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Reduction in Red Blood Cell Lysis by Polymer Intervention During Rodent Liver Normothermic Machine Perfusion

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Background. Normothermic machine perfusion (NMP) has emerged as a promising strategy to improve the preservation and utilization of donor livers, particularly from extended criteria donors and those donated after circulatory death. Despite its advantages, hemolysis remains a major challenge, leading to free hemoglobin release, oxidative stress, and endothelial injury, ultimately affecting graft viability. This study evaluated the use of poloxamer 188 (P188) and Ficoll PM70 as membrane-stabilizing agents to mitigate hemolysis in rodent liver NMP models. We assessed their effects on red blood cell (RBC) stability and grafts during machine perfusion in both a model of donation after brain death and a model of warm ischemia injury. **Methods.** Rat livers were subjected to NMP with RBC-based perfusates containing P188 and/or Ficoll PM70, or an untreated control. Hemolysis was measured using a NanoDrop spectrophotometer to quantify the free hemoglobin concentration. Hemodynamic stability, liver injury markers, and histological analysis were analyzed to determine the overall graft viability. **Results.** Treatment with P188 or Ficoll PM70 significantly reduced circulating free hemoglobin in donation after brain death livers, minimizing hemolysis. Similarly, the combination of P188 and Ficoll PM70 significantly reduced hemolysis in warm ischemia livers and showed preserved liver sinusoidal endothelial cells. However, the reduction in hemolysis did not result in improved parameters measured during *ex vivo* machine perfusion. **Conclusions.** The use of Poloxamer 188 and Ficoll PM70 in NMP shows promise in reducing RBC hemolysis without compromising perfusion hemodynamics. Additionally, both polymers demonstrated improved preservation of liver sinusoidal endothelial cells, as evidenced by histological analysis. However, some findings suggest that P188 may induce the release of proinflammatory cytokines in the perfusate. Overall, these findings highlight the potential of P188 and PM70 in enhancing NMP protocols for liver transplantation, though further studies are necessary to confirm their efficacy and safety before clinical translation.

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INTRODUCTION

Liver transplantation is a life-saving treatment for patients with end-stage liver disease. However, the limited availability of donor organs leaves many patients on the organ waitlist for extended periods. Recent advancements in organ preservation via normothermic machine perfusion (NMP) have emerged as a promising solution to increase organ utilization, particularly for expanded criteria donors such as organs donated after circulatory death (DCD) that were previously deemed unsuitable for transplantation.[1,2] Preservation via NMP maintains organs under near-physiological conditions by ensuring oxygen and nutrient delivery, reducing ischemic injury, and enabling real-time graft evaluations. It allows the acquisition of valuable assessment markers, such as lactate clearance, bile

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production, and hemodynamic stability, making it particularly valuable for enabling transplantation of DCD livers. By enabling graft assessment and improving organ viability, NMP has led to increased utilization of DCD livers, better donor-recipient matching, shorter in-hospital stays, and lower graft retransplantation rates.[3]

To ensure adequate oxygenation during NMP, various oxygen carriers, including red blood cells (RBCs), whole blood, and hemoglobin-based oxygen carriers (HBOC-201), are commonly utilized.[4-6] Although, HBOC-201 offers logistical advantages owing to its ease of storage and increased availability, its clinical significance is hindered by its progressive transition to methemoglobin. Methemoglobin is formed when hemoglobin is oxidized to ferric iron, making it unable to bind oxygen, which significantly compromises oxygen delivery during perfusion. Unlike RBCs, HBOC-201 lacks methemoglobin reductase, a nicotinamide adenine dinucleotide-dependent enzyme necessary for converting methemoglobin to hemoglobin. This characteristic poses strict limits in HBOC-201 in NMP, as prolonged perfusion times increase the risk of functional anemia and tissue ischemia.[7] Additionally, HBOC-201 has been associated with oxidative stress, vasoconstriction, and increased risks of inflammation and cardiac and renal injury in other clinical applications.[6-9] Although these factors currently pose challenges for their use in ex vivo perfusion, ongoing advancements in oxygen-carrier technology may help mitigate these limitations and improve their applicability in the future.

