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MAIN TEXT

The effects of nitric oxide on coagulation and inflammation in ex vivo models of extracorporeal membrane oxygenation and cardiopulmonary bypass

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Abstract

Background: Extracorporeal life support (ECLS) has extensive applications in managing patients with acute cardiac and pulmonary failure. Two primary modalities of ECLS, cardiopulmonary bypass (CPB) and extracorporeal membrane oxygenation (ECMO), include several similarities in their composition, complications, and patient outcomes. Both CPB and ECMO pose a high risk of thrombus formation and platelet activation due to the large surface area of the devices and bleeding due to system anticoagulation. Therefore, novel methods of anticoagulation are needed to reduce the morbidity and mortality associated with extracorporeal support. Nitric oxide (NO) has potent antiplatelet properties and presents a promising alternative or addition to anticoagulation with heparin during extracorporeal support.

Methods: We developed two ex vivo models of CPB and ECMO to investigate NO effects on anticoagulation and inflammation in these systems.

Results: Sole addition of NO as an anticoagulant was not successful in preventing thrombus formation in the ex vivo setups, therefore a combination of low-level heparin with NO was used. Antiplatelet effects were observed in the ex vivo ECMO model when NO was delivered at 80 ppm. Platelet count was preserved after 480 min when NO was delivered at 30 ppm.

Conclusion: Combined delivery of NO and heparin did not improve haemocompatibility in either ex vivo model of CPB and ECMO. Anti-inflammatory effects of NO in ECMO systems have to be evaluated further.

KEYWORDS

anticoagulation, cardiopulmonary bypass, extracorporeal life support, extracorporeal membrane oxygenation, mechanical circulatory support, nitric oxide, thrombosis

1 | BACKGROUND

Extracorporeal life support (ECLS) encompasses the broad application of mechanical circulatory support used to manage acute, reversible cardiac or pulmonary failure.[1] ECLS includes cardiopulmonary bypass (CPB), extracorporeal membrane oxygenation (ECMO), extracorporeal lung assist (ECLA), extracorporeal cardiopulmonary resuscitation (ECPR), and extracorporeal carbon dioxide removal (ECCOR). A standard extracorporeal circuit comprises drainage and reinfusion vascular access cannulae, polymeric circuit tubing, a mechanical blood pump and a hollow fiber membrane oxygenator. The large surface area of extracorporeal circuits (~1.8 m^2) stimulates significant systemic coagulopathic and inflammatory responses.[2]

Although ECMO and CPB share technical similarities there are some distinct differences in between them. Modern ECMO generally uses a centrifugal pump, while CPB uses a roller pump. The membrane oxygenators used in ECMO are generally composed of polymethyl pentene (PMP), while a more cost-effective polypropylene material is used for CPB. ECMO is a closed system, whereas CPB has a venous reservoir with a sizeable air–blood interface. CPB is used for short-term applications (i.e. hours) in the operating room during cardiothoracic surgery to replace heart and lung function.[3]

Unfractionated heparin (UFH) is the standard anticoagulant used in ECMO and CPB. Despite heparin administration, thrombus formation occurs due to contact activation of blood components especially within the CPB circuit's large surface area and air-blood interface in the venous reservoir.[4] Patients placed on CPB during cardiac surgery are also at significant risk of postoperative bleeding and often require transfusion therapy.[5,6]

ECMO and CPB are lifesaving therapies; however, both are associated with thrombotic and hemorrhagic complications which significantly increase morbidity and mortality.[7–9] Maintaining a balance between preventing thrombus formation and bleeding is a crucial priority in extracorporeal circulation and efforts to improve anticoagulation management and develop biocompatible materials have been made. Several coatings have been developed to reduce platelet adhesion and fibrin deposition. However, these coatings still require the use of systemic anticoagulation.[10]

Alternative anticoagulant agents to UFH, are low molecular weight heparin (LMWH) and direct thrombin inhibitors.

However, it is a still a priority to discover alternatives to the modern standards of practice for systemic anticoagulation. Thus, there is ongoing effort to improve the management of systemic anticoagulation, and study the effects of readily available compounds, including nitric oxide (NO) gas, on anticoagulation.

Inhaled NO is used in severe acute respiratory failure to improve oxygenation.[11] NO also has antithrombotic and vasoprotective properties, shown in extracorporeal support.[11,12] The antithrombotic effects of NO have led to an increased effort to create NO-releasing polymer technologies as an alternative to systemic anticoagulation during extracorporeal circulation.[12,13]

Nitric oxide exerts antiplatelet effects by increasing cyclic guanosine monophosphate (cGMP) production, leading to decreased intracellular calcium levels and downregulation of the coagulation cascade.[14] The coupling of nitric oxide synthase (NOS) to a reductase leads to an increased NO production in the endothelium, leading to altered vascular tone and platelet activity. NO decreases platelet activation and adhesion and white blood cell activation at the endothelial surface. Thus, it is theorized that the addition of NO to the sweep gas of the oxygenator may reduce platelet activation and adhesion, and therefore thrombosis. This study aimed to evaluate the effects of NO via sweep gas on platelet adhesion and activation, thrombus formation, and inflammatory activation and evaluate its potential efficacy in a CPB and an ECMO model.

2 | MATERIALS AND METHODS

The study included two separate ex vivo models, including an ECMO model and a CPB model. All methods were approved and performed following the guidelines and regulations set forth by the Metro-North Ethics Committee (HREC/16/QPCH/320). Informed consent for study participation was obtained from the Australian Red Cross Blood Service (17-11QLD-05).

