

**Background:** Endothelium-derived microparticles (EMPs) are submicron vesicles released from the plasma membrane of endothelial cells in response to injury, apoptosis or activation. We have previously demonstrated EMP-induced acute lung injury (ALI) in animal models and endothelial barrier dysfunction in vitro. Current treatment options for ALI are limited and consist of supportive therapies. We hypothesize that standard clinical continuous venovenous hemofiltration (CVVH) reduces serum EMP levels and may be adapted as a potential therapeutic intervention.

**Materials and methods:** EMPs were generated from plasminogen activation inhibitor-1 (PAI-1)-stimulated human umbilical vein endothelial cells (HUVECs). Flow cytometric analysis was used to characterize EMPs as CD31- and annexin V-positive events in a submicron size gate. Enumeration was completed against a known concentration of latex beads. Ultimately, a concentration of ~650,000 EMP/mL perfusate fluid (total 470 mL) was circulated through a standard CVVH filter (pore size 200 μm, flow rate 250 mL/hr) for a period of 70 minutes. 0.5 mL aliquots were removed at 5- to 10-minute intervals for flow cytometric analysis. EMP concentration in the dialysate was measured at the end of 4 hours to better understand the fate of EMPs.

**Results:** A progressive decrease in circulating EMP concentration was noted using standard CVVH at 250 mL/hr (a clinical standard rate) from a 470 mL volume modelling a patient’s circulation. A 50% reduction was noted within the first 30 minutes. EMPs entering the dialysate after 4 hours were 5.7% of the EMP original concentration.

**Conclusion:** These data demonstrate that standard CVVH can remove EMPs from circulation in a circuit modelling a patient. An animal model of hemofiltration with induction of EMP release is required to test the therapeutic potential of this finding and potential of application in early treatment of ALI.

**Keywords:** acute lung injury; microparticles; dialysis; endothelium
The presence of endoplasmic reticulum proteins (e.g. BIP-2) and externalized annexin V suggest that EMP release is a deliberate compensation by stressed ECs (59,60). Endothelial dysfunction resulting from EMP exposure has been demonstrated both in vitro and in vivo (6,15,61). We have previously shown that EMPs increase pulmonary endothelial permeability and decrease eNOS phosphorylation, diminishing NO production (5,6).

Multiple studies of ALI in animal models and humans suggest a benefit of hemofiltration on lung function in the setting of ALI (62–68). This effect has been largely attributed to a decrease in venous pressure, an increase in circulatory hydrostatic pressure and clearance of inflammatory cytokines. We hypothesized that the CVVH benefit seen in ALI recovery may be attributable to the removal of EMPs from circulation. As a first step, this report tests our hypothesis by examining the efficacy of standard clinical CVVH dialysis in removing EMPs from a modelled patient circulation.

**Methods**

**EMP generation, identification and enumeration**

EMPs were generated using previously published protocols (6). Human umbilical vein endothelial cells (HUVEC passage 4–6; Clonetics, Walkersville, MD) were grown to confluence, then stimulated with plasminogen activation inhibitor-1 (PAI-1, 10 ng/mL; American Diagnostica Inc., Stamford, CT). Supernatant was collected 3 hours after stimulation and centrifuged (145 g, 8 minutes) to remove cell debris. The supernatant was subsequently ultracentrifuged (100,000 g, 6 minutes, 4 degrees) and pelleted. EMPs were re-suspended in phosphate buffered saline (PBS) and stored at −80 °C. Flow cytometric analysis was used to characterize and quantify EMPs based on CD31- and annexin V-positivity occurring in a size gate less than 1 μm. This size gate was set on forward versus side scatter using latex standard beads measuring 0.84 μm (Spherotech FP-0856-2). Enumeration was completed against a known concentration of 7.6 μm polystyrene beads placed within the sample (Spherotech PPS-4). The flow rate used for analysis was 12 μL/min (Figs. 1 and 2).

As a separate and confirmatory measure, transmission electron microscopy (TEM) was used to describe particle size distribution. A 25-μL drop of EMP in solution was pipetted onto a Formvar plastic coated grid (Ted Pella, Inc.) and allowed to air dry for 1 hour. The microparticle grid was examined and photographed by TEM with a JEOL 100 CX microscope (Fig. 3).

**CVVH dialysis**

EMPs (300 × 10⁶) were introduced to an IV bag (model patient) containing 300 mL of 0.9 NS (model patient) and manually agitated for 3 minutes. The IV bag was connected to a primed CVVH circuit containing 170 mL of 0.9 NS and mixed (~650 × 10⁵ EMP/mL final mixed perfusate concentration) as shown in Fig. 4. After mixing, the perfusate was circulated through the CVVH filter (NxStage® PUREMA®, 200 μm pore size, 35 μm wall thickness, rate 250 mL/hour) for a period of 70 minutes. 0.5 mL aliquots were removed for flow cytometric analysis at 5–10 minute intervals for 70 minutes of dialysis. EMP levels were recorded and expressed as a fraction of starting concentration after mixing occurred. Three runs were completed and the fraction of EMPs versus time was plotted with standard error of the mean (SEM). At the end of 4 hours filtration, dialysate EMP concentration was measured using flow cytometry methods as previously described.

