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Hemadsorption extracorporeal therapy removes cytokines ex vivo in horses

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OBJECTIVE
Plasma cytokine adsorption has shown benefit as an adjunctive therapy in human sepsis but has yet to be investigated in horses. We hypothesized that ex vivo filtration of equine plasma with a novel cytokine adsorption device would significantly reduce concentrations of lipopolysaccharide-stimulated cytokines. We also hypothesized that the device would adsorb medications commonly used to treat sepsis.

ANIMALS
8 horses owned by North Carolina State University.

METHODS
Four liters of heparinized whole blood was collected from healthy adult horses (n = 8) and stimulated with lipopolysaccharide (100 ng/mL) for 6 hours (37 °C.) from June 4, 2023, to December 15, 2023. Plasma was filtered through a cytokine adsorption device or sham circuit. Samples were collected at 11 time points for multiplex cytokine analysis. Chemistry analysis was performed before and after filtration. To investigate the impact of the device on medication concentrations, equine plasma containing potassium penicillin, gentamicin, and flunixin meglumine was filtered through the cytokine adsorption device or sham for 6 hours. Drug concentrations before and after filtration were determined by ultra-high-performance liquid chromatography. Prefiltration versus postfiltration sample concentrations were analyzed by Student paired *t* test using GraphPad Prism 9.0 ($P < .05$).

RESULTS
Filtration of lipopolysaccharide-stimulated equine plasma (n = 8) for 6 hours resulted in significant mean reductions in the cytokines IL-10, IL-5, IL-8, tumor necrosis factor-α (TNF-α), and IL-1β, as well as albumin. Drug concentrations of potassium penicillin, gentamicin, and flunixin meglumine were also significantly reduced by filtration.

CLINICAL RELEVANCE
This work provides proof of concept for further investigation of extracorporeal cytokine adsorption as a potential adjunct treatment for equine sepsis.

Keywords: extracorporeal, equine, hemadsorption, cytokines, dialysis

Sepsis is defined as an exaggerated systemic inflammatory response to infection and is a common condition in horses. Despite substantial advances in medical management, sepsis continues to be a leading cause of illness and death in these patients.[1,2] Sepsis can occur in both very young and adult horses as a complication of a variety of conditions. In adult horses, sepsis is a potential sequela to overwhelming infection or tissue injury, such as pneumonia, cellulitis, or colic. Similar to adult horses, foals can also develop sepsis secondary to infection or tissue injury or, more commonly, as a complication of complete or partial failure of passive transfer of immunity.

A key component in the pathophysiology of sepsis is altered systemic concentrations of cytokines, which can lead to deleterious effects on immune and organ function and patient outcomes.[3] Tumor necrosis factor-α (TNF-α) and IL-1β are 2 cytokines that are increased early in equine sepsis and are known to contribute to altered systemic perfusion and increased vascular permeability.[4–6] Increased concentrations of these cytokines have been identified in foals and adult horses with naturally occurring sepsis, and marked increases in TNF-α have been

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linked to a higher rate of mortality in cases of equine colic and sepsis in foals.[4,7,8] Dysregulation of cytokines in horses has also been linked to renal failure and hyperinsulinemia, which may drive laminitis.[9,10] Current management of sepsis in neonatal and adult large animals is limited to antimicrobial and supportive therapy, with no interventions that specifically target cytokine removal.

Plasma cytokine adsorption through a novel extracorporeal therapy device (CytoSorb; CytoSorbents Corp) has shown promise as adjunctive therapy in humans with sepsis, decreasing mortality rates in patients with secondary conditions including vasoplegic shock, respiratory, cardiac, and renal failure.[11,12] This company produces a parallel product for veterinary patients called VetResQ (CytoSorbents Corp). CytoSorb technology works by removing hydrophobic substances that weigh below 60 kDa using a concentration-dependent gradient. With this mechanism, substances with high plasma concentrations are cleared more efficiently than those with low concentrations. This concentration-dependent removal helps to diminish the "cytokine storm," while also avoiding the complete elimination of cytokines from systemic circulation. This is a key feature of this therapy, since the complete elimination of cytokines could have deleterious effects on host immune function.