2.1 | Extracorporeal circuit preparation

Ex vivo ECMO circuits were constructed using the MAQUET PLS-I oxygenator, ROTAFLOW centrifugal pump, and BIOLINE (heparin and albumin) coated polymeric tubing as previously published by our group[15] (Figure 1).

Nitric oxide sweep gas was added to the ex vivo system using an INOmax apparatus (Mallinckrodt, Staines-upon-Thames, United Kingdom) connected to the sweep flow with modified tubing connected to the membrane oxygenator gas inlet. CO_2 enhanced gas (5% CO_2, 21% O_2, 74% N_2) was added to the NO as sweep gas at 2.5 L/min.

Ex vivo CPB circuits were constructed in a similar fashion. However, blood drained from the venous drainage line was fed in-line to the venous reservoir, where it was stored and exposed for an air-blood interface for up to 6 h (see Figure 1iii). Detailed description of the models are given in the supplementary material.

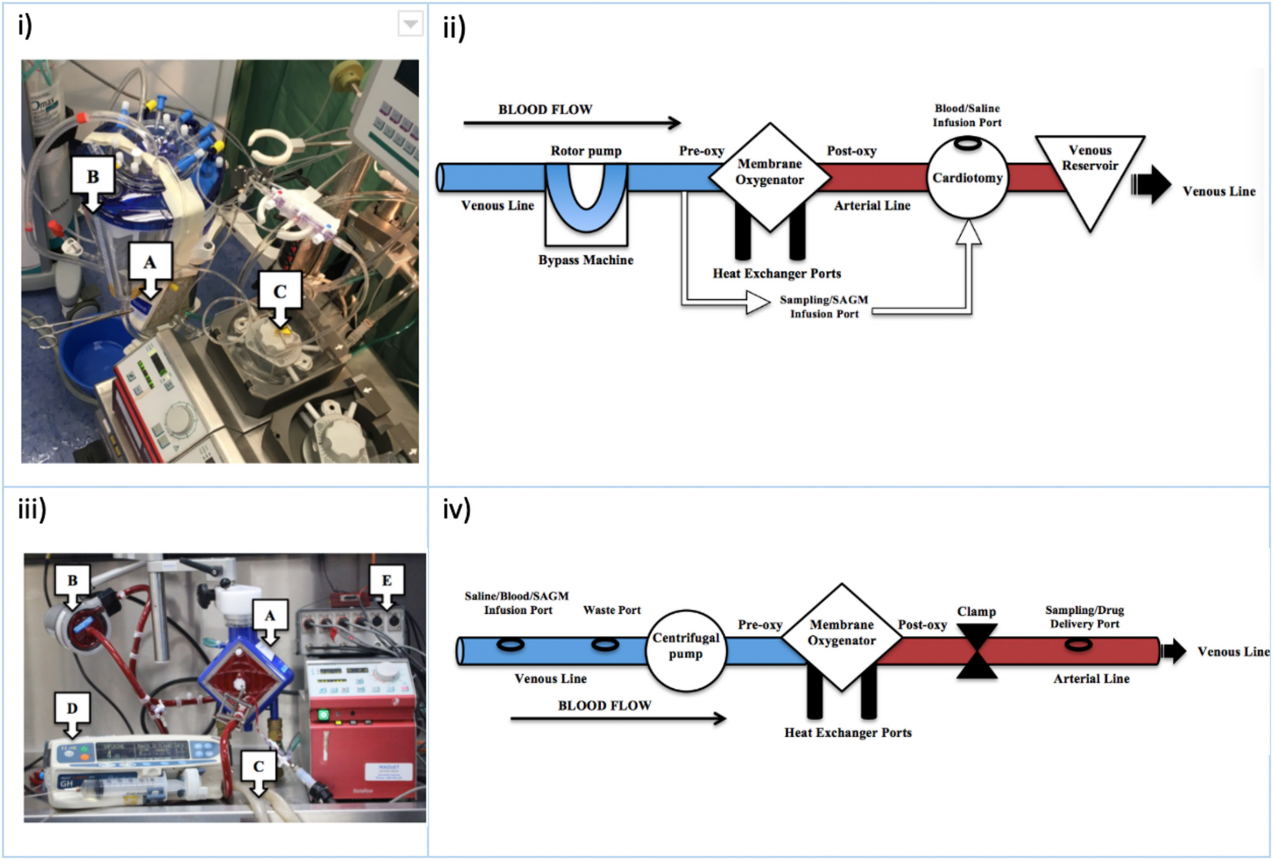

FIGURE 1 (i) Ex vivo ECMO circuit model, featuring the MAQUET PLS ECMO set (A) and ROTA FLOW (B); Alaris Guardralis plus infusion kit (C); Jostra Heat Cooler unit (D) and Lab Jack monitoring system (E). (ii) 2D schematic of ECMO circuit to demonstrate pathway of blood flow in developed ex vivo circuit loop. (iii) Ex vivo CPB circuit model, constructed using the MAQUET QUADROX-I membrane oxygenator (A), venous cardiotomy reservoir (B) and rotor pump (C), and BIOLINE tubing. (iv) CPB circuit: 2D representation of blood flow pathway in developed ex vivo circuit loop.

2.2 | Patients and circuit study design

Preliminary ex vivo circuits revealed that the trace levels of citrate remaining in the whole blood sample from the collection bags affected decreased calcium levels during the ex vivo perfusion. After baseline measurements, we injected a 10-mL aliquot of calcium chloride to bind the free citrate in the blood to maintain adequate serum calcium levels (>2.5 mmol/L). The pH was adjusted using 12.5 mL of 8.4% sodium bicarbonate to maintain a pH range of 7.2 and 7.5. Two preliminary ECMO circuits have been run with NO in the sweep gas without further anticoagulation.

