

Thrombography Reveals Thrombin Generation Potential Continues to Deteriorate Following Cardiopulmonary Bypass Surgery Despite Adequate Hemostasis

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Abstract: The intrinsic and extrinsic activation pathways of the hemostatic system converge when prothrombin is converted to thrombin. The ability to generate an adequate thrombin burst is the most central aspect of the coagulation cascade. The thrombin-generating potential in patients following cardiopulmonary bypass (CPB) may be indicative of their hemostatic status. In this report, thrombography, a unique technique for directly measuring the potential of patients' blood samples to generate adequate thrombin bursts, is used to characterize the coagulopathic profile in post-CPB patients. Post-CPB hemostasis is typically achieved with protamine reversal of heparin anticoagulation and occasionally supplemented with blood product component transfusions. In this pilot study, platelet poor plasma samples were derived from 11 primary cardiac surgery patients at five time points: prior to CPB, immediately post-protamine, upon arrival to the intensive care unit (ICU), 3 hours post-ICU admission, and 24 hours after ICU arrival. Thrombography revealed that the Endogenous

Thrombin Potential (ETP) was not different between [Baseline] and [PostProtamine] but proceeded to deteriorate in the immediate postoperative period. At the [3HourPostICU] time point, the ETP was significantly lower than the [Baseline] values, 1233 ± 591 versus 595 ± 379 nM.min (mean \pm SD; $n = 9$, $p < .005$), despite continued adequacy of hemostasis. ETPs returned to baseline values the day after surgery. Transfusions received, conventional blood coagulation testing results, and blood loss volumes are also presented. Despite adequate hemostasis, thrombography reveals an underlying coagulopathic process that could put some cardiac surgical patients at risk for postoperative bleeding. Thrombography is a novel technique that could be developed into a useful tool for perfusionists and physicians to identify coagulopathies and optimize blood management following CPB. **Keywords:** cardiopulmonary bypass, blood transfusion, thrombin generation, blood coagulation, postoperative care. JECT. 2011;43:19–25

The exposure of blood to artificial surfaces, hemodilution, systemic heparin anticoagulation, and surgical trauma are unique to cardiopulmonary bypass (CPB) patients and subjects them to increased risks for postsurgical coagulopathy (1,2). Bleeding following heart surgery is a major

complication leading to one of the highest transfusion rates among all major surgical procedures (3,4). The challenge of attaining hemostasis and avoiding the deleterious effects of blood product transfusions is particularly dire with high risk patients (repeat procedures, elderly, etc.) who undergo complex surgeries such as multiple valve or combined graft/valve procedures, ventricular assist device implants, and heart transplants (5). Understanding the mechanisms of the hemostatic perturbations associated with CPB requires ongoing study to optimize transfusion therapy and ultimately, reduce morbidity and mortality.

The central participant in the formation of blood clots is the thrombin molecule as there are no enzymatic

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pathways that bypass it (6). Activation pathways converge at the point where prothrombin is converted to thrombin. Activated thrombin goes on to trigger both positive and negative feedback pathways, convert fibrinogen into fibrin and it is the most potent activator of platelets. The ability of blood to generate an adequate thrombin burst is crucial in order for blood clots to form in a timely and adequate manner (6,7). This ability can be assessed *in vitro* by measuring thrombin generation curves (thrombograms) using patients' plasma samples first described in 1953 (8,9). Early methods involved measuring thrombin activity from repeated, manual subsamplings of recalcified plasma. Thromboelastography (TEG[®], Haemoscope Corp., Niles, IL) provides a surrogate measure of thrombin generation by deriving "velocity curves" (10) from the resistance caused by the formation and polymerization of fibrin strands in the sample cup. Since fibrinogen is depleted before 5% of all thrombin is formed (11) a precise attribution to thrombin generation cannot always be assumed. Calibrated automated thrombography (CAT, Thrombinoscope BV, Maastricht, The Netherlands) represents an advancement, which automates and simplifies the direct measurement of thrombin generation curves (12).

Thrombin generation in CPB patients has been reported in the past using indirect techniques such as measuring prothrombin 1.2 or thrombin-antithrombin complexes (13,14). These methods provided useful but distinctly different information as compared to what thrombography produces. Measurements of thrombin activation by-products provide indications of thrombin generation that has already occurred. Thrombograms plotted using CAT whereas, measure the remaining capacity of blood to generate a thrombin burst when adequately activated, as indicated by substrate cleavage. Thrombography continues to monitor the decrease in substrate cleavage as antithrombins neutralize the activated thrombin.

