCT concentration was increased (hypercoagulable state). We also observed that an anaemic state produced prolongation in the rate of clot formation. Therefore, HCT values should be addressed in order to obtain a correct interpretation of the TEG tracings. This explains why in severe anemia where the rheology of blood flow alters the centripetal flow of RBC within a vessel wall thereby decreasing the platelet-platelet interaction leading to a weak fibrin clot. Although few studies in dogs have been carried out [31,32] to the best of our knowledge we are the first to report the effect of RBC on R and G values of TEG.

Conclusion

The modified TEG with a reduced TCT of 7 mins reported earlier by us can be used for measurement of platelet function without loss of sensitivity to changes in concentrations of platelets, fibrinogen and RBC. While platelet concentration had no impact on the reaction time, the shear elastic modulus of TEG-rTT increases progressively with increasing concentrations of platelets with major changes occurring at platelet concentrations below 100,000/ μ l. Increases in the G values with increasing platelet concentration are attenuated with decreasing concentration of fibrinogen and increasing concentration of RBCs. Hence TEG-rTT will be useful in the evaluation of platelet function during and post cardiac surgery if haematocrit and concentrations of platelets and fibrinogen are determined simultaneously. Studies to determine the effect of platelet activating factor (PAF) and platelet inhibitors like aspirin, c7E3 (ReoPro \ddot{a}) on TEG-rTT parameters have been assessed and will be reported later.

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Ethics:	There is no ethical violation as it is based on voluntary anonymous interviews
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Guarantor:	Dr. Kesav Jagadeesan will act as guarantor of this article on behalf of all co-authors.

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