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Journal

ASAIO Journal, 60(4)

ISSN

1058-2916

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Publication Date

2014-07-01

DOI

10.1097/mat.0000000000000079

Peer reviewed

HHS Public Access

Author manuscript

ASAIO J. Author manuscript; available in PMC 2015 July 01.

Published in final edited form as:

ASAIO J. 2014 ; 60(4): 429–435. doi:10.1097/MAT.0000000000000079.

Biocompatibility Assessment of the Levitronix® Centrimag® Adult ECMO Circuit in a Model of Acute Pulmonary Hypertension

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Abstract

Extracorporeal membrane oxygenation (ECMO) is rarely utilized in patients with severe pulmonary hypertension (PH) as a bridge to lung transplantation. In this study we assess the blood biocompatibility of the integrated Centrimag® Novalung ® ECMO system (veno arterial) in an acute model of PH. Severe PH (≥ 2/3 systemic) was induced in eight ovines through progressive ligation of the main pulmonary artery. System performance, platelet activation, thromboelastography parameters, fibrinogen, plasma free hemoglobin, and total plasma protein were measured at initiation, 3 and 6 hrs of support in the ECMO (N=4) and sham (N=4) groups. A stable ECMO flow (2.2 ± 0.1 L/min), low trans-membrane pressure gradient and steady blood O_2 and CO_2 levels were maintained. Platelet activation was low (< 4%) in both the groups whereas platelet responsiveness to agonist (platelet activating factor) was reduced in the sham group when compared to the ECMO group. There were no differences in the TEG parameters, fibrinogen concentration, plasma free hemoglobin (< 10 mg/dL) and plasma total protein between the two groups. The findings of low levels of platelet activation and plfHb suggest adequate blood biocompatibility of the integrated Centrimag® Novalung® circuit use for short term support in a model of PH.

Introduction

Extracorporeal membrane oxygenation (ECMO) has been a useful method to provide cardio-respiratory support in patients with severe lung dysfunction[1]. Since its first clinical report in 1972, ECMO has been increasingly used in patients undergoing lung transplantation for pulmonary allograft dysfunction or as a bridge to lung transplant[2]. The ideal support system

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to be utilized in patients with pulmonary hypertension (PH) leading to pulmonary failure is yet to be defined.

Despite recent medical advances, patients with PH are still a challenging population to treat. Severe right ventricular failure makes PH a progressive disease with a dismal prognosis[3]. Significant improvements with the use of intravenous or inhaled pulmonary vasodilators have been made in the medical treatment of patients with primary PH delaying the natural cause of the disease[4,5]. However, lung transplantation is the only option for patients refractive to pharmacological therapies[6]. Literature on the use of ECMO in adult patients with PH is rare. Only few reports with the use of veno-arterial (VA) ECMO to rescue patients with idiopathic pulmonary artery hypertension after listing for lung transplant have been presented in the adult population[3]. However, ECMO is frequently used in infants with both primary and secondary PH to treat congenital abnormalities[7].

Despite widespread use, the duration of support for ECMO is often limited to 2–3 weeks and is associated with significant complications including bleeding, thrombosis, inflammation, hemolysis and infection[8]. Elevation in cellular activation (such as activated platelets and leukocytes), cytokines and other humoral factors have been reported during ECMO support[9–11]. These complications lead to significant morbidity and mortality. Moreover thrombosis of the pulmonary arterial system during veno-arterial (VA) ECMO support (where the blood bypasses the lungs) is a feared potential complication that could affect this patient population.

The introduction of ECMO support using magnetically levitated pumps, and low resistance coated oxygenators have enhanced the efficiency and biocompatibility of the ECMO systems[12,13]. The use of such pumps and oxygenators has reduced trauma to blood, reducing the risks of hemolysis and thrombosis and lowering complement activation[14].

Very scarce data is available on the use of ECMO in models of severe PH with elevated PAP resulting in Right ventricular strain and failure. Most of the studies have used other mechanisms (such as exposure to monochrotaline and shunting) to induce PH[15,16]. Also, measuring the red blood cell damage (hemolysis) and platelet activation are essential parameters to assess the biocompatibility of artificial organs and have been utilized extensively in evaluating mechanical pump design[17,18]. Hence evaluating the blood biocompatibility is a vital aspect in the design of medical devices.

