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Electron Microscopy as a Tool for Assessment of Anticoagulation Strategies During Extracorporeal Life Support: The Proof Is on the Membrane

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Abstract

Extracorporeal life support (ECLS) is fast becoming more common place for use in adult patients failing mechanical ventilation. Management of coagulation and thrombosis has long been a major complication in the use of ECLS therapies. Scanning electron microscopy (SEM) of membrane oxygenators (MOs) after use in ECLS circuits can offer novel insight into any thrombotic material deposition on the MO. In this pilot study, we analyzed five explanted MOs immediately after use in a sheep model of different acute respiratory distress syndrome (ARDS). We describe our methods of MO dissection, sample preparation, image capture, and results. Of the five MOs analyzed, those that received continuous heparin infusion showed very little thrombosis formation or other clot material, whereas those that were used with only initial heparin bolus showed readily apparent thrombotic material.

Keywords

extracorporeal membrane oxygenation; extracorporeal life support; anticoagulation; heparin; acute respiratory distress syndrome

Extracorporeal membrane oxygenation (ECMO) is a mode of extracorporeal life support (ECLS) that improves survival in patients with severe cardiac or respiratory failure.¹ A typical ECMO circuit is composed of vascular catheter access cannulas (dual-site or single-site), tubing, pump, and a membrane oxygenator (MO), all of which are made of

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nonbiological polymers. Despite its expanding utilization, ECMO continues to cause thrombotic complications because of the activation of blood by either contact with polymer circuit components and/or, shear stress from the extracorporeal blood pump.²⁻⁴ In addition to the gas exchange properties, the MO functions as a sieve and filters out thrombotic deposits. Similar to pulmonary emboli, these thrombi may eventually reduce oxygenator function leading to MO failure, which forces emergency replacement of the MO.⁴ Therefore, it is standard practice to provide systemic anticoagulation with a continuous heparin infusion to prevent thrombotic complications and subsequent device failure.⁵ Reports in the literature document successful ECLS therapy with reduced or no heparin anticoagulation, and our laboratory have recently documented normal coagulation function in an animal model using low-dose heparin anticoagulation during ECLS for acute respiratory distress syndrome (ARDS).⁶

The best way for real-time monitoring of anticoagulation is a challenge, as bedside assays such as activated clotting time (ACT) and thromboelastography (TEG); monitoring of antithrombin, D-dimer, and other functional coagulation parameters; have failed to accurately predict bleeding or clotting complications.⁵ Ideally, anticoagulation therapy during ECLS would prevent circuit-related clot and bleeding problems by inhibiting platelet and coagulation factor activation.

Unfractionated heparin (UFH) remains the most commonly used anticoagulant.⁵ Unfractionated heparin, hereafter simply referred to as heparin, prevents the generation of thrombin by binding antithrombin to prevent the activity of factor Xa. Because each manufacturer's lot of heparin has different effects on coagulation function, and each coagulation analysis method introduces variability due to reagent and device differences, no standardized method for providing anticoagulation exists. Different ECLS programs must rely only on their clinical experience, guidance from the Extracorporeal Life Support Organization (ELSO), and published literature to develop their own anticoagulation protocols. These anticoagulation strategies may not work for trauma and combat-related applications of ECLS, as they often require one-to-one monitoring and are resource intensive.

Scanning electron microscopy (SEM) assessment of the MO of ECLS systems can define the clot burden and overall effectiveness of performed anticoagulation during ECLS. Work as early as 1988 described SEM analysis of ECLS circuitry, including punch biopsies of portions of the membrane. Lehle *et al.*⁹ in 2008 described a new approach in which the membranes are fully disassembled after use and were subjected to SEM. That group used oxygenators from human patients treated for severe ARDS. Thrombotic deposits were found among the oxygenators, but it could not be identified if the deposits were from the patient's preexisting state (disseminated intravascular coagulopathy, thrombocytopenia, etc.) or were a result of the blood interacting with the ECMO circuitry.⁷

In this pilot study, we undertook a similar approach in that we evaluated the recovered ECMO membranes (Quadrox HLS Advanced 5.0, Maquet GmbH, Rastatt, Germany) from sheep that underwent different heparin usage strategies during ECMO. This is the first electron microscopy documentation of MOs used in an ovine model of oleic-induced ARDS

supported with ECMO. To improve future testing of ECLS devices for trauma, we report our initial experience with SEM analysis and describe the potential role that it can play in comprehensive evaluation of anticoagulation strategies after ECLS therapy.