While there are reports in the literature on hemodialysis[13] and plasmapheresis in horses,[14–16] use of extracorporeal therapy in large animals is exceedingly rare. To date, there are no studies investigating the impact of cytokine adsorption in large animal patients and the feasibility of these therapies is unknown. For this study, we hypothesized that ex vivo filtration of equine plasma with a novel cytokine adsorption device (VetResQ) would significantly reduce concentrations of lipopolysaccharide (LPS)-stimulated cytokines. We also hypothesized that the device would adsorb medications commonly used to treat sepsis. Our goals were to use an ex vivo filtration system to obtain proof-of-concept data on the effects of filtration using a novel cytokine adsorption device on equine plasma cytokine concentrations, biochemistry parameters, and concentrations of medications commonly used to treat sepsis, including flunixin meglumine, potassium penicillin, and gentamicin.

Methods

Animals

This study was approved by the North Carolina State Institutional Animal Care and Use Committee (IACUC 23-110). Four liters of whole blood from 8 healthy adult mixed-breed horses owned by North Carolina State University was collected via a 14-G jugular catheter and heparinized with 5 µg/mL of unfractionated heparin. Horses' health status was evaluated through physical exam, CBC, and chemistry analysis. Whole blood was stimulated with 100 ng/mL LPS from *Escherichia coli* 055:B5 (Sigma-Aldrich) for 6 hours at 37 °C on an orbital shaker. Following LPS stimulation, blood was allowed to settle for 30 minutes at 37 °C, and RBCs were drained off. Plasma was harvested by centrifugation at 3,500 rpm (2,058 X *g*) for 20 minutes. After the baseline aliquots were collected, 2 liters of plasma was filtered through a cytokine adsorption device using an extracorporeal blood pump circuit at a rate of 130 mL/min. In 1 horse, an additional 2 L of LPS-stimulated plasma was harvested and filtered through a sham circuit with no filtration device connected. Aliquots of plasma were collected throughout filtration or sham at 11 time points (0, 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 minutes).

Cytokine analysis

Quantitative analysis of cytokines was measured using an equine-specific Milliplex Map Magnetic Bead Panel (EMD Millipore) per the manufacturer's protocol using a previously validated method.[17] Samples were analyzed in duplicate. The measured cytokines and chemokines with significant stimulation in this ex vivo model included IL-1β, IL-5, IL-8, IL-10, and TNF-α. Four measured cytokines either failed to stimulate or were below the limit of detection for this assay: IL-6, IL-18, eotaxin, and CCL5. Briefly, for the assay, 96-well plates were washed using kit wash buffer before use. Background (assay buffer), standard, and control wells were loaded onto the plate. Next, 25 µL of each sample and assay buffer was plated in sample wells, and each sample was plated in duplicate. Plates were covered and incubated on an orbital shaker overnight at 4 °C in the presence of magnetic beads coated with fluorescently labeled capture antibodies for each analyte. Beads were then washed and incubated with biotinylated detection antibodies, followed by the addition of streptavidin phycoerythrin. Beads were washed again before resuspension in 150 µL of sheath fluid and sample analysis using a minimum count of 50 beads per well was used for inclusion in the analysis. Standard curves were analyzed and deemed to be appropriate. The mean of the duplicate samples was used for analysis. The plate was run on a Bio-Rad Luminex Bio-Plex 200 Suspension Array Stem (Luminex), and Bioplex Manager Software (Bio-Rad, version 6.2) was used for analysis.

Chemistry analysis

Biochemistry analysis was performed on baseline and 6-hour postfiltration samples by a Stago Chemistry Analyzer (Stago) housed in the North Carolina State University clinical pathology laboratory. Glucose, urea, creatine, phosphorus, calcium, magnesium, total protein, albumin, globulin, triglycerides, bilirubin, alkaline phosphatase, AST, GGT, sorbitol dehydrogenase, creatinine kinase, sodium, potassium, chloride, and bicarbonate were all included in the analysis. All values on the chemistry were analyzed for statistical significance and deviations from normally accepted ranges.[18]

Pharmaceutical analysis

Medication "spiked" blood sample—A 14-gauge catheter (Angiocath, Becton Dickinson) was placed

into the jugular vein for the collection of blood samples. Two 60-mL aliquots (bag A and bag C) of heparinized whole blood collected from 1 healthy adult horse were spiked with 50 µg/mL potassium penicillin, 70 µg/mL gentamicin, and 5 µg/mL flunixin meglumine and incubated for 30 min at 37 °C while protected from light. Selected concentrations were based on the 30-minute plasma concentrations at standard clinically accepted recommended dosages reported in prior pharmacokinetic studies[19,20] for each medication.