For the ECMO group, one control and four treatment groups were studied:

1. No anticoagulation ($n=4$).
2. Heparin 400 IU ($n=4$), as a positive control.
3. NO = 80 ppm + 400 IU Heparin ($n=3$).
4. NO = 50 ppm + 400 IU Heparin ($n=3$).
5. NO = 30 ppm + 400 IU Heparin ($n=3$).

For the CPB group, one control and three treatment groups were studied:

1. No anticoagulation ($n=4$).
2. Heparin 400 IU ($n=3$).
3. NO = 80 ppm + 400 IU Heparin ($n=3$).
4. NO = 30 ppm + 400 IU Heparin ($n=3$).

Circuits assigned to the heparin group were injected with 400 IU UFH following baseline measurements. An INOmax DS-IR delivery system was used to continuously deliver NO gas at 30, 50 and 80 ppm, via the membrane oxygenator, using the sweep gas line and an end-to-end connector.

2.3 | Sampling

The set time points for collecting blood probes included t = baseline, 15, 30, 60, 120, 240 and 480 min. Routine blood gas measurements were collected using an Abbott

iSTAT Point of Care analyzer to monitor pH, pO_2, pCO_2, O_2 saturation, hematocrit (Hct) and suitable electrolyte and ion levels in the sample. Furthermore, a complete blood cell count panel was performed using the Beckman Coulter Counter for samples at time points: baseline, 60, 240 and 480 min. Approximately 66.5 mL of blood was removed throughout the experiment at time points: baseline, 15, 30, 60, 120 and 240 min, in the CPB model. The volume lost to sampling in the ECMO model was 81.5 mL, as it involved an additional time point at 480 min. Additive saline–adenine–glucose-mannitol (SAGM) solution was infused at a rate of 4 mL per hour to replace volume lost to blood sampling in the ECMO model.

Activated Clotting Time (ACT) was measured at each time point to monitor anticoagulation status, using the Hemochron Blood Analyzer. Using the multiplate analyzer, platelet function and aggregation in response to adenosine-diphosphate (ADP) (0.2 mM), ristocetin (RISTO) (10 mg/mL), and thrombin receptor activating peptide test (TRAP) (1 mM) agonists using the technique of impedance aggregometry was characterized at the time points: baseline, 60, 240 and 480 min. The rotational thromboelastometry (ROTEM) delta system was used to analyze citrated blood collected at baseline, 60, 240 and 480 min for tests on the extrinsic pathway (EXTEM), the functionality of fibrinogen (FIBTEM) and for detection of Heparin (HEPTEM).

Plasma specimens were analyzed using custom Luminex Magpix-based assays (Luminex Corp., Austin, TX, USA) following the manufacturer's instructions. Samples measured included ADAMTS13, myeloperoxidase (MPO), P-selectin (Milliplex Human Cardiovascular Disease Magnetic Bead Panel 2) (Millipore Corp., Billerica, MA, USA), and cytokines IL-8 and Tumor necrosis factor-α (TNF-α) (Human Cytokine/Chemokine Magnetic Bead Panel) (Millipore Corp., Billerica, MA, USA). Concentrations were calculated using a 5-parameter logistic standard curve corrected for background readings. Plasma samples were assayed in duplicate, and quality controls supplied by the manufacturer were used to determine assay accuracy. Assay sensitivities, including minimum detectable concentrations, for each sample that was analyzed were ADAMTS13: 0.339 ng/mL, MPO: 0.036 ng/mL, P-Selectin: 0.051 ng/mL, IL-8: 0.4 pg/mL, TNF-α: 0.7 pg/mL.

2.4 | Scanning electron microscopy

Control circuits were prepared for scanning electron microscopy (SEM) to visualize the rate of cellular uptake on oxygenator fibers. Following completion of the study, membrane oxygenators were thoroughly flushed out with 0.9% normal saline solution to remove blood. Sections of oxygenator fiber were cut with an industrial band saw and stored in 4% paraformaldehyde (PFA). The samples were sent externally to the University of Queensland Centre of Microscopy and Microanalysis for imaging (Figure S1).

2.5 | Statistical analysis

Data were statistically analyzed using GraphPad Prism Software. The data are presented as the mean ± standard error of the mean (SEM). In addition, the datasets for each anticoagulant treatment were compared using two-way ANOVA with Tukey's multiple comparisons (GraphPad Prism, GraphPad Software, Inc, CA, USA).

3 | RESULTS

3.1 | Preliminary findings for incorporating NO into extracorporeal circulation

The study was commenced using NO delivered at 80 ppm in both the ex vivo models. The initial observation was that NO alone was not sufficient in preventing thrombus formation for up to 8 h, leading to the combined delivery of 400 units of UFH in addition to NO. The use of combined delivery of NO and UFH was justified by the thrombus formation observed in the ECMO circuits given NO without UFH ($n = 2$) compared to those without anticoagulation ($n = 4$). Furthermore, the mean blood flow rate for NO delivered at 80 ppm decreased after 60 min.

The first attempt to introduce NO at 80 ppm into the ex vivo CPB circuit was unsuccessful. Sizable thrombus formation in the venous reservoir occurred before 240 min leading to the termination of the experiment. These preliminary findings were instrumental in optimizing the ex vivo models for both ECMO and CPB. The data suggest that NO via sweep gas given as the sole anticoagulant during extracorporeal support is insufficient in sustaining anticoagulation and the longevity of the ex vivo circuits up to 8 h.