Methods

Animal Study Design

The membranes analyzed in this manuscript were convenience samples removed at the conclusion of a separate study on ECMO use in a trauma model of ARDS.⁸ In that work, a prospective cohort laboratory study was performed to compare low versus standard heparin anticoagulation therapy on the coagulation function in sheep induced into ARDS with an oleic acid (OA) injection and then supported with venovenous (V)-ECMO as adjunct to mechanical ventilation.¹⁰ Time on ECMO was divided into a preinjury period, before the induction of lung injury (5 hours), and a postinjury period (9 hours). Sheep were randomized to the standard heparin dose group (group H+, n = 2) or the low heparin dose group (group H-, n = 3).

The US Army Institute of Surgical Research Institutional Animal Care Use Committee approved the study. This study was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the *Guide for the Care and Use of Laboratory Animals*. The primary study design from which these membranes were collected is described in detail elsewhere.¹⁰ Crossbred female sheep (45 ± 6 kg) were studied in an awake, conscious state, and administered an OA-produced lung injury, while having respiratory support provided by ECLS (Cardiohelp, Maquet GmbH, Rastatt, Germany). We evaluated five membranes used in this experiment. Sheep received a tracheostomy and had a pulmonary artery catheter placed in the left jugular vein to administer medications. A 23 F bicaval dual-lumen catheter (Avalon Elite, Maquet, USA) was placed through the right internal jugular vein¹¹ and connected to the VV-ECMO system previously primed with normal saline. Blood flow through the MO was constant throughout the study at approximately 2 L/min. Sheep were awakened and weaned from mechanical ventilation to continuous positive airway pressure of 8 cm H₂O provided through the tracheostomy. The fraction of inspiratory oxygen (FiO₂) was set at 0.5 before injury and at 1.0 after injury. To achieve adequate sedation, buprenorphine (Reckitt Benckiser, UK), and Midazolam (Hospira, USA) were administered.¹⁰

To induce ARDS, lungs were injured by intravenous injection of 0.1 ml/kg of OA mixed in 20 ml of whole blood and 300 IU of heparin, repeated up to three times, to achieve a PaO₂ to FiO₂ ratio less than 200.¹⁰ Thus, OA injections also contributed to the heparinization of all study animals. In the standard heparin group, H+, a continuous heparin (APP Pharmaceuticals, USA) infusion maintained the ACT between 160 and 180 s after an initial bolus of 150 IU/kg during the dual-lumen catheter insertion.^{6,10} In the low heparin group, H-, sheep received only the initial 150 IU/kg heparin bolus during ECMO catheter placement and another heparin bolus during the OA injection (300-900 IU). No continuous heparin administration took place in this group. Heparin administration before OA injury was 287.14 ± 45.3 IU/kg in the H+ group, and 150 ± 0 IU/kg in the H- group. Total heparin

administration in the H+ group was 386.26 ± 89.32 IU/kg, whereas the H- group total was 159.37 ± 1.89 IU/kg. Other coagulation data are listed in Table 1.

Materials

The MOs used in this pilot study were convenience samples obtained at the conclusion of the study described earlier.¹⁰ During the study, ECLS therapy was carried out using the Cardiohelp ECLS System (Maquet GmbH, Rastatt, Germany). The Cardiohelp System utilized the Quadrox HLS Advanced 5.0 MO (Maquet GmbH, Rastatt, Germany). All components of the ECLS circuit are factory lined tip-to-tip with heparin coating (Bioline Coating, Maquet GmbH, Rastatt, Germany). The coating is a heparin–albumin combination used to coat all surfaces of the ECMO circuit and oxygenator.¹² Five MOs, two from the H+ group and three from the H- group, were recovered at the conclusion of the experiment after approximately 14 total hours of use (5 hours before induced lung injury and nine hours after injury).