In this study we assessed the blood biocompatibility of the Levitronix CentriMag® – NovaLung® (Figures 1A & 1B) integrated adult ECMO circuit in an acute model of PH. Circulating activated platelets, thromboelastogram (TEG) parameters, fibrinogen, plasma free hemoglobin and total plasma protein (TPP) were assessed. The purpose of performing these measures was to assess blood biocompatibility, although the overall purpose was focused on defining performance variables of each device, rather than specifically evaluating the blood compatibility. We hypothesized that this integrated ECMO circuit with the magnetically levitated CentriMag pump and low resistance Novalung oxygenator would limit platelet activation and hemolysis in an acute PH model. We believe that this is the first study in evaluating platelet activation and hemolysis in a PH model supported with ECMO

in large animals and could serve as a point of comparison for other newer integrated systems under development.

Methods

Study Plan

All animal testing was performed under an approved protocol from the University of Pittsburgh Institutional Animal Care and Use Committee. A total of eight animals were included in the study. Four animals (N=4) underwent surgical initiation of ECMO at 2.5 L/min. Sham studies was performed on four animals (N=4) and maintained in the state of PH without ECMO treatment. Complete anticoagulation was maintained during the study period with a continuous infusion (or bolus) of unfractionated heparin.

Surgical Procedure

All animals were premedicated with atropine and anesthetized with a bolus of sodium thiopental (15 mg/kg initial bolus, followed by 0.5 mg/kg/h administered intravenously). Ventilation was provided through endotracheal intubation with a 40% FiO_2 at a frequency of 12–15 breaths/min and a tidal volume of 6–10 ml/kg/min. Ventilator settings were adjusted by maintaining arterial partial carbon dioxide pressure between 35–40 mmHg. The left carotid artery was cannulated for recording aortic pressure and sampling blood for blood gas and electrolyte analysis. The left jugular vein was also cannulated for measuring central venous pressure, right ventricular pressure and the pulmonary artery pressures through a Swan-Ganz catheter. Animals were heparinized (150 U/kg) to maintain the whole blood activated clotting time (ACT) between 180–240 seconds.

To create the acute hypertension model, a lateral thoracotomy in the fourth intercostal space, removal of 5^{th} rib and pericadiotomy was performed. The great vessels were dissected and a umbilical tape was placed around the pulmonary trunk above the pulmonary valve similar to a PA-banding procedure. A pressure catheter was placed distal to the pulmonary band. The Swan-Ganz catheter was pulled back until the distal end placed proximal to the PA banding. Basal echocardiographic measurements were performed with a GE-Vingmed Vivid system (GE Vingmed Ultrasound, Horten, Norway). The examination was recorded on digitally. Standard 2D views, pulsed and continuous wave Doppler measurements were obtained as per current recommendations and modified as needed for this open-chest animal model

The pulmonary artery snare was tightened gradually until the peak pulmonary artery pressure reached 70% of the systemic arterial pressure. Cannulation for VA ECMO was performed through the right internal jugular vein and right common carotid artery. ECMO support was initiated once the PA pressure goal was attained or in case of hemodynamic deterioration during PA snaring. ECMO flows were maintained at 2.5 L/min for four animals.

Blood collection

Preoperative whole blood was collected from each animal by jugular venipuncture using an 18-gauge 1.5-in needle with syringe. The first 3 mL were added to sodium heparin tubes

(VacuTainer, Becton Dickenson, NJ) for plfHb, TPP and fibrinogen. An additional 2.7 mL of blood was drawn into monovettes containing 0.3 mL of 0.106 trisodium citrate (Sarstedt, Newton, NC, USA) for platelet activation and TEG assessment. Postoperative samples were collected at initiation (0 hr), 3 and 6 hours of support in the ECMO and sham group.

