

Assessment of Antiplatelet Agents and Platelet Activating Factor on Modified Thromboelastography

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ABSTRACT

Thromboelastography (TEG) is a powerful technique for the prediction of coagulation disorders in a wide variety of clinical settings and offers many advantages over conventional blood coagulation tests. We have earlier reported a modified TEG activated with recombinant tissue thromboplastin (rTT) (TEG-rTT) with relatively shortest test completion time (TCT) than standard TEG system (TEG-0) and commercially used celite activated TEG (TEG-Celite) system. The TEG-rTT was found suitable for the measurement of platelet function without loss of sensitivity to changes in concentrations of platelets, fibrinogen and RBC in blood. In this work the sensitivity of TEG-Celite, TEG-rTT and TEG-0 with and without the addition of platelet agonist collagen in detecting strong platelet inhibition by c7E3 Fab (ReoPro[®]) (GPIIb/IIIa receptor blockade) in citrated platelet rich plasma (C-PRP) and a weak platelet inhibition by aspirin (ASA) (platelet thromboxane A² suppression) was evaluated in C-PRP and citrated whole blood (C-WB). The effect of platelet activating factor (PAF) on the sensitivity of TEG-rTT in C-PRP was also determined. The results demonstrated that with TEG-rTT neither G nor R was affected by ASA in concentrations that completely inhibited platelet aggregation (PAG) induced by 2.5mM arachidonic acid (AA) in C-WB or C-PRP containing normal or mildly decreased platelet concentrations. Also the addition of collagen did not produce any significant change in G value but it reduced the R value which was statistically significant. In both PRP and C-WB, 200µM (FC) of ASA had no significant effect on R or G values in both the presence and absence of collagen. With TEG-0, TEG-Celite and TEG-rTT, G was moderately sensitive to inhibition by the strong platelet inhibitor c7E3 Fab (ReoPro[®]) as compared to PAG induced by 5mM Adenosine diphosphate (ADP) while R was not affected by the same. PAG and 14C-serotonin (5HT) release were affected by increasing concentrations of PAF whereas it had no effect on TEG-rTT. The various activated or conventional TEG systems are sensitive only to detect moderate to severe platelet dysfunction and they are unable to detect mild platelet disorder.

Keywords: TEG-rTT, Aspirin, c7E3 Fab (ReoPro[®]), platelet activating factor, Tissue factor

Introduction

Transient impairment of platelet function is one of several important hemostatic abnormalities associated with cardiac surgery involving cardiopulmonary bypass (CPB) which may contribute to excessive microvascular bleeding (EMB) [1-3]. Availability of a reliable point-of-care (POC) test to detect CPB associated platelet dysfunction is very important for the early identification of patients with EMB or those likely to develop EMB so that they can benefit from transfusion of platelets and/or desmopressin (DDAVP) [4,5]. Patients with major coagulation defects requiring administration of plasma and/or cryoprecipitate will also benefit immensely if a rapid and sensitive POC test is available to assess platelet function during cardiac surgery [6,7]. It will also help to avoid administration of these expensive and potentially risky agents in patients with a surgical source of bleeding requiring re-exploration [8,9].

One criteria for such a POC test is that it should (1) have a short (< 20 minutes) TCT even with abnormal results (2) be sensitive to detect moderate to severe platelet dysfunction

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associated with CPB (3) not be affected by ASA (4) not be affected by mild to moderate thrombocytopenia and/or anaemia (5) be reasonably robust and reproducible and (6) be reasonably inexpensive. TEG is one such non-invasive test that provides quantitative measurement of the ability of whole blood to clot [10,11]. Disturbances in haemostasis and coagulation is indicated by deviation of TEG parameters from reference values [12,13]. We have earlier reported a study which to our best of knowledge is the first modified TEG system that used rTT as the activator at a low concentration of 0.025% w/v [14]. When compared with TEG-0 and commercially available TEG-Celite system, the TEG-rTT had a reasonable TCT of 7 mins for detecting coagulopathies as compared to the longer turnaround time of standard laboratory tests and other TEG activated systems. Since rTT activates the clotting mechanism via the physiologic pathway unlike commercial activators like celite, it will be very useful for the accurate diagnosis of haemostasis abnormalities during cardiac surgery with CPB. The findings that 0.025% of rTT activator shortened the rate of clot formation (R) and increased the elastic strength of the clot (G) significantly when compared to unmodified TEG indicated that the 0.25% rTT-TEG will enable the robust assessment of both qualitative and quantitative function of platelets and fibrinogen.