In vivo medication administration blood sample—The same horse as above was administered 22,000 IU/kg of potassium penicillin, 6.6 mg/kg of gentamicin, and 1.1 mg/kg of flunixin meglumine, IV. At 30 minutes postadministration, 600 mL of blood (bag B) was collected.

Bag A and bag B were filtered through individual cytokine adsorption devices using an extracorporeal blood pump circuit. Bag C was spiked with the same drug concentrations as bag A and run through an extracorporeal blood pump circuit with no filter (sham) to determine medication breakdown and adsorption by materials over time. Samples were collected at 11 time points as described for cytokine analysis.

Quantitative analysis was performed by the Analytical Chemistry Research Laboratory at the

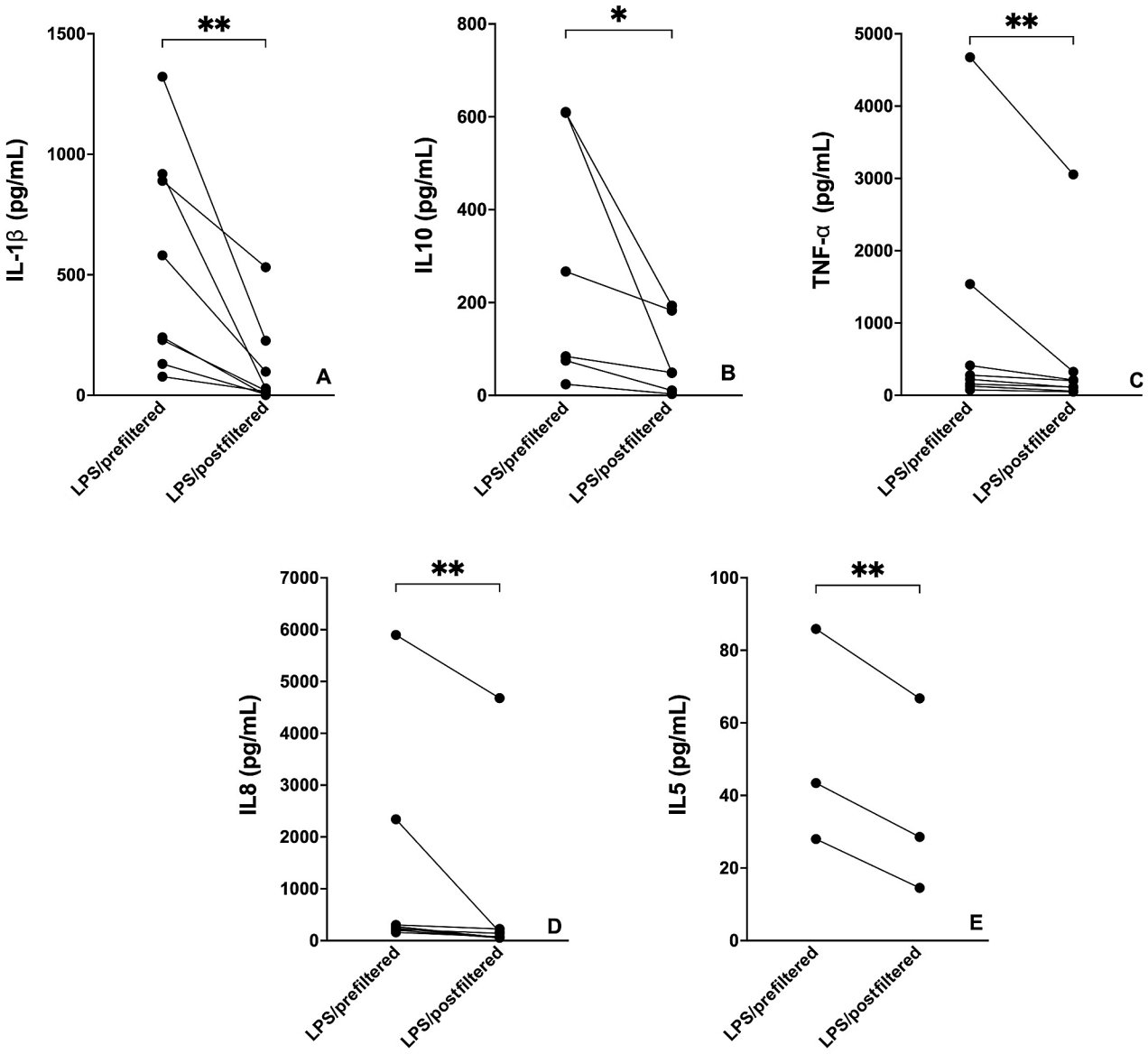

Figure 1—Mean concentrations of IL-1β (A), IL-10 (B), tumor necrosis factor-α (TNF-α; C), IL-8 (D), and IL-5 (E) before and after 6 hours ex vivo filtration with a cytokine adsorption device (VetResQ; CytoSorbents Corp) in plasma obtained from heparinized whole blood samples, each collected from a healthy adult horse (n = 8) and then stimulated for 6 hours with 100 ng/mL lipopolysaccharide from *Escherichia coli* 055:B5 between June 4, 2023, and December 15, 2023. For each time point, each circle represents the pre- and postfiltration mean results for 1 horse. Lines connect results for the same horses. Not all cytokines were quantified for each sample. Student *t* test were used for parametric data and Wilcoxon signed-rank testing was used for nonparametric data. *$P < .05$. **$P < .01$.

Virginia-Maryland College of Veterinary Medicine. Concentrations of penicillin, gentamicin, and flunixin were determined by ultra-high-performance liquid chromatography with tandem mass spectrometry. With the use of this method, calibration curves were made in blank equine plasma fortified with stock drug solution over a plasma concentration range of 0.09 to 84.4 µg/mL for penicillin and gentamicin and 0.018 to 16.9 µg/mL for flunixin. The coefficient of determination (R^2) for each curve was > 0.99, and all concentrations were within ± 15% of the actual concentration. The system had a limit of detection of approximately 0.03 µg/mL for penicillin and gentamicin and 0.001 µg/mL for flunixin meglumine. The