Membrane Oxygenator Preparation

At the conclusion of the animal experiment, circuit tubing was clamped and cut to separate it from the catheters, then disconnected from the animals and reconnected to form a loop. One thousand units of heparin were then injected into this loop, and circuit flow was in brief reinstated to ensure mixing. The MO was then separated from the Cardiohelp System pump and the blood was drained manually. A gravity-fed 0.9% saline rinse (1–2 L/min [total 10 L]) was used to clear the circuitry while retaining any clots that were present in the MO. The MO was drained of saline and secured into a custom built manifold. Using a handheld electric saw, the four sides of the membrane case were cut exposing the inner sandwich of polymethylpentene (PMP) fibers (Figures 1 and 2). The cut MOs (as viewed from direction of blood flow through the membrane, z axis, see Figure 2) were digitally photographed for later clot density analysis. This analysis quantified as a percentage of the total surface area of the initial interface sheet (arrow A, Figure 2) covered with clot material, measured by the analysis of still digital imagery utilizing ImageJ software (National Institute of Mental Health, Bethesda, MD, USA). After disassembly, the MO sheets were fixed in 4% paraformaldehyde for later imagery by SEM. Time from Cardiohelp removal to digital image capture and fixation of separated membrane sheets was approximately 20 min. Scanning electron microscope images were captured at various depths of the MO (along the z axis, see Figures 2 and 3). The Quadrox Advanced HLS 5.0 MO is composed of alternating sheets of temperature-regulating fluid-carrying fibers and gas-exchange fibers. The membrane is internally separated into two halves by a rigid plastic sheet at the mid-point of the membrane. Once cut free from the membrane housing, these halves were removed, and each half was further divided into three segments containing the same number of sheets.

To prepare samples of the MO sheets for SEM, we fixated hollow fiber sheets and dehydrated them in a progressive series of cold ethanol and water mixture (50%, 70%, 80%, 90%, 95%, and 100% ethanol) for 10 min each. With gentle rocking motions, clot material on membranes was treated with a mixture of 50% ethanol and 50% hexamethyldisilazane (HMDS; Electron Microscopy Sciences, Hatfield, PA) for 5 min. This was then repeated

with 100% HMDS for 10 min. After liquid dehy-dration, the samples were placed into an exhaust hood and allowed to completely dry under mild air flow. We analyzed samples at the Pathology Electron Microscopy Facility at the University of Texas Health Science Center, San Antonio, TX, for image capture. The samples were mounted using carbon disks, and sputter-coated using a Denton Vacuum Desk IV (Moorestown, NJ) with a 50% each gold and palladium target compound. We observed surface topography of each sample using a JEOL 6610LV Scanning Electron Microscope (Peabody, MA). Nine representative images per group are documented in Figures 4-7.

Results

Still imagery examination of H+ membranes revealed no thrombus formation on gross examination (Figures 1 and 2). In contrast, H- membranes had visualized thrombus formation on the venous surface of the MOs (Figure 3). Density analysis using ImageJ software revealed a clot burden of $26.5 \pm 12.3\%$ on the blood entry point of the H- group oxygenators. Analysis of H+ group photographs documented negligible deposits, with no evidence of thrombotic material (Figure 4). Membrane oxygenators in the H- group appeared to have clot deposition in membranes 1 and 2 of this group (Figure 5). Clots found in the MO were preferentially located closer to the blood-entry point (venous side) along the z axis, and decreased further along the z axis of the membrane toward the middle of the membrane. Thrombi in the MOs typically exhibited parallel fibrin threads with abundant adherent platelets and red blood cells in the field of view (Figure 6A, B). Within the clot structure, the fibrin strands layered deep, interspersing heteroge-neously with red blood cells. The structure of each clot was unique to its discovery depth in the MO. For the H- membranes that showed clot formations at the initial interface, clot structure appeared rich in fibrin strands with a maximum width of $0.75 \mu\text{m}$ (Figure 6A). Further along the z-axis, deeper within the MO structure, clot structure became unidirectional. Fibrin strands at this level measured a maximum of $0.50 \mu\text{m}$ in diameter (Figure 6B). Closer SEM investigation of a spiral-shaped thrombus that formed at the mid-line separator (Figure 7) revealed fewer fibrin fibers and cross-linked fibers, but more platelets (Figure 8).