We also investigated the potential usefulness of the TEG-rTT as a POC test for CPB associated platelet dysfunction by simulating in vitro blood alterations commonly encountered in patients undergoing cardiac surgery. 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 were analysed. Based on the results we reported that the TEG-rTT was suitable for measurement of platelet function without loss of sensitivity to changes in concentrations of platelets, fibrinogen and RBC [15].

However there are conflicting reports as to whether TEG is sensitive to ASA-induced platelet dysfunction [16-19]. The aim of this work was to determine the sensitivity of (i) TEG-Celite; (ii) TEG-rTT; and (iii) TEG-0 with and without the addition of platelet agonist collagen in detecting strong platelet inhibition (GPIIb/IIIa receptor blockade by c7E3 Fab (ReoPro®)) and a weak platelet inhibition (aspirin induced platelet thromboxane A² suppression).

ASA is an antiplatelet drug used in preventing coronary artery disease (CAD). It acts by blocking cyclooxygenase pathway and inhibiting the conversion of arachidonic acid to thromboxane A² thereby preventing platelet aggregation. In order to mimic mild platelet dysfunction, the blood samples were spiked with known concentration of aspirin and the effect on TEG parameters were investigated.

c7E3 Fab (ReoPro®) also known as abciximab is a monoclonal antibody which binds to the glycoprotein IIb/IIIa-receptor of platelets [20,21]. The platelet IIb/IIIa-receptor blockade by c7E3 Fab (ReoPro®) inhibits platelet activity and prevents platelet aggregation [22,23]. Hence in this work c7E3 Fab (ReoPro®) was utilised to mimic strong platelet dysfunction and its effect on TEG parameters were studied.

We also studied the effect of platelet activating factor (PAF) on TEG-rTT parameters. PAF is a phospholipid which produces potent aggregation of platelets indicating platelet aggregate response. It acts as a stimulant and can provide a measure of platelet activity. HemoSTATUS^a device is used widely to monitor coagulation during cardiac surgery with CPB and it utilises PAF as an agonist for activation of blood coagulation [24]. There are no previous studies on the effect of PAF on TEG parameters. Hence we utilised PAF in various concentrations to assess its effect on R and G values of TEG-rTT.

Materials and Methods

The protocol for this study was approved by the Institutional Ethical Committee of K.J. Research Foundation, Chennai, India and was carried out after written informed consent was obtained from all volunteers. Each study group consisted of four adult volunteers in the age group of 30 to 45 years from whom blood samples were collected. Subjects with known history of bleeding abnormalities, receiving any medications like nonsteroidal anti-inflammatory drugs (NSAIDs), aspirin / aspirin like effect drug or oral contraceptives for the past two weeks were excluded from the study.

In this work 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 based on our previous report [14]. Citrated platelet free plasma (C-PFP) was obtained by centrifuging C-PRP at 12,000g 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.

ASA (Sigma Chemical Co, St. Louis, MO) solution was prepared by dissolving equimolar amounts of ASA and NaHCO₃ to yield a concentration of 40mM, adjusting the pH to 7.34 with 1M HCl. Then 20mL of this freshly prepared ASA solution or saline (control) was added to 4 ml of citrated whole blood (C-WB) or C-PRP (250,000 and 100,000 platelets/mL) to yield a final ASA concentration of 200mM. This concentration of ASA completely inhibited platelet aggregation induced by 2.5mM (FC) of AA (Helena Laboratories, Beaumont, TX) which was examined using a turbidimetric platelet aggregometer (PAP-4, BioData, Horsham, PA).