limit of quantification was set at the lowest concentration on the individual calibration curves (0.09, 0.018, and 0.006 µg/mL for penicillin, gentamicin, and flunixin, respectively). Quality control was performed on at least 4 replicates of high, medium, and low concentrations of each analyte. Accuracy and precision for each compound are reported **(Supplementary Table S1)**. Additional information on the method is available **(Supplementary Material S1)**.

Statistical analysis

Data were analyzed using GraphPad Prism (GraphPad Software LLC). The number of donor horses (n) utilized for the experiment was based on adequate power of statistical comparison (α = 0.05). For a cytokine to be included in analysis at least 3 horses had to show stimulation (at least twice baseline). Each cytokine included in the analysis was assessed for normality at the baseline with no LPS addition versus LPS-stimulation point and at time point 0 and time point 360 minutes post-filtration using a Kolmogorov-Smirnov test. For normally distributed data, cytokines were evaluated using a paired *t* test, and for nonnormally distributed data, cytokines were compared using the Wilcoxon signed-rank test. Finally, cytokines were visually inspected by graphing to assess the decrease in cytokine concentrations over the course of filtration. Statistical significance was set at $P < .05$. Chemistry was evaluated for statistically and clinically relevant changes in the same manner as cytokine analysis.

Results

Animals/cytokine inclusion

Cytokines included in the final analysis for this study were IL-1β, TNF-α, IL-8, IL-5, and IL-10, with "n" for each cytokine ranging from a maximum of 8 to a minimum of 3 individual horses.

Cytokine concentrations

Plasma concentrations of IL -1β (n = 8), TNF-α (8), IL-8 (8), IL-5 (3), and IL-10 (6) were significantly reduced ($P < .05$) following 6 hours ex vivo filtration with a novel cytokine adsorption device (VetResQ) **(Figure 1)**. The cytokine with the greatest mean reduction was IL-1β at 84.3%, while the cytokine with the lowest mean reduction was TNF-α at 44.6%. Percent reduction for the other cytokines was 57.2% (IL-8), 57.8% (IL-5), and 72.9% (IL-10). Subjectively, the rate of cytokine removal was cytokines highest in the first hour of filtration **(Figure 2)**.