Discussion

This report adds to the accumulating evidence that SEM examination of recovered ECMO membranes is a useful tool to assess efficiency of systemic anticoagulation during ECLS. To our knowledge, this is the first SEM evaluation of membranes used in a trauma ARDS model in ovines. Systematic evaluation by SEM may prove useful in establishing a database that collects information on the interaction of different disease states, anticoagulation therapy, thrombotic events, and oxygenator performance. Further studies may improve this proposed database with analysis of the percentage of surface area obstructed by clots serving to inform future clinicians of potential clot complications.

Group H+ membranes that underwent continuous heparin infusion revealed minimal material collection within the membrane. Group H- membranes had clot formation primarily at the initial interface where blood enters the MO. Although the heparin administration data are limited in this early work, the focus of this effort was to highlight the

capabilities of SEM to identify and characterize thrombus formation, or lack thereof, within membranes after usage. The SEM images proved a useful tool for definitive analysis not only of the quantity, but also the composition of, debris remaining on the MO after usage. The difference in heparin administration is thus an important, yet not focal, finding in this preliminary work. The difference in thrombus formation noted between the heparin strategies is consistent with data showing high shear stress within a MO results in increased platelet activation and thrombus formation as blood enters the membrane.¹³ Platelet activation initiates from shear stress applied from the blood pump. The shear-activated platelets then roll along the oxygenator surface, generating increased von Willebrand factor binding to GPIb and outside-in signaling.¹⁴⁻¹⁶ As this initial work was undertaken with convenience samples, comprehensive analysis of the coagulation profile within the membranes was not performed. The data that were able to be learned from the SEM analysis has highlighted the critical role coagulation studies play in these studies, and such analyses should be planned for in future work.

Previous studies have indicated that continuous heparinization may not be necessary during ECLS. Kundu *et al.*¹⁷ utilized three groups of rabbits on ECMO: one with a standard systemic heparinization regimen; the second group without any heparin; and the third group with the ECMO circuitry coated with the Maquet Bioline albumin-heparin surface to prevent thrombosis. Using SEM image analysis, they documented no significant differences between the ECMO membranes after use in either group. This demonstrated that heparin-free ECMO in this short-term model may work without complications. This article also showed much higher platelet activation and attachment to the MO in the group that received systemic heparinization. In a combined experimental and clinical study, Whittlesey *et al.*¹⁸ showed no histological evidence of thromboembolism in lambs supported with ECMO up to 96 hours when compared with lambs that received systemic heparinization. In this study, fibrinogen level and platelet counts were lower with no anticoagulation therapy and this did not lead to clinically detrimental effects. Our group recently published that ECMO without continuous heparinization could sustain a circuit up to 10 hours.⁶ Moreover, several case reports have concluded that it is safe to discontinue systemic heparinization in traumatic bleeding patients requiring ECMO.¹⁸

We documented the first SEM analysis of MOs using a trauma-related ARDS ECMO model. This information may lead to the knowledge on when to replace MOs. This is important because emergent circuit or MO exchange can be dangerous and techniques vary widely among centers.⁵ Advances in state-of-the-art antithrombogenic coatings have led to much decreased systemic anticoagulation needs.¹⁸⁻²⁰ One case report documents that it was possible to maintain a patient for up to 151 days of continuous ECMO use without continuous heparinization and subsequent thrombotic events.²⁰ In some cases, such as traumatic brain injury, ECMO therapy has been historically contraindicated because of increased bleeding risk from systemic anticoagulation, although data show that, when dictated by clinical needs, ECMO can be safely applied in these cases with minimal anticoagulation.¹⁹ We proposed that this may be a result of newer polymers, hollow-fiber MOs, and antithrombogenic coatings. None of these have been systematically studied for their anticoagulation potential in real world situations such as trauma-related ARDS.