Alternatively, blood-based oxygen carriers, such as packed RBCs and whole blood, replicate physiological oxygen transport more closely than synthetic alternatives such as HBOC-201. However, shear stress from the perfusion apparatus (ie, tubing and pump) leads to RBC rupture, making hemolysis during NMP a significant issue. RBCs lysis is known to release free hemoglobin, a potent proinflammatory molecule that increases endothelial permeability and activates inflammatory signaling pathways.[10] Elevated levels of inflammatory signals have been associated with poor clinical outcomes in other applications of extracorporeal circulation similar to NMP, including increased mortality rates in extracorporeal membrane oxygen support patients[11] and increased development of acute kidney injury during cardiopulmonary bypass.[12] Despite significant efforts to optimize NMP circuits, hemolysis remains an unavoidable challenge, irrespective of the pump type (ie, roller pump, centrifugal, or pulsatile).[13-17]

Therefore, in addition to pump mechanical optimization, membrane-stabilizing agents have been explored as strategies to mitigate RBCs lysis during extracorporeal circulation. Various compounds, such as pentoxifylline, polyethylene glycol, melatonin, and simvastatin, have been shown to have protective effects against shear-induced RBCs damage in cardiopulmonary bypass models.[18-20] These agents are thought to reinforce membrane stability, allowing RBCs to better withstand nonphysiological shear forces. Among the various agents explored herein, Poloxamer 188 (P188), a triblock copolymer, has been extensively studied for its membrane-stabilizing properties, which help protect RBCs against shear-induced hemolysis.[21] Studies suggest that P188 integrates into the cell membrane, reducing membrane permeability, stabilizing osmotic balance, and preventing rupture in high-stress environments.[22] Additionally, P188 has been shown to reduce ischemia–reperfusion injury (IRI) in both lungs[23] and cardiomyocytes,[24] and presents a potential pharmacological intervention capable of ameliorating organ damage caused by IRI.[25] Similarly, Ficoll PM70, a polysaccharide-derived compound, has been widely used in blood separation protocols because of its ability to prevent RBCs aggregation and to preserve cellular function during prolonged storage.[26,27] Therefore, both compounds have the potential to maintain RBCs integrity and prevent significant structural damage during NMP. The implementation of membrane-stabilizing agents such as P188 and Ficoll PM70, along with optimized perfusion techniques, provides a potential strategy to reduce RBCs injury and improve the effectiveness of machine perfusion preservation.

This study aimed to investigate the effects of P188 and Ficoll PM70, and their ability to reduce hemolysis in rodent liver NMP. The effects of these additives on NMP were tested via incorporation into the perfusate and assayed in both a model of donation after brain death (DBD) and a model of warm ischemia injury. The addition of these additives to DBD livers allowed us to establish baseline data on the positive and negative effects of P188 and Ficoll PM70 without the confounding effects of ischemic injury. As no adverse effects from polymer treatment were observed in nonischemic livers, the additives were subsequently tested in warm ischemia organs, a relevant model given their higher susceptibility to IRI and increased risk of graft dysfunction. Using a 1-h warm ischemia threshold allows us to evaluate the protective potential of polymer additives under conditions of heightened vulnerability, whereas previous studies have shown that livers subjected to this duration can be recovered and rendered transplantable with ex vivo perfusion,[28] reinforcing the relevance of this model for assessing strategies to preserve expanded criteria grafts. Outcomes such as perfusion biochemistry, metabolic recovery, and liver injury assessment were analyzed to determine the overall benefits of these interventions in preserving RBC cellular integrity and improving organ viability.

MATERIALS AND METHODS

Ethical Statement

All research complied with ethical standards and regulations approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital (Boston, MA) and Shriners Children's Hospital (Boston, MA).

