Original Article

An In Vitro Model for Hemostasis Monitoring During Simulated Cardiopulmonary Bypass

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Keywords: cardiopulmonary bypass, activated clotting time, coagulation, hemostasis, thrombelastograph

Presented as a poster at the American Society of Extra-Corporeal Technology 36th International Conference, March 12-14, 1998, Philadelphia, Pennsylvania

ABSTRACT

The optimum model for hemostasis monitoring during cardiopulmonary bypass (CPB) is the evaluation of physiologic changes in the intact organism. This is often logistically difficult and expensive. The purpose of this study was to design an in vitro model of blood coagulation for use in simulated CPB.

Human expired blood components within 4 days of outdating were reconstitituted as follows: 4 units of packed red blood cells, 4 units of platelets, and 3 units of fresh frozen plasma. The mixture was circulated in a simulated extracorporeal circuit. Blood samples were drawn every 30 minutes over a 2 hour period, recalcified, and analyzed for platelet count (PLT), fibrinogen concentration (FIB), prothrombin time (PT), activated partial thromboplastin time (aPTT), celite and kaolin activated clotting times (ACT), and thrombelastography (TEG).

In the four different coagulation monitors utilized, there were no significant changes in celite or kaolin ACTs. PT increased from 15.8 ± 1.1 sec to 25.2 ± 7.8 sec and aPTT from 62.1 ± 15.9 sec to 78.9 ± 36.5 sec (p = NS). There were no changes in either PLT count or FIB concentration. Both celite and tissue factor activated TEG values trended towards hypocoagulability.

In conclusion, the results show that the in vitro model is stable over 120 min of recirculation time within a simulated cardiopulmonary bypass circuit in regards to platelet count, hematocrit, total protein, PT, aPTT, and ACT. Further examination will be necessary to establish the effects of the model in regard to platelet function.

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Article available at https://ject.edpsciences.org or https://doi.org/10.1051/ject/1998303127