Our report confirms and extends the work by Lehle *et al.*⁹ on the potential utility of SEM to define the thrombotic burden after ECLS support. The Lehle study is very important because it included both SEM and functional MO performance data in human use. The ECMO membranes recovered were because of decreased performance days of use in critically ill patients in one of the largest ECMO centers in the world.⁹ The Lehle study and this study were both driven by an emerging clinical need to document the efficacy of anticoagulation strategies during ECLS. Rigorous analysis of recovered MOs will provide useful information on the performance and efficiency of systemic anticoagulation of ECLS support.⁹ In the Lehle paper, membranes were rinsed with normal saline and a phosphate buffer; then a fixative solution, and then a cryoprotectant solution. We rinsed our membranes with heparin, saline, and then paraformaldehyde. The Lehle group froze their MOs at -80°C until later analysis; our group dissected and analyzed the membranes immediately after ECLS termination. The Lehle group dissected MOs by band saw into 1 cm thick slices to be processed for SEM. Our dissection involved removal of the outer casing of the membrane by reciprocating saw, and manual dissection of individual sheets of PMP fibers which were manually cut into strips for processing. The Lehle SEM analysis showed deposits of red blood cells and platelets embedded on the membrane fibers, particularly adjacent to the strands holding the gas exchange fibers together.⁹ The nature of the Lehle design allows for time between explantation and analysis. Their approach enables analysis at a time convenient to the researchers, and would allow large groups of membranes to be examined at once. Our method also does not include a cryoprotective step because we wished to process our MO immediately after removal, and requires dissection and analysis relatively quickly after explantation. Our approach of sequential removal of the PMP sheets allowed for an increased quantitative assessment of the clot formation within the membrane, along the z axis. We document that clot formation lined preferentially along the PMP sheets and in greater density at the blood inlet of the membrane. We propose that clot formation occurs before the MO within in the circuit, letting the MO acts as a sieve. Based on other studies the change in shear stress applied to blood as it enters the MO activates the clotting cascade facilitating the greatest potential for clots.⁶

Both methods achieved the goal of SEM analysis of the PMP fibers, allowing for a more conclusive post-ECMO assessment of the MOs. Although both methods differ in basic approach, we are confident that they are both useful in providing insight into the dynamics of clot formation on MOs. Future standardization of MO preparation, dissection, and SEM analysis may inform future ECMO strategies on how to reduce oxygenator failure and serve as a net assessment of the biological effects of anticoagulation. Correlation of SEM analysis with early warning signs of oxygenator failure, such as increasing pressure differential across the MO, may allow clinical teams to better understand the effects of life-threatening complications associated with ECLS. In addition, future studies of SEM analysis on MOs coated with novel antithrombogenic coatings may prove a vital tool in the assessment of emerging methods of clot prevention.

Our analysis of still images also differs from techniques used by other groups²¹ in that we opted for a percentage of clot size relative to the total membrane surface area, instead of a volumetric analysis. Our group feels that a percentage of clot size is a more clinically relevant tool, as a bedside provider may easily and quickly estimate percentages of

membrane area inundated with clot material. With a repository of clot information as described earlier, a quick estimation of clot percentage on the visible surface of the MO may enable providers to make an estimation of potential membrane failure and correlate it to documented MO performance at similar percentages. Two papers by Dornia *et al.*,^{21,22} in which multidetector computed tomography (MDCT) images were collected on MOs after removal from patients, with findings of the MDCT confirmed by SEM according to the Lehle method, adds to the growing arsenal of tools available to analyze thrombotic deposits within MOs. The MDCT technique was confirmed by SEM as in the Lehle paper; however fails to provide high resolution imagery of the separate blood components found within the MO. It also fails to provide details about the structure of thrombi only available with a high-power magnification such as with SEM. Future studies are warranted to further develop the relationship between MDCT and SEM findings. MDCT, with more detailed SEM congruence, may prove a valuable tool in the clinician's bag for close-to-bedside analysis of failing MOs.