Liver Procurement and Storage

Male and female Lewis rats (200–250 g, Charles River Laboratories) were anesthetized with 3%–5% isoflurane in 1 L/min of oxygen. The animal was placed in the supine position, and the abdominal area was shaved for hepatectomy. Once the depth of anesthesia was confirmed by the absence of reflex via the toe pinch, a transverse midline incision was made to expose the hepatic vessels and bile duct. The bile duct was cannulated with a 22G polyethylene tube, and the hepatic artery and associated gastric and splenic branches were ligated with silk sutures. After cannulation and ligation, 30 U of sodium heparin was injected into the suprahepatic inferior vena cava to prevent clotting. The portal vein was cannulated with a 16G catheter and flushed with 60 mL of heparinized saline, and the liver was excised, as shown in Figure 1A. Livers subjected to warm ischemia were incubated

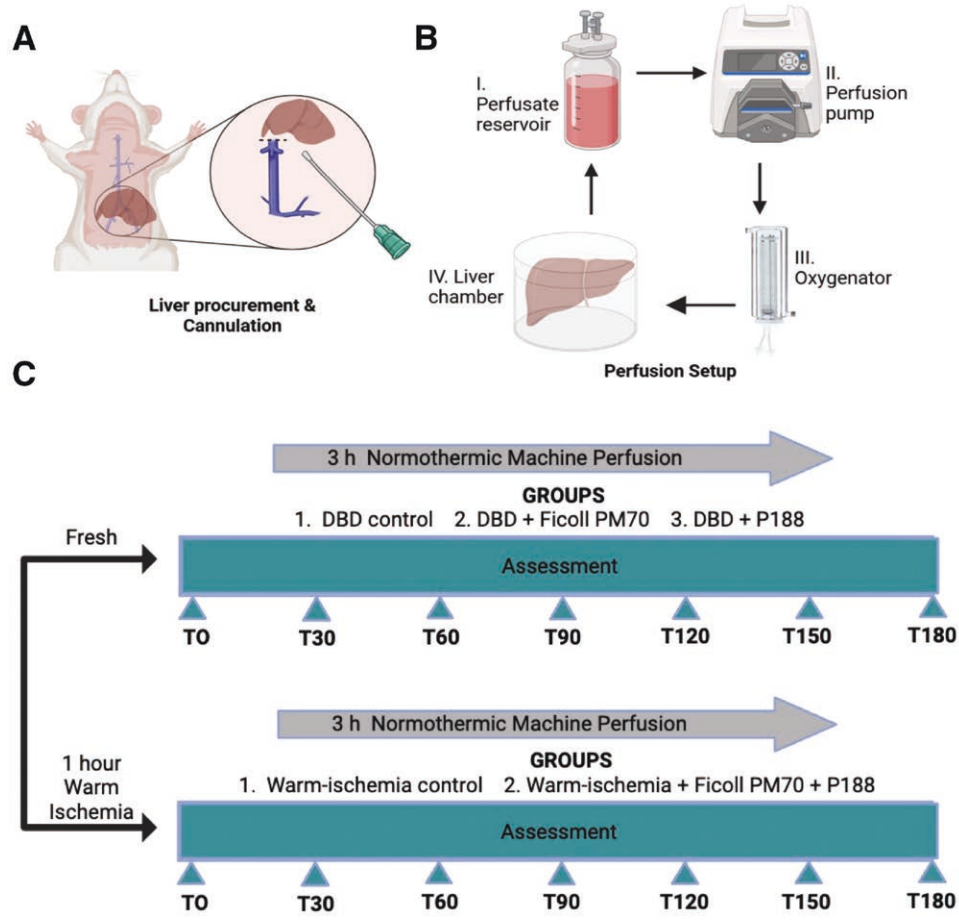


FIGURE 1. Schematic representation of the experimental setup and study design for liver perfusion assessment. A, Schematic of rodent liver procurement and cannulation. B, Diagram of the ex vivo liver perfusion system. The system consists of (I) a perfusate reservoir, (II) a perfusion pump, (III) an oxygenator, and (IV) a liver chamber for housing the organ during perfusion. Perfusate circulates through the system in a closed-loop cycle. C, Experimental groups. Livers were subjected to either DBD (nonischemic) or warm ischemia conditions, followed by 3 h of NMP. Groups included controls and treatments with Ficoll PM70 or P188 (n = 3-4 for all groups). DBD, donation after brain death; NMP, normothermic machine perfusion; P188, Poloxamer 188. Image created on Biorender.com.