Platelet activation analysis

Platelet activation was assessed using an established flow cytometric analysis[19]. Briefly, blood (5 μL) was transferred from tubes into 12 × 75 mm polystyrene tubes with 5 μL of CAPP2A (7.5 μg/mL; Veterinary Medical Research and Development; VMRD, Pullman, WA, USA), 5 μL of goat anti-mouse IgG-phycoerythrin (60 μg/mL; Invitrogen, Carlsbad, CA, USA), and 35 μL of Tyrode buffer (Electron Microscopy Services, Hatfield, PA, USA) with 1% bovine serum albumin (BSA) and 0.106 M sodium citrate and incubated for 20 min. Stimulated samples were prepared as above using 25 μL of Tyrode's buffer with BSA, and 5 μL of either 200 μM adenosine diphosphate (ADP; Calbiochem, San Diego, CA, USA) or 100 μM platelet activating factor (PAF; Calbiochem, San Diego, CA, USA), and 5 μL of 20-mM GPRP (Anaspec). Samples were washed with 1 mL of Tyrode's buffer with 1% BSA and 0.106 M sodium citrate (washing buffer) and mixed. Samples were centrifuged for 10 min at 132 × g. The supernatant was discarded and the pellet resuspended. IgG1-Alexa Fluor 488 (isotype control; 5 μL of 25 μg/mL; MCA928A488, AbD Serotec), MCA2418-Alexa Fluor 488 (5 μL of 25 μg/mL; MCA2418-A488), and/or MCA2419-Alexa Fluor 488 (5 μL of 25 μg/mL; anti-human CD62P-clone Psel.KO.2.7, AbD Serotec) were added to tubes and incubated for 20 minutes. Samples were mixed with 1 ml of washing buffer and centrifuged at 132 × g. Following removal of the supernatant, 1% paraformaldehyde was used to fix the samples. Flow cytometric analysis was performed as previously described[19].

Thromboelastography (TEG)

In addition to the ACT, thromboelastography (TEG, Haemonetics, IL, USA) was used to assess the thrombogenic status of the blood with and without the effects of heparin. TEG is a methodology used clinically to measure the kinetics of whole blood coagulation and provides a comprehensive assay of the overall clotting process by measuring the rate and strength of blood clot formation[20]. Briefly, 20 μL of 0.2 M calcium chloride was added to a TEG sample cup. Blood samples (1000 μL) were mixed with kaolin (3455, Heamonetics, IL) and 340 μL of blood was added to the sample cup before initiating the TEG. R-time (time until the first significant levels of detectable clot formation), k-time (time from the measurement of R time until a fixed level of clot firmness is reached), Alpha (rate at which the clot or fibrin develops), MA (represents maximum clot strength) were measured.

Hemolysis and Fibrinogen measurement

Heparinized blood (3 mL) samples were used in hemorhelogical assays to measure blood parameters including plfHb, TPP, and fibrinogen concentrations. Blood samples were centrifuged at 9,500 × *g* for 15 min at room temperature (ML Vanguard V6-500, Marketlab, Inc., Caledonia, MI, USA). The plasma supernatant was then transferred to a 1.5 mL microcentrifuge tube and centrifuged again at 15,000 × *g* for 12 min. The supernatant was

placed into a 1.5 mL microcentrifuge tube and then centrifuged again at 20,800 × *g* for 20 min. The resulting plasma was then transferred to disposable semi-micro cuvettes (Thermo Fisher Scientific, Inc.) for measurement of plfHb, TPP and fibrinogen concentrations were assessed using a bench top refractometer (Kernco Instruments Co., Inc., El Paso, TX, USA). Total plasma protein concentration was used as an indirect measure of blood dilution and is also generally related to liver function.

Statistical Analysis

Continuous variables are shown as mean with standard deviation. Two-way analysis of variance (ANOVA) was performed to compare the ECMO group with the sham group. Tukey's post hoc test was used for pair wise comparisons. Values of $p < 0.05$ were considered statistically significant for each analysis.

Results

System performance

All animals completed the study protocol. ECMO pump flow was consistently maintained at 2.2 ± 0.1 L/min throughout the study period for 6 hr as shown in Figure 2A. All ECMO animals showed optimal respiratory support during the study period with mean arterial pO_2 and pCO_2 values of 390.95 ± 56.03 mmHg and 39.08 ± 4.46 mmHg, respectively (figure 2B). The transmembrane pressure gradient was low throughout the study (5.7 ± 1.46 mmHg, Figure 2C). The sham group had a decreased mean arterial pO2 value (figure 2b) of mm Hg 196±38.7 mm Hg ($p<0.05$).

Hemodynamics and Hematological parameters

The hematological values and hemodynamics are presented as shown in Table 1 and 2 respectively. No differences were observed between the ECMO and the sham group in any of the parameters listed ($p > 0.05$) except pulmonary arterial pressure (PAP), Central venous pressure (CVP) and Right ventricular pressure (RVP) (ANOVA, $p <0.01$). All the animals were all within the same weight (41.9±4.6 kg) with a cardiac output of 4.4±1.7 L/min.