The effect of different concentrations of c7E3Fab (ReoPro®) (Abciximab, Centocor, Malvern, PA) on TEG parameters were analysed and were also examined simultaneously using PAG induced by 5mM ADP (Sigma Chemical Co, St Louis, MO). PAG study with agonist ADP (1.25 and 2.5µl/ml) was performed using a Chronolog platelet aggregometer.

Recombinant human lyophilized PAF (Sigma Chemical Co, St Louis, MO) was reconstituted with tris tyros albumin buffer to yield a stock solution of 1.86×10^{-5} M concentration of PAF. This stock solution was serially diluted to yield PAF concentrations of 5, 10, 20µM. Measurement of secretion of serotonin was performed according to the method of H. Holmsen and C. A. Danglemaier [25]. Platelet procoagulant activity (PCA) was determined with Stypven time protocol [26] using Russell's Viper Venom (Sigma Chemical Co.)

Statistical Evaluation

For the statistical evaluation of the effects of different platelet inhibitors and activator on TEG measurements analysis of variance (software package Instat, version 7.0) was applied. The results were expressed as mean \pm 1 standard deviation and a p value of ≤ 0.05 was considered statistically significant.

Results and Discussion

Effect of ASA

Fig.1 shows the effect of ASA on G and R values of TEG-rTT with and without the addition of collagen in C-PRP (250,000 and 100,000 platelets/mL) and C-WB. It is the most widely

used antiplatelet drug in patients undergoing CABG. ASA produces antiplatelet effect by blocking the cyclooxygenase pathway and thereby producing the inhibition of platelet aggregation. In this work a final concentration of 200µM of ASA was used as it produced complete inhibition of arachidonic acid induced PAG. The graph shows the results of the effect of mild inhibitor aspirin on TEG-rTT parameters. The elastic shear modulus "G" of TEG-rTT is plotted on the left of Y axis and expressed in dynes/cm². The rate of clot formation "R" in mm is plotted on the right of Y axis. Two different concentrations of C-PRP (100,000/µL and 250,000µL) and whole blood samples were tested with and without aspirin in the presence and absence of collagen. As previously noted, increase in platelet concentration produced an increase in the G value and no significant changes in the R value [15].

In both PRP and whole blood the addition of collagen did not produce any significant change in G value but it reduced the R value which was statistically significant. In both C-PRP and C-WB, 200µM (FC) of ASA had no significant effect on R or G values of TEG-rTT in the presence and absence of collagen.

It can be seen from Fig. 1, that neither G nor R was detectably affected by ASA in concentrations that completely inhibited PAG induced by 2.5mM AA (results not shown). This was regardless whether the experiments were carried out in recalcified C-WB or C-PRP containing normal or mildly decreased platelet concentrations. Maximum inhibition of

