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Fabrication and In vivo Thrombogenecity Testing of Nitric Oxide Generating Artificial Lungs

Kagya Amoako
University of New Haven, kamoako@newhaven.edu

Patrick Montoya
Medarray Inc.

Terry C. Major
University of Michigan - Ann Arbor

Ahmed B. Suhaib
University of Michigan - Ann Arbor

Hitesh Handa
University of Georgia

See next page for additional authors

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Author line: Kagya A Amoako[1,4], Patrick J Montoya[2], Terry C Major[1], Ahmed B Suhaib[1], Hitesh Handa[1], David O Brant[1], Mark E Meyerhoff[3], Robert H Bartlett[1], Keith E Cook[1,4]

Institutional Affiliation:

[1]Departments of Surgery, University of Michigan Medical Center, Ann Arbor, MI USA

[2]Medarray Inc., Ann Arbor, MI USA;

[3]Department of Chemistry, University of Michigan, Ann Arbor, MI USA;

[4]Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI USA

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Reprint/Negotiation/Corresponding Authors: Keith E Cook, PhD and Kagya A Amoako, PhD

Departments of Surgery and Biomedical Engineering

University of Michigan

1150 W Medical Center Drive

B560B MSRBII

Ann Arbor/MI 48109-0686

USA

Phone: 734 615 5357

Fax: 734 615 4220

keicook@umich.edu

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Abstract

Hollow fiber artificial lungs are increasingly being used for long-term applications. However, clot formation limits their use to 1-2 weeks. This study investigated the effect of nitric oxide generating (NOgen) hollow fibers on artificial lung thrombogenicity. Silicone hollow fibers were fabricated to incorporate 50 nm copper particles as a catalyst for NO generation from the blood. Fibers with and without (control) these particles were incorporated into artificial lungs with a 0.1 m^2 surface area and inserted in circuits coated tip-to-tip with the NOgen material. Circuits (N=5/each) were attached to rabbits in a pumpless, arterio-venous configuration and run for 4 hrs at an activated clotting time of 350-400s. Three control circuits clotted completely, while none of the NOgen circuits failed. Accordingly, blood flows (ml/min) were significantly higher in the NOgen group (95.9 ± 11.7, $p < 0.01$) compared to the controls (35.2 ± 19.7), and resistance, (mmHg/mL/min), was significantly higher in the control group after 4 hours (15.38 ± 9.65, $p<0.001$) than in NOgen (0.09 ± 0.03). On the other hand, platelet counts and plasma fibrinogen concentration expressed as percent of baseline in control group (63.7 ± 5.7%, 77.2 ± 5.6% [$p<0.05$]) were greater than those in the NOgen group (60.4 ± 5.1%, 63.2 ± 3.7%). Plasma copper levels in the NOgen group were 2.8 times baseline at 4 hours (132.8 ± 4.5 µg/dl) and unchanged in the controls. This work demonstrates that NO generating gas exchange fibers could be a potentially effective way to control coagulation inside artificial lungs.

Introduction

Hollow fiber artificial lungs are increasingly being used for long-term applications. These applications include extracorporeal membrane oxygenation, pumpless arteriovenous carbon dioxide removal, and thoracic artificial lungs. However, clot formation limits their use to 1-2 weeks. Blood contact leads to clot formation, increased resistance, and decreased gas exchange efficiency [1-4]. Furthermore, shed thromboemboli from these devices can cause organ dysfunction. Antithrombotic coatings for blood-contacting surfaces including Membrane lungs are available [5-10], but these coatings have not worked well enough to markedly reduce clot formation or eliminate the need for systemic anticoagulation.

One possible solution to this problem is the use of NO flux from the surfaces of the artificial lung. Nitric oxide (NO) is a short-acting, potent platelet inhibitor that is normally produced by endothelial cells [11]. The half-life of NO is only 2-5 sec in blood [12]. As a result, NO delivery from polymer surfaces has been examined as a means to focus anticoagulation solely at the biomaterial surface without systemic effects or cell damage [13]. Accordingly, previous studies have shown that platelet adhesion is reduced on polymers that either release stored NO or generate it from NO donors in blood if the NO flux exceeds that of the endothelium [14,15].

The goal of this study was to examine the effect of NO generating (NOgen) surfaces in artificial lungs for the first time. Silicone (polydimethylsiloxane) gas exchange fibers were thus manufactured to incorporate Cu particles. The Cu particles catalyze NO formation in blood via decomposition of circulating s-nitrosothiols via the mechanism in Figure 1 [16-18]. NO generation and clotting have both been shown to be

linearly related to surface expression of Cu [19]. These fibers were incorporated in miniature artificial lungs, which were inserted into a circuit that was similarly coated tip-to-tip with the NOgen material. The circuit was then evaluated for thrombogenicity during for a period of 4 hour in a pumpless arterio-venous circulation model in rabbits.

Materials and Methods

Circuit Components

Radial flow artificial lungs were constructed with NOgen or pure silicone hollow fibers (Figure 2A). Both fiber types were constructed at Medarray, Inc (Ann Arbor, MI) using a proprietary two-part silicone formulation (MedArray Inc, Ann Arbor MI). NOgen fibers were doped with 10 weight percent (wt%) of 50 nm Cu particles (Sigma Aldrich, St Louis MO). The hollow fibers had an average inner and outer diameter of 100 and 160 μm. Each fiber bundle had a path length, axial length, and void fraction of 1.1 ± 0.2 cm, 2.7 ± 0.5 cm, and 0.37 respectively. The prime volume and surface area were 20 ml and 0.09 m^2.