Thrombin generation has been found useful in the detection and quantification of thrombotic tendencies (e.g., protein S or C deficiencies), bleeding tendencies (e.g., congenital factor deficiencies, thrombasthenia, or thrombopenia), and the control of procoagulant therapy in hemophiliacs and oral antithrombotic therapy (e.g., low molecular weight heparins, vitamin K antagonists, and platelet inhibitors) (11). Acquired hypercoagulable states such as in acute myocardial infarction patients were also detectable on thrombograms (15). Schols et al. reported that thrombin generation parameters improved in patients who responded to postoperative plasma transfusion therapy but deteriorated in those who did not (16). Schols et al. also used thrombography and TEG to characterize dilutional coagulopathy (17).

In this pilot study, we explore the use of CAT following cardiac surgery as a first step towards initiating systematic

investigations to examine the thrombin mediated physiologic mechanisms in the recovery of CPB disrupted hemostatic systems.

METHODS

Patients and Blood Sample Collection

Enrollment to this minimal risk protocol study was initiated following Institutional Review Board approval (June 4, 2008). All adult participants undergoing primary CPB surgery were eligible to be enrolled. Exclusion criteria included emergency operations, patients with known coagulopathies, and patients on anticoagulant therapy within 48 hours of surgery. Patients were otherwise randomly selected according to investigator availability.

Cardiopulmonary bypass was conducted using roller pumps (Stockert S5, Sorin Group USA, Inc., Arvada, CO), with hollow fiber oxygenators (RX25) and X-coated tubing (Terumo, Ann Arbor, MI). Prime fluid consisted of Plasmalyte A (Baxter, Deerfield, IL) with 10,000 units of heparin (Porcine-APP Pharmaceuticals LLC, Schaumburg, IL). The heparin table dose (300 U/kg) was administered via central line and the activated clotting time was maintained above 480 seconds (HMS Plus System, Medtronic, Minneapolis, MN) with heparin boluses as needed. Pump prime was reduced by hemoconcentration as volume allowed. Moderate hypothermia (32°C) was instituted routinely during a majority of the period when the heart was arrested. Protamine was administered at a dosage recommended by the heparin assay method (HMS Plus System). Any blood remaining in the pump post-CPB was processed with a cell saver and returned to the patient by the anesthesia personnel in the operating room. Decisions to transfuse blood products were made empirically or based on surgical site oozing, standard coagulation test results post-heparin reversal, preoperative patient information, and complexity of the surgical procedure. All patients received aminocaproic acid for antifibrinolytic prophylaxis at standard dosing: 4–5 gm intravenously during the first hour followed by a continuous infusion: 1–1.25 gm/h.

Samples collected were as follows: [Baseline] - collected pre-surgical incision; [PostProtamine] - collected 3–5 minutes after protamine administration, prior to fresh frozen plasma (FFP), platelet, or cryoprecipitate administration—this sample was redrawn after more protamine was given if full heparin reversal was not confirmed (using the HMS Plus System) when the first sample was drawn; [PostICU] - upon intensive care unit (ICU) arrival; [3HrPostICU] - 3 hours after ICU arrival; and [24HrPostICU] - 24 hours after ICU arrival. Standard coagulation function tests including prothrombin time (PT), activated partial thromboplastin time (aPTT), platelet count, and fibrinogen concentration

were conducted per institutional protocol: pre-operative, post-protamine, and 3 hours after ICU arrival.

Blood samples were collected from central venous lines or arterial lines after drawing back 10 mL of blood. The blood was transferred immediately to non-vacuum, 3.2% citrate-containing sample tubes and placed on ice. The samples were centrifuged twice at 2900 g for 10 minutes, the platelet poor plasma (PPP) was extracted and placed in cryotubes for storage at -80°C . Samples were thawed in batches at 37°C for CAT analysis.

Thrombin Generation Measurements

In the United States, CAT equipment and reagents are distributed by Diagnostica Stago, Inc. (Parsippany, NJ). Hardware required for CAT analysis included a microplate fluorometer, Fluoroskan Ascent[®] FL (Thermo Fisher Scientific, Inc., Waltham, MA), equipped with a 390/460 nm filter set (excitation/emission) and a dispenser as well as a desktop computer with the Thromboscope[™] analysis software installed.