Circulating Activated Platelets-CD 62P expression

Resting platelets—Post-operative platelet activation (unstimulated) in both the ECMO and the sham group remained low (<10%) during the 6 hr period and remains similar ($p > 0.05$) for both the groups (Figure 3).

Platelet response to agonist—The platelet response to ADP stimulation was significantly increased (60–70%, $p<0.01$) when compared to the unstimulated samples ($p < 0.05$). However no difference can be seen in the ADP stimulated samples between the ECMO and the sham group ($p = 0.46$). Activation of platelets in response to PAF (Figure 3) shows a statistically significant decrease in platelet activation in the sham group when compared to the ECMO group ($p = 0.01$, ANOVA), indicating reduced platelet functionality in the sham group (No ECMO). However the pair wise comparisons using Tukey's test did not show any significant difference.

Hemostatic and blood damage markers

The ACTs for both groups were maintained above 200 sec throughout the 6 hour study period. The thrombosis markers R-time, K-time, Angle (α) and MA were measured using the TEG and were not statistically different between the ECMO and the sham group as shown in figure 4. The overall fibrinogen concentration (mg/dl) remained within the normal range (100–200 mg/dl) and similar in both the groups. PlfHb and TPP (Figure 5) were also within the normal range and similar in both the groups ($p > 0.05$). PlfHb was found to be lower than 10 mg/dl in both the groups.

Discussion

ECMO has been an established support for cardiac and respiratory failure and is frequently used to salvage patients in advance cardiopulmonary failure as a bridge to transplant or recovery[21]. Patients with severe PH are a challenging population with progressive and fatal disease. Pharmacological therapies using vasodilators such as epoprostenol and bosentan have provided excellent results. However lung transplantation remains the only beneficial option for patients refractory to optimal vasodilator treatment[6]. The experimental use of right ventricular assist devices (RVAD) in PH patients has resulted in the elevation of the PA pressure leading to parenchymal bleeding and death in spite of temporary hemodynamic improvements. Some authors such as Berman et al. have suggested that VA ECMO is the most optimal strategy during RV failure as it decreases PA pressure, increases left sided pressures and facilitates preservation and recovery of end-organ failure[22].

Previously a venous-venous Centrimag® Novalung® combination has been used successfully for respiratory support in a model of PH for more than 72 hours without inducing hemodynamic, coagulatory, or inflammatory imbalances[13]. In our study we have used a veno-arterial CentriMag-Novalung circuit in an acute PH model to evaluate biocompatibility. The results of the biocompatibility testing showed few differences in platelet activation and thrombosis markers, which is somewhat challenging to conventional thinking. However, experience with the CentriMag®, a third generation blood pump with a fully magnetic bearingless technology, has already shown minimal heat generation and reduced blood cell damage in ventricular assist device (VAD)[19]. The addition of the Novalung, a low resistance gas exchange device with a biocompatible heparin - albumin coated surface, did not change the measured platelet activation or hemostatic parameters over published values for the CentriMag device itself. The Novalung has been previously reported as a pumpless CO_2 removal technology for lung transplantation[13,23]. Applications of the Novalung in a pumpless setting are limited to CO_2 removal due to the limited flow that can be shunted across into the venous circulation. However, coupling the Novalung with a centrifugal pump in a venous-venous setting has previously been shown to provide adequate oxygenation in pigs[13]. In our study a sufficient pO_2 value of 391 ± 56 mm Hg and pCO_2 of 39 ± 4.5 mm Hg were achieved confirming the adequate oxygenation capabilities of the integrated system. New oxygenators with integrated pumps have been developed in the past to provide excellent oxygenation[24,25]. It will be very interesting to compare the results obtained from this study with these newer systems currently available.

While ECMO has provided patients with respiratory and cardiac support, these devices are associated with a myriad of complications including bleeding, thromboembolism, and infection and can lead to debilitating strokes, sepsis and multi-organ failure[26,27]. These biocompatibility limitations in ECMO are some of the major reasons leading to patient complications. Thus addressing the issues of biocompatibility becomes a necessity while designing medical devices.

