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The Effect of Air Exposure and Suction on Blood Cell Activation & Hemolysis in an *In-Vitro* Cardiectomy Suction Model

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Abstract

Cardiopulmonary bypass (CPB) elicits a systemic inflammatory response. The cause may include surface-induced leucocyte activation and hemolysis. An *in-vitro* study was designed to describe the effects of both suction and an air-blood interface independently, and in combination on leucocyte and platelet activation, and hemolysis in an *in-vitro* model.

Fresh human blood was drawn and tested under 4 different conditions including: control (A), 10 minutes of – 600 mmHg suction (B), 10 minutes of blood exposure to room air at 100 ml/min (C), and 10 min of simultaneous suction and air flow (D). Samples were analyzed by flow cytometry (platelets and leucocytes) and plasma free hemoglobin (PFHb). Leucocyte CD11b expression and platelet P-selectin (CD62P) were analyzed by flow cytometry.

Compared to baseline, granulocytes were significantly activated by air (Group C, $p=0.0029$), and combination (Group D, $p=0.123$) but not by suction alone (Group B). Monocytes and platelets were not significantly activated in any group. The PFHb increased significantly in Group C ($p<0.001$) and Group D ($p<0.001$).

This study suggests that the inflammatory response and associated hemolysis during CPB may be related to air exposure, which could be reduced by minimizing the air exposure of air to blood during cardiectomy suction.

Keywords

leucocytes; hemolysis; inflammatory mediators; cardiopulmonary bypass; platelets

Introduction

Cardiopulmonary bypass (CPB) is commonly used to support the circulation during operative repair of acquired and congenital heart diseases. However, CPB is associated with certain pathophysiologic side effects that are related to the time on bypass and the complexity of the underlying operation. Systemic inflammation, hemolysis and coagulation dysfunction have all been described as major complications.^{1–3} Many pathways and factors contribute to these CPB associated morbidities and include: contact of the blood components with artificial surfaces and air exposure, ischemic-reperfusion injuries, operative trauma and

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suction.⁴ Much research has centered on the causes of systemic inflammation, coagulation abnormalities and hemolysis during CPB and cardiotomy suction (CS) use, but the exact cause of these side effects is not completely understood.

Cardiotomy suction allows better visualization in the operative field by returning shed blood back to the heart-lung machine by using negative pressure applied to a suction wand or device. However, CS creates dynamic conditions that expose blood to air, suction and additional exposures to plastic surfaces. These factors may cause a pronounced inflammatory response, hemolysis and activation of certain coagulation and humoral factors in blood that has been exposed to conditions created by CS.⁵ Reinfusion of CS blood may contribute to secondary organ dysfunction and other complications including renal failure, respiratory failure, neurologic dysfunction, bleeding disorder and myocardial injury.^{6,7} Eliminating CS may reduce these side-effects; however CS is required for many cardiovascular procedures including complex intracardiac and great vessel procedures as well as valve replacements and congenital repairs. Avoiding the use of CS may increase the need for homologous blood transfusions postoperatively.^{5,8}

Previously, this laboratory described an in-vitro red cell damage model where room air, suction and a combination of the air and suction were exposed to static blood samples.⁹ In both sheep and human blood, hemolysis was dose related to the amount of suction and air flow in combination when tested together. The maximum test conditions were -600mmHg (suction) and 100mL/min (air flow). Isolated exposure to either air or suction alone showed minimal changes in hemolysis. The current study was designed to study to effects of these conditions on platelets and leucocytes

Materials and Methods

After the approval from the Institutional Internal Review Board and subject consent, 20 mL of fresh blood from 23 healthy human volunteers (n=23 for platelets, n=10 for leucocytes), was individually acquired through venipuncture and transferred into citrated tubes (BD Lavender Vacutainer Sodium Citrate 0.109M, 3.2%) and used to conduct the experiment at room temperature (24–26° C). Five mL aliquots from each sample were individually transferred into 50 cc polystyrene centrifuge tubes (Thermo Fisher Scientific Inc., NY) that were tested under 4 different and unique conditions.