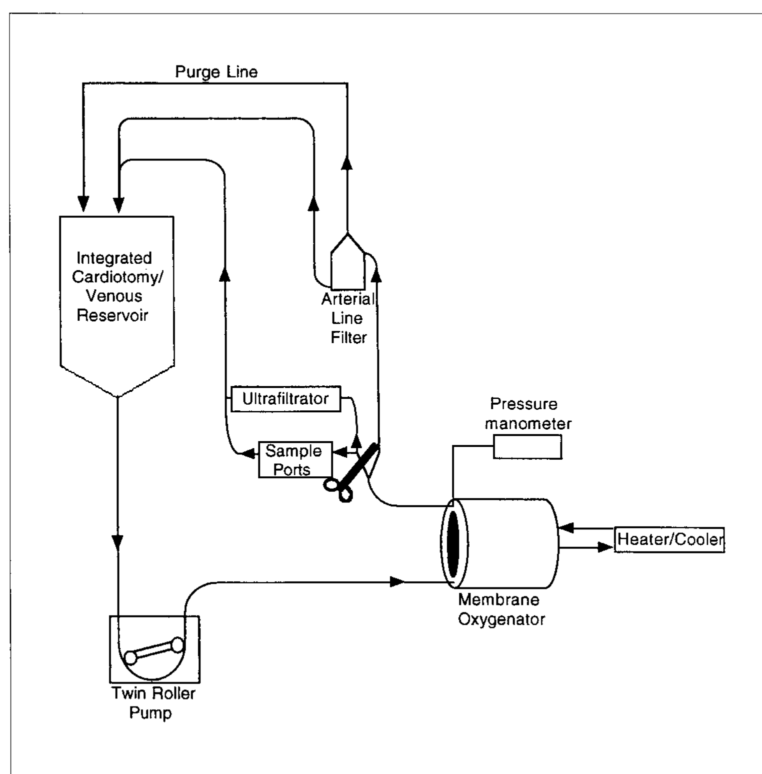
INTRODUCTION

It is commonly accepted that a delicate balance exists between coagulation and fibrinolytic systems which prevents excessive bleeding or thrombosis in normal individuals. The process of cardiopulmonary bypass (CPB) utilized during cardiac surgery disrupts the interaction of the various elements involved in hemostasis (1). Subsequent coagulopathic complexities continue to plague clinicians by adversely affecting patient outcomes despite advances in surgical technique and improved device biocompatibility (2-4). Improving patient hemostasis post-CPB can be achieved by a complete understanding and evaluation of coagulation phenomena. Perioperative hemostatic deficiencies of idiopathic origin can be most thoroughly diagnosed using a multidisciplinary approach that includes the assessment of vascular integrity (5), abnormal plasma levels of coagulation proteins, qualitative and quantitative platelet deficiencies (6), and fibrinolytic activity (2).

Rapid diagnosis of hemostatic deficiencies during cardiothoracic surgery is paramount for effective intervention of bleeding. The diagnosis is coupled with pharmacological intervention and therapeutic protocol including replacement therapy strategies (7). However, with the increasing awareness of transfusion related complications such as immune mediated reactions, acute lung injury, and infection (hepatitis C, HIV, etc.), efforts to limit blood and blood product transfusions should be maximized (8). Consequently, transfusion protocols based on laboratory data and coagulation assessment have been shown to reduce microvascular bleeding and reduce blood product needs and are vitally important in directing interventional strategies that affect patient outcomes (9).

Research on coagulation determination and assessment is complicated by numerous factors, including the complexity of the intact hemostatic system, inter-patient variability, methodological variation between studies, and the obtainment of informed consent for the completion of prospective research on humans. Many of the challenges involved with coagulation research could be overcome by establishing an in vitro model utilizing reconstituted expired human blood. Establishment of a direct linear correlation between the experimentally created in vitro model and the coagulation phenomena that occur in vivo would open the arena of research requiring the use of human blood and blood products by allowing a marked increase in the validity of device study and therapeutic protocol assessment. Additionally, laboratory expense can be significantly reduced by the use of expired human blood products which would otherwise be wasted.

Figure 1: Circuit diagram



The objective of this study was to evaluate reconstituted expired whole human blood products through a prospective, randomized ex-vivo laboratory trial. A comprehensive panel of tests was performed throughout a period of CPB, with the results used to delineate changes in hemostatic state. The goal was to determine if a linear coagulation model could be defined that would correlate to coagulation phenomena expected in an in vitro preparation.

MATERIALS AND METHODS

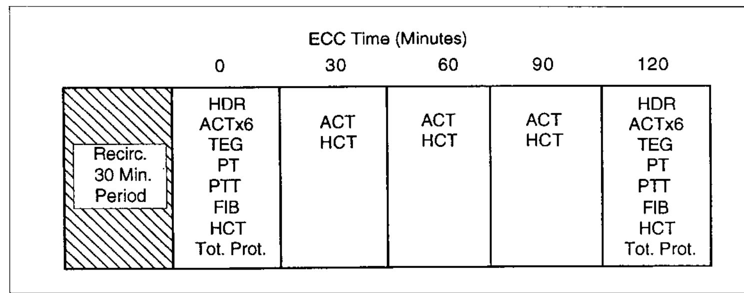
A CPB circuit was constructed to mimic that used in extracorporeal circulation during cardiac surgery (Figure 1). The extracorporeal circuit consisted of 3/8 in polyvinyl chloride tubing, a cardiotomy/venous reservoir[a], a membrane oxygenator with integral heat exchanger[b], a sample port from which to draw blood samples, an arterial line filter[b], and an ultrafiltrator[c]. A positive displacement roller pump adjusted to a "just non-occlusive" setting was used. The circuit was primed with 1 L of normal saline.

a Baxter Healthcare Corp., Bentley Lab Division, Irvine, CA 92714
b C.R. Bard, Inc., Billerica, MA 01876
c Sarns 3M Healthcare, Ann Arbor, MI 48103