The test circuit was a pumpless arteriovenous (AV) shunt (Figure 3). The inlet to outlet circuit components were each a 16 (inlet) or 14 (outlet) gauge angiocath, 1/4" luer lock PVC connector, 3" long 1/4" inner diameter (ID) tygon tubing, a 1/4" - 1/4" luer lock straight polycarbonate connector, and another 3" long 1/4" ID tygon tubing section. The NOgen shunts were coated tip-to-tip with either the two-part silicone or tygon (Fisher Scientific, Pittsburg PA) with10 wt% of 50 nm Cu(II) oxide particles (Sigma Aldrich, St Louis MO, Product number 544868) in tygon polymer. NO-gen silicone was used to coat the angiocaths and connectors in either silicone using the synthesis procedure

described previously [19]. To coat tubing, tygon pellets chopped up from tygon tubing were dissolved in tetrahydrofuran (THF) (Sigma Aldrich, St Louis MO) at, 1g pellets per 3mL THF by vortexing the mixture for 30 minutes. Cu particles were then suspended in the solution and sonicated for 30 minutes. The resulting mixture was then coated onto the circuit tubing and cured at room temperature for 48 hours.

Measurement of NO Flux from Fibers

NO generation was measured from 1 cm long tubing samples (NOgen and non-NOgen surfaces, N=5 ea), and from 1 cm long fibers (NOgen and non-NOgen, N=5 ea) using a Seivers nitric oxide analyzer (NOA), model 280 (Boulder, CO) according to previously described methods [19]. In brief, S-nitrosoglutathione (GSNO, 1 μM), 30 mM glutathione and 5 mM Ethylenediaminetetraacetic acid, all purchased from Sigma Aldrich, were added to an amber reaction vessel containing phosphate-buffered saline (PBS, pH= 7.34) at 37˚C. The solution was purged with nitrogen gas and the output gas was swept to a nitric oxide analyzer (GE Analytical Instruments, Boulder CO) at 200 ml/min. Baseline measurements were taken for 5 minutes before samples were introduced into the GSNO-rich solution. The NO generated from the reaction was continuously measured, and a peak NO flux was calculated by dividing the peak NO generation rate by the sample surface area.

Rabbit Thrombogenecity Model for Testing Extracorporeal Circulation (ECC) Circuits

The animal handling and surgical procedures were approved by the University Committee on the Use and Care of Animals in accordance with University of Michigan

and federal regulations. A total of 10 ECC circuits (N=5/group) were tested for thrombogenecity using 10 adult New Zealand male rabbits (Myrtle's Rabbitry, Thompson's Station, TN). All rabbits (2.5-3.5 kg) were initially anesthetized with intramuscular injections of 5 mg/kg xylazine injectable (AnaSed Lloyd Laboratories Shenandoah, Iowa) and 30 mg/kg ketamine hydrochloride (Hospira, Inc. Lake Forest, IL). Procedures for maintenance rabbits under anesthesia, maintaining normal blood pressure, surgical procedure for placement of the AV circuit, measuring blood gases (arterial blood pH, pCO_2, pO_2, total hemoglobin and methemoglobin), and measuring coagulation have all been previously published [14,15,20].

The circuit was primed with saline solution and 6U/ml of heparin sulfate and placed into position by cannulating the left carotid artery for circuit inflow and the right external jugular vein for circuit outflow. The rabbits were given a heparin bolus (300U/kg, IV). Activated clotting time (ACT) was measured with a hemochron blood coagulation system model 801 (International Technidyne Corp. Edison, NJ) using 0.4 ml of blood. Once ACT was within 350-400s, the circuit was unclamped and a 10U/kg/hr heparin infusion was initiated. In addition, 0.12 μmol/kg/min infusion of the NO donor, S-Nitroso-N- acetylpenicillamine (SNAP), was started immediately after the ECC blood flow was initiated to replace any lost NO donors in blood. Blood flow was monitored with an ultrasonic flow probe and flow meter (1/4" ME6PXN and HT207, respectively; Transonic, Ithaca, NY). Circuit inlet and outlet pressures were measured using fluid coupled pressure transducers (Hospira Inc. Lake Forest, IL) and a data acquisition system (Biopac Systems In, Aero Camino Goleta, CA). Pressures and flow were recorded at the onset of blood flow and every 30 minutes thereafter. In addition, blood

samples were collected every hour for measurement of blood gases, platelet and total white blood cell (WBC) counts, plasma fibrinogen concentration, activated clotting time (ACT), and platelet aggregation as performed at baseline. After four hours, the rabbits were euthanized with Fatal Plus (130 mg/kg sodium pentobarbital; Vortech Pharmaceuticals Dearborn, MI). The circuits were then fixed in 2% gluteraldehyde and autopsied for clot inspection on their gas exchange fibers using scanning electron microscopy (Philips XL30).

Data and Statistical Analysis

Resistance was calculated in the standard fashion as the average pressure drop across the circuit divided by the average flow rate. Mixed model analysis with repeated measures was used to determine the effect of circuit type (NOgen or control) and time on platelet count, plasma fibrinogen, and resistance using SPSS (Chicago, IL). A p-value < 0.05 is regarded as significant. Kaplan Meier analysis was used to estimate the survival of circuit type, and statistical differences in all baseline data between circuit types were analyzed using a student t-test.

Results

In Vitro NO Flux from Fibers

The NO flux from fibers and tubing containing 10 wt% Cu particle (50 nm) were 12 ± 4 x 10^{-10} mol cm^{-2} min^{-1} and 14.7 ± 2.5 x 10^{-10} mol cm^{-2} min^{-1} respectively. Addition of the control fibers and circuits to the resulted in no additional NO release over baseline readings.