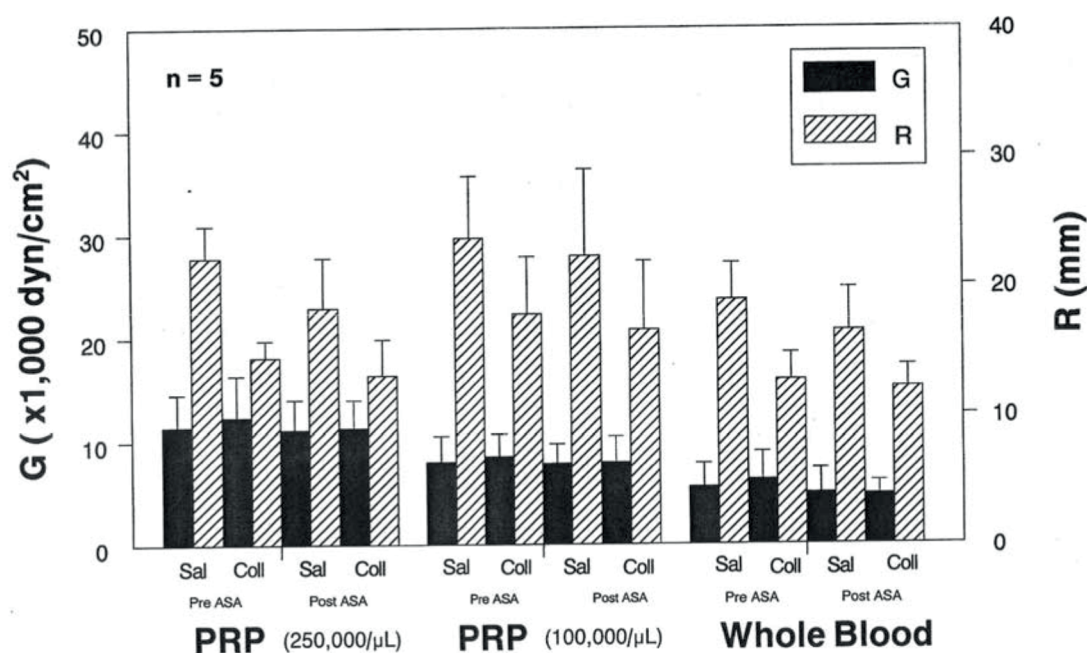


Fig. 1: Effects of Aspirin (ASA) on G and R of TEG-rTT with and without collagen in C-PRP and C-Whole Blood. ASA (200µM f.c.) was added to aliquots of either C-PRP with different concentrations of platelets or C-whole blood to yield complete inhibition of arachidonic acid-induced turbidimetric platelet aggregation in C-PRP

platelet function by ASA could not be detected in any of the TEG systems used.

Effect of c7E3 Fab (ReoPro®)

Fig.2 shows the comparison of the effects of platelet glycoprotein IIb/IIIa-receptor blockade by c7E3 Fab (ReoPro®) in C-PRP on G and R values of TEG-0, TEG-Celite, TEG-rTT, TEG-rTT with collagen and PAG induced by 5mM ADP. The data confirms the previously published observations indicating that with TEG-0, TEG-Celite and TEG-rTT, G was moderately sensitive to inhibition by the strong platelet inhibitor, c7E3 Fab (ReoPro®) as compared to PAG induced by 5mMADP, while R was not affected by the same. However, Khurana et al have used maximum concentration of rTT in their work [23]. Whereas in this study an optimal concentration of rTT was selected which reduced the TCT significantly to 7mins and also produced the optimal G value [14].

In this study the platelet concentration was maintained at 250,000/ μ L and effect of the increase in concentrations of c7E3 Fab (ReoPro®) on G and R values of TEG-0, TEG-rTT with and without collagen, TEG-Celite and PAG induced by 5 μ M ADP was studied. Fig.2a shows the % inhibition of c7E3 Fab (ReoPro®) on G value of different TEG systems and PAG study with increasing c7E3 Fab (ReoPro®) concentration on the X axis in μ g/ml. Fig. 2b shows the rate of clot formation i.e., R in mm on the Y axis and increasing concentration of c7E3 (ReoPro®) in μ g/ml on the X axis. The results showed that the PAG was the most sensitive test to pick up the inhibition by c7E3 Fab (ReoPro®) attaining complete inhibition (100%) at 4 μ g/ml of c7E3 Fab (ReoPro®). The TEG-0 system

was less sensitive compared to all other types of activated TEG systems at all concentrations of c7E3 Fab (ReoPro®) which were tested. Among all TEG systems, the most sensitive was the TEG-rTT followed by TEG-celite which showed greater sensitivity to inhibition at all concentrations of c7E3 Fab (ReoPro®). We were able to achieve 78% platelet inhibition in TEG-rTT at 8 μ g/ml of c7E3 (ReoPro®). While the TEG-Celite system was about 65% sensitive, at the same concentration the TEG-rTT with collagen was only 60% inhibited. The effect of increasing concentrations of c7E3 Fab (ReoPro®) on different TEG systems analysed demonstrated that there was no effect on the R value of any of the activated TEG systems. Whereas the TEG-0 system showed an increase in R value from 44 to 50mm at a c7E3 Fab (ReoPro®) concentration of 2 μ g/ml, beyond which the increase in concentration of the inhibitor did not produce any change in the R value of the TEG-0 system.