MONITORS

Five different monitors were utilized to observe coagulation parameters for this experiment. The Hemostasis Management System (HMS)[d] was used to perform the Heparin Dose Response (HDR) as well as an activated clotting time (ACT). The Hemochron 8000, 801, and Jr.[e] were all used to perform ACTs. In the 8000 and the 801, both kaolin and celite activated samples were run at each time point. The Thrombelastograph (TEG)[f] was described by Hartert in 1947 and is manufactured by the German company Hellige. It is used to measure the viscokinetic properties of an ex vivo developing clot by measuring the shear elasticity of clot formation. Two different types of platelet activators were utilized for the TEG samples. Celite (TEG_c) is a surface-activating agent that acts to increase the platelet surface area, while recombinant human tissue factor (TEG_{tf}) acts to increase platelet aggregation. Both of these reagents accelerate clot formation. Coagulation monitors were calibrated according to manufacturers' specifications, and quality controls were performed at the beginning of each day of data collection.

Figure 2: Sampling time line



ACT = activated clotting time; FIB = fibrinogen; HCT = hematocrit; HDR = heparin dose response; aPTT = activated partial thromboplastin time; PT = prothrombin time; Tot. Prot. = total protein; TEG = thrombelastograph

RECONSTITUTION OF BLOOD

The reconstitution of blood products was completed by combining 4 units of expired packed red blood cells (PRBC), 3 units of expired fresh frozen plasma (FFP), and 4 units of expired platelets. Blood products were combined from all blood types and Rh factors to give an approximate volume of 1660 ml, and ultrafiltration was utilized to achieve a final hematocrit (HCT) of 39-42%. These expired products were obtained from the American Red Cross and the University of Nebraska Medical Center (UNMC) hospital blood bank and were used within 4 days of expiration. All experiments were completed within 3 h of the reconstitution process.

Reconstitution occurred in the cardiotomy reservoir, and blood was introduced into the extracorporeal circuit using a "chase method," in which the prime volume was chased out of the circuit into a waste bag until the entire circuit was filled with only the reconstituted blood. This served to minimize the hemodilution of the reconstituted blood mixture, which could have inadvertently altered laboratory results.

A circulation time of 30 min (flow rate of 4 L/min) was used for circuit equilibration at 100 mmHg of pressure created by the use of a variable resistor. During this 30 min circulation time (flow rate of 4 L/min), temperature was adjusted to 37 ± 10°C.

SAMPLING

Total extracorporeal circulation time was 150 min with sampling completed over a 120 min time period. Samples were drawn at times 0, 30, 60, 90, and 120 min (Figure 2). A baseline hematocrit and total protein were drawn after the first 30 min of circulation and at each sampling point. At each sampling point, 2 ml of blood was drawn into each 3 ml syringe that was required for the tests to be performed during that point on the sampling timeline. To recalcify each sample, 0.75 ml of 0.025 M $CaCl_2$ was added to each syringe and the syringe was inverted 10 times.

Following recalcification, the following assays were performed: HDR at 0 and 120 min; ACT at 0, 30, 60, 90, and 120 min using the HMS Hepcon ACT, Hemochron 8000, 801, and the Hemochron Jr. A spun hematocrit was performed in duplicate on the $CaCl_2$ diluted samples as well as the whole blood samples in order to identify the percentage of packed red blood cell mass.

TEG profiles were obtained at 0 and 120 min using both celite and tissue factor as activators. An additional 3 ml of blood was introduced into a 3 ml blue top vacutainer[g] citrate tube (0.105 M sodium citrate) to measure prothrombin time (PT), partial thromboplastin time (PTT), and fibrinogen. A 4 1/2 ml sample of blood was dispensed into a lavender top vacutainer tube containing EDTA (ethylene diamine tetraacetic acid) to measure platelets. After the blood was introduced into the tubes, they were refrigerated at 4°C and then sent to the UNMC Pathology lab within a 2 h period to be evaluated. Total protein was determined by the use of a refractometer[h].

STATISTICS

All data were collected and loaded onto a personal computer in spreadsheet format. Data were analyzed with an unpaired Student's t test and are presented as mean ± standard deviation of the mean. Statistical significance was accepted at the $p \leq 0.05$ level. Statistical analysis was performed using a commercially available statistics program[i].