General Rabbit Physiology

Table 1 presents general rabbit physiology in each group. Baseline data on PaO_2, pH, and mean arterial pressure (MAP) were significantly different between circuit types at p = (0.01, 0.008, 0.04) respectively. As shown in Table 1, baseline MAP in both NOgen and control groups were lower than normal rabbit MAP. In addition, baseline data on blood flow, $PaCO_2$, and heart rate were not different between circuit group at p = (0.28, 0.58, and 0.42) respectively. Over the course of the study, heart rate and MAP were significantly higher in the control group (223.0 ± 25.6 bpm, 64.5 ± 25.0 mmHg) than in the NOgen group (194.7 ± 6.1 bpm, 42.7 ± 7.7 mmHg) ($p<0.05$) due the vasodilatory effect of NO on blood vessels. Partial pressures of CO_2, O_2 and pH were relatively normal and stable in the control (30.1 ± 2.9 mmHg, 125.8 ± 3.1 mmHg, 7.39 ± 0.05) and NOgen (35.0 ± 8.10 mmHg, 289.8 ± 15.2 mmHg, 7.33 ± 0.08) groups. There was minor acidosis in the NOgen group, which may be due to greater AV shunt flow and resultant reduced peripheral perfusion (see below).

ECC Blood Flow and Artificial Lung Resistance

The Kaplan-Meier survival for flow in control and NOgen circuits is shown in Figure 4. All NOgen circuits remained patent for the entire test duration. In contrast, two control circuits had no blood flow after 30 minutes and one more had none after 60 minutes. For the remaining controls, one maintained at least baseline flows while the other had flows significantly less than baseline levels ($p < 0.01$). On the other hand, blood flow increased from baseline levels almost approaching significance ($p = 0.07$; Figure 5) in the NOgen group. This is due to a combination of no significant change in resistance

(see below) and the increase in mean arterial pressure that occurs over the course of the experiment.

The decrease in flow in the control group was due to an increase in resistance due to thrombus formation. The resistance in the NOgen circuits (black bars) and control circuits (white bars) is shown in Figure 6. Resistance did not change significantly with time in the NOgen group ($p < 0.01$). In the control group, all resistances from 30-240 minutes were significantly higher than baseline resistance ($p < 0.01$). Resistance in the control group rose from 0.08 ± 0.06 mmHg min/mL at baseline to 21 ± 9 mmHg min/mL at 30 minutes. Thereafter, devices with infinite resistance (zero blood flow) were removed from the data as they failed, but resistance values remained over 5.5 ± 2.5 mmHg min/mL for the two devices that retained some blood flow. Autopsy results from control and NOgen lungs, shown in Figure 7 (gross, fiber level view) and Figure 8 (fine, surface topography), revealed significantly less clot formation on the NOgen's lungs' gas exchange fibers compared to controls. It can be seen quite starkly that clot formation on the control lungs led to their increased resistance to blood flow, decreased flow, and failure. Moreover, as expected, it can be seen that clot formation is more severe in control devices that failed than in those that did not fail.

Hematology

Activated clotting times were generally higher in NOgen than in control group. At baseline ACT in NOgen (362.0 ± 97.2) was not significantly higher than control (385.8 ± 82.3, p=0.68). It also did not increase or decrease significantly after 4h of blood flow in NOgen (376.5 ± 86.9, p=0.76) and control (307.5 ± 86.9, p=0.3) groups respectively.

Methemoglobin levels remained below 0.9 ± 0.3% in all circuits. In both control and NOgen groups, platelet counts dropped significantly ($p < 0.05$) from baseline to 58.3 ± 5.6% and 53.5 ± 4.3% respectively after an hour of extracorporeal circulation (ECC). See Figure 9. In addition, the duration of blood flow had an effect on platelet count ($p<0.01$) but not plasma fibrinogen ($p=0.21$). Between hour 1 and 4, platelet counts and plasma fibrinogen did not change in control group now of size N=2, whereas only platelet count was significantly lower at hour 4 in NOgen group ($p<0.05$) compared to hour 1. See Figure 9.

Plasma Copper Concentration

Serum copper level at the onset of blood flow in control group (132.8 ± 4.5 µg/dl) was not significantly different from the NOgen group (134.7 ± 22.5 µg/dl, $p < 0.01$). As expected, baseline copper level was maintained for 4 hours in the control group ($p < 0.01$). However, plasma copper levels in the NOgen group significantly increased to 2.8 times baseline levels ($p < 0.001$).

Discussion

The aim of this study was to develop a copper-mediated, NO generating, hollow fiber membrane lung and evaluate its thrombogenicity. These fibers were created successfully and capable of $12 \pm 4 \times 10^{-10}$ mol cm^{-2} min^{-1} of NO flux. Previous 10 wt% surfaces with either 3 µm [19] and 50 nm [21] copper particles produced 9×10^{-10} and 15×10^{-10} mol cm^{-2} min^{-1}, respectively, under identical *in vitro* conditions. Thus, the fiber surfaces performed as expected. Due to the NO flux, the NOgen ECC lungs were

less thrombogenic than their non NOgen controls. The NO generating ECC lungs showed markedly less surface clot formation and were thus all patent for the duration of the study. In contrast, 60% of the control lungs clotted off enough to completely eliminate blood flow after an hour of circulation.

