Original Article

The Effects of Nitric Oxide on Coagulation During Simulated Extracorporeal Membrane Oxygenation

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ABSTRACT

Extracorporeal membrane oxygenation (ECMO) is associated with profound alterations in hemostasis, with platelet dysfunction often being implicated as a causative factor for transfusion. Nitric oxide (NO) has shown to be a rapid yet temporary inhibitor of platelet function. The purpose of this study was to evaluate the effects of NO on platelet number and function in an in vitro ECMO model.

Eight silicone membrane oxygenators were primed with fresh, heparinized, bovine blood and allowed to circulate for 48 hours. The treatment group (NO) consisted of four oxygenators that had an end concentration of 20 ppm NO applied to the sweep gas. Platelet counts, methemoglobin levels, plasma-free hemoglobin levels, activated clotting times, and thromboelastographic (TEG) studies were performed at baseline, 1, 6, 12, and 24 h. Scanning electron microscopy (SEM) was performed on sample areas from each oxygenator.

The treatment group maintained an average of 25% higher platelet counts than the control group (85.1 ± 32.0, × 10^3 versus 66.5 ± 30.9 × 10^3) although statistical significance was not achieved. Methemoglobin levels were significantly elevated in the treatment circuit at hours 12 and 24 (p< .05). This could be attributed to the lack of a biological interaction that would break down this toxic by-product. TEG indices steadily declined in both groups from baseline (−0.4 ±3.6) to (−17.2 ± 3.3 p <.0007) treatment and (−20 ± 4.5, p<.0001) control, with the treatment circuit maintaining only slightly improved indices over the most of the study. SEM data showed increased fibrin and cellular deposits in the control group (p< .05) when compared with the treatment group.

NO added to the sweep gas of a simulated ECMO circuit at 20 ppm had little effect on the maintenance of platelet number and function.

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INTRODUCTION

Patients undergoing extracorporeal membrane oxygenation (ECMO) therapy are at increased risk for intracranial hemorrhage, first because of the specific disease state and the resulting prolonged hypoxia and acidosis, and second from iatrogenic coagulopathies. Subsequently permanent neurological dysfunction can be the result (1–3). In addition to systemic heparinization, hemostasis is altered because of the continual consumption of platelets by the extracorporeal circuitry, specifically the membrane lung (4, 5). The use of coatings to mask the extracorporeal circuit from the natural activation processes of blood has not been a widely accepted treatment, and its clinical effectiveness continues to be disputed. Because of this controversy, new themes for biological compatibility are continually being sought. NO, a powerful vasodilator used primarily to treat pulmonary hypertension and right ventricular heart failure is known to be implemented in many of the body’s vital functions (6, 7). Of particular interest is NO’s ability to temporarily inhibit platelet adhesion and aggregation (6, 8).

Nitric oxide is naturally released from intact vessel endothelium when stimulated with acetylcholine at muscarinic receptors (6). NO is produced in the endothelial cells via the oxidation and cleavage of a nitrogenous group from the amino acid L-arginine. The enzyme responsible for this oxidation and cleavage is termed nitric oxide synthase (NOS). After its production in the endothelium, NO enters tissues and cell membranes through the process of simple diffusion, which is facilitated by concentration gradients and NO’s lipophilic nature. In vivo NO readily binds the ferrous portions of heme groups within the intracellular matrix. Heme groups found intracellularly are concentrated within the enzyme guanylate cyclase. This binding of NO to guanylate cyclase causes a formational change that activates the enzymatic properties of the molecule. The activated enzyme guanylate cyclase catalyzes the conversion of intracellular magnesium guanosine triphosphate to cyclic guanosine monophosphate (cGMP) (6). The primary effect of cGMP is the profound relaxation of the vasculature to which it is introduced. Further studies have shown cGMP also plays a major role in platelet aggregation and adhesion (6, 9, 10). In vitro studies by Ignarro, 1989, showed inhibition of platelet adhesion and aggregation with prolonged bleeding times in animals who were exposed to inhaled nitric oxide (iNO) (6). It was discovered that the molecule that accounted for the vasodilatory effects of NO was also responsible for NO’s unique effect on platelet function.