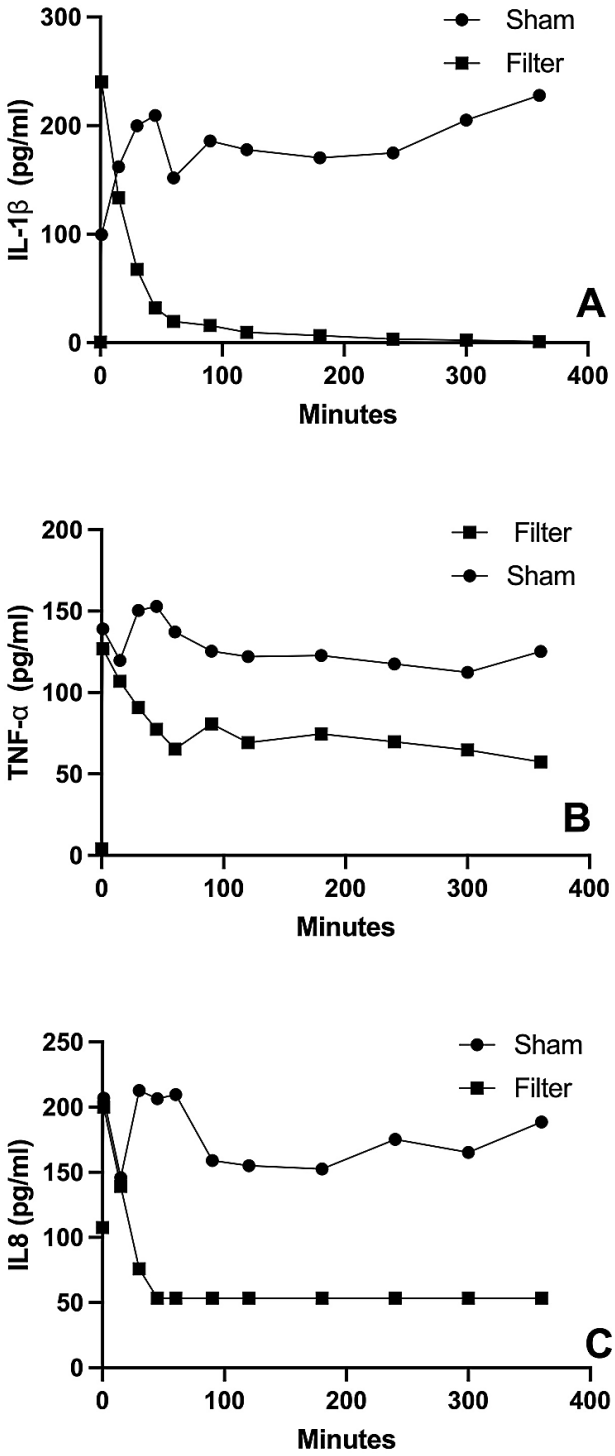

Figure 2—Comparisons of the concentrations of IL-1β (A), tumor necrosis factor-α (TNF-α; B), and IL-8 (C) in plasma after filtration with a sham device (Sham) versus a cytokine adsorption device (Filter) as described (Figure 1) for plasma obtained at 0, 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 minutes of lipopolysaccharide stimulation of a single sample of equine whole blood.

In 6-hour sham-filtered plasma (n = 1), there was no significant reduction in TNF-α (+11%), IL-8 (+10%), or IL-5 (+0%), and there were marked increases in IL-1β (+56%) and IL-10 (+19%) (Figure 2).

Biochemical analysis

Significant decreases in biochemistry parameters in VetResQ-filtered plasma (n = 8) included albumin (median, 2.4; range, 2.0 to 2.7 g/dL; *P* = .0078; 100% of horses), globulins (median, 2.6; range, 2.1 to 3.2 g/dL; *P* = .008; 38% of horses), potassium (median, 3.5; range, 3.2 to 4.1 mmol/L; *P* = .0002; 100% of horses), and calcium (median, 9.75; range, 8.6 to 11.0 mg/dL; *P* = .001; 88% of horses). No significant increases were observed **(Figure 3)**.

In 6-hour sham-filtered plasma (n = 1), there were no significant changes in plasma biochemistry parameters **(Supplementary Table S2)**.