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d Hemotec Inc., Englewood, CO 80134
e International Technidyne Corp., Edison, NJ 08820
f Haemoscope Corporation, Skokie, IL 60053
g Becton Dickenson and Company, Franklin Lakes, NJ 07417
h TS METER, Cambridge Instruments, Buffalo, NY 14201
i Super ANOVA, Abacus Concepts, Berkeley, CA 94704

RESULTS

A total of 3 trials was completed utilizing this model. Within these trials, there were found to be no significant differences between kaolite and celite ACT values of the Hemochron 801 and 8000 at any time point (Table 1).

The mean celite ACT values for the Hemochron 801 was 132.5 ± 3.5 sec, while the mean kaolin ACT value was 134.8 ± 4.8 sec. The Hemochron 8000 had an average celite ACT value of 133.8 ± 22.0 sec and a mean kaolin ACT value of 146.4 ± 5.4 sec. The HMS mean ACT value was 111.1 ± 4.8 sec, considerably lower as compared with the other monitors. However, this was only statistically different when compared with the 8000 kaolin mean ACT values (p = 0.009). The Hemochron Jr. had an elevated mean ACT value of 190.2 ± 19.2 sec when compared with the previous monitors. The mean ACT value for the Hemochron Jr. was statistically significant (p = 0.0001) when compared with the HMS, 801, and 8000 (Figure 3).

Both the PT and aPTT times increased over the experimental period, but were not statistically significant when compared with baseline values. These values are illustrated in Figure 4.

The baseline values for HDR began at 2.3 ± 0.0 mg/kg and decreased to 1.3 ± 0.3 mg/kg at the 120 min sampling period (p = NS), indicating a decreased tendency for coagulability.

The hematocrit for the undiluted samples averaged 40.3 ± 1.5%, while the diluted samples had an average value of 35.7 ± 0.6%. This dilutional factor is important, since the concentration of fibrinogen and the platelet count would be 11.4% more due to the recalcification volume change. A statistical difference (p = 0.02) between the diluted hematocrit at 120 min and undiluted hematocrit at 30 min has been shown (Table 2).

Baseline total protein values were 7.4 ± 1.0 g/dL and decreased to 7.1 ± 1.8 g/dL at the 120 min time period (p = NS). The values at both time periods fell within accepted normal time protein values for non-diluted human samples (Figure 5).

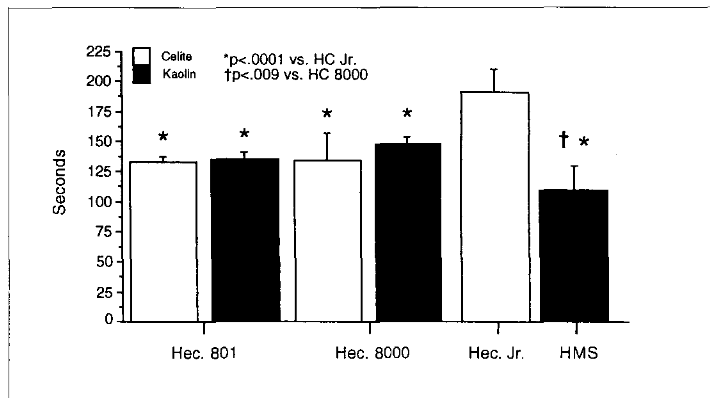
All measured platelet counts fell within normal range of 150,000/mm^3 to 350,000/mm^3. The mean baseline and 120 min platelet counts were 189.7 ± 41.6 1000/mm^3 and increased to 190.0 ± 55.1 1000/mm^3, respectively (Figure 6). Fibrinogen remained within the cited normal human values of 150-350 mg/dL (Figure 5), and was 188.3 ± 25.2 mg/dL at the start of the experiment and 186.0 ± 18.5 mg/