Platelet surface adhesion receptors glycoprotein IIb/IIIa, and p-selectin are inactivated by increasing levels of cGMP and. In addition, an increase in cGMP causes the platelet cytoskeleton to undergo a temporary structural change reducing platelet efficiency. (6, 11, 12) However, platelets are thought to regain full function within 3–6 sec because of NO’s strong affinity for the ferrous heme molecules within red blood cells (8). It is this inactivation and almost immediate reactivation that may prove to be beneficial to patients undergoing ECMO therapy. By introducing NO into the sweep gas of a membrane lung, the ability of platelets to adhere to the membrane may be reduced. In addition, the temporary effects of NO may allow for rejuvenation of full platelet function upon exit from the NO ventilated oxygenator.

The purpose of this study was to determine if the addition of nitric oxide to the sweep gas of a silicone membrane oxygenator, in an in vitro model, would result in increased platelet preservation and better hemostasis overall during simulated ECMO.

MATERIALS AND METHODS

For each of four studies, an in vitro test circuit was constructed (Figure 1). Each test circuit consisted of two coiled silicone membrane oxygenators (0800-2A), two stainless steel heat exchangers, two 500-mL reservoirs to simulate patient blood volume, and the necessary amount of nonheparin-coated polyvinylchloride tubing to complete the circuit.

Both circuits, treatment (TRT) and control (CTR), were initially connected by means of a bridge conduit to allow for complete mixing of the prime constituents. Upon initiation of each study, the circuits were separated by means of a tubing clamp placed on the connecting bridge. A single roller pump was used to propel the perfusate through both circuits. The circuits were primed with 1 L of 5% dextrose solution and 25 mL sodium bicarbonate. Each oxygenator was primed via a vacuum source connected to the gas outlet port of the oxygenator. The circuits were thoroughly debubbled and warmed to 37°C.

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Figure 1: Circuit diagram.

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If the initial hematocrit was below the desired level, the volume was repeatedly chased out of both circuits with whole blood. Baseline lab values were obtained before division of the test circuits. Upon initiation of each test, the circuits were divided and NO 1:800 NO:N2 was added to the sweep gas via a Y connector at a final concentration of 20 ± 5 ppm to the treatment circuit. (Figure 1). NO content was analyzed utilizing a commercially available NO monitoring analyzer. The gas flow rate was maintained at 1LPM (room air) throughout the experiment via an air compressor. The perfusate was maintained at a flow rate of 300 mL/min, HCT of 40 ± 5% and at a physiologic temperature of 37.0 ± 2.0°C. Because of loss of water vapor via the gas outlet ports of the membrane lungs, normal saline 0.9% was added to the individual circuits when necessary to maintain HCT at the predetermined level.

Each study consisted of a 48-h time period during which data were obtained at baseline, 1, 6, 12, 24, and 48 h. Samples were removed from each circuit via a stopcock placed distal to the heat exchanger and proximal to the blood reservoir.

COAGULATION MONITORING

Specific coagulation tests consisted of the following: platelet (PLT) counts, plasma-free hemoglobin levels (PFH), and methemoglobin levels (Met Hgb), thromboelastographic (TEG) profiles, celite activated clotting times (ACT), and spun HCT.

The TEG is a measure of whole blood coagulation reflecting the interactions of all aspects of thrombus formation. TEG parameters recorded were as follows: R time (marks the beginning of clot formation and reflects whole blood coagulation kinetics), K time, and alpha angle (depict the rate of clot growth and are a reflection of platelet and plasma factor function), maximum amplitude (MA) (depicts clot strength and correlates directly with platelet function and plasma factor interaction), and TEG index (an over-all evaluation of the TEG profile mathematically incorporating all of the TEG variables depicting over-all thrombus dynamics.

SCANNING ELECTRON MICROSCOPY

After 48 h of continual circulation, the silicone membranes were dissected and 1-in × 1-in samples were taken from the proximal, middle, and distal locations. Fixing of the samples was completed by immersion in a solution of glutaraldehyde. The fixed samples were desiccated to remove all moisture and mounted onto aluminum pins. Samples were sputter coated with a thin layer of gold to allow for reflection of the electron beam. Evaluation and comparison of the samples was completed at 4,000× and 40,000× magnification. A positive (Figure 2) or negative (Figure 3) result was determined by the over-all presence or absence of clot and fibrin strands covering the membrane filaments.