Thrombin generation was measured as previously described (12). Briefly, 20 μL of PPP Reagent (containing phospholipids and 5 μM of Tissue Factor), was pre-loaded in Immulon 2HB, round bottom 96-well plates (Thermo Fisher Scientific Inc., Waltham, MA). In separate wells, 20 μL of Human Thrombin Calibrator was pre-loaded to provide reference thrombin curves. In all wells, 80 μL of thawed sample plasma was pipetted in. Samples were run in triplicates for both PPP Reagent and calibrator wells. Thrombin activation in all wells was initiated when the fluorometer automatically dispensed 20 μL of the FluCa mixture. The FluCa mixture consisted of the fluorescent substrate Z-GGR-AMC (2.5 mM) freshly dissolved in HEPES buffer, pH 7.35 (20 mM HEPES, 140 mM NaCl, 100 mM CaCl_2 and 60 mg/mL bovine serum albumin). The analysis software produced the following parameters with normal ranges (mean \pm standard deviation) provided (18):

- 1) Lag Time (min): Reflects the initiation phase of thrombin generation, i.e., the time taken for the explosive phase of thrombin generation to initiate. Most clotting time tests measure only the time taken for this event to take place. Normal: male = $2.63 \pm .59$; female = $2.49 \pm .39$.
- 2) Peak (nM): The maximal concentration of thrombin generated in that sample. Normal: male = 318.5 ± 52.9 ; female = 293.4 ± 48.5 .
- 3) Time to Peak (ttP, min): Indicative of the rate of thrombin generation. Normal: male = $5.82 \pm .88$; female = $5.52 \pm .85$.
- 4) ETP or Endogenous Thrombin Potential (nM.min): Provides an overall measure of thrombin functionality in clot formation because it is the product of thrombin

concentration and time. Normal: male = 1745 ± 259 ; female = 1803 ± 241 .

The propagation phase of thrombin generation is reflected by the mean rate of thrombin generation (19). This velocity index is derived from the above parameters by the formula $\text{Peak}/(\text{ttP-Lag Time})$, and is expressed in nM/min.

Data Handling and Statistics

Patient demographics and perioperative clinical data were extracted from their electronic charts and tabulated. Preoperative transfusion risk scorings were calculated (5). Data are plotted as mean \pm SD unless otherwise noted. For each parameter being analyzed, analysis of variance with repeated measurements was used to detect significant differences between measurements at various time points. Post hoc testing was done using Bonferroni/Dunn's method to make pairwise comparisons between baseline and the different post-CPB time points. A *p*-value of less than .05 was considered significant unless otherwise indicated.

RESULTS

Demographics and preoperative clinical data of the cohort of patients studied are summarized in Table 1. No patients required surgical re-exploration for bleeding. All patients recovered from their operations and were discharged from the hospital. On Table 2 we present each patient's predicted probability of receiving homologous blood transfusions based on pre-operative information to provide a standardized method of gauging this cohort's relative risks. The TRACK (Transfusion Risk and Clinical Knowledge) system is a validated risk model that assigns scores for each of five predictors (age, weight, pre-operative hemoglobin, gender, and surgical complexity), which are then added up and used to generate the probabilities (5). The measured amount of chest tube drainage (3 hour and 24 hour totals) as well as the number and type of blood products administered are also presented on Table 2.

Calibrated automated thrombography produces curves as shown in Figure 1 for a representative patient over the study duration. Patient F had a normal baseline

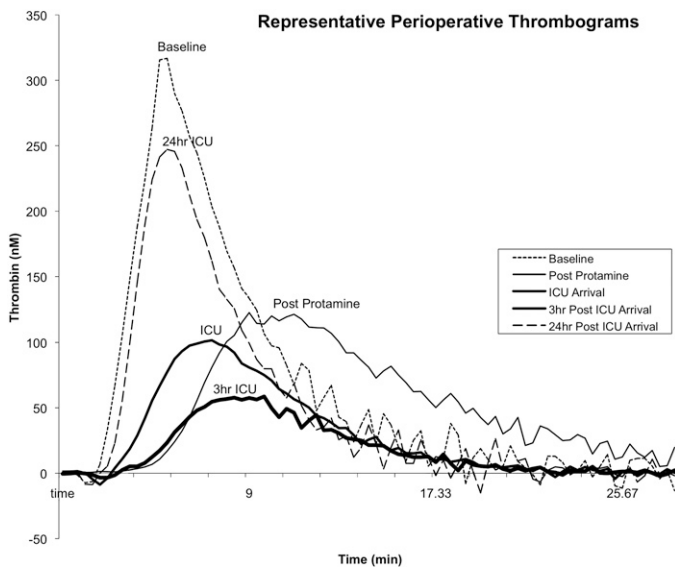
Table 1. Demographics and surgical data.