Medication concentrations

VetResQ filtration resulted in similar removal of medications from both the "spiked" and 'in vivo" samples. In the ex vivo spiked sample (bag A), final gentamicin, potassium penicillin, and flunixin meglumine concentrations were decreased by 80.5%, 97.2%, and 55.2%, respectively, in 6-hour postfiltration versus prefiltration plasma samples. In the in vivo drug administration sample (bag C), final gentamicin, potassium penicillin, and flunixin meglumine concentrations were decreased by 89.5%, 91.4%, and

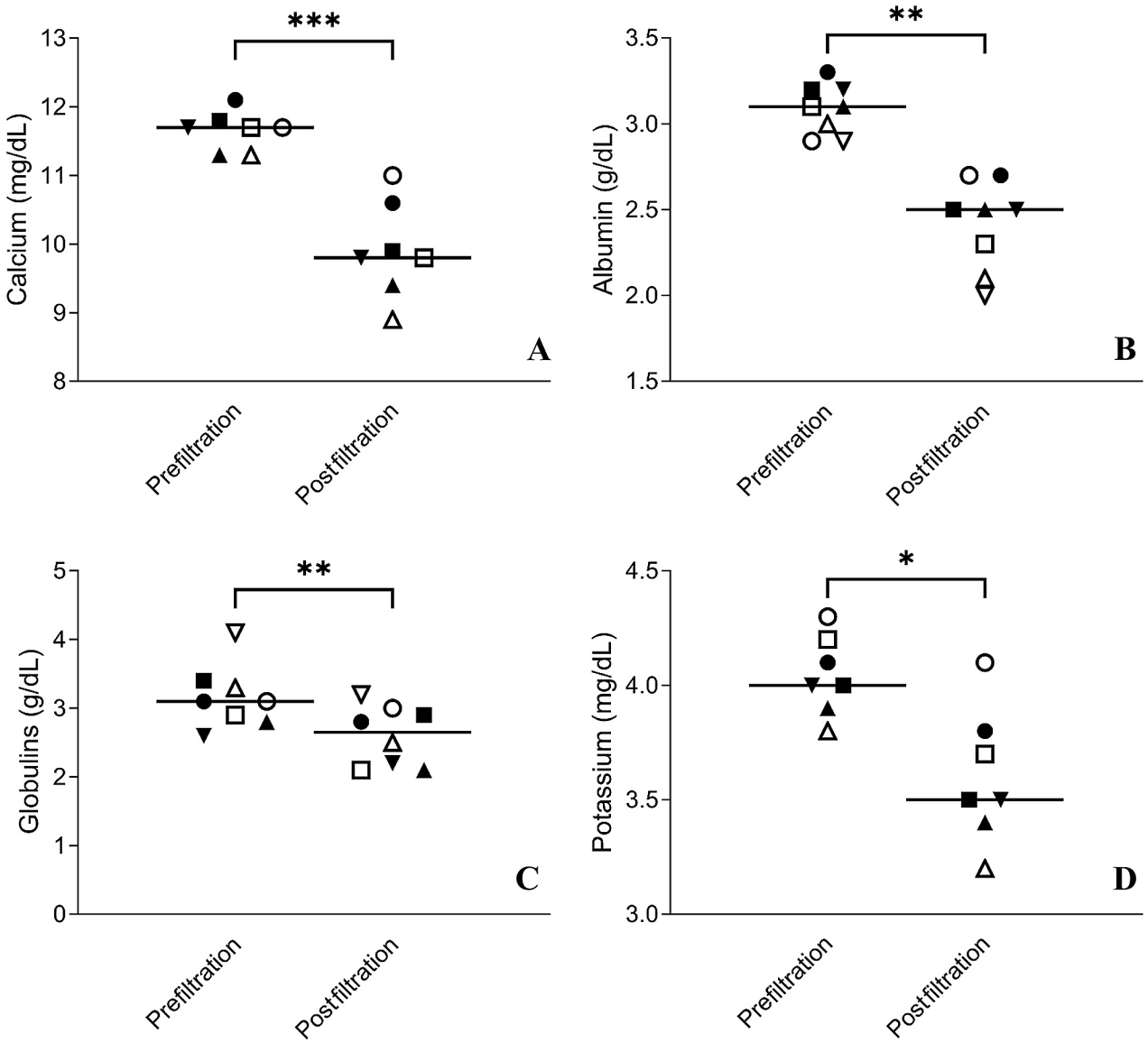

Figure 3—Mean concentrations of calcium (A), albumin (B), globulins (C), and potassium (D), before and after 6 hours ex vivo filtration with a cytokine adsorption device (filter) as described (Figure 1). For each time point, each shape on the graph represents the pre- and postfiltration results for one horse. Student *t* test were used for parametric data and Wilcoxon signed-rank testing was used for nonparametric data *$P < .05$, **$P < .02$, ***$P < .001$.