In-Vitro CS Model Apparatus

The experiment was tested on an apparatus consisted of a test chamber made from 50 mL polyethylene centrifuge (Thermo Fisher Scientific Inc., NY) a vacuum pump used to generate a suction -600 mmHg (Model ME4, Vacuubrand Inc., Essex, CT) and a Matheson 602 series precision gas flow meter (Matheson Trigass, Montgomeryville, PA) to control a consistent 100 mL/min room air flow through blood sample. These conditions were selected because they elicited maximal red cell damage in previous work and allowed for indirect comparison to similar test conditions. The gas flow meter was connected to an air line with the tip positioned just above the bottom of the test chamber. As air bubbles traveled through the blood sample, an air-blood interface was generated and a blood-air bubble column expanded from the base of the test chamber. The air-blood column was contained within the test chamber by a polyurethane sponge plug coated with antifoam A (Sigma-Aldrich, St. Louis, MO) and a rubber stopper. The antifoam-A sponge isolated the reaction to the test chamber by debubbling the foam that was created by the reaction to air. The pressure within the circuit was monitored continuously with a digital pressure indicator (Model DPI 705, Omega Engineering Inc., Stamford, CT). All experiments were conducted at room temperature [Figure 1].

Experimental Conditions

Blood from each sample was tested in 4 different conditions (Table 1); each condition was applied individually for 10 minutes in time. Group A consisted of baseline conditions (Control). Suction at – 600 mmHg was applied to the blood sample and designated as Group B, which was generated and regulated by the vacuum pump. Group C blood was exposed to air at 100 mL/min, generating a frothing blood–bubble mix. Group D was a combination of air and suction exposure of described in Groups B and C. The specific air and suction settings were chosen because they elicited maximal hemolysis in previous work and they represent conditions that can occur during clinical use of CS.⁹ One physical factor that does exist in all blood and flow models are shear forces. Models do exist for calculating shear, however there is no practical way to calculate shear in this test model.

Supplemental Antifoam A Testing

The potential did exist for an artifact related to the exposure of blood to Antifoam A to activate the blood independent of the individual test conditions. Additional pieces of Antifoam A-coated sponge used in this test model were taken from a commercially available cardiac bypass reservoir (Terumo, Tokyo, Japan) and added to samples of blood from each group to determine if polyurethane antifoam-A caused activation. Surface marker expression was then quantified.

Hemolysis

After running the different experimental conditions, 3 mL of blood from each aliquot was analyzed to quantify hemolysis. The measurement of plasma free hemoglobin (PFHb) was made using a spectrometer with absorbent readings at 577nm, 560nm and 593nm.¹⁰

Indirect Immunofluorescence Flow Cytometry Preparation

Monocyte and granulocyte CD11b expression was used as a marker for leucocyte activation. Also, CD62P (P-selectin) expression was measured on platelets as a marker of platelet activation.

Platelet measurements—The conditioned blood aliquots were diluted 10 μ L to 990 μ L with HBSS (Hank's Balanced Salt Solution without CaCl₂, MgCl₂, MgSO₄, phenol red) in polypropylene 12 $\times$ 75 tubes and gently mixed. 100 μ L of the diluted blood was added to another 2 labeled tubes (P1 and P2). As a control, 10 μ L of FITC-mouse IgG1 and 10 μ L of PE-mouse IgG1 were added to P1 tube and for the experimental tube, 10 μ L of CD61-FITC and 10 μ L of CD62P-PE were added to P2 tube. Then both tubes were incubated at room temperature in dark for 15 minutes. Later on, 700 μ L of 1% formaldehyde buffer (1% formalin in dPBS) was added to each tube, vortexed, and transferred to 12 $\times$ 75 polystyrene tubes and stored for 24 hours in refrigerator at 4°C. Samples were then run using a flow cytometer, and data were collected and analyzed.

Leucocyte measurements—For leucocyte CD11b measurement, 100 μ L of the conditioned aliquot was transferred individually to polystyrene tubes (W1) and directly labeled with 10 μ L of antibody for PE-mouse antihuman CD11b and FITC-mouse anti-pig CD14. As controls for FACS analysis, 10 μ L of FITC-mouse IgG2b negative control and PE-mouse IgG2a negative control were used in the other tube (W2). Once stock solutions were applied, tubes were stirred and incubated in the dark at 4°C for 30 minutes. 2 mL of BD RBC lysis buffer was added to each tube and incubated for 10 min at room temperature in the dark. Tubes were then centrifuged for 5 minutes at 1100 rpm. Following the removable of supernatant, 2 mL of washing solution buffer (dPBS + 0.1% sodium azide + 0.5% BSA) was added to each tube. Tubes were centrifuged a second time for 5 min at 1100

rpm. Following the removal of supernatant, 250 μ L of 1% formaldehyde buffer was added to each tube. Tubes were vortex mixed, transferred to 12 $\times$ 75 polystyrene tubes and stored for 24 hours in a refrigerator at 4°C.