It should be noted that the artificial lung fiber bundle design presents an exceedingly challenging test for evaluating biocompatibility. The fiber bundles were packed very densely to maximize artificial surface and hasten coagulation. The void fraction was only 33%, compared to a typical value of 50% in a commercial oxygenator. The linear fiber density was thus 50 fibers/cm, resulting in only a 200 μm space per fiber. Given the fiber diameter of 160 μm, there is only an average of 40 μm between adjacent fibers and, moreover, adjacent fiber layers are directly touching with no space between them. Thus, even a small amount of thrombus can markedly occlude the blood flow path. In the NO lungs, platelet binding appears to be largely eliminated, keeping these narrow channels open.

Despite these positive results, systemic platelet counts did not differ between the NOgen and control groups. For this to be true, all experiments must have resulted in the same loss of platelets. Yet, SEMs indicate very little platelet binding to the NOgen lungs and significant binding on the control lungs. Several issues might explain this paradox. First, only two control devices remained patent at one hour. Thus, after baseline, platelet data in the control group reflects the least procoagulant of those devices. These devices had a lesser amount of clot formation and platelet binding. It may also be that NO reduced platelet binding to the lungs but did not fully eliminate platelet activation in flowing blood due to surface-generated, pro-coagulant molecules such as

thrombin. In this scenario, platelets would continue to pass through the lung but would then be removed in the rabbit by the mononuclear phagocyte system. This is possible because NO does not reduce protein adsorption to the fiber surface. Thus, contact system proteins such as FXII and kallikrein can still be adsorbed, initiate the coagulation cascade, and generate thrombin.

To that point, fibrinogen adsorption was larger in the NO-generating group than in controls. Greater fibrinogen adsorption has been observed in previous blood studies where copper particles were coated onto circuit tubing [15]. This effect could be due to a rougher NOgen surface or due to charge interaction between the polymer surface, where an oxidation-reduction reaction is constantly taking place, and the polar terminals of plasma fibrinogen. The mechanisms of interaction among the NOgen surface, NO generation and fibrinogen activation is, however, still not clear [14,22,23]. Future studies, therefore, should examine protein adsorption and activation at the surface in more detail and seek to reduce it. To reduce it, surface coatings could be employed that create a smoother and less adsorptive surface. This top-coat must be thin, however, such that it does not significantly inhibit hydration, corrosion, and ionization of copper at the blood/polymer interface.

The main disadvantage of NOgen is leaching of copper into blood. Although copper is an essential trace element present in normal diet, excess of it in serum can be toxic. Potential adverse effects of copper toxicity include irritation of the eyes, mouth, and nose; nausea; liver and kidney failure; and even loss of life after a high intake. According to the food and drug administration (FDA), about 2mg of copper per day is required by the average adult with an acceptable daily intake of 0.5mg per kg body

weight. Thus, the acceptable daily total Cu intake could be 37mg for a 75kg man with 5L total blood volume. If absorbed all at once, this would lead to a blood copper concentration of 750 µg/dl. The amount of copper in the blood was 333 ± 3.9 µg/dl after 4 hours but does not include any copper diffusing into tissues. It is unclear if this level would lead to toxic effects. Ultimately, long-term studies are required to examine this. If this is proven to be a problem, alternate or mixed catalysts such as organoselenium could be explored [24].

Conclusion

This study evaluated the first Cu-mediated NO-generating hollow silicone fiber lung in an ECC setup. The results indicate that NO-generating hollow fiber lungs significantly reduce blood coagulation compared to their non NO- generating controls. The resistance of the NO generating artificial lungs did not change significantly over the course of 4 hours, while the 60% of the control lungs occluded completely. Accordingly, the control group had significant lower blood flow and significantly higher resistance due to occlusive clot formation.

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Legend

Figure 1 Model of Cu-mediated NO generation from circulating S-nitrosothiols by hollow fiber membrane lungs for platelet inhibition

Figure 2 Design of radial flow ECMO oxygenator (Borrowed with permision from Medarray Inc) A, Prototype of nitric oxide generating hollow silicone fiber oxygenator B, and NOgen silicone fiber surface showing copper catalysts C.

Figure 3: Extracorporeal circulation circuits: Control (clear) and NO-generating (bottom)

Figure 4: Survival of control and experimental ECC circuits after flow initiation

Figure 5: Time course blood flow in control and NOgen ECC circuits

Figure 6: Time course blood flow resistance in control and NOgen ECC circuits

Figure 7: Scanning electron micrographs of the artificial lung fibers showing clot formation on the outer layers of fibers from A) a failed control lung, B) a control lung that survived 4 hours, and C) a NOgen lung.

Figure 8: Scanning electron micrographs of the artificial lung fiber surfaces of A) control and B) NOgen fibers taken from fiber layers in the middle of the device. Control surfaces contain far more platelet deposition than NOgen surfaces.

Figure 9: Levels of platelet consumption and plasma fibrinogen concentration during extracorporeal circulation