3.2 | Ex vivo circuit performance

ECMO circuits assigned to the control group were not successful in performing for 8 h at the flow conditions set at baseline. These findings were reflected by the decrease in mean run time (4.8 h) compared to circuits treated with heparin or NO + heparin. The circuit mean run time for the heparin group was 6.75 h. Therefore, circuits that failed before 8 h were terminated when the flow decreased below 1.5 L/min. Increasing NO concentration did not significantly affect overall circuit function, as circuits for

NO = 30, 50, and 80 ppm + heparin groups were run for 8, 7.8, and 7.5 h, respectively. We did not observe a clear improvement in circuit function when using heparin or NO during CPB studies, as all circuits reached the 4 h endpoint.

3.3 | Blood gas and hematological parameters

Arterial blood gas and complete blood cell count variables for ECMO and CPB ex vivo circuits collected at baseline and endpoint are displayed in Table 1. Adding NO in addition to heparin over the study period did not significantly affect these variables at 240 min for CPB or for most coagulation parameters at 480 min in the ex vivo ECMO model. The exception was PaO_2 in the ECMO circuit, which decreased significantly at 480 min when NO was delivered at a concentration of 80 ppm ($p = 0.015$).

3.4 | Activated clotting time measurements, platelet aggregometry and platelet count

Results for activated clotting time (ACT) measurements, platelet aggregometry and platelet count for both models are displayed in Figure 2. ACT measurements in both models did not show significant differences, only at 240 min, treatment groups with NO + heparin delivered at 50 and 80 ppm demonstrated significantly higher ACT values compared to the control group ($p < 0.05$) (Figure 2ii). There was a dose-dependent reduction in maximal platelet aggregation in the ECMO model in the TRAP-6 subgroup across NO delivered at 30, 50, and 80 ppm + heparin groups. Compared to the heparin group, the decrease in platelet aggregation of the NO delivered at 80 ppm + heparin group was significantly different ($p < 0.008$), but not for the NO delivered at 30 ppm + heparin or NO delivered at 50 ppm + heparin groups. As measured by RISTO and ADP assays, platelet aggregation did not elicit a significant difference between groups. In the CPB arm of the study, multiplate analysis demonstrated that platelet aggregation was not affected by the addition of NO gas.

3.5 | Protein assays

Luminex Magpix-based assays have been performed for ADAMTS-13, P-selectin, and MPO, all results are displayed in Figure 3. In the ECMO arm of the study, all groups demonstrated gradually decreasing concentrations of ADAMTS-13 over time. There was no significant interaction between the effect of the method of anticoagulation and time ($p = 0.98$). Also in the CPB arm, there was neither a significant interaction between the method of anticoagulation and time ($p = 0.68$), nor were there any significant differences between anticoagulation methods ($p = 0.41$).

In the ECMO arm of the study, all groups demonstrated a gradual increase in the concentration of P-selectin except the heparin group, which gradually decreased (Figure 3iv). No significant interaction was found between the effect of anticoagulation and time ($p = 0.94$). Simple primary effect analysis of the method of anticoagulation showed no significant differences ($p = 0.5$) and no significant main effect of time ($p = 0.18$).

For concentrations of MPO in both the CPB and ECMO arms, there was a statistically significant increase in concentration over time. In addition, there was a significant difference within each group (CPB: $p = 0.014$, 0.0002 and 0.0002 for heparin, NO delivered at 30 and 80 ppm + heparin, and ECMO: $p = 0.0002$ for heparin, NO delivered at 30, 50, and 80 ppm + heparin, respectively (Figure 3v,vi)). However, there was no significant difference in MPO concentration between methods of anticoagulation.

3.6 | Cytokines IL-8 and TNF- α

In both models the concentration of IL-8 increased gradually from baseline to 60 min. It then increased at an accelerating rate from 60 to 120 min. In the ECMO arm, the concentration of IL-8 in the heparin group was considerably higher than NO delivered at 30, 50, and 80 ppm at 120 min ($p = 0.002$, 0.0026, and 0.013, respectively). In the CPB model at 120 min, the concentration of IL-8 in the heparin group was significantly higher than NO delivered at 80 ppm + heparin ($p = 0.024$). There were no other significant differences between groups.

In both models the concentration of TNF- α increased for all groups up to 120 min (Figure 4iii,iv). At the 120 min time point, there were significant differences between the heparin group and the NO delivered at 30, 50, and 80 ppm + heparin groups ($p = 0.0036$, 0.0038 and 0.0071).

4 | DISCUSSION AND CONCLUSION

Anti-thrombotic therapy for extracorporeal life support (ECLS) requires a careful balance between sufficiently

TABLE 1 Arterial blood gas parameters for (a) ECMO and (b) CPB circuits.