The presence of circulating activated platelets has been suggested as a marker for increased risk of thrombotic complications[28]. Platelet activation has been measured in patients with blood contacting devices such as stents, mechanical heart valves and VADs, as well as in patients without blood contacting devices that are experiencing from acute myocardial infarction, and ischemic stroke[29]. Previously several flow cytometric assays to quantify circulating activated bovine and ovine platelets were developed and applied to pre-clinical studies with VADs[19,28]. The application of similar assays during ECMO implantation yields a greater understanding of device effects on ovine platelet activation during pre-clinical testing as well as in the development of new surface coating technologies. Major et al. used such assays to measure the CD62P expression on platelets in an ECMO circuit coated with a nitric oxide (NO) releasing polymer[30]. The NO releasing polymer significantly reduced thrombus formation in the ECMO circuit compared to polymer control after 4 hr blood exposure and platelet CD62P expression increased upon collagen stimulation of blood.

In our study, the platelet activation in both the groups (ECMO and sham) remained very low (<10%). Furthermore the platelets were able to respond to agonists such as ADP and PAF, indicating that they were not dysfunctional. In the sham group, response to PAF was diminished compared to ECMO group ($p < 0.05$); this reduced response to PAF suggested mild platelet dysfunction in the sham group. The inability of platelets to respond to stimulation suggests that substantial levels of dysfunction have occurred. Functional abnormalities of platelets have occurred in patients with chronic PH[31,32]. This could be related to specific inhibition of signaling pathway(s) involved in platelet response to PAF under PH again under chronic conditions. Though there is no clear evidence of such abnormalities in acute conditions, we speculate that a 6 hr time frame could be the onset of platelet abnormalities in severe PH. Moreover the increased response of platelets to PAF in the ECMO group could be due to the effect of extra corporeal circulation. Interestingly, the average number of platelets was similar throughout the study in both the groups as seen in Table 1. This is in contrast to previous studies in other oxygenator devices that show a sharp drop in platelet counts following initiation of ECMO[33].

Hemolysis has been a common complication associated with ECMO. Nonphysiological shear stresses, flow paths, pressure gradients, and blood contacting surfaces within the pump and oxygenator are some of the common factors associated with the occurrence of hemolysis in ECMO[34]. In our study, the level of plfHb and TPP were low indicating almost no hemolysis throughout the study in both the groups.

Preventing platelet activation and fibrin formation are extremely essential in successful clinical outcomes and provide a need to decrease thrombus formation in patients with extracorporeal life support (ECLS). In the past, circulating activated platelets and hemolysis

has been quantified in bovines and ovines implanted with VADs under development. These assays have demonstrated its utility in assessing the performance and design of mechanical pumps and also evaluate various surface coatings to improve device hemocompatility[17,18,28,35]. We believe that by evaluating the performance and hemocompatibility (via platelet activation, functionality and hemolysis) of the Centrimag-Novalung device, the results may aid in the process of improving the device design and also serve as a basis for clinical trials in PH patients.

The objective of this study was to assess the biocompatibility of the CentriMag-Novalung integrated ECMO circuit as an alternative to other ECMO system available. In the ovine model these devices showed promising results with minimal hemostatic alterations. We understand that few important parameters such as inlet and outlet pO2 (marker for oxygenator failure) for the ECMO circuit under consideration were not measured. This was primarily due to the short term nature of this study. As mentioned previously, one of the primary goals of this study was to measure the blood biocompatibility focusing only on the general performance variables of the device.

Surgical sham studies were performed to characterize the effects of surgery on temporal platelet activation and hemolysis. Platelet activation and hemolysis elevated relative to surgical sham controls can be directly attributed to the device under investigation. A control group of a less modern circuit system may have provided additional insight for the relative benefits of the integrated circuit studied. It is also acknowledged that the power of this study with n=4 is low and also the fact that 6 hours is a short time frame to look for cellular activation. Also, the PA banding model in this study is more comparable to RV failure in severe pulmonary embolism and does not strongly represent an acute on chronic event. Despite this, the proposed study demonstrated that the VA ECMO used in an acute PH model showed lower platelet activation, preserved platelet function and lower incidence of hemolysis. Future studies should be conducted in a long term-support model of PH so that more extensive information can be obtained using the same markers of activation in the preclinical ovine model.

Conclusions

CentriMag–NovaLung ECMO was successfully implemented in an acute ovine model of PH. Stable pump flow of 2.25 LPM over 6 hrs with no system failures was observed with adequate oxygenation and CO_2 removal. Low levels of cellular and haemostatic activation, adequate platelet response to external stimuli (ADP and PAF), and minimal hemolysis in the ECMO group indicate promising biocompatibility. Further studies should be considered for long-term support as well as clinical application.