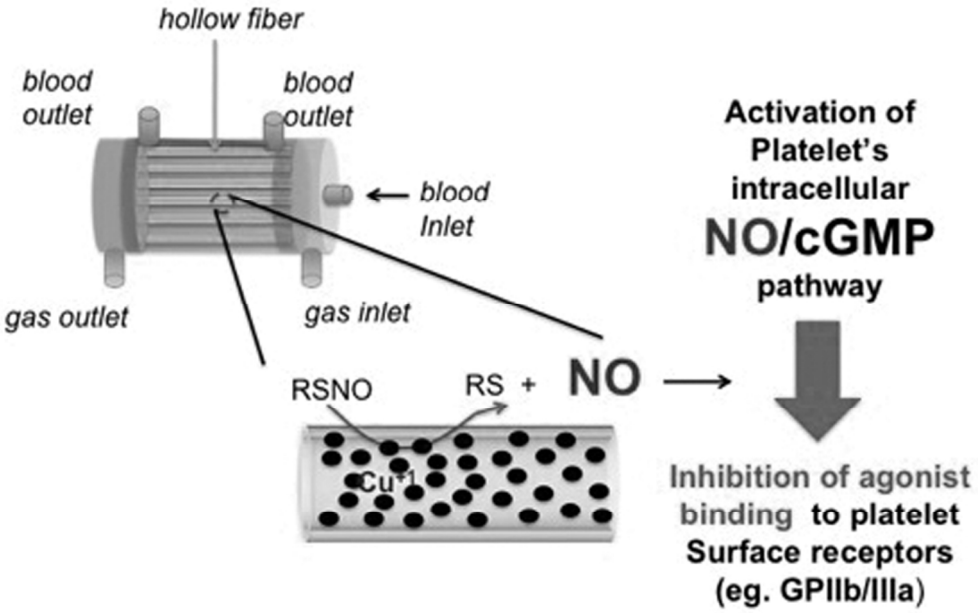

Figure 1: Model of Cu-mediated NO generation from circulating S-nitrosothiols by hollow fiber membrane lungs for platelet inhibition.
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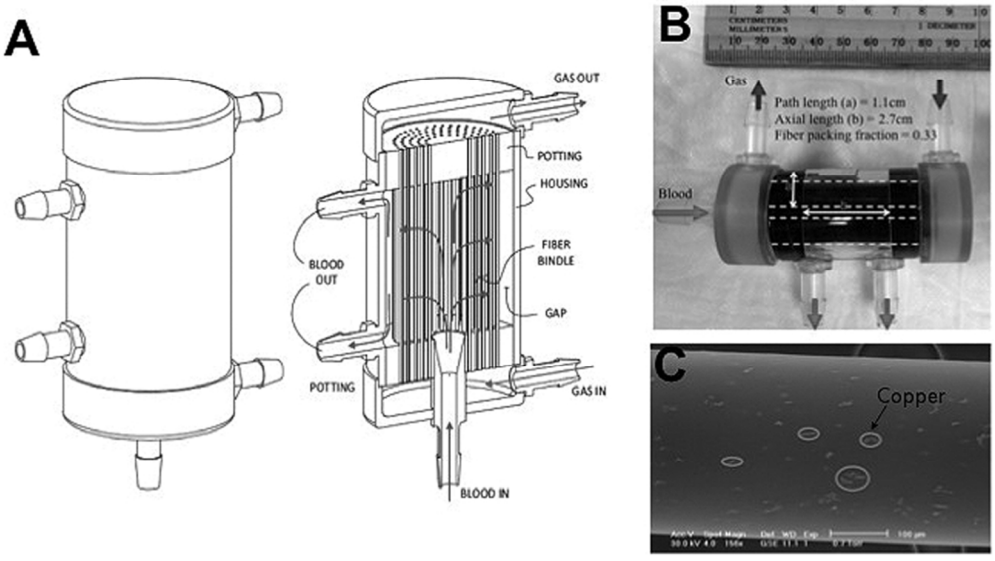

Figure 2: Design of radial flow ECMO oxygenator (Borrowed with permision from Medarray Inc) A, Prototype of nitric oxide generating hollow silicone fiber oxygenator B, and NOgen silicone fiber surface showing copper catalysts C.
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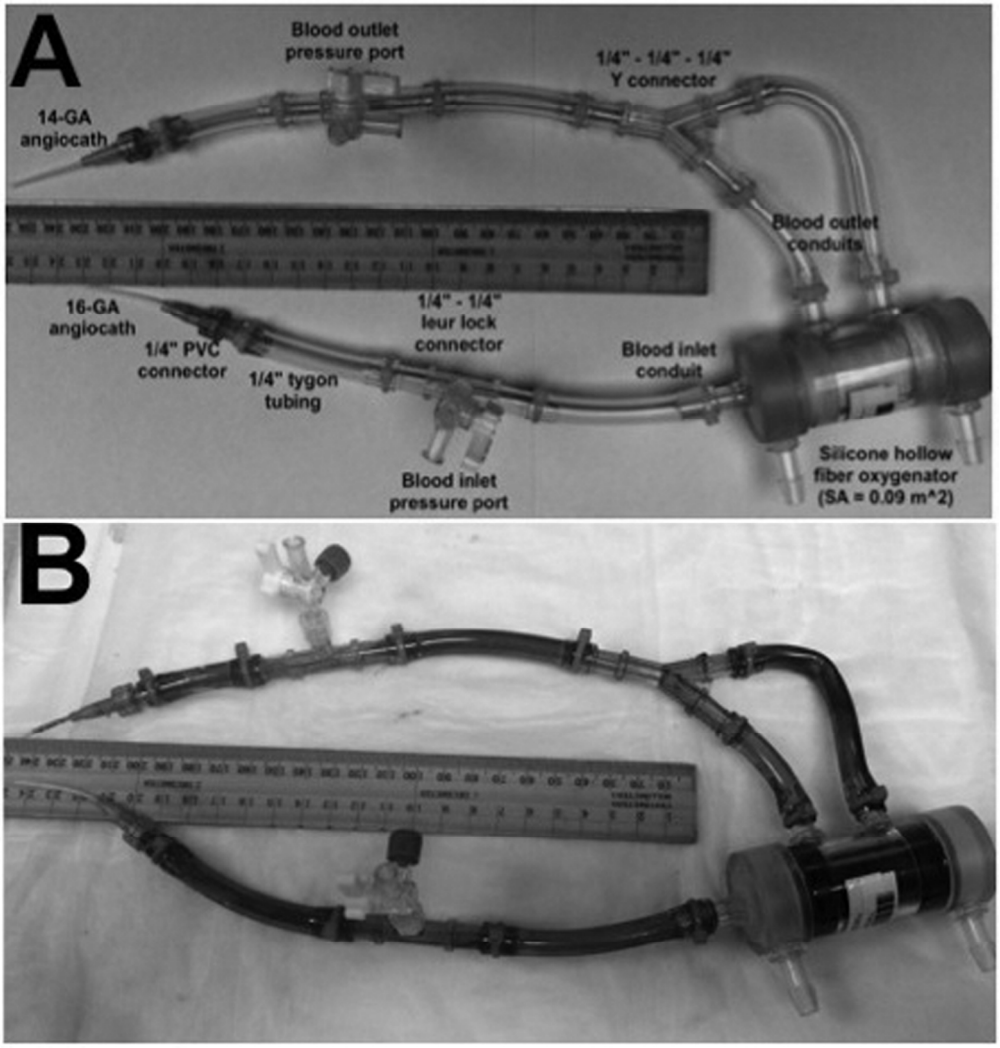