Table 1: Mean ACT values over 120 min extracorporeal circulation time

Test	Baseline	30 min	60 min	90 min	120 min
801 cACT	138.0 ± 41.3	132.3 ± 34.2	132.0 ± 30.4	128.3 ± 31.0	132.0 ± 15.6
801 kACT	133.0 ± 34.6	142.3 ± 32.9	136.5 ± 44.5	131.0 ± 25.9	131.0 ± 25.9
8000 cACT	161.5 ± 9.2	142.3 ± 22.1	119.0 ± 45.1	141.0 ± 28.5	105.0 ± 40.2
8000 kACT	150.7 ± 36.1	153.0 ± 41.6	142.7 ± 32.7	140.0 ± 34.4	145.7 ± 17.6
Jr. ACT	208.5 ± 44.5	188.0 ± 83.6	177.7 ± 81.3	210.3 ± 46.3	166.3 ± 61.1
HMS ACT	106.0 ± 21.7	107.0 ± 24.0	117.0 ± 30.8	115.0 ± 28.8	110.3 ± 24.6

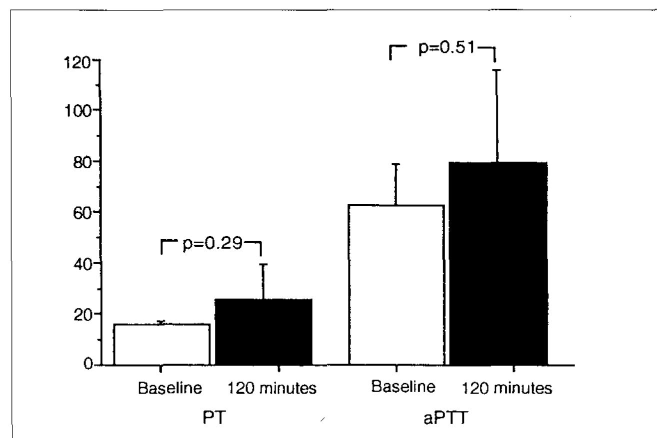
Note: cACT = celite activated clotting time (sec); kACT = kaolin activated clotting time (sec); Jr. ACT = Hemochron Jr. activated clotting time (sec); and HMS ACT = Hemostasis Management System activated clotting time (sec)

Figure 3: Mean ACT values for Hemochron 801, Hemochron 8000, and Hemochron Jr.



Hec. 801 = Hemochron 801; Hec. 8000 = Hemochron 8000; Hec. Jr. = Hemochron Jr.; HMS = Hemostasis Management System; * = p = 0.0001; † = p = 0.009

Figure 4: Mean PT and aPTT values for baseline and 120 min sampling period



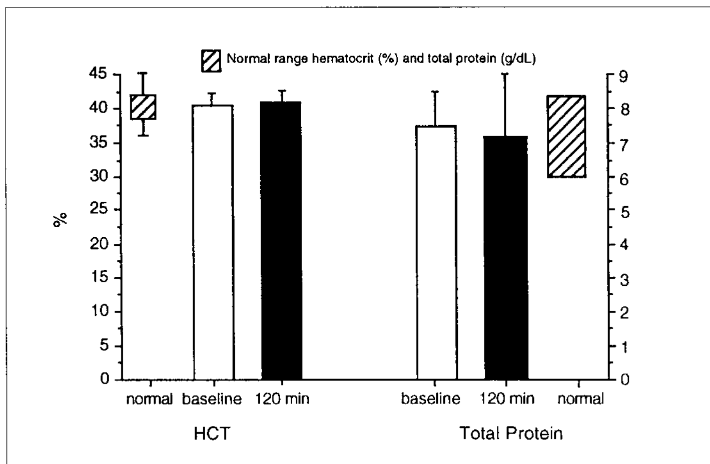
aPTT = activated partial thromboplastin time; PT = prothrombin time

Table 2: Mean hematocrit values over 120 min extracorporeal circulation time

Test	Baseline	30 min	60 min	90 min	120 min
Dil. HCT	35.7 ± 0.6	36.7 ± 1.5	36.3 ± 1.2	35.7 ± 1.5	34.7 ± 2.1[a]
Undil. HCT	40.3 ± 1.5	41.0 ± 1.7	40.3 ± 1.5	40.3 ± 1.5	40.3 ± 1.5