Acknowledgments

The authors would like to thank the McGowan Institute Center for Pre-clinical Studies for their animal care and management. This work was supported by Levitronix who also provided us with the ECMO system.

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A B

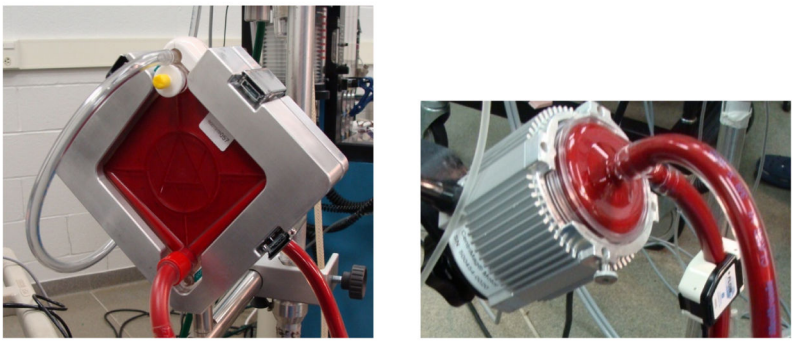

Figure 1.
A) Novalung oxygenator; B) The Levitronix Centrimag pump

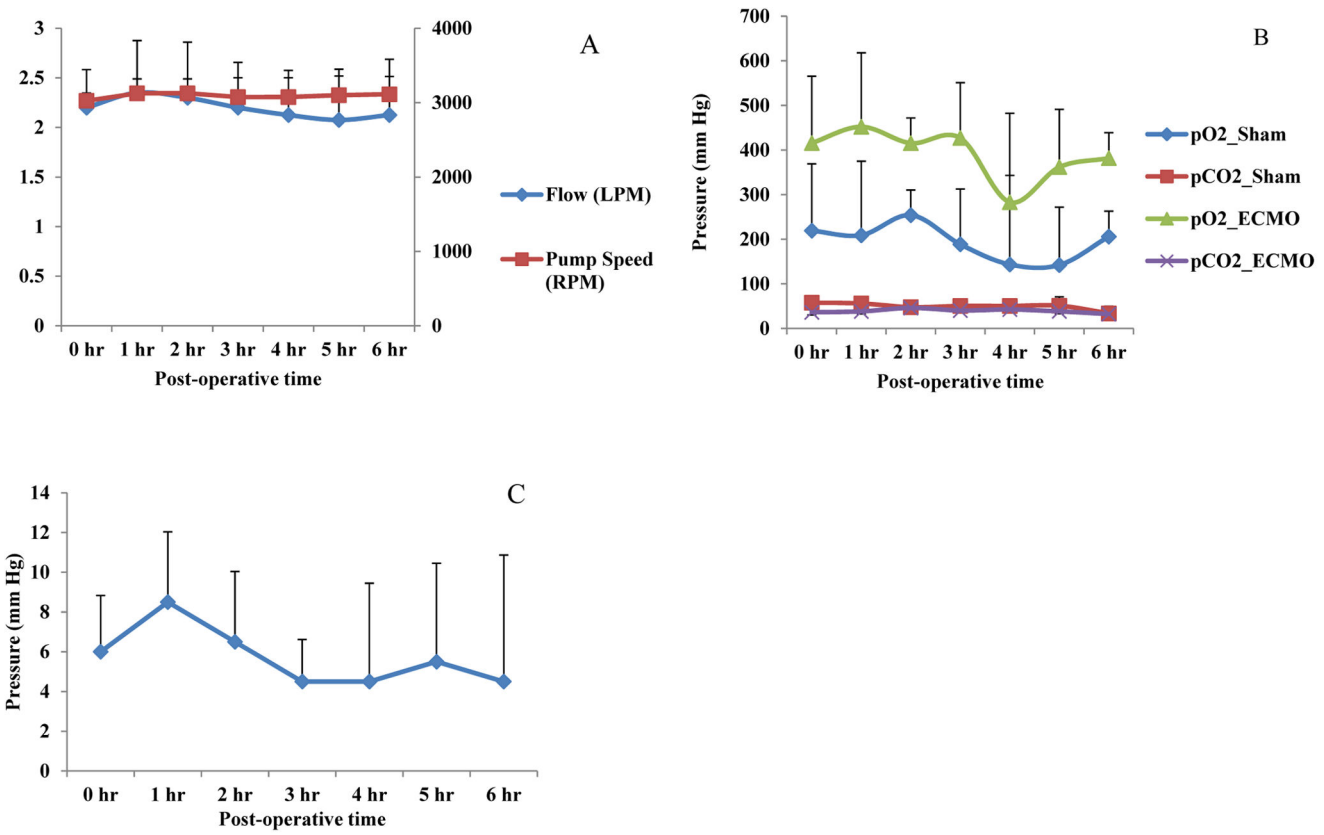

Figure 2.