(a) ECMO							
Time	**Parameter**	**Control (4)**	**Heparin (4)**	**NO = 30 ppm (3)**	**NO = 50 ppm (3)**	**NO = 80 ppm (3)**	***p***
Baseline	pH	6.96 (0.03)	6.94 (0.02)	6.96 (0.04)	6.94 (0.059)	6.82 (0.02)	ns
	pO_2 (mm Hg)	71.25 (17.26)	57 (6.89)	102 (27.95)	74 (11.37)	89.33 (24.63)	ns
	pCO_2 (mm Hg)	85.93 (5.91)	84.03 (8.76)	68.57(10.32)	97.40 (16.41)	82.27 (16.47)	ns
	RBC ($\times 10^{12}$/L)	3.98 (0.20)	3.95 (0.23)	3.93 (0.219)	4.50 (0.48)	3.47 (0.73)	ns
	WBC ($\times 10^9$ /L)	4.58 (1.02)	5 (0.39)	4.83 (0.74)	3.2 (0.06)	4.77 (1.29)	ns
	Hct (%)	34 (0.91)	33.5 (1.04)	32.33 (3.18)	33.33 (1.20)	29.33 (6.17)	ns
	Hb (g/dL)	115.25 (4.70)	116.5 (9.67)	113.68 (6.84)	124.67 (9.84)	98 (14.73)	ns
	Plt ($\times 10^9$ /L)	139.25 (22.50)	156.25(21.08)	135.33(4.91)	102.33 (28.72)	91 (22.91)	ns
480 min	pH	7.39 (0.02)	7.27 (0.01)	7.28 (0.02)	7.30 (0.03)	7.18 (0.04)	ns
	pO_2 (mm Hg)	155.5 (9.50)	155.00 (3.81)	143.33 (1.86)	142.33 (1.20)	97.68 (0.33)*	0.015
	pCO_2 (mm Hg)	16.33 (9.44)	35.60 (0.55)	32.33 (0.63)	32.47 (1.35)	37.50 (2.82)	ns
	RBC ($\times 10^{12}$/L)	3.00 (0.17)	2.68 (0.10)	3.15 (0.22)	3.42 (0.18)	2.52 (0.43)	ns
	WBC ($\times 10^9$/L)	2.20 (1.20)	2.50 (0.04)	3.00 (0.60)	2.70 (0.32)	2.07 (0.64)	ns
	Hct (%)	21.00 (2.00)	17.75 (0.75)	19.00 (1.73)	20.33 (0.88)	18.00 (0.00)	ns
	Hb (g/dL)	86.00 (1.00)	90.00 (6.82)	91.33 (6.33)	101.00 (6.56)	75.67 (11.00)	ns
	Plt ($\times 10^9$ /L)	57.00 (47.00)	71.25 (5.98)	130.33 (14.75)	70.67 (12.00)	58.67 (22.26)	ns
(b) CPB							
Time	**Parameter**	**Heparin (3)**		**NO = 30 ppm (3)**		**NO = 80 ppm (3)**	***p***
Baseline	pH	6.98 (0.05)		6.93 (0.05)		6.92 (0.05)	ns
	pO_2 (mm Hg)	83.50 (39.34)		81.63 (28.22)		88.33 (140)	ns
	pCO_2 (mm Hg)	47.07 (6.97)		46.87 (5.49)		59.80 (15.67)	ns
	RBC ($\times 10^{12}$/L)	3.25 (0.93)		2.38 (0.12)		2.76 (0.25)	ns
	WBC ($\times 10^9$/L)	2.87 (0.58)		3.90 (0.40)		3.10 (0.42)	ns
	Hct (%)	20.87 (1.62)		20.33 (2.36)		23.67 (6.27)	ns
	Hb (g/dL)	97.00 (31.48)		69.67 (6.12)		70.00 (3.22)	ns
	Plt ($\times 10^9$ /L)	48.67 (2.44)		75.00 (16.44)		86.67 (5.70)	ns
240 min	pH	7.36 (0.13)		7.45 (0.16)		7.24 (0.03)	ns
	pO_2 (mm Hg)	150.33 (2.03)		154.00 (13.00)		144.33 (4.06)	ns
	pCO_2 (mm Hg)	21.50 (10.77)		9.00 (9.00)		26.80 (0.10)	ns
	RBC ($\times 10^{12}$/L)	2.17 (0.26)		2.07 (0.13)		2.44 (0.30)	ns
	WBC ($\times 10^9$/L)	1.53 (0.55)		2.15 (0.15)		2.10 (0.44)	ns
	Hct (%)	16.70 (0.89)		10.78 (5.42)		15.00 (0.00)	ns
	Hb (g/dL)	64.33 (9.21)		59.00 (4.00)		72.00 (8.54)	ns
	Plt ($\times 10^9$/L)	42.67 (21.18)		53.50 (35.50)		72.00 (4.58)	ns

* significance $p < 0.05$

inhibiting platelet and coagulation factor activation to minimize circuit thrombus formation while maintaining sufficient endogenous procoagulant activity to prevent hemorrhage in the patient. The ongoing high incidence of hemorrhagic and thrombotic complications associated with CPB and EMCO reinforces the need to develop novel anti-thrombogenic strategies.[8,16]

At present UFH is the most widely used systemic anticoagulant during ECLS. The only frequently used alternatives are direct thrombin inhibitors.[17] Both prostacyclin and NO, exogenously added to extracorporeal circuits along with UFH to inhibit the interaction between platelets and extracorporeal surfaces, have been shown to reduce platelet activation, adhesion, and consumption.[18,19]

The present study aimed to evaluate the effects of NO via sweep gas on hemostasis, platelet activation, and inflammatory pathways during extracorporeal support.