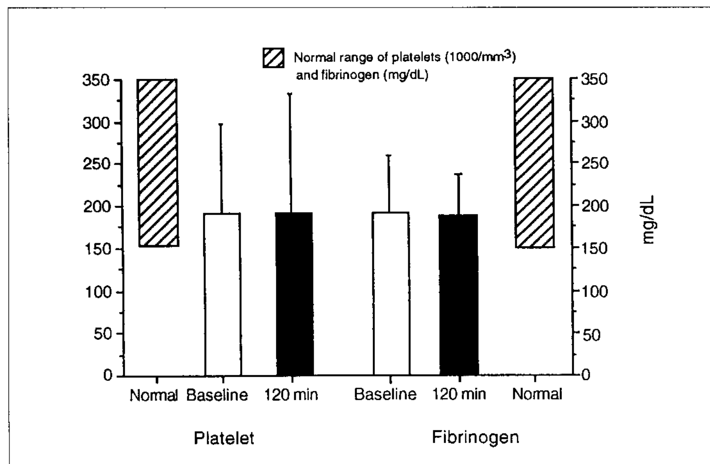
Note: Dil. HCT = dilutional hematocrit (%) in 0.75 ml of 0.025 M $CaCl_2$ in 2.0 ml of blood; Undil. HCT = undiluted hematocrit (%); a = $p < 0.05$ versus undiluted 30 min time period

Figure 5: HCT and total protein valuees: normal, baseline, and 120 min sampling period



HCT = hematocrit

Figure 6: Platelet and fibrinogen values: normal, baseline, and 120 min sampling period



dL at 120 min.

TEG profiles exhibited reduced coagulopathy when compared with expected normal celite and tissue factor values (Figure 7) from freshly drawn blood. The TEG_c for R time, normally 10 to 14 mm, was 20.2 ± 33.2 mm at the 120 min sampling time. The TEG_c MA at the 120 min sample was 3.3 ± 1.8 mm, while the normal values were close to 70 mm. Finally, the TEG_c alpha angle, normally 54° to 67° was 22.0 ± 0.0° at 120 min.

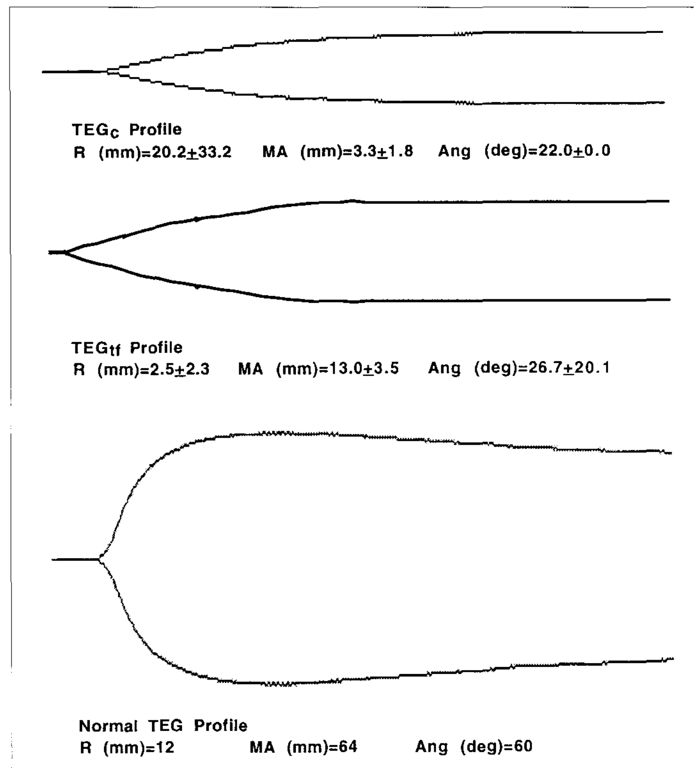
Baseline values for the TEG_{tf} were as follows: R = 2.5 ± 2.3 mm, MA = 13.0 ± 3.5 mm, and alpha angle = 26.7 ± 20.1°. These values were dramatically lower than the normally freshly drawn blood values of R = 4.0 to 6.5 mm, MA = 59.0 to 68.0 mm, and alpha angle = 60.0 to 74.0. The 120 min values for R and MA were also depressed when compared with the normal values (Figure 7).