STATISTICS

All data was recorded and entered into a computer database. Statistical analysis was performed utilizing a commercially available statistics program. Statistical differences for all parametric data were determined by Student’s t-test analysis and represented as mean ± standard deviation of the mean. Nonparametric data were evaluated utilizing a Pearson’s Chi Squared Test. Statistical significance was accepted at the p ≤ .05.

Ohmeda Inc., Liberty Connors, NJ
Dragerwerk AG, Lubeck, Germany
Timeter Instrument Corp., Lancaster, PA
Haemoscope Corp., Skokie, IL
Hemochrom 8000, International Technidyne Corp., NJ
RESULTS

Results represent only 24 h of the entire 48-h period because of degradation (complete hemolysis) of the perfusate at sample times after 24 hours. Platelet counts remained higher in the TRT circuit at all time periods, and while the TRT group maintain a 25.4 ± 12.3% higher platelet count than the CTR overall, statistical significance was not achieved at any of the sample times (Figure 4). The most rapid decline in platelet counts occurred between the baseline value (203 ± 16.7 mm$^3$) and the 1-h sample period (124.3 ± 73.5 mm$^3$ TRT and 101.5 ± 65.1 mm$^3$ control (CTR) $p = .654$). There was a similar but less pronounced decline at each sample period, with the widest variation between groups occurring at the 24-h time period (48.8 ± 36.1 mm$^3$ TRT and 27.8 ± 26.8 mm$^3$ control, $p = .386$).

The results for plasma-free hemoglobin are shown in Figure 5. Differences between the TRT and CTR groups were similar at all time periods, and statistical significance was not achieved.

There was a rapid increase in both TRT and CTR beginning at the 1-h time period. Plasma-free hemoglobin values at each time period, excluding the baseline value, were excessive. This was likely attributable to the rapid degradation of the perfusate at 37.0 ± 2.0°C.

The changes in methemoglobin levels are shown in Figure 6. TRT and CTR values were significantly different at the 12-h sample time, TRT (1.8 ± 0.4%) and CTR (1.3 ± 0.3%) $p = .042$. Met Hgb levels steadily increased from baseline to 6 h in both TRT (0.93 ± 0.2% –1.8 ± 0.3%) and CTR (0.93 ± 0.2% –1.4 ± 0.3%). Hours 6 to 12 showed a stabilization of Met Hgb levels in the TRT circuit and a small decline in the CTR circuit. Following the 12-h sample time, the TRT circuit rose from 1.8 ± 0.3% at the 12-h time to 2.4 ± 0.8% at the 24-h sample time. Statistical significance was again obtained at the 24-h sample time between TRT (2.4 ± 0.8%) and CTR (1.4 ± 0.2%), $p = .032$.

Activated clotting time values began within the range of 180–220 sec. Although this is within the normal range for heparinized neonates on extracorporeal life support, a correlation across species cannot be made because of the many differences in hematological factors. By the 1-h sample time, the ACT in both circuits had moved out of the normal range (412.0 ± 393.8 sec TRT) and (420 ± 387.9 sec CTR). ACT values continued to increase rapidly throughout the experiment and were >1000 sec in both groups by the 24-h sample time. There were no statistical differences found among any of the recorded ACT data.

There were no significant differences found between the TRT and CTR groups for any of the TEG data. TEG R time results showed no delay in initial clot formation in the TRT circuit over the first hour; whereas, the CTR circuit R times increased by 19 ± 10% over baseline ($p = .225$). The R time continued to increase in both circuits becoming significantly lengthened over baseline at the 12-h sample time ($p = .043$).
The largest variation between TRT and CTR occurred at the 24-h sample time (24.9 ± 7.0 sec TRT and 31.9 ± 9.4 CTR). However, this was not shown to be statistically significant. The K time data were extremely varied, showing an increased K time overall in both circuits. MA values showed little variation in either circuit from baseline (62.4 ± 17.6 mm) to 1 h (61.0 ± 14.2 mm TRT and 60.8 ± 23.8 mm CTR), and PLT function was interpreted to be normal throughout this time period. MA values declined in both circuits at an almost equal ratio and were not significantly reduced from baseline at any time period (Figure 7). The alpha angle data mirrored the K time data and were also extremely varied. Figure 8 represents PLT counts and the TEG Index data. Note the inverse relationship, as platelet counts decreased the TEG index increased in a more negative direction. Statistical significance was not achieved between groups.