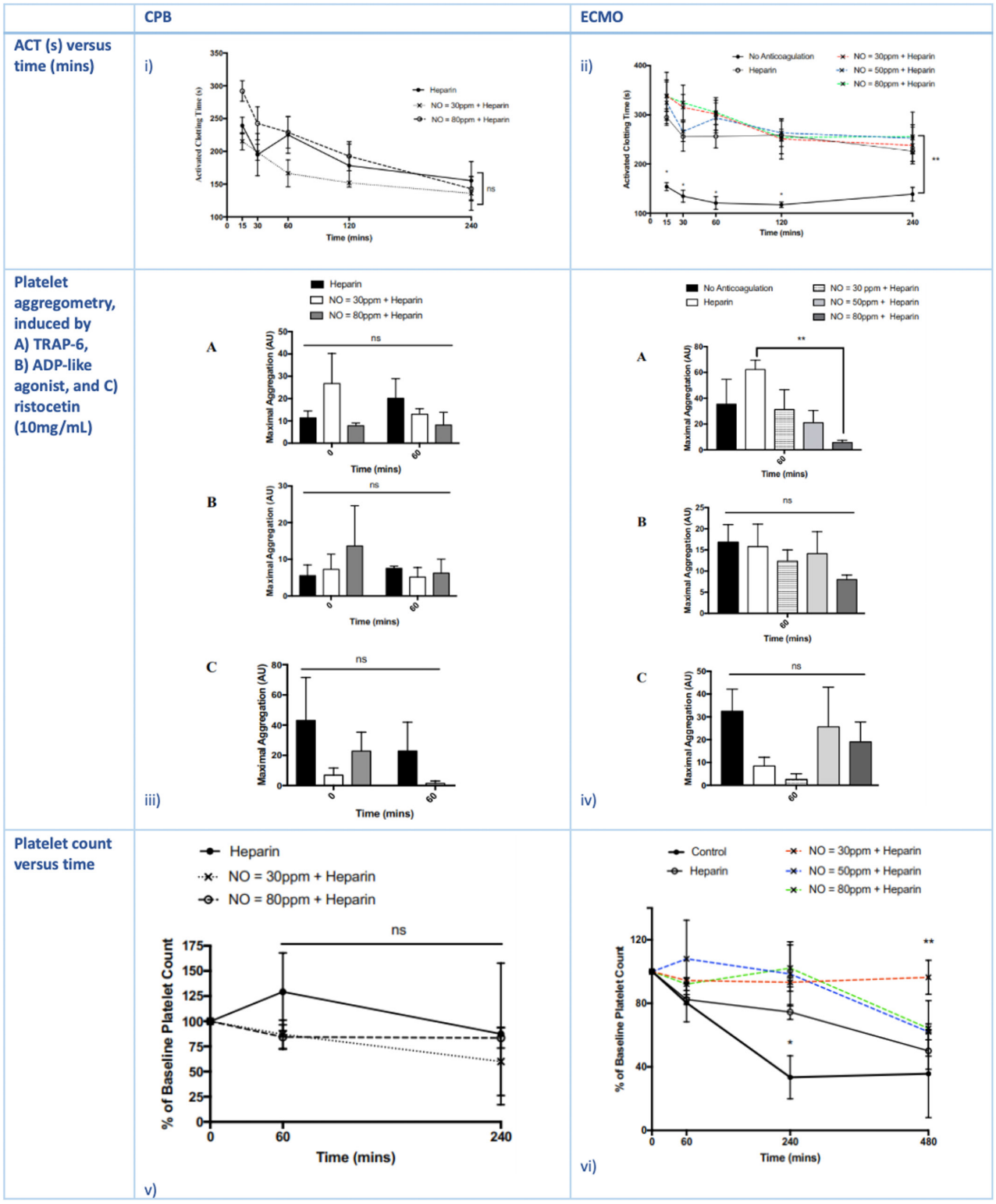

FIGURE 2 Comparison of ACT versus time for ECMO (i) and CPB (ii) circuits, in vitro platelet aggregometry versus time for ECMO (iii) and CPB (iv) circuits, and platelet count versus time for ECMO (v) and CPB (vi) circuits. For ACT, values at baseline have been excluded from this figure as all circuits reported values above the cut off of 1000s. For the ECMO group in (i), data for $t = 480$ min (trial endpoint) has been excluded as not all control and heparin circuits ran to predetermined endpoint at 4 L/min. *p value <0.05 versus all ECMO treatments and **p value <0.05 versus NO = 50 and 80 ppm. For platelet aggregometry in ECMO (iii): **p value = 0.008, calculated by 2-way ANOVA with Tukey's multiple comparison. For platelet count versus time in ECMO (v): *p value <0.005 versus all treatment groups and **p value <0.0002 versus control.