DISCUSSION

Cardiopulmonary bypass associated coagulopathies have been, and continue to be, a challenge to the successful outcome of cardiac surgical procedures. The management of abnormal post-surgical bleeding may necessitate pharmacologic intervention, blood product administration, and surgical reexploration. All of these treatments come with their own extensive list of increased risks and costs. DelRossi and associates have reported that open heart patients are responsible for 25% of all transfused blood products in some institutions (10). The risks of these transfusions involve both infections agent transmission and non-infectious transfusion reactions. The risk of hepatitis transmission is estimated in 1 in 200,000 transfused units, while the risk of post-transfusion HIV infection is reported as 1 in 420,000 transfusions (11). While the consequences of transfusion related viral infection are obvious, the consequences of transfusion reactions are more subtle in their effect on morbidity and increased cost. Non-infectious transfusion reactions include accute immune mediated hemolysis, febrile nonhemolytic reactions, hyper- and hypokolemia, and transfusion related acute lung injury (11).

Establishment of an in vitro model for blood coagulation during CPB will allow simplified study of the various mechanical and physiological changes induced by the extracorporealization of the blood. This simplification results from better control of experimental variables involved with clinical trials. Variables that confound the experimental study of coagulation in cardiac surgery utilizing CPB can include the complexity of the intact hemostatic system, inter-patient variability, methodological variation between studies and difficulty obtaining informed consent for the completion of prospective research on humans. Many clinical trials also require the cooperation of nu-

Figure 7: TEG profiles: celite, human tissue factor, and normal



TEC_c = celite thrombelastograph; TEG_{tf} = tissue factor thrombelastograph; R = R time (mm); MA = maximum amplitude (mm); ANG = alpha angle (degrees)

merous personnel outside the operation suite such as pre, intra, and postoperative nursing personnel. This model utilizes expired human blood products, a resource that is readily available and is simply discarded. Research utilizing these products provides an extension of usefulness for this vital resource.

The excellent stability of total protein and hematocrit shows minimal alteration in red cell structure throughout the trials. Any excessive red cell lysis would cause significant increase in total protein and decrease in hematocrit. This suggests that any cellular trauma induced by the simulated circuit had minimal effect on red cell number and function. Platelet counts done at baseline and at 120 min also showed no significant decrease. This lack of alteration in platelet number suggests that the simulated circuit had limited effect on the total amount of platelets, although platelet function as assessed by TEG, was depressed. Studies have shown that platelet number decreases dramatically upon initiation of bypass and remains relatively steady throughout the bypass run (12). Much of this decrease can be attributed to hemodilution and initial deposition on the artificial surfaces of the circuit (13). Since our baseline platelet counts were drawn after allowing the reconstituted blood products to recirculate for 30 min, the stability of our results is consistent with this observation. The difference between undiluted and diluted hematocrit can be used to quantify the dilutional factor of fibrinogen levels and platelet count. Hematocrit of diluted samples was 11.4 ± 0.2% lower than the valves of undiluted samples. This indicates that fibrinogen and platelet counts are actually 11.4 ± 0.2% higher taking dilution into account.

Our study provided ACT results that showed a strong degree of linear correlation throughout the experimental time span. Analysis of the ACT results from the HemoTec HMS, Hemochron 8000, and Hemochron 801 showed no significant difference between the three devices regarding the celite activated samples. Statistically significant difference was found between the kaolin activated ACT results for the Hemochron 8000 and the HemoTec HMS. Previous studies have confirmed this finding (14). The lower values given by the HMS have been shown to be due to differing monitoring device sensitivity and the liquid state of the reacting kaolin. Although previous studies have shown good correlation between Hemochron Jr. ACT and other Hemochron devices, the Hemochron Jr. mean ACT values in our study were consistently higher than the mean values of the 8000 and 801 (15). Despite this statistically significant difference between the Hemochron Jr. and other monitors, the ACT results remained consistent over time within all trials. The linearity of the results from each of the ACT monitors is evidence of the stability of our coagulation model.