Figure 3: Extracorporeal circulation circuits: Control (clear) and NO-generating (bottom).
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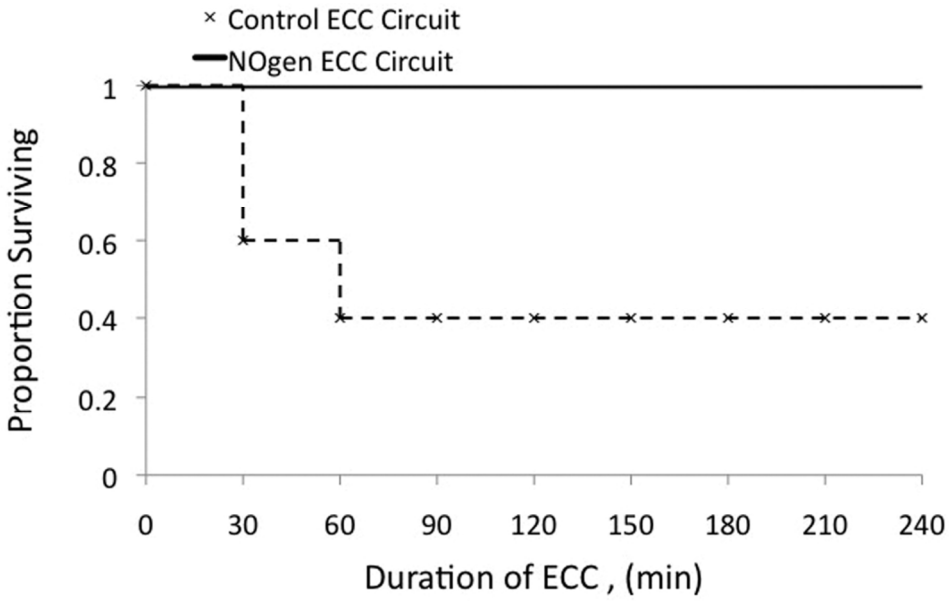

Figure 4: Survival of control and experimental ECC circuits after flow initiation.
62x38mm (600 x 600 DPI)

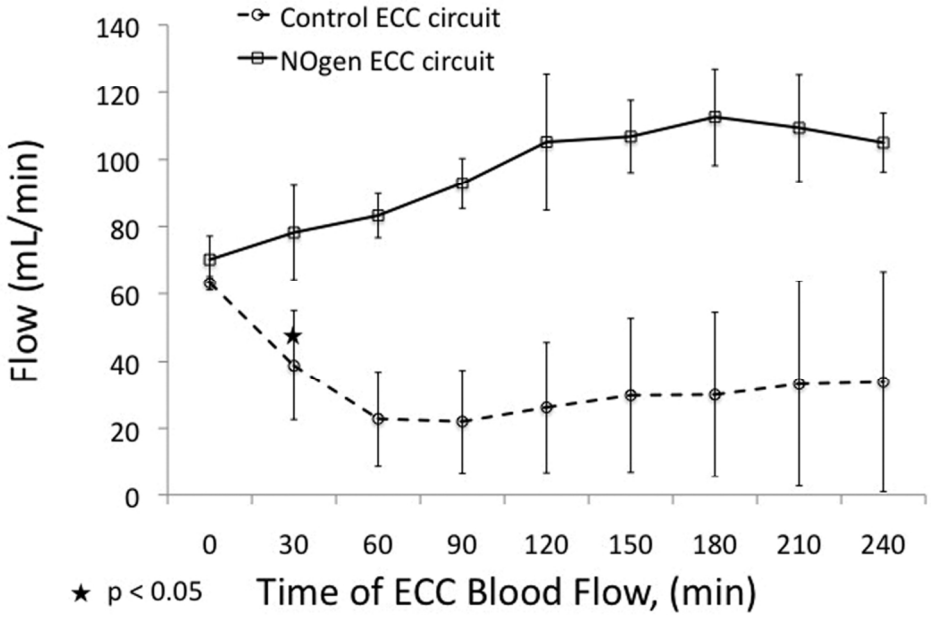

Figure 5: Time course blood flow in control and NOgen ECC circuits.
69x47mm (600 x 600 DPI)