Scanning electron microscopy (SEM) data showed a significant difference between TRT and control, with the CTR circuit having higher levels of fibrin and cellular debris deposited on the silicone membrane mesh ($p = .05$) (Figures 2, 3). PLT aggregations were unidentifiable in either treatment or control. There were no statistical differences between any of the sample locations on the membrane.

**DISCUSSION**

Because of the vast surface area contained within the artificial lung, the activation of the blood components passing through this device contributes significantly to the overall effect of the ECMO circuit on blood hemostasis. It is, therefore, theorized that reduction in the activation of blood within the oxygenator alone would lead to improvements in hemostasis. The hematological abnormalities associated with the implication of ECMO are far from being overcome. Because of the lack of biocompatibility of the extracorporeal circuitry, the risk of disrupting the hemostatic balance in these patients is inevitable. A single study by Plötz FB, et al. has shown that the largest contributor to this insult on hemostasis is the membrane lung (13).

Mellegren et al. 1996, showed a significant reduction in the amount of platelet trapping, in vitro by the membrane lung (hollow fiber) with the addition of NO to the sweep gas at 15 ppm (14). In addition, in 1998 Mellegren showed a reduction of platelet trapping by hollow fiber membrane oxygenators with the addition of NO at 40 ppm during adult CPB (5). However, statistical significance was not achieved. The results of these studies showed a trend toward the efficacy of NO when added to the sweep gas of a hollow fiber membrane lung. However, the majority of ECMO protocols often incorporate a silicone membrane lung in place of the hollow fiber lung. In 1996, Mellegren showed that silicone membrane lungs have a significantly increased ability to activate and trap platelets leading to decreased platelets counts overall when compared with the hollow fiber lung (15). Therefore, it was necessary to define NO’s ability to effect hemostasis, particularly in platelet number and function, across this type of device.

In our study, both TRT and CTR showed significant reduction in platelet number and function over time, suggesting platelet activation and trapping by the in vitro circuitry. The mechanism for platelet interaction and consumption is a very complex one and in our study, introduction of NO gas showed no significant improvement over the control. This seems to be in contrast to studies conducted utilizing the hollow fiber lungs. This discrepancy may be attributed to the manner in which the blood products were collected, whether traumatically (slaughterhouse) or atraumatically (venipuncture), and the differences in reactivity between human and bovine blood to both the membrane lung and to NO. In addition, the life span of blood at normothermia in the absence of a biological system to interact with is very limited, and this could have contributed...
to the unpredictability of the trends observed past the 6-hour datapoint.

TEG results showed decreasing platelet dysfunction over time and mirrored the reduction in platelet number. With a single exception at the 6-h time period, the TRT circuit maintained higher platelet numbers and increased platelet function over the control. Although our results were not shown to be significant it seems that a trend does exist, and further studies utilizing human blood products and an increased number are warranted.

After 24 h of circulation, MetHgb a toxic by product of the metabolism of NO, was significantly elevated over the control. Although this result shows the potential for elevated MetHgb levels resulting in methemoglobinemia, toxic levels of MetHgb (>2.5 mg/dL) were not reached in the treatment circuit.

SEM evaluation of the silicone membranes showed increased layers of fibrin and other media adhered to the CTR circuit. However, these results are descriptive in nature and although difference between positive and negative results were easily defined in many of the samples, three were difficult to quantify. Although this seems to add precedence to the efficacy of NO overall, the results cannot be compared without a definitive grading system. These results again suggest a very complex interaction that the addition of NO was unable to block.

In conclusion, our analysis has been unable to show significant improvement in platelet number or function with the use of NO in an in vitro ECMO circuit containing a silicone membrane lung. Although SEM data suggest that the use of NO reduce fibrin deposits overall, further studies are warranted to determine the over-all efficacy of a treatment of this type. In addition to the standard TEG protocol, a simultaneous sample was run with the addition of Abciximab (Reopro®) 0.01μM to the cup before addition of the blood sample. Reopro is a PLT inhibitor that binds glycoprotein IIb/IIIa on the surface of PLTs.

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