**Results**

EMP clearance from our modelled patient can be seen in Fig. 5. The decline in EMP levels due to dialysis is rapid with 50% of clearance being seen in the first 30 minutes. Within 40 minutes, the circulating EMP levels were reduced to 29% of starting concentration and this level stayed relatively constant over the duration of the study. To determine the fate of the cleared EMPs, we measured EMP levels in the dialysate. The dialysate contained 5.7% of the original EMP concentration (~650 × 10⁵ EMP/mL).

![Fig. 1. An EMP size gate was first defined using 0.84 μm latex beads. FSC vs. SSC was used to create this gate denoted as P2. 7.6 μm latex beads are shown in gate P1 and allowed for enumeration of EMPs.](image-url)
at the end of 4 hours of uninterrupted dialysis. This suggests that the filter or tubing is entrapping EMPs.

Discussion
The data presented here demonstrate that counter-current dialysis using a standard clinical CVVH filter and under clinical conditions removes EMPs from a modelled patient’s circulation. Given our previous evidence of EMP-induced ALI, these findings support the idea of CVVH as a meaningful therapeutic intervention in ALI due to clearance of EMPs from the patient circulation. The experimental conditions achieve the same plasma level of EMPs as seen in human clinical disease (1 × 10⁶ per mL).

While this work demonstrates that a standard 200-μm CVVH circuit and filter entraps EMPs, there are significant limitations to the model presented. The observed EMP clearance is likely a saturable phenomenon, which would explain the relatively constant EMP concentration from 40 to 70 minutes and low dialysate EMP levels. The relative volume of human circulation (~5 L) to CVVH circuit (~200 mL) is much higher in the clinical condition as compared to our model (300 and 170 mL, respectively). While this would not affect a steady state of dialysis, a saturable filter EMP-binding would become relevant sooner in the clinical condition. Additionally, EMPs may continue to be generated in a patient and a non-saturable clearance method would be required to

Fig. 2. Within gate P2, events positive for annexin V (P3) and CD31 (P4) were enumerated against P1 to quantify EMP levels. The same settings were used to quantify EMPs in perfusate samples.

Fig. 3. EMP size distribution as measured by TEM (10,000 × magnification) accompanied by a representative micrograph. A 1 μm size reference is shown in the bottom right of the micrograph.
achieve a lower steady state EMP concentration. Further, this model examined only the diffusion force across a CVVH filter. Convection and pressure gradient forces were not modelled, as these interventions require replacement fluid and this would confound EMP measurement. However, if EMP charge and size are amenable, forced hindered diffusion via the application of convective and pressure forces coupled with replacement by EMP-free fluid would likely sustain clearance. In conjunction with particle trapping by the filter, the first order kinetics of EMP clearance could be prolonged at a minimum. Additionally, EMP interactions in vivo with plasma oncotic elements (circulating cells, albumin, etc.) are not modelled in this system. We do not feel that such elements have a major impact upon the steady state of EMPs in human disease states using flow cytometry have identified the $0.5 \times 10^6$ per mL threshold injury value using flow cytometry of submicron events occurring in platelet-free plasma (54). Thus, the absence of these elements in our model is representative of the steady state of unbound plasma EMPs present in human disease.

The low dialysate EMP level and plateauing circulation EMP level beyond 40 minutes suggest that EMP sequestration occurs within the CVVH filter or tubing and that this is a saturable phenomenon. Frequent changing of the CVVH filter is a logical step that may overcome this limitation. Additionally, more efficient and selective EMPs clearance can be realized by conjugating endothelium-specific antibodies to commercially available specialty CVVH filters (Aethlon ADAPTTM). This approach would provide more selective binding sites and higher affinity for circulating EMPs. By combining a selective filter with convection and pressure gradients, EMP clearance could likely be substantially prolonged. Clinical data generally support the protective role of CVVH in ALI management. A clinical randomized trial and several animal trials demonstrate that the achievement of negative fluid balance is associated with improved outcomes from ALI (62–65),(67,68). In addition to its salutary effects on EMP levels, early CVVH may prevent or minimize volume overload that is commonly observed with the resuscitation phase of critical illness, particularly in persons suffering concomitant acute kidney injury (AKI). Thus, CVVH could be used as an effective tool targeting both the molecular machinery and iatrogenic components of ALI.

As a translatable next step, we plan to assay patients pre- and post-dialysis to observe EMP concentration effects. Ultimately, the application of dialysis to rodent models of EMP-induced ALI would examine efficacy in...
on-going diseases and EMP thresholds associated with worse injuries. While the utility of ultrafiltration can be tested in an animal model, diffusion appears to be enough to remove EMPs due to their small size: standard dialysis alone was effective in this in vitro model.

Conflicts of interest and funding
The authors have not received any funding or benefits from industry or elsewhere to conduct this study.

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CVVH Clearance of EMPs
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