73%, respectively, in 6-hour postfiltration versus prefiltration plasma samples **(Figure 4)**.

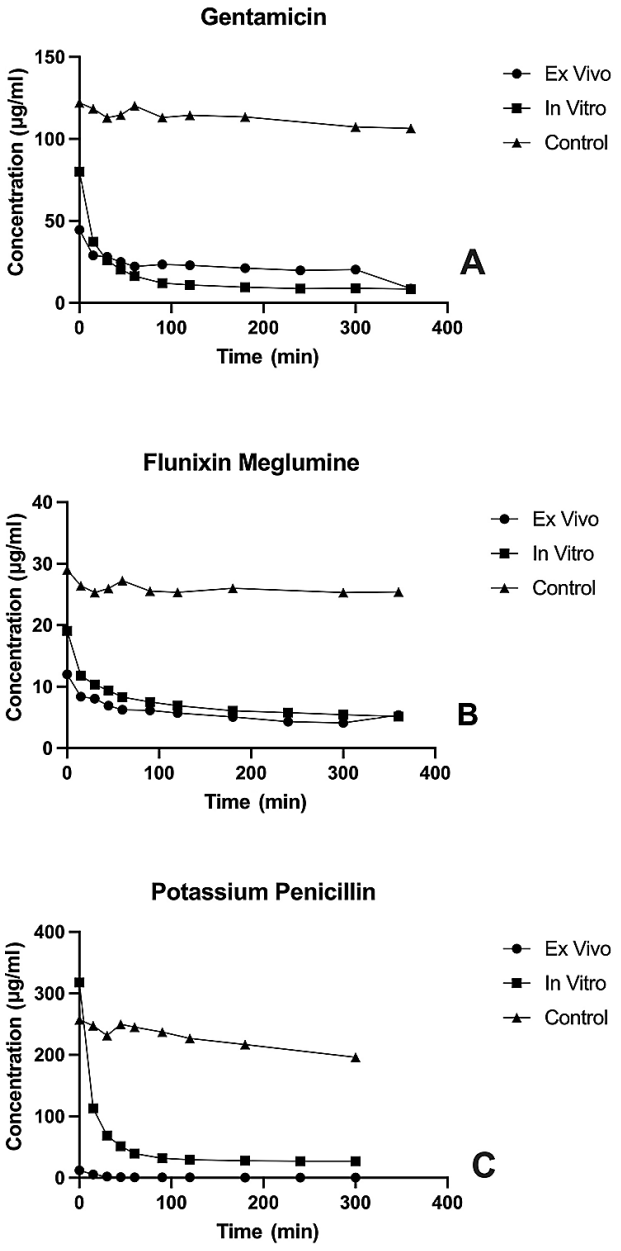

Figure 4—Comparisons of the concentrations of gentamicin (A), flunixin meglumine (B), and potassium penicillin (C) in plasma after filtration with a sham device (control) versus a cytokine adsorption device with either ex vivo or in vivo stimulation as described (Figure 1) for plasma obtained at 0, 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 minutes of filtration a single sample of equine whole blood.

With 6 hours of sham filtration, final gentamicin, potassium penicillin, and flunixin meglumine concentrations were decreased by 13%, 22.78%, and 12.4%, respectively, in 6-hour postfiltration versus prefiltration plasma samples (Figure 4).

Discussion

For this study, we hypothesized that ex vivo filtration of equine plasma with a novel cytokine adsorption device (VetResQ) would significantly remove both LPS-stimulated cytokines, as well as medications commonly used to treat septic patients. This is the first proof-of-concept study to demonstrate the significant removal of equine cytokines following ex vivo filtration of equine plasma with a novel hemadsorption device. The novel hemadsorption cartridge is filled with bio- and hemocompatible polymer beads covered in tiny pores that enable the adsorption of molecules and substances up to 60 kDa. All equine cytokines analyzed in this study were significantly reduced by filtration. The cytokine with the greatest mean reduction was IL-1β, while the cytokine with the lowest mean reduction was TNF-α. Interestingly, the largest and smallest molecular weight cytokines, TNF-α (homotrimer, 52 kDa) and IL-8 (11 kDa), respectively, displayed slightly less mean reduction than IL-1β (24.2 kDa), IL-10 (20.45 kDa), and IL-5 (15 disulfide-linked homodimer, approx 30 kDa), which could suggest that molecules at the upper and lower limits of device adsorption are not eliminated to the same degree as mid size molecules. Another explanation for the differences in reduction of cytokine concentrations is likely due to the concentration present in the blood after stimulation, since the hemadsorption filter quickly removes substances at high concentrations but minimal amounts of substances at low concentrations. With this mechanism, high plasma concentrations of cytokines are cleared more efficiently than lower levels, which helps to restore cytokine balance without the complete elimination of cytokines. This is essential as a balanced cytokine response is essential for optimal host immune function.[21] Finally, the sham-filtered group showed increasing levels of IL-1β over the 6-hour time period, potentially due to incomplete removal of leukocytes. Ongoing production, which contributes to a higher sustained plasma concentration, could have contributed to an overall greater rate of removal for IL-1β.