Effect of PAF

Till now the PAG study is the gold standard method to detect platelet dysfunction. Fig.3 gives the results of various concentrations of PAF on TEG-rTT parameters. While G and R are plotted on the left of y axis and the effect of various concentrations of PAF on PAG, % 14C serotonin release and % PCA are plotted on the right of y axis. X axis shows the concentration of PAF varying from 5 μ M to 20 μ M FC. From the results it can be observed that increasing PAF concentrations did not produce any significant decrease in TEG parameters, G and R values.

Whereas at 5 μ M concentration and 20 μ M concentration, PAF produced 38% platelet aggregation and 55% aggregation

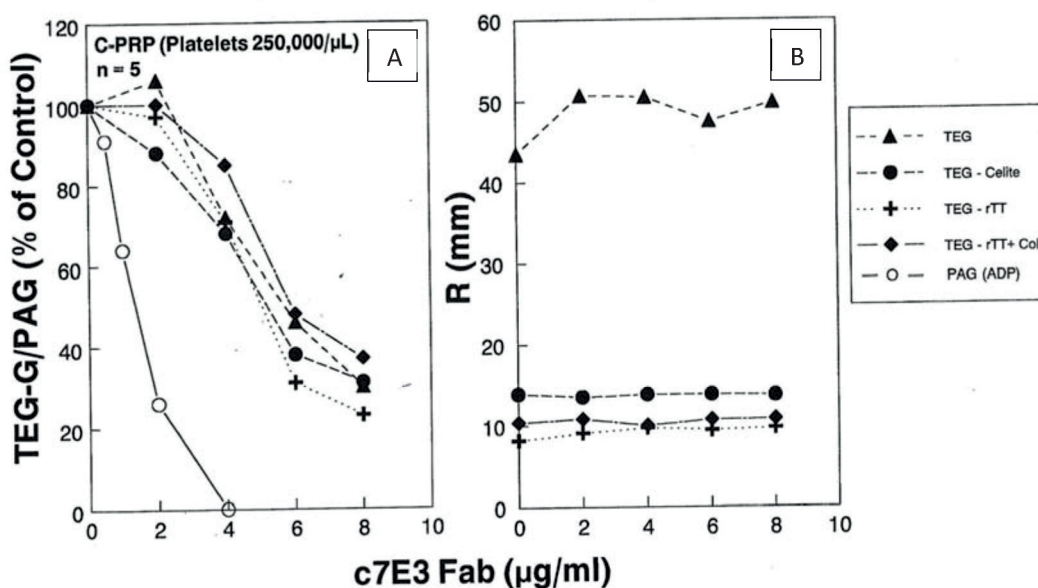


Fig. 2: Comparison of the effects of platelet GP IIb/IIIa-receptor blockade by c7E3 Fab (ReoPro®) on G and R in standard TEG and TEG with addition of Celite, rTT or rTT plus Collagen and turbidimetric platelet aggregation induced by 5 μ M ADP.