Cell Counts—For cell counts, the surface expression of CD14 on the leucocyte was used to approximate the WBC count. The surface expression of CD61 on platelets was used as a measure of platelet count.

Statistical Analysis—To account for variances between human subjects, all measured parameters were compared as a percentage of baseline to values obtained following each test condition. Baseline measurements for each marker displayed a non-parametric distribution; therefore the non-parametric Wilcoxon signed-rank test was used to determine statistical significance for surface marker expression. Data were compared to baseline values for each blood sample and presented in percentile value where baseline was 100%. Because multiple variables were being tested, the Bonferroni correction was applied. A significant p -value was defined as $p < 0.0125$.

Results

The present study compares the effects of air exposure and suction on blood cell activation and hemolysis using an *in-vitro* CS model. In this model the air-blood interface caused leucocyte activation and hemolysis.

Hemolysis

Whole blood hemolysis was significantly higher in Groups C and D, corroborating previous studies.⁹ The mean PFHb in Groups A and B were similar ($p=0.741$). In Groups C and D, PFHb increased to an average of 50.55 ± 2.91 mg/dL ($p < 0.001$) and 99.59 ± 4.53 mg/dL ($p < 0.001$), respectively [Figure 2].

Leucocyte and Platelet Counts

The leucocyte and platelet counts showed no difference between control and any experimental condition. There were no statistically significant differences in leucocyte and platelet counts between groups as determined by the Wilcoxon signed-rank test. The mean total leucocyte counts compared to baseline were $94 \pm 13\%$ ($p=0.655$) in Group B, $95 \pm 14\%$ ($p=0.715$) in Group C and $95 \pm 9\%$ ($p=0.735$) in Group D. Platelet counts were similarly unchanged in all conditions with average of $105 \pm 34\%$ ($p=0.5130$) in Group B, $105 \pm 24\%$ ($p=0.466$) in Group C and $116 \pm 36\%$ ($p=0.860$) in Group D [Figure 3].

Inflammatory Response Markers

The results showed an increase in the expression of CD11b in human granulocytes with a mean of $304 \pm 230\%$, ($p=0.0029$) in Group C and a mean of $232 \pm 169\%$, ($p=0.0123$) in Group D. No significant changes occurred in Group B with mean of $116 \pm 35\%$ ($p=0.7623$) [Figure 4].

The expression of CD11b in monocytes showed no significant changes, group B with mean $110 \pm 26\%$ ($p=.9214$), group C with mean $126 \pm 28\%$ ($p=.51$), and group D with mean $114 \pm 25\%$ ($p=.6926$) [Figure 4].

Platelet activation

The expression of P-selectin was not significantly different from baseline in Groups B ($99 \pm 30\%$, $p=.9633$), C ($147 \pm 68\%$, $p=.0166$) and D ($122 \pm 44\%$, $p=.2103$) [Figure 4].

Supplemental Antifoam A Testing Result

Samples of group A blood with no additive showed no significant difference in granulocyte, monocyte, or platelet activation compared to group A blood exposed to Antifoam A covered polyurethane; $p=.439$, $p=.1130$, and $p=.0303$, respectively.

Discussion

This study evaluated the effect of air and suction on leucocytes and platelets in a simple *in-vitro* model. The air-blood interface used in this test model with and without suction caused leucocyte activation and hemolysis, which confirmed earlier studies from this laboratory that the combination of air and negative pressure causes hemolysis.⁹ The goal was to evaluate the different effects of air exposure and suction independently, and in combination on blood components and how this may contribute to leucocyte activation and hemolysis. Air exposure caused activation of granulocytes, but not monocytes or platelets.

In this study, blood samples were anticoagulated with citrate which may have had a modulating effect on leucocyte and platelet activation. Further studies will be conducted with heparin anticoagulation to determine any effects that occur as a result of using different anticoagulants. It is possible that heparin alone can activate an inflammatory pathway or the chelation of calcium ion by citrate may decrease activation.¹¹

Cardiotomy suction has been recognized as a major source of hemolysis during cardiac operations.¹² Cardiotomy suction may play a significant role in inducing a systemic inflammatory response.⁵ The influence of CS and on postoperative systemic inflammatory response is not fully understood. Many studies have demonstrated elevated levels of hemolysis and inflammatory mediators in CS blood and therefore concluded that re-transfusion of CS blood contributes to the postoperative response.¹³ However, these studies do not clarify the cause of this activation.