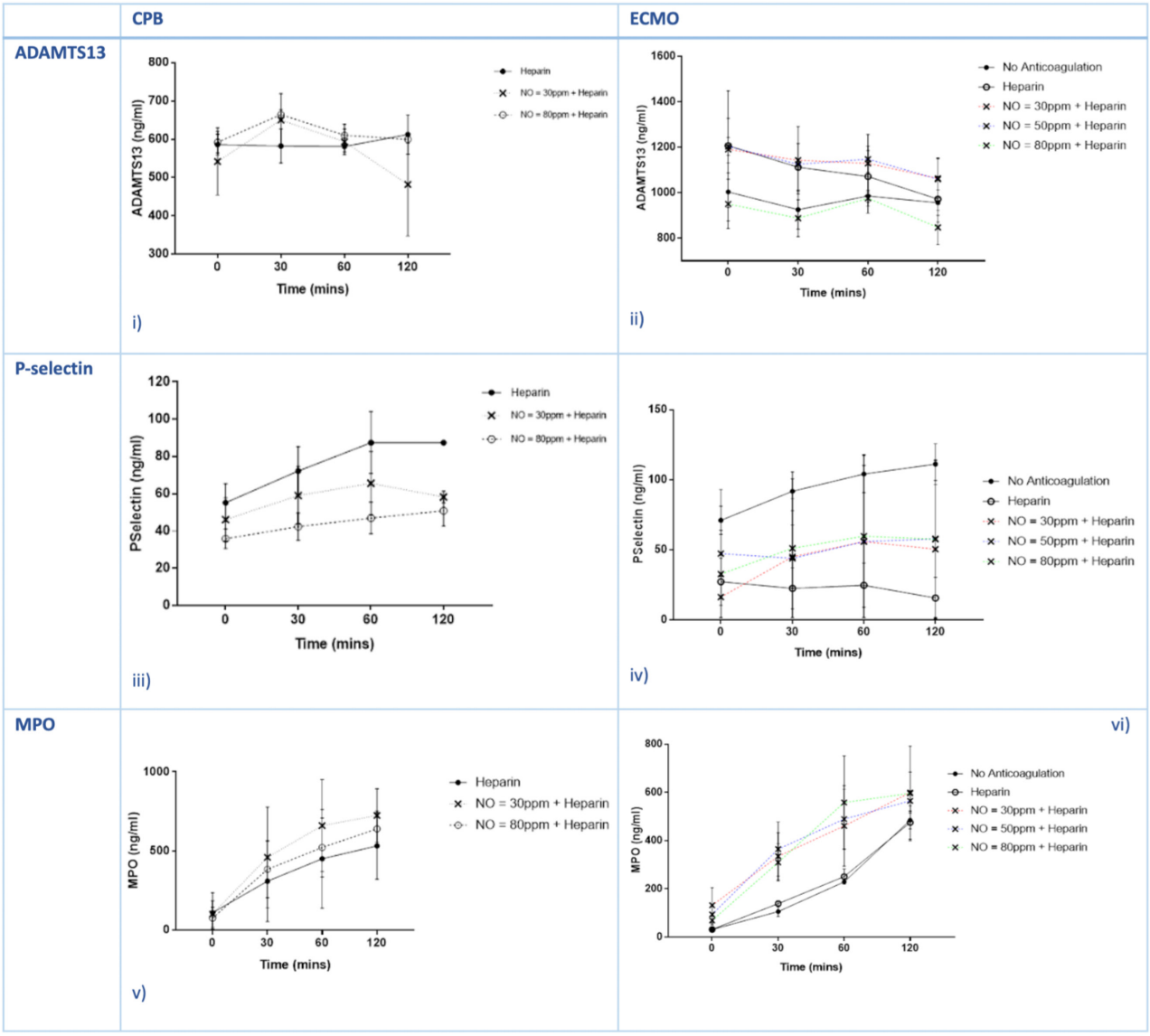

FIGURE 3 Luminex Magpix-based assay graphs of ADAMTS13 versus time for (i) ECMO and (ii) CPB, P-selectin versus time for (iii) ECMO and (iv) CPB and MPO versus time for (v) ECMO and (vi) CPB.

Several key findings emerged from this study. Firstly, in both the ECMO and CPB, the addition of NO to heparin did not consistently produce a significant anticoagulant effect over and above that of UFH alone as measured by ACT. In the ECMO group at 240 min, only the treatment groups with higher doses of NO (50 and 80 ppm) maintained a significantly higher ACT value than the control group. The higher dose NO delivered at 80 ppm + heparin group demonstrated a higher ACT than the NO delivered at 30 ppm + heparin group in the CPB group. However, the finding was not statistically significant.

Secondly, a dose-dependent effect on maximal platelet aggregation in the TRAP-6 assay was observed in the ECMO circuits and a possible impact of NO on platelet count. NO has been previously demonstrated as a potent antiplatelet agent in vivo, both directly and with NO-driven cyclooxygenase activation and prostaglandin I2.[20] From this perspective, the present study demonstrated inconsistent results between the ECMO and CPB circuits. The air–blood interface can potentially explain such a discrepancy in CPB, which further activates clotting factors. There was a proportionate decline in platelet function in the ECMO group with an increasing dose of NO, as measured by the TRAP-6 assay. Platelet aggregation was significantly reduced at the highest amount of NO delivered at 80 ppm + heparin group than heparin alone ($p = 0.008$), but not at lower doses of NO delivered. Similar results with no difference on platelet activation in infants on CPB with 20 ppm of NO have been reported.[21]

No difference was observed between groups on ADP-like agonist or RISTO assays. In the CPB group, across all