Demographics	Mean	Minimum – Maximum
Age (years old)	69.9	45–85
Body surface area (m^2)	2.0	1.87–2.33
Gender (male/female)	9/2	NA
Surgical data		
Cross-clamp time (min)	82.9	48–158
CPB time (min)	118.6	75–230
On-CPB urine output (mL)	285	107–807
Cell saver processed volume returned (mL)	623	242–1213

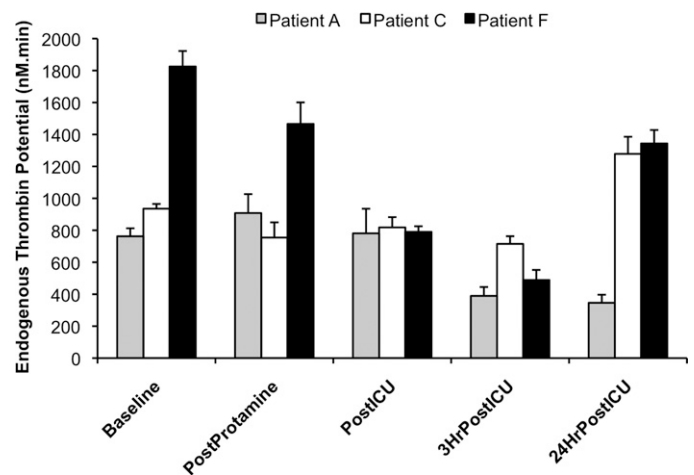
Table 2. Transfusion risks, blood loss, and transfusions received.

Patient	TRACK Risk	3 Hr CTD (mL)	24 Hr CTD (mL)	PRBC (Unit)	FFP (Unit)	Platelets (Conc)	Cryo (10 pack)
A	87.5%	305	1285	2	4	1	0
B	81.0%	130	332	3	0	1	0
C	58.5%	81	366	1	0	1	1
D	72.3%	590	890	0	3	1	0
E	55.5%	300	950	2	4	1	1
F	31.8%	268	551	1	4	2	1
G	46.3%	200	595	2	5	1	0
H	67.1%	415	1595	4	6	2	1
I	49.4%	910	2370	0	6	2	1
J	61.5%	235	905	1	3	1	0
K	52.4%	230	850	1	4	1	1
Mean	60.3%	333	972	1.5	3.5	1.3	.5

Cryo, cryoprecipitate concentrates; CTD, chest tube drainage; FFP, fresh frozen plasma; PRBC, packed red blood cells; TRACK, transfusion risk and clinical knowledge.

**Figure 1.** Changes in thrombin generation curves from Patient F over the study period.

thrombogram. After CPB was terminated and systemic heparin neutralized, the thrombin generation curve was observed to have shifted to the right and had a lower peak. The shift to the right was reflected in the extended lag time while the decreased slope to reach the peak was quantified by calculating the propagation rate. Despite the dramatic difference in the shape of the curve, the area under the curve, representing the ETP, was not too different from baseline for this patient (see Figure 2). Administration of four units of FFP, two platelet concentrates, and a 10 pack of cryoprecipitate seemed to help the lag time improve at arrival to the ICU, but the improvement was not sustained 3 hours later. The thrombogram from the sample taken at 3 hours after ICU arrival was in fact the poorest of all the samples obtained for this patient. By the next day, the thrombogram was beginning to look similar to the one generated from the baseline sample.

**Figure 2.** Changes in Endogenous Thrombin Potential in Patients A, C, and F over the study period. Each sample was analyzed in triplicate. Error bar = standard deviation.