for 1 h in a temperature-controlled chamber filled with saline bath at 37 °C.[28] DBD livers were immediately re-perfused in the perfusion system.

RBC Collection

Whole blood was collected from healthy donor rats via cardiac puncture. Donor animals were obtained from the Charles River Laboratories and were age-matched to the liver donors. They were not weight-matched, as larger animals were required to obtain sufficient blood volume. Although animals used in the study included both males and females, donor blood was not sex-matched to the grafts. Donor rats were anesthetized as described earlier and placed in the supine position. The area around the lower sternum was shaved and the skin was disinfected. An incision was made to expose the heart, and a 23G needle attached to a 10-mL syringe containing 189 U of sodium heparin was inserted into the left ventricle at a 45-degree angle. Blood was drawn from the left ventricle, and the needle was slowly retracted to avoid heart collapse. This procedure was performed until no more blood could be drawn. A total of 6–10 mL of blood was collected from each donor.

Whole blood was centrifuged at 2200 rpm for 10 min at 20 °C. The resulting layers of the plasma and buffy coat were removed. The remaining RBCs were resuspended in perfusate, and the process was repeated 2 more times. Isolated RBCs were transferred to sterile storage tubes and preserved at 4 °C for a maximum of 5 d.

Normothermic Machine Perfusion

This study investigated the effects of Poloxamer 188 (P188) and/or Ficoll PM70 on hemolysis in a liver model of, both, donation after brain death (nonischemic livers) and after warm ischemia injury. Nonischemic livers perfused without additives (control), with Ficoll PM70 or P188 (n = 4 each), whereas warm ischemic livers were perfused with or without (WI control) a combination of Ficoll PM70 and P188 (n = 3, each) (Figure 1C). The livers were perfused through the portal vein at 37 °C in a double-jacketed organ chamber for 3 h with Williams' E base medium supplemented with 20 mL of packed RBCs (hematocrit 15%–20%), 100 U/mL insulin, 1000 U/mL heparin, 24 mg/L dexamethasone (Sigma-Aldrich), 2 mM Gluatamax (ThermoFisher Scientific, Waltham, MA), 10 mg/mL Bovine Serum Albumin (ThermoFischer Scientific), and 1% penicillin-streptomycin (ThermoFischer Scientific) for a total volume

of 100 mL. Hematocrit levels were analyzed using a Sysmex XP-300TM Automated Hematology Analyzer (Sysmex, Kobe, Japan). For grafts perfused with biological polymers, P188 (Sigma-Aldrich) and/or Ficoll PM70 (Cytiva) were added to the perfusate at concentrations within the effective and previously established safe ranges, as demonstrated by prior studies (1% and 4%, respectively).[27,29] These concentrations were chosen to balance membrane protection, inhibition of RBC aggregation, and optimal perfusate viscosity for ex vivo liver perfusion.

The perfusate was oxygenated with 95% O_2 and 5% CO_2 using a porous silicone coil oxygenator (Radnoti LLC, Covina, CA) and circulated through a bubble trap (Radnoti LLC) using a roller pump (Masterflex L/S, Vernon Hills, IL, Figure 1B). After procurement or warm ischemia, the liver was weighed and connected to a perfusion system at a flow rate of 6 mL/min. The flow rates were increased until either a maximum pressure of 6 mmHg or a maximum flow of 30 mL/min was reached. Details of the technical aspects of the machine perfusion protocol can be found elsewhere.[30]