In this study, the rate of cytokine reduction was highest in the first hour of filtration and slowed thereafter. This could be indicative of the cartridge nearing saturation within the first hour or simply be the result of the closed model system, which did not model ongoing cytokine production as occurs in vivo. As the device relies on a concentration gradient, fewer available cytokines in the circuit should result in a lower rate of elimination. These results are similar to the results noted in human studies[22] where the rate of elimination diminished throughout the 6-hour filtration period as cytokines concentrations decreased in the patient.

While there were statistically significant changes in biochemical parameters, the values obtained in this ex vivo model would not have been considered clinically significant alterations in a patient. Albumin (64 to 68 kDa), potassium, and calcium loss are commonly seen in humans and small animals undergoing both dialysis and hemoperfusion.[15,23] In humans,

albumin loss is linked to the removal of middle-weight molecular toxins that fall in the same 60- to 64-kDa range as albumin and is viewed as an acceptable side effect of toxin removal.[23] In both humans and small animals, the return to normal biochemistry parameters typically occurs within 24 hours after adsorption treatment for potassium and calcium.[24] The analyte loss experienced in our model is most likely due to the closed nature of our system with no access to additional stores of albumin, globulin, potassium, or calcium. Additional studies are needed to determine the potential impact of this treatment on levels of albumin, potassium, and calcium in horses in vivo, as further decreases in these parameters in patients whose values are already low could require replacement therapy.

This study examined 3 medications commonly used in the treatment of equine sepsis: penicillin (low lipophilicity, moderate protein binding), gentamicin (low lipophilicity, low protein binding), and flunixin meglumine (high lipophilicity, high protein binding. The medication elimination rates experienced in this study suggest that medications, regardless of solubility and protein binding capacity, are removed at moderate to high rates. This finding mimics the medication removal findings in humans undergoing extracorporeal therapy.[25] In human medicine, dose adjustment for patients treated with dialysis is part of the normal standard of care.[26] Our preliminary findings suggest that equine patients will also require modified dosing regimens, intervals, or therapeutic drug monitoring when receiving extracorporeal therapy. Our results show that flunixin meglumine and gentamicin were removed at a moderate rate, suggesting that extracorporeal hemadsorption therapy may also serve as a treatment option for equine patients experiencing acute toxicity from nonsteroidal anti-inflammatory or aminoglycoside overdoses. This use of extracorporeal hemadsorption therapy has already been documented in dogs with successful treatment of acute intoxications such as NSAID overdoses and ibuprofen overdoses[27,28] and may hold promise for other drug intoxications.

Regarding study limitations, variable LPS response has been previously reported in horses.[6,29] We also experienced variation in individual animal responses to ex vivo LPS stimulation. This inconsistency led to differences in sample number for the various cytokines (n = 3 to 8) and limited the number of cytokines we could analyze for the effects of filtration. Finally, while these proof-of-concept data are promising, it remains to be determined whether in vivo filtration with a novel hemadsorption cartridge will effectively and significantly adsorb equine cytokines in the face of ongoing production and improve clinical outcomes in septic patients.

In the present study, VetResQ successfully filtered equine cytokines implicated in sepsis with minimal changes seen in biochemical parameters. Ongoing evaluation of cytokine adsorption in healthy equines and equines with LPS-induced endotoxemia will further inform the potential clinical utility of this novel adjunctive sepsis therapy.

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Disclosures

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Supplementary Materials

Supplementary materials are posted online at the journal website: avmajournals.avma.org