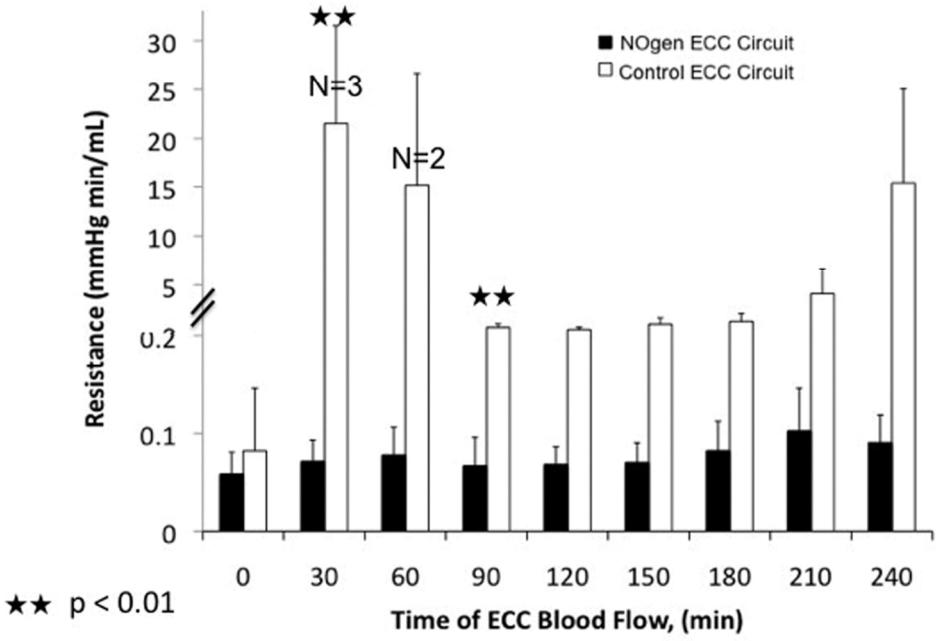

Figure 6: Time course blood flow resistance in control and NOgen ECC circuits.
66x43mm (600 x 600 DPI)

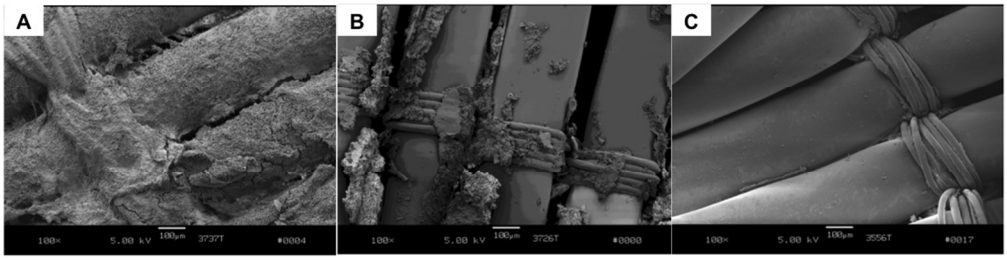

Figure 7: Scanning electron micrographs of the artificial lung fibers showing clot formation on the outer layers of fibers from A) a failed control lung, B) a control lung that survived 4 hours, and C) a NOgen lung.
406x103mm (72 x 72 DPI)

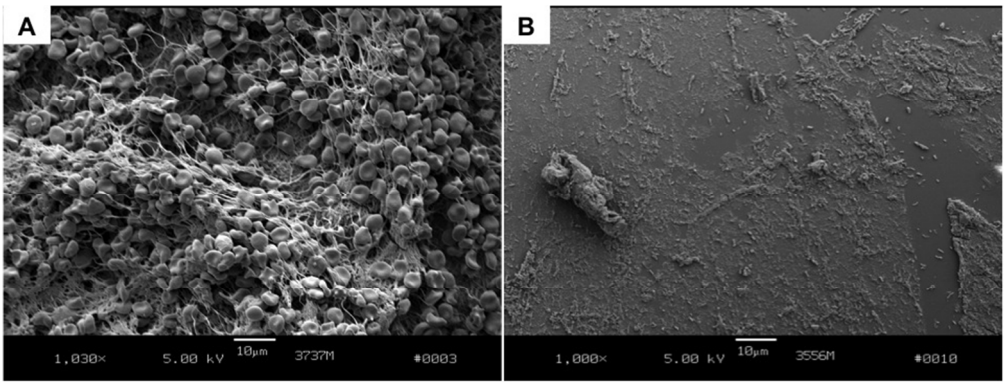

Figure 8: Scanning electron micrographs of the artificial lung fiber surfaces of A) control and B) NOgen fibers taken from fiber layers in the middle of the device. Control surfaces contain far more platelet deposition than NOgen surfaces.
338x127mm (72 x 72 DPI)