Perfusion Data Acquisition and Processing

Oxygen and lactate measurements were performed every 30 min using a blood gas analyzer (Siemens Medical Solutions, Malvern, PA). Hepatic injury markers (alanine transaminase [ALT] and aspartate transaminase [AST]) were measured hourly using a Piccolo Xpress Chemistry Analyzer (Abbott, IL). The liver graft was weighed before and after perfusion to determine weight change, a proxy for edema and dehydration. The pressures and flow rates were recorded, and portal vein resistance was determined by dividing pressure by flow rate and correcting for the weight of the liver after procurement. Perfusate samples were collected, centrifuged at 4000*g* for 10 min, and the supernatant was stored at –20 °C for free hemoglobin assessment.

The liver tissues were fixed in 10% formaldehyde and transferred to 70% ethanol. Fixed tissues were processed for terminal deoxynucleotidyl transferase dUTP Nick end labeling (TUNEL), and hematoxylin and eosin (H&E) staining was performed by the Massachusetts General Hospital Histology Molecular Pathology Core (Charlestown, MA).

Characterization of Free Heme Release

The levels of free hemoglobin were quantified using a NanoDrop One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, ND-ONE-W), employing a standardized hemoglobin custom method provided by the manufacturer. We focused on determining oxyhemoglobin concentrations, utilizing an analysis wavelength of 414 nm, an extinction coefficient of 524 280 M^{-1} cm^{-1}, and a molecular weight of 64.5 kDa. The accuracy and reliability of the hemoglobin custom method were validated following the protocol outlined in the manufacturer's manual (ThermoFisher Scientific, Waltham, ND-ONE-W). Before perfusion, baseline hemolysis values were obtained, and all subsequent time-point samples were normalized to this baseline to account for any preexisting or storage-related RBC lysis. After validation, 2 µL of supernatant from the centrifuged perfusate samples was loaded onto a clean pedestal for absorbance measurements.

Perfusate-based Proinflammatory Markers

Proinflammatory markers were measured using a bead-based immunoassay (Luminex, Austin, TX) by Eve Technologies Corp. (Alberta, CA). Briefly, the supernatants of the outflow perfusate samples at the start of perfusion (T0) and 3 h (T180) were collected, frozen, and shipped on ice for processing. Analysis was performed using a Luminex 200 system according to the manufacturer's instructions (Millipore Sigma, Burlington, MA). Intracellular adhesion molecule (ICAM-1) was measured via a bead-based immunoassay following the manufacturer's instructions using a Luminex XMAP Intelliflex system (PPX-07; ThermoFisher Scientific). Only samples above the limit of detection were included in the inflammatory marker analysis, resulting in all groups in the ICAM panel and DBD+P188 in the interleukin (IL)-2 panel having lower n-number compared with the rest of the results.