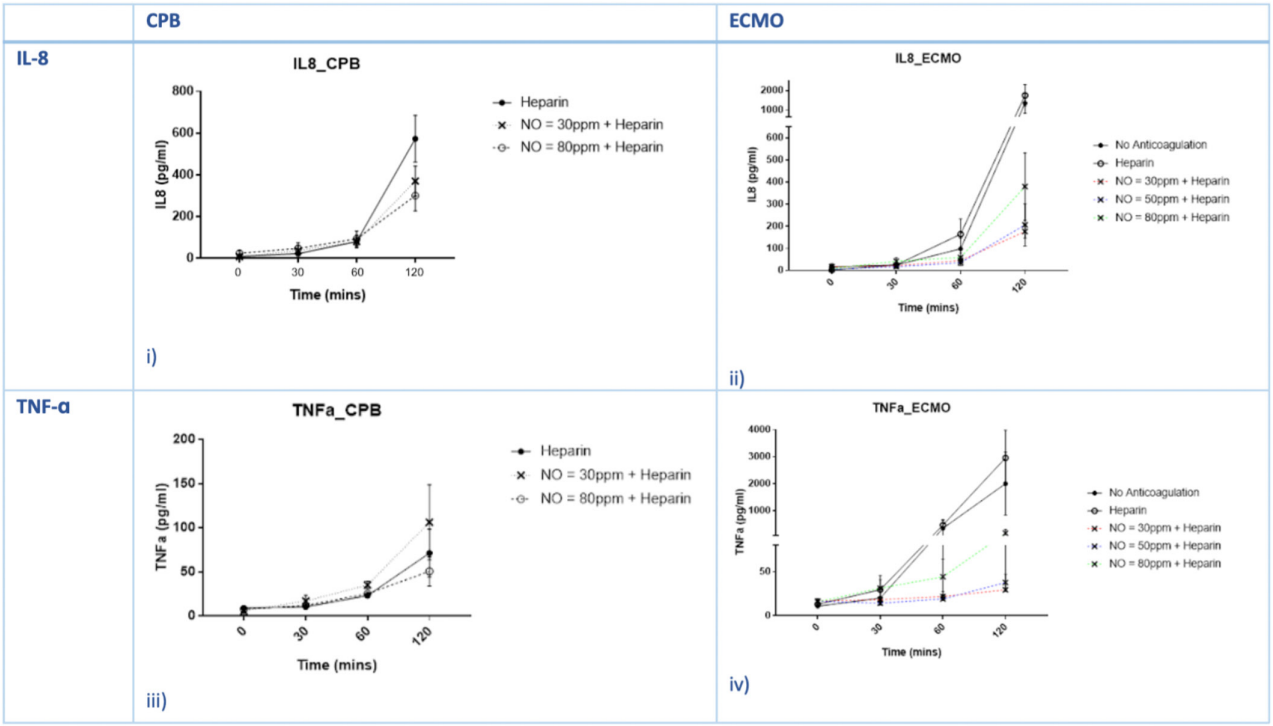

FIGURE 4 Luminex Magpix-based assays IL-8 versus time for (i) ECMO and (ii) CPB, and TNF-α against time for (iii) ECMO and (iv) CPB circuits.

assay types, no difference in platelet aggregation was detected, in any assay type, with increasing doses of NO, nor between NO and heparin groups. Again, a potential explanation for this discrepancy is the air–blood interface in the CPB circuit. NO is a highly volatile substance, and therefore its concentration is likely reduced with time as the circuit continues to run. This is supported by literature data, with one study by Gianetti et al. not only reiterating the ease with which NO crosses air–blood barriers, but also that NO below 80 ppm concentrations are not expected to produce pro-oxidant effects.[21]

In the ECMO circuit, platelet count remained stable and was maintained significantly higher by the lowest dose of NO delivered at 30 ppm + heparin group compared to the control experiments ($p < 0.0002$). However, this finding could not be replicated in any other dosage groups. In the CPB circuit, the inverse was true, with the platelet count remaining stable in the highest dose of NO delivered at 80 ppm + heparin group. However, there was no significant difference between groups. If NO has a dose-dependent effect on platelet function, this should be further investigated. One possible explanation may be differences in pump mechanics between the two groups. A centrifugal pump is used in the ECMO circuit versus a roller pump in the CPB circuit. One previous study by Steines et al. demonstrated that specific centrifugal pump designs had been associated with severely inhibited platelet function in vitro.[22]

Interestingly, increasing doses of NO appeared to influence inflammatory biomarkers. In the ECMO arm of the study, the addition of NO at all concentrations appeared to be associated with lower concentrations of the inflammatory biomarkers IL-8 and TNF-α, compared to the heparin and control groups. However, this effect was only observed at higher doses of NO delivered at 80 ppm in CPB. As stated previously, this could potentially result from losses of volatile NO. Therefore, if this is the case, a higher initial dose of NO would be required to sustain a significant anti-inflammatory effect due to the constant NO diffusion out of the perfusate. In our experiment, the effects of NO on inflammatory signaling pathways reflect the influence on circulating immune cells. To what extent these results change if NO also acts on endothelial cells remains unclear.

Several limitations were identified in this study. First, the ex vivo design of the study apparatus meant that important biological feedback mechanisms, such as the interplay between local vascular tissue inflammatory responses and coagulation pathways, were not able to be assessed. Second, the serum concentration of NO within the circuit could not be continuously monitored.

The effect of NO on platelets was likely to be influenced and/or convoluted by the effect of heparin. This likely explains why we could not detect a dose-dependent response to NO. Further investigation is required to delineate these

mechanisms and determine the clinical benefit of using NO in combination with other agents that influence hemostasis for applications in ECMO and CPB. For further research the anti-inflammatory effects and the influence on platelet aggretometry in an in vivo model would be of great interest.

AUTHOR CONTRIBUTIONS

Conceptualization: Maximilian V. Malfertheiner, Jacky Y. Suen and John F. Fraser; methodology: Maximilian V. Malfertheiner, Ashlen Garrett, Margaret Passmore, Richard I. Webb, Viktor Von Bahr, Jonathan E. Millar, Debra Black, Mahe Bouquet, Nicole Bartnikowski and Jacky Y. Suen; analysis: Maximilian V. Malfertheiner, Ashlen Garrett, Margaret Passmore, Richard I. Webb, Bailey A. Schneider, Debra Black, Mahe Bouquet and Jacky Y. Suen; writing—original draft preparation: Maximilian V. Malfertheiner, Ashlen Garrett, Andrew B. Haymet, Bailey A. Schneider and Jacky Y. Suen; writing—review and editing: Margaret Passmore, Richard I. Webb, Viktor Von Bahr, Jonathan E. Millar, Nchafatso G. Obonyo, Debra Black, Mahe Bouquet, Nicole Bartnikowski and John F. Fraser; supervision: Maximilian V. Malfertheiner, Margaret Passmore, Jacky Y. Suen and John F. Fraser; project administration: John F. Fraser; funding acquisition, John F. Fraser. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of The Prince Charles Hospital, Australia (HREC/16/QPCH/320). Written informed consent was obtained from the Australian Red Cross Blood Service (17-11QLD-05) from all subjects who donated blood. The consent form is available for review by the Editor-in-Chief.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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