Measuring granulocyte and monocyte CD11b expression provide a means to identify an early-onset inflammatory response.¹⁴ Data exists that shows an increase in pro-adhesion potential, as indicated by an increase in granulocyte and monocyte CD11b.¹⁵ In this study, the results indicate that granulocyte CD11b expression was increased during isolated air-blood interface and combined air-suction conditions. On the other hand, no changes in the expression of CD11b were observed in isolated suction condition alone. These data support the idea that the inflammatory response caused by CS may be related to the excess amount of air exposure but not isolated suction. Granulocyte activation causes a release of cytokines which mediate the inflammatory response. In future studies this model will be used to measure the cytokine levels and correlate these with CD11B expression.

Cardiopulmonary bypass induces a degree of hemolysis as a result of a combination of mechanical trauma and biological mechanisms. This study shows that the source of hemolysis is primarily from factors associated with an air-blood interface and suction as have been previously described.⁹ The data describe a significant increase in PFHb in isolated air-blood interface, even when combined with suction. This observation corroborates other investigations that reported an increase of red blood cell damage during the use of CS.^{12,16}

Cardiotomy suction could cause platelet damage or activation due to aspiration of air and blood. Excessive use of CS may result in a decreased ability of platelets to aggregate.¹⁷ P-selectin (CD62P) is a component of the α -granule membrane of resting platelets that is expressed only on the platelet surface during and after platelet degranulation and secretion.¹⁸ Platelet surface P-selectin is considered to be the "gold standard" marker of

platelet activation.¹⁹ Despite the fact that many studies have presented alterations in platelet count, clot formation and platelet activation during CPB,²⁰ the results from this study did not indicate any statistically significant increase of P-selectin expression, or any significant change in platelet count under any test condition. Platelet function was not evaluated, but function will be measured in future experiments.

There are several limitations to this study. An in-vitro test does not clearly reproduce the in-vivo setting in which variations in patients or additional factors such as temperature, shear forces and other artificial organs such as filters and the gas exchange device may induce either minor or profound changes. A negative pressure of -600 mmHg and 100 cc/min of air flow used may be considered excessive for the length of time (10 minutes) in which the blood was tested. These conditions are routinely reached or exceed during clinical CPB. Further studies will vary the amount of suction and air to determine if a "dose-response" effect on leucocytes exists. The experimental model evaluated static blood, thus the application of these parameters in an active blood flowing model would be a better representation of in-vivo use.

In clinical application an air-blood interface is not only observed in cardiomy suction but also observed when intermittent or continuous streams of air siphon down the venous line towards the venous reservoir. The amount of air is related to the siphon pressure or the amount of air being entrained at the venous cannulation site in the heart. Although this air is removed in the venous reservoir it is certainly possible the air-blood interface could enhance cellular activation or injury. Minimizing air entrainment in the venous line or eliminating the air-blood surface in the venous reservoir could minimize blood damage.

The findings from this study indicate that air exposure and suction during simulated cardiomy suction is a cause of hemolysis and granulocyte activation. The response could be minimized by reducing air exposure to the blood and minimizing suction pressures. Clinical use of cardiomy suction varies widely amongst individual practitioners, yet it is widely accepted that blood damage occurs, especially when there are large amounts of suction air, foam, and blood. "Sucker bypass" is one such extreme when massive amounts of air, blood and suction are used in an emergent setting of massive hemorrhage. It is certainly feasible that shed blood from the operative field can be discarded or salvaged by a red cell washing device. However immediate re-transfusion is necessary there is large blood loss from the operative field. When a coronary suction tip becomes occluded, an extreme negative pressure can be created which can meet or exceed the values tested in this model. A pressure limited suction wand that vents in atmosphere would reduce extremes in negative pressure if the wand became occluded. In addition, large amounts of air are exposed to blood if the suction pump is on and blood is contained in the suction tubing. Hemolysis and granulocyte activation caused by CS could be minimized by limiting air exposure and suction pressure.

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Figure 1.
Experiment apparatus: A: gas flow meter. B: vacuum pump. C: blood sample. D: pressure regulator. E: antifoam-A sponge.

Figure 2.
Changes in hemolysis for each experimental condition.

Figure 3. Changes in platelet and leucocyte counts compared to baseline for each experimental condition.

Figure 4. Changes in monocytes, granulocytes and platelets compared to baseline for each experimental condition.

Table 1

Summary of Study Conditions

Group	Volume	Group Description	Study Condition	Testing Time
Group A	5mL	Baseline	Normal condition (control)	0 minutes
Group B	5mL	Suction	Suction at -600mmHg	10 minutes
Group C	5mL	Positive pressure	Air exposure at 100mL/min (bubbling)	10 minutes
Group D	5mL	Combination	Both suction and air exposure	10 minutes