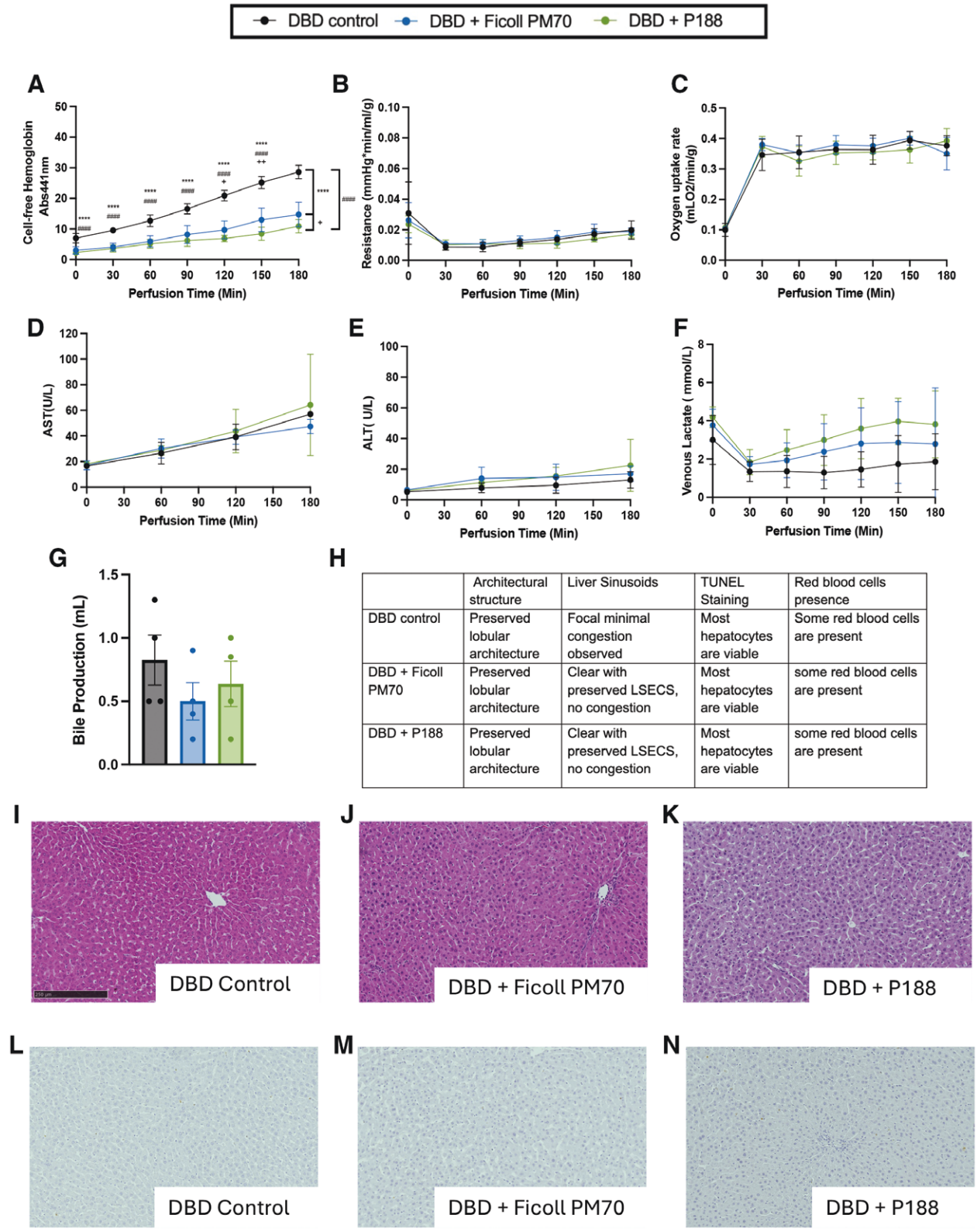
Statistical Analysis

All statistical analyses were performed using Prism 10 (GraphPad Software Inc., La Jolla, CA). Outliers were first identified using the ROUT method with Q set to 1%. Variables that varied over time were analyzed using a mixed-effects model, with time and group as fixed effects. Variables that were only studied at the final time point were analyzed using one-way analysis ANOVA to study the effect of groups. Multiple comparisons were performed using Tukey's test.

RESULTS

P188 and Ficoll PM70 Mitigate Hemolysis Without Compromising Hemodynamics in a DBD Liver Model