System performance: A) ECMO flow maintained at 2.2±0.1 LPM and 3091±36 throughout the 6 hr period. B) A constant CO_2 and O_2 level maintained throughout the study. The pO2 and pCO2 for the sham group is also shown. C) A low transmembrane gradient of 4–8 mmHg is maintained throughout the 6 hr period.

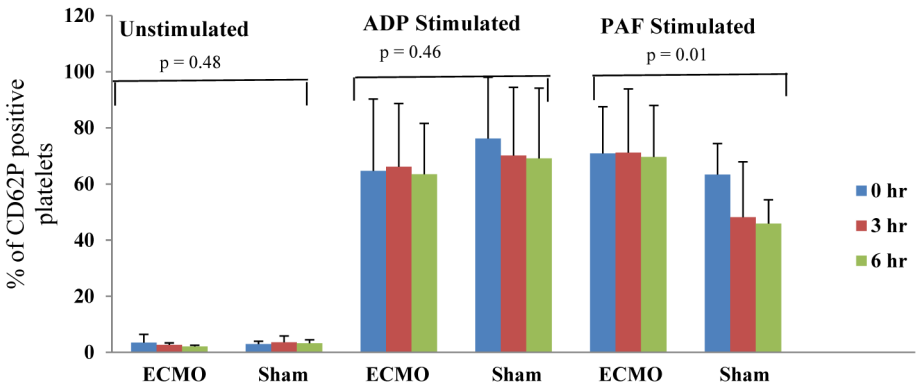

Figure 3.

CD 62P expression (unstimulated, ADP stimulated and PAF stimulated samples) on platelets at 0, 3 and 6 hr.

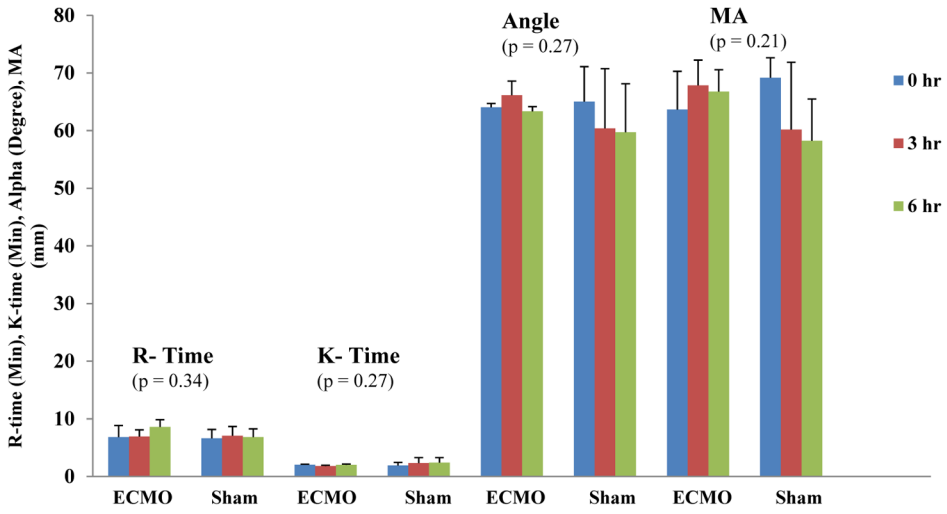

Figure 4.

TEG parameters (R-time (in Min), K-time (Min), Alpha (Degree), MA (in mm)) were measured at initiation (0 hr), 3 hr and 6 hr in the ECMO and the sham(No ECMO) group.