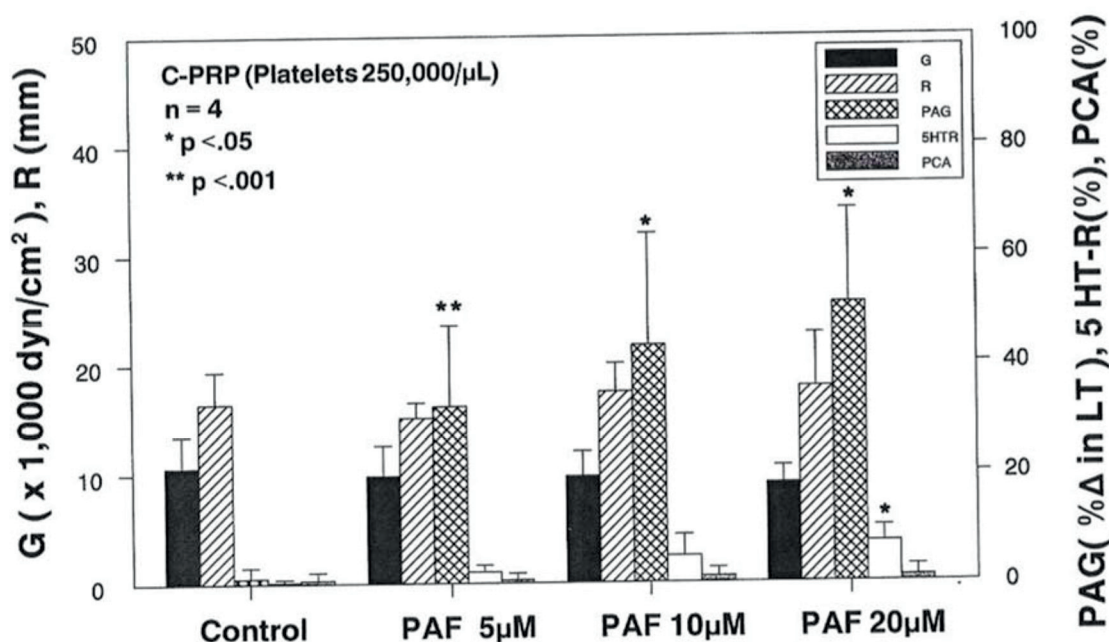


Fig. 3: Effects of platelet activating factor (PAF) in G and R of TEG-rTT, turbidimetric platelet aggregation (PAG), 14C-serotonin (5HT) release and platelet procoagulant activity (PCA) in PRP. PCA was measured with the Stypven time using Russell's Viper Venom (Sigma Chemical Co.).

respectively in the PAG study. It was observed that with increase in concentration of PAF there was an increase in the % of 5HT released but it was significant only at 20µM concentration of PAF. There was no significant change in the PCA with increase in PAF concentration. This study of the effect of PAF on TEG parameters concurred with the fact that TEG being a low shear system would not be affected by increasing amounts of PAF whereas PAG study is a high shear system and hence sensitive enough to produce aggregation at low concentrations of PAF itself.

In this investigation the effect of ASA and different concentrations of c7E3 Fab (ReoPro®) was studied on different TEG parameters of various TEG systems. To the best of our knowledge this is the first study comparing the effects of two types of platelet inhibitors, aspirin and c7E3 Fab (ReoPro®) on different types of TEGs like standard and modified TEGs as well as PAG induced by 5µM ADP. Different TEG systems were moderately sensitive (reduction of G) to inhibition by the platelet glycoprotein IIb/IIIa blocking agent, c7E3 Fab (ReoPro®).

Conclusion

In a cardiac setup, it can be argued that TEG not being sensitive to ASA like platelet defect is advantageous since most patients are on ASA prior to surgery. At the same time in non-cardiac settings it is worrisome that mild platelet dysfunction (aspirin induced platelet defect) may not be picked up by TEG-0 or activated TEG systems. The addition

of collagen did not improve the sensitivity of TEG-rTT to detect ASA like defect. This study also revealed that the modified TEG system (TEG-rTT) was the most sensitive to pick up platelet dysfunction produced by the addition of c7E3 Fab (ReoPro®) compared to standard TEG and activated systems.

Conflict of Interest:	All authors declare no COI
Ethics:	There is no ethical violation as it is based on voluntary anonymous interviews
Funding:	No external funding
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