Changes in ETP over the study duration for three patients are shown in Figure 2. Patient A experienced the longest pump run amongst this study cohort at 230 minutes. This patient did bleed substantially (>1000 mL over 24 hours) postoperatively despite blood product transfusions. His ETP was the only one that did not recover robustly on the following day. Patient C received one unit each of platelets and cryoprecipitate but did not receive any FFP. This patient's ETP remained near abnormally low baseline levels over the study duration, but showed improvement the next day. The pattern of ETP changes shown in Figure 2 for Patient F was representative of most patients in this study cohort. Six of 11 patients produced ETPs where the post-protamine samples were near baseline levels but deteriorated in the ICU, only to recover the next day. The lowest measured ETP was noted in the first ICU sample in two patients while this occurred at 3 hours post-ICU arrival in the other four patients.

Clotting function as reported by PT testing was significantly worse after CPB as shown in Figure 3A. The

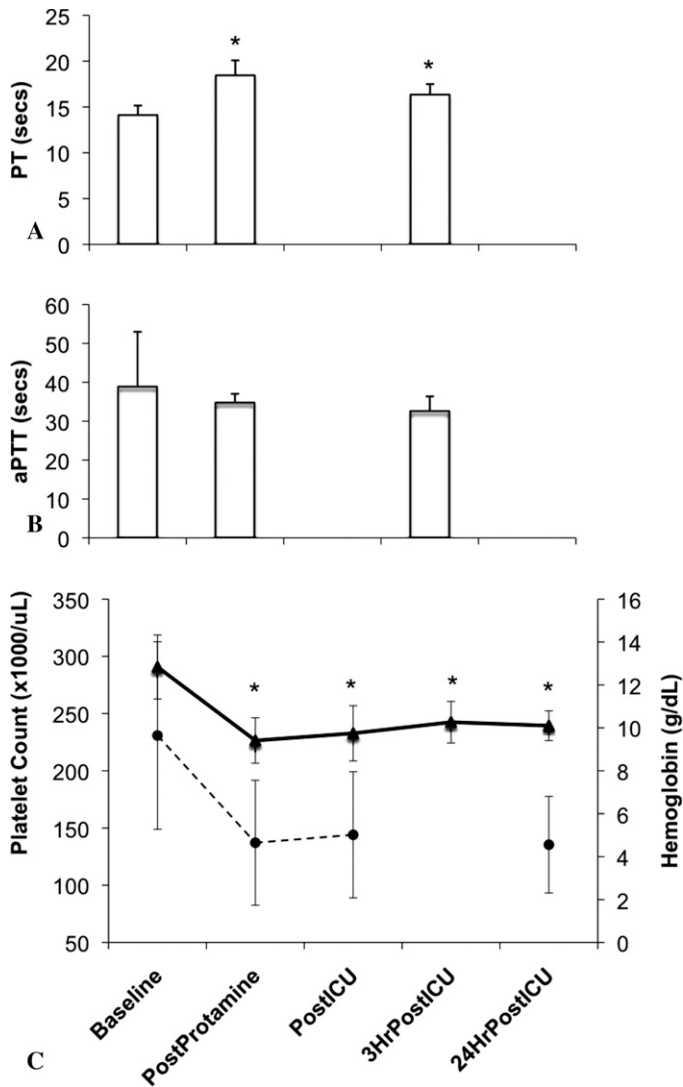


Figure 3. Clotting function tests, hemoglobin, and platelet count changes over the study period. A: Prothrombin Time; B: Activated Partial Thromboplastin Time; C: Platelet Count (filled circles) and Hemoglobin (filled triangles). $n = 11$ for most time points. Mean \pm SD * $p < .005$ compared to baseline.

aPTT, however, did not change on average (Figure 3B). Hemoglobin concentration and platelet counts (Figure 3C) were significantly reduced from baseline levels and this was sustained over the study duration despite most patients receiving transfusions of cellular blood components following CPB.