The absorbance at 414 nm was used to quantify cell-free hemoglobin in the spun-down perfusate, capturing the contributions from oxyhemoglobin, deoxyhemoglobin, methemoglobin, and free heme species. High levels of cell-free hemoglobin were observed in the DBD control livers, as indicated by the absorbance values in the perfusate over the 3-h perfusion period (Figure 2A). This effect was significantly mitigated by the addition of Ficoll PM70 or P188 to the perfusate, as shown by a significant reduction in hemolysis ($P < 0.05$). Perfusion parameters, such as vascular resistance, oxygen uptake rate, venous lactate levels, liver transaminases (AST and ALT), and bile production, are critical indicators of liver graft health during ex vivo perfusion. These metrics collectively provide insights into graft viability, metabolic activity, tissue oxygenation, and hepatocellular injury.[31] No statistical difference was observed in the vascular resistance between the DBD control and treated groups ($P > 0.05$, Figure 2B). Similarly, no statistical differences were observed in the oxygen uptake rate, which followed a similar trend across all groups, stabilized at 30 min, and remained constant during the 3-h perfusion duration (Figure 2C). Liver transaminases (AST and ALT), a proxy for liver damage, were not significantly different between the control and treated groups, with both ALT and AST levels increasing over time (Figure 2D and E). Interestingly, DBD control livers produced the lowest lactate levels over time when compared with DBD treatment with P188 or Ficoll PM70, although the difference was not statistically significant (Figure 2F). The total bile production measured at the end of perfusion showed no significant differences among the 3 groups (Figure 2G). Blinded pathological analysis of H&E-stained sections (Figure 2H) revealed pronounced vascular congestion in the DBD non-polymer-treated livers (Figure 2I). However, polymer-treated livers showed clear sinusoids with no congestion within liver



	Architectural structure	Liver Sinusoids	TUNEL Staining	Red blood cells presence
DBD control	Preserved lobular architecture	Focal minimal congestion observed	Most hepatocytes are viable	Some red blood cells are present
DBD + Ficoll PM70	Preserved lobular architecture	Clear with preserved LSECS, no congestion	Most hepatocytes are viable	some red blood cells are present
DBD + P188	Preserved lobular architecture	Clear with preserved LSECS, no congestion	Most hepatocytes are viable	some red blood cells are present

FIGURE 2. Evaluation of hemolysis, histological changes, cell death, and perfusion parameters DBD livers perfused with Ficoll PM70 or P188. A, Quantification of hemoglobin in perfusate of DBD livers with and without polymer intervention. B, Vascular resistance (mm Hg/mL/min/g) during perfusion for DBD groups: DBD control, nonischemic + Ficoll PM70, and nonischemic + P188. C, Oxygen uptake rate (mL O_2/min/g) over time for the DBD liver groups. D and E, Levels of AST (U/L) and (ALT, U/L) measured during perfusion. F, Venous lactate levels (mmol/L) during perfusion in the nonischemic liver groups. G, Bile production (mL) for DBD liver groups over the perfusion duration. H, Summary table of histological findings across groups. I–K, Representative H&E images for the DBD control, DBD + Ficoll PM70 and DBD + P188, respectively. L–N Representative TUNEL images highlighting cellular apoptosis in the DBD liver groups. Hemolysis levels at each time point were normalized to baseline measurements taken prior to perfusion .Statistical significance is indicated as follows: $P < 0.05$ (+), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (**** or ####). Scale bar = 250 μm. ALT, alanine transaminase; AST, aspartate transaminase; DBD, donation after brain death; H&E, hematoxylin and eosin; P188, Poloxamer 188; TUNEL, terminal deoxynucleotidyl transferase dUTP Nick end labeling. Images created with Biorender.

sinusoid endothelial cells (Figure 2J and K). No hepatic death was observed in either group, despite the presence of vessel blockage in nontreated livers, as determined by TUNEL staining (Figure 2L–N).

Analysis of Cytokine Expression and Immune Modulation in DBD Livers Treated With Ficoll 70 and P188

Adhesion molecule-1 (ICAM-1), a marker of endothelial activation, was elevated in the P188 group compared with both the DBD control and Ficoll PM70 groups (Figure 3A), although these differences were not statistically significant. Notably, Ficoll PM70 treatment resulted in the lowest ICAM-1 level. Proinflammatory cytokine IL-6 was significantly elevated in the P188 group compared with both the control ($P = 0.0015$) and Ficoll PM70 groups ($P = 0.0011$), indicating a pronounced inflammatory response associated with P188 treatment (Figure 3B). In contrast, IL-1β levels remained comparable across the groups, with no significant differences observed (Figure 3C). IL-2 levels were highest in the DBD control group and slightly lower in both treatment groups, but the difference was not statistically significant (Figure 3D).