Limitations of our study to define the thrombotic burden in MO after trauma induced ARDS supported with ECMO include the small sample size, the lack of a no heparin experimental arm, and short time of support. The nature of the injury induced in the animal, via OA mixed with heparin, is a confounding factor, which will not be the same when human MOs are inspected. Our preliminary results are hindered by a short ECMO time of approximately 14 hours in each case. Future studies with greater numbers of membranes, longer periods of ECMO support (days vs. hours), along with consistent hematological and coagulation monitoring at specified time intervals, are required to refine and standardize the technique of MO analysis by SEM.

Conclusion

We recommend further exploration of SEM as a tool for studying the effect of anticoagulation strategies used in ECLS. This approach provides the ultimate assessment of the efficiency of systemic anticoagulation and may improve our ability to reduce levels of systemic heparinization in ECMO patients.

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Figure 1. Membrane oxygenator, group H $\pm$. Dashed lines denote areas where the MO was cut. MO, membrane oxygenator.

Figure 2.

Membrane oxygenator, group H+. Arrow “A” shows the initial interface from which SEM samples were taken. Arrow “B” shows the mid-line separator in the middle of the membrane sheets. Arrow “Z” shows the direction of blood flow through the membrane (referred to throughout the paper as the *z* axis. The “deeper” through the membrane, the further away from the initial interface, and closer to the blood exit at the end of the *z* axis. SEM, scanning electron microscope.

Figure 3.
Representative membrane oxygenator from group H-.

Figure 4.

Representative SEM images of MO from group H+ membranes. Images are arranged from left to right, then top to bottom. The nine levels of image capture described in the methods section apply here. **A:** The initial venous blood/membrane interface membrane sheet sample; subsequent images represent deeper membrane layers of the MO. **I:** The last membrane sheet sample of the MO stack. Vertical lines represent gas exchange sheets of the membrane. Horizontal lines represent the alternate temperature control sheets. MO, membrane oxygenator; SEM, scanning electron microscope.

Figure 5.

Representative scanning electron microscope images of MO from group H- membranes. Images are arranged from left to right, then top to bottom. The upper left image (A) shows deposits at the initial blood/membrane interface; the five subsequent images (B-F) show clot formation through the depth of the membrane. G: A large artifact on the midline plastic separator. The last two images (H, I) appear very similar to the images from the group H+ membranes showing that there was no penetration of clotting through all sheet layers as images H and I are clear and similar to those taken in group H+. MO, membrane oxygenator; SEM, scanning electron microscope.

Figure 6. Scanning electron microscope images of clot structure within the MO in group H-. **A:** Thrombus formed at the blood inlet surface; **(B)** thrombus formed at one-quarter of the depth of the MO along the z line. MO, membrane oxygenator.

Figure 7. Scanning electron microscope image thrombus located at the mid-point separator of the MO. MO, membrane oxygenator.

Figure 8.
Detail of spiral thrombus. Less cross-linked fibers but more platelets.

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Table 1.

Heparin Administration Data

Group	Heparin Before Injury	Heparin Before Injury (IU/kg)	Total Heparin	Total Heparin (IU/kg)
H+	14,635.00 ± 2,785.00	287.14 ± 45.30	19,735.00 ± 5,185.00	386.26 ± 89.32
H−	5,940.00 ± 357.91	150.00 ± 0.00	6,306.67 ± 347.20	159.37 ± 1.88