Changes in cohort CAT parameter averages are presented in Figure 4. As shown in Figure 4A, the lag time had deteriorated in post-CPB samples. Compared to baseline, the increase in lag time became significantly worse at the 3-hour post-ICU arrival time point (Figure 4A). The propagation rate and ETP, however, were preserved near baseline after heparin reversal but also became significantly worse on average in the ICU (Figure 4B, C). The lowest

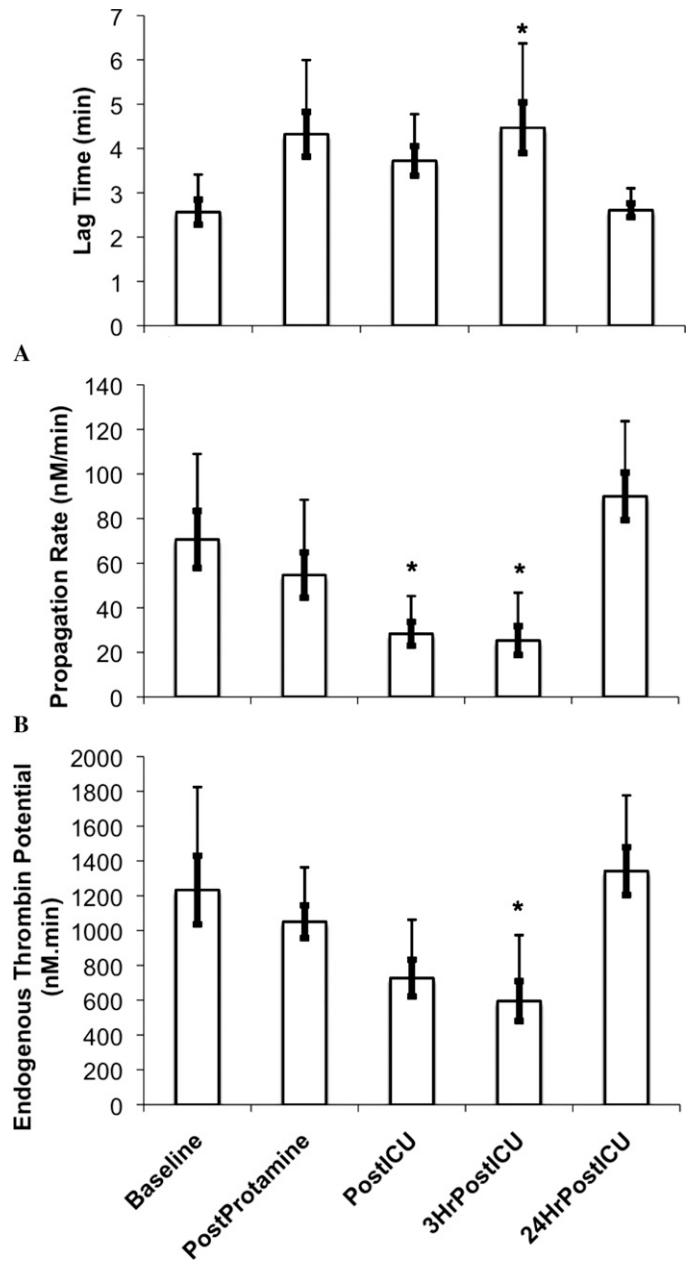


Figure 4. Thrombogram parameter changes over the study period. A: Lag Time; B: Propagation Rate; C: Endogenous Thrombin Potential. $n = 11$ for most time points. Mean \pm SD. Bold error bars: standard error of mean. * $p < .005$ compared to baseline.

average ETP occurred 3 hours following ICU admission, 1233 ± 591 versus 595 ± 379 nM.min (mean \pm SD; $n = 9$, $p < .005$). All CAT parameter averages in the cohort recovered to baseline levels by the next day after surgery.

DISCUSSION

In this pilot study, perioperative thrombograms were obtained in a small series of cardiac surgery patients.

Endogenous thrombin potentials and propagation rates were preserved near baseline in most patients after heparin was neutralized post-CPB. In contrast, lag times were found to be increased over the same period. Since the lag time represents the same aspect of blood clotting as in standard coagulation testing, its deterioration along with those of PTs after heparin reversal should be expected. Elevated PTs, being less than twice reference values, are not considered abnormal for immediate post-CPB samples (20–22). When hemostasis was deemed adequate, chest closure and transfer to the ICU commenced. Subsequently, ETP and propagation rates deteriorated for many patients during recovery in the ICU despite all patients having earlier received hemostatic blood component transfusions and despite the lack of severe bleeding (<150 mL/h of chest tube drainage) in most patients. Standard coagulation function test results remained typical for postoperative cardiac surgery patients (<1.5× normal) (20,23). Several postulations may be raised by this phenomenon:

- 1) Heparin Rebound—thrombograms are certainly highly sensitive to the presence of small amounts of heparin; however, aPTTs were not significantly elevated. The value of aPTTs as an indicator of heparin rebound has recently been questioned (24) by studies showing poor correlation between elevated aPTTs and elevated anti-Xa levels. Future study designs would require other tests to either confirm or rule out heparin rebound, such as anti-Xa assays and/or TEG with and without heparinase.
 - 2) Consumption of clotting factors—since active clot formation likely continues at the microvascular level during postsurgical recovery, it is possible that consumption had exceeded coagulation factor synthesis by the liver in many patients. In patients lacking severe bleeding, stable, adequate clots may have already formed prior to factor depletion. Moreover, recent reports raise the possibility that transfusions, as often occurred in this cohort, are known to contain elevated cytokines and platelet activating factor, leading to a paradoxical increase in consumption of proteins and perhaps even subclinical bleeding (22,24).
 - 3) Dilutional Coagulopathy—*in vitro* thrombogram studies have previously been shown to result in reductions of ETP rather than in lag times (17). Possible exacerbations of plasma dilution after CPB may be attributable to packed red blood cell transfusions, the return of cell saver processed volumes, as well as injudicious use of crystalloid solutions by anesthesia personnel. Schols et al. also demonstrated improvements in ETPs when diluted plasma samples were supplemented with prothrombin complex concentrates (17). Factor concentrates, unlike FFP, do not contain anticoagulant factors and may thus be more efficient at treating dilutional
- coagulopathy. The same group had previously demonstrated deficient thrombin-generating potential in patients who were non-responsive to FFP transfusion (16).
- 4) “Pendulum” theory—As discussed and presented by a recent industry sponsored hemostasis advisory panel (25) there may be a physiologic series of shifts in risks between hemostasis and thrombosis following any surgical procedure. The necessary procoagulant state immediately postsurgery, which promotes clot formation and hemostasis, gives way to an anticoagulant state during the recovery period. The authors suggest that the anticoagulant phase may be a necessary physiologic response due to the need to prevent uncontrolled clot formation and to promote recovery. Thrombograms in this study seem to support this “pendulum” theory although future studies may need to tease out the contributory effects of the aforementioned possibilities.

Chandler and Velan studied and characterized the kinetics of thrombin activation during and immediately after CPB by measuring thrombin activation markers supplemented with computer models (13). Three time points in that study are in correspondence with the present study, namely: baseline, post-protamine, and 2 hours after surgery (roughly post-ICU arrival in this study). The activation studies noted that thrombin and soluble fibrin generation spiked upon CPB initiation, during myocardial reperfusion and after protamine administration. Two hours following surgery, thrombin activation and fibrin generation were observed to be returning to baseline values. At the post-protamine time point, the present study suggests that ETP was preserved and available to generate the activity noted in the previous study. At the 2 hour postsurgical time point this study raises the question of whether the lowered thrombin activation markers measured in the previous study could possibly be as a result of the depletion of thrombin-generating capacity as indicated by ETPs.

Conclusive findings based on this pilot study are severely restrained by the small patient cohort and the lack of comparison groups. Most obviously interesting would be a comparison between patients receiving and not receiving hemostatic blood component transfusions, along with their respective bleeding statuses. In this observational study, no effort was made to use a standardized, validated transfusion protocol. Incidences of prophylactic transfusions will have to be eliminated from any future study since they tend to confound the meaning of any test system with bleeding being the end point. An effort to do so using TEG-Guided Therapy with platelet mapping has been initiated at our institution. Simultaneous thrombography and modified thromboelastography may complement each other well

as they are both capacitive assays (as opposed to kinetic testing with clotting time assays) (17). The former provides information on thrombin-generating capacity while the latter would provide information on fibrin clot formation as well as platelet functionality. Thrombogram analysis can be performed with platelet rich plasma (26), but requires overcoming more logistical challenges than the available resources allocated to this pilot study.

Serial thrombograms obtained following cardiac surgery have not previously been reported. Its potential utility as a guide to manage blood component transfusions is unknown. Future studies carried out in conjunction with the use of protocol driven transfusion management algorithms may enable thrombography to better reveal the pathophysiological changes in the postoperative cardiac surgery patient. Better understanding of these changes may elucidate which changes are relatively benign while alerting clinicians to those that require treatment.

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