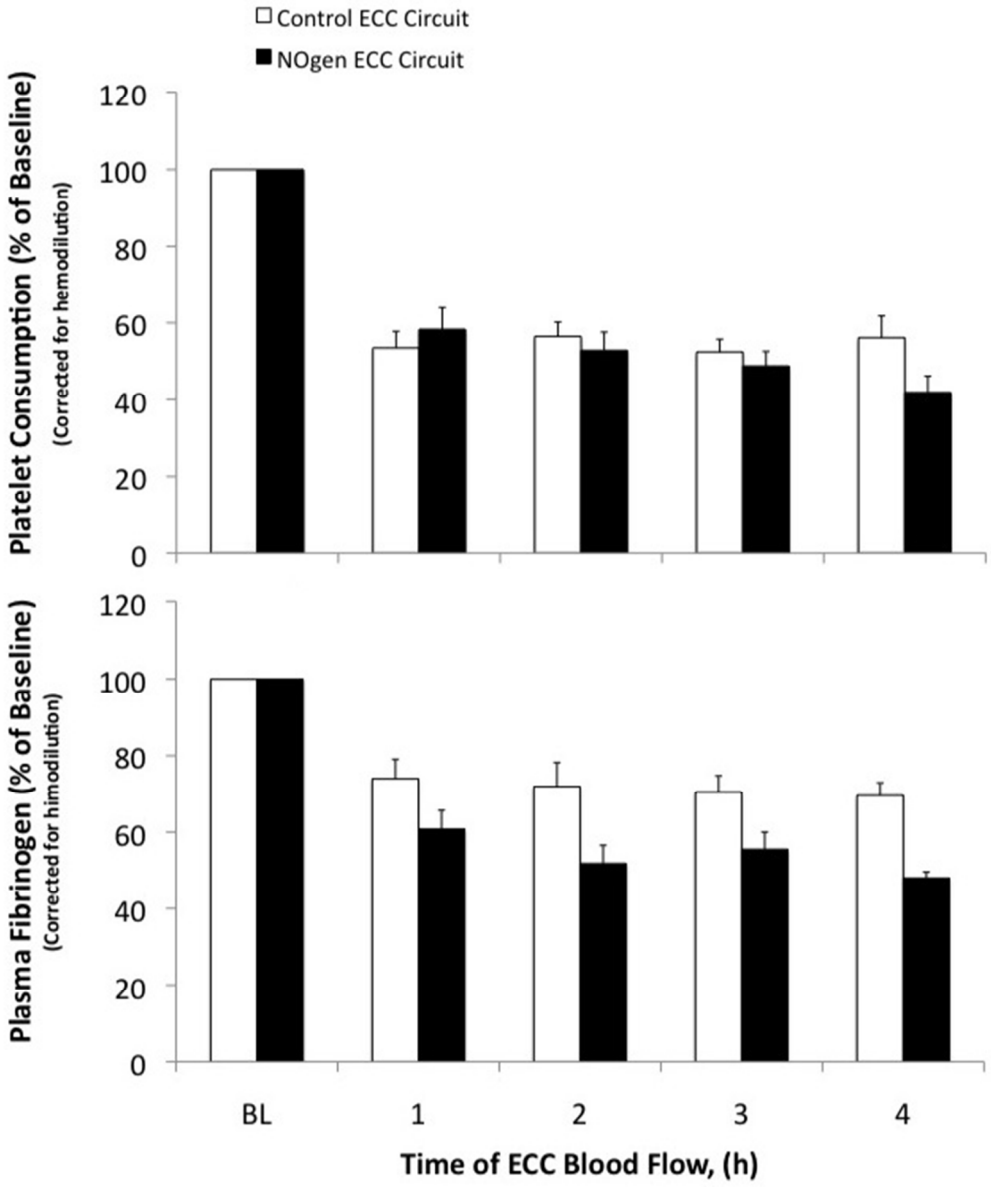

Figure 9: Levels of platelet consumption and plasma fibrinogen concentration during extracorporeal circulation.
222x264mm (72 x 72 DPI)

Table I

Effects of NO generating surface on hemodynamic parameters of the extracorporeal circulation (ECC) circuits and rabbits

Treatment	Parameter	Baseline[#]	Time on ECC (hours)			
			1	2	3	4
Control ECC	MAP	39.0 ± 6.0	41.80 ± 18.80	59.50 ± 23.30	89.0 ± 45.30*	93.50 ± 43.10*
	HR	201.80 ± 30.30	208.80 ± 22.5	253.50 ± 14.80*	248.50 ± 13.40	204.0 ± 14.10
	ECC BF	63.20 ± 2.10	22.80 ± 14.0*	26.20 ± 19.40*	30.0 ± 24.40*	33.80 ± 32.80*
	ACT	331.0 ± 36.70	337.0 ± 58.0	314.5 ± 19.3	276.50 ± 18.0	307.50± 38.90
	$PaCO_2$	30.33 ± 5.80	29.60 ± 4.0	31.0 ± 0.90	30.8 0± 1.10	28.70 ± 2.54
	pH	7.48 ± 0.01	7.44 ± 0.10	7.3.0 ± 0.10	7.33 ± 0.11	7.32 ± 0.01
NOgen ECC	MAP	48.60 ± 6.20	34.40 ± 2.60*	46.80 ± 28.60	54.0 ± 16.20	52.20 ± 22.70
	HR	185.80 ± 30.40	193.60 ± 12.80	195.20 ± 17.60	194.40 ± 10.90	202.60 ± 5.10
	ECC BF	70.50 ± 6.90	83.20 ± 6.50	105.20 ± 20.40*	112.60 ± 14.40*	105.0 ± 8.70*
	ACT	362.0 ± 43.40	401.80 ± 45.60	393.20 ± 26.90	371.20 ± 20.90	376.80 ± 19.40
	$PaCO_2$	35.92 ± 5.70	35.10 ± 9.89	33.70 ± 10.07	34.76 ± 7.98	35.66 ± 6.84
	pH	7.43 ± 0.02	7.32 ± 0.11	7.31 ± 0.11	7.28 ± 0.09	7.31 ± 0.05

Values are means ± SEM

*$p < 0.05$ vs baseline; ANOVA with Tukey's post-hoc analysis

[#] Values are just after establishing flow in ECCs. MAP = mean arterial pressure (mm Hg), HR = heart rate (beats/min), BF = blood flow (ml/min), ACT = activated clotting time (sec), $PaCO_2$ = arterial partial pressure of CO_2.