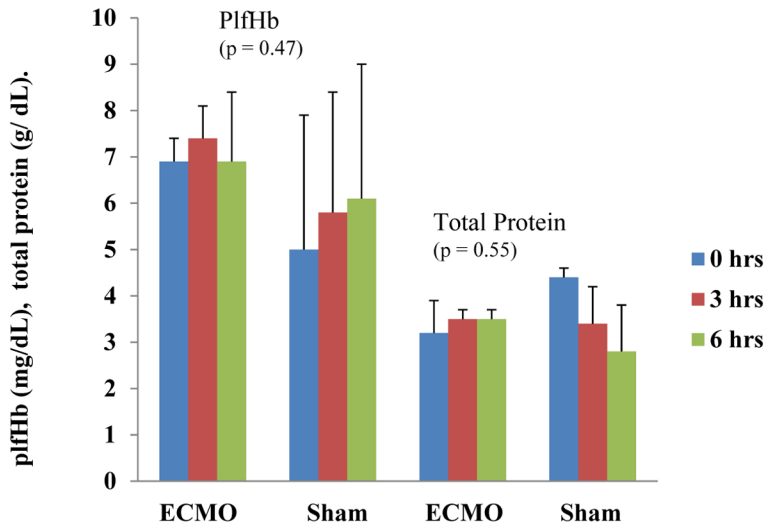

Figure 5.

A) Concentration of plfHb (mg/dL) and B) Concentration of total plasma protein (g/dL).

Table 1

Hematological Parameters

		ECMO			Sham		P-Value
	0 hr	**3 hr**	**6 hr**	**0 hr**	**3 hr**	**6 hr**	
WBC X10^3 u/L	3.32±1.55	4.97±1.16	5.1±0.8	4.32±2.03	4.75±1.98	4.52±2.29	0.43
RBC 10^6 u/L	7.15±0.94	7.29±0.69	7.20±0.89	8.78±1.02	8.23±1.41	7.81±2.07	0.51
Hgb gm/dl	6.8±1.0	6.92±0.51	6.85±0.68	9.02±0.89	8.52±1.07	8.07±1.89	0.24
HCT %	21.75±2.71	21.85±1.5	21.60±1.76	28.85±3.48	26.77±2.13	25.37±4.3	0.19
Platelets ×10^5 u/L	292±164	351.5±170.0	344.75±153.00	352.25±304.00	307.25±334.00	274.25±274	0.55
MCV	30.50±0.57	30.00±1.41	30.25±1.89	33.00±2.16	33.00±2.94	33.25±3.68	0.41
MCH	9.5±0.3	9.52±0.28	9.52±0.28	10.3±0.6	10.42±0.57	10.4±0.5	0.23
MCHC	31.2±1.4	31.7±0.2	31.7±1.1	31.35±0.71	31.75±1.63	31.55±2.20	0.61
Neutrophils %	49.5±15.5	55±9.8	69±7.1	37.25±16.76	48.5±4.2	64.5±13.7	0.66
Lymphocytes%	48.5±15.5	42.75±11.08	29±7.1	61.75±16.87	49±5	33.0±13.3	0.44
PT (sec)	25.77±5.13	30.22±8.43	29.12±7.48	29.12±14.84	23.70±7.62	27.40±9.09	0.52

Table 2

Hemodynamics

	PAP(mm Hg)		CVP(mm Hg)		RVP(mm Hg)		ABP(mm Hg)	
	ECMO	Sham	ECMO	Sham	ECMO	Sham	ECMO	Sham
0 hr	16.3±2.9	21.5±8.4	9.5±1.3	12.5±7.5	26.3±11.5	27±11.9	62±9.3	77.8±14.7
1 hr	12.7±1.2	21.3±11.4	10.3±4.6	13±7.4	25.5±3.7	27.5±6.5	58.8±8.4	67.5±7.3
2 hr	14.8±3.9	19±6	7.5±4.7	16.3±5.1	20.8±8.4	30.8±5.6	64.5±9.4	61.3±15.3
3 hr	15.3±3.6	20.8±3.8	6.8±3.0	21.5±9	22±9.5	29.3±1.3	67.3±14.5	55.3±16.1
4 hr	15±1.2	22.5±7.3	6.8±2.8	19.5±6.6	22.3±9.7	30±3.0	64.3±9.7	53.8±11.7
5 hr	15.5±0.6	22.3±5.3	7.3±1.7	22.3±6.8	24±13.6	33.3±9.3	65.3±7.3	45.8±10.8
6 hr	14.5±2.4	21.3±6.2	8.3±3.7	21.8±5.6	20±5.3	25±2.8	60±10.4	44±10.7