Prothrombin time and activated partial thromboplastin time results increased over time in each of the trials. This increase, although not statistically significant, corresponds to some coagulation factor depletion throughout its circulation time. This factor depletion was not significant enough to cause decreases in ACT or fibrinogen levels. Both ACT and fibrinogen levels remained quite stable throughout each of the three trials.

Heparin Dose Response test results were obtained for baseline and 120 min on trial 1 and at 120 min on trial 3. Although not significant, a decrease was found. This difference corresponds to a mildly decreased response to heparin. This is further evidence of mild factor depletion occuring over the time of circulation.

The results of the TEG analysis showed deficit in platelet function, although no significant alteration in platelet number was found. The TEG provides a global assessment of hemostatic mechanisms involving coagulation factors and platelets. It provides an evaluation of initial platelet-fibrin interaction, clot strengthening, and fibrin cross linkage (16). Maximum amplitude (MA) is a measure of the function of platelets in establishing the maximum clot stiffness (17). The MA of our celite activated specimens at 120 min was 3.3 ± 1.8 mm. Normal values for celite activated TEGs have been quoted as 50-60 mm. These results suggest failure of the platelets to provide clot strength, but they fail to correlate with the consistent level of coagulation

evidenced by the other tests performed in this study. It is well documented that stored platelets show rapid reduction in their responsiveness to aggregating agents (18). This decrease in platelet adhesion, aggregation, and secretion is collectively known as platelet storage lesion (19). It has been shown that platelets are inhibited by release of neutrophil proteolytic enzymes (20), increased complement (C3a, C4a, C5a) levels (21), and release of nucleotides AMP and diadenosine tetraphosphate during storage of platelets for periods up to 5 days (22).

It seems unlikely that platelet storage lesion induced deficits in function should account for our poor TEG results. Several studies have shown that despite these proven pathological changes, platelets rapidly recover function after transfusion and also after incubation in fresh plasma (23, 24). The platelets in our study were in a plasma rich medium upon reconstitution although the temperature was allowed to reach room temperatures (approximately 22°C) before reconstitution. Although we cannot explain why platelet function was so poor, it was later found that the TEG device may have been malfunctioning.

The TEG was calibrated with a two point mechanical technique described in the manufacturer literature. Immediately following the study, a malfunctioning thermometer within the temperature regulated chamber of the TEG device was discovered. The temperature autoregulating device which normally kept the sample well temperature at 37°C was actually 42.3°C. This produced hypocoagulable profiles very much like those produced earlier in our study, although now device dysfunction was found at that time. Results from all other parameters showed no statistically significant difference over time, although differing from established normal values. The reduced coagulation potential seen by thrombelastography may have resulted from mechanical interference with the device. Further work is in progress to reassess TEG evaluation of this model as well as provide precise control of such variables as pH, ionized Ca^{2+}, and glucose, all of which have been shown to affect platelet function (18).

Our study may receive some criticism because it involves in vitro work. In vitro studies by definition lack the bioactivity of an intact human body. This fact, in and of itself, should not bring into question the validity of such studies. Recent publications have shown the importance of in vitro coagulation study (25, 26). In vitro experiments, along with critical and in vivo studies have been, and continue to be, and important part of the study of extracorporeal blood-surface interactions. Although in vitro models are imperfect predictors of the complex interactions of the human body, they can offer insight into the processes that occur in vivo without eliciting harm to actual patients. Many of these insights can then be extended to studies that involve the human system directly. An in vitro model is not a substitute for in vivo experiments, but rather an adjunct to them.

In conclusion, we found our reconstituted expired whole blood model to be stable over 120 min of recirculation time within a simulated cardiopulmonary bypass circuit in regards to platelet count, hematocrit, total protein, PT, aPTT, and ACT. The validity of this model is still in question due to the results of TEG analysis. It will be necessary for further examination to be performed to establish platelet function. This model can be used to examine the effects of endless variables within the simulated extracorporeal circuit if the stability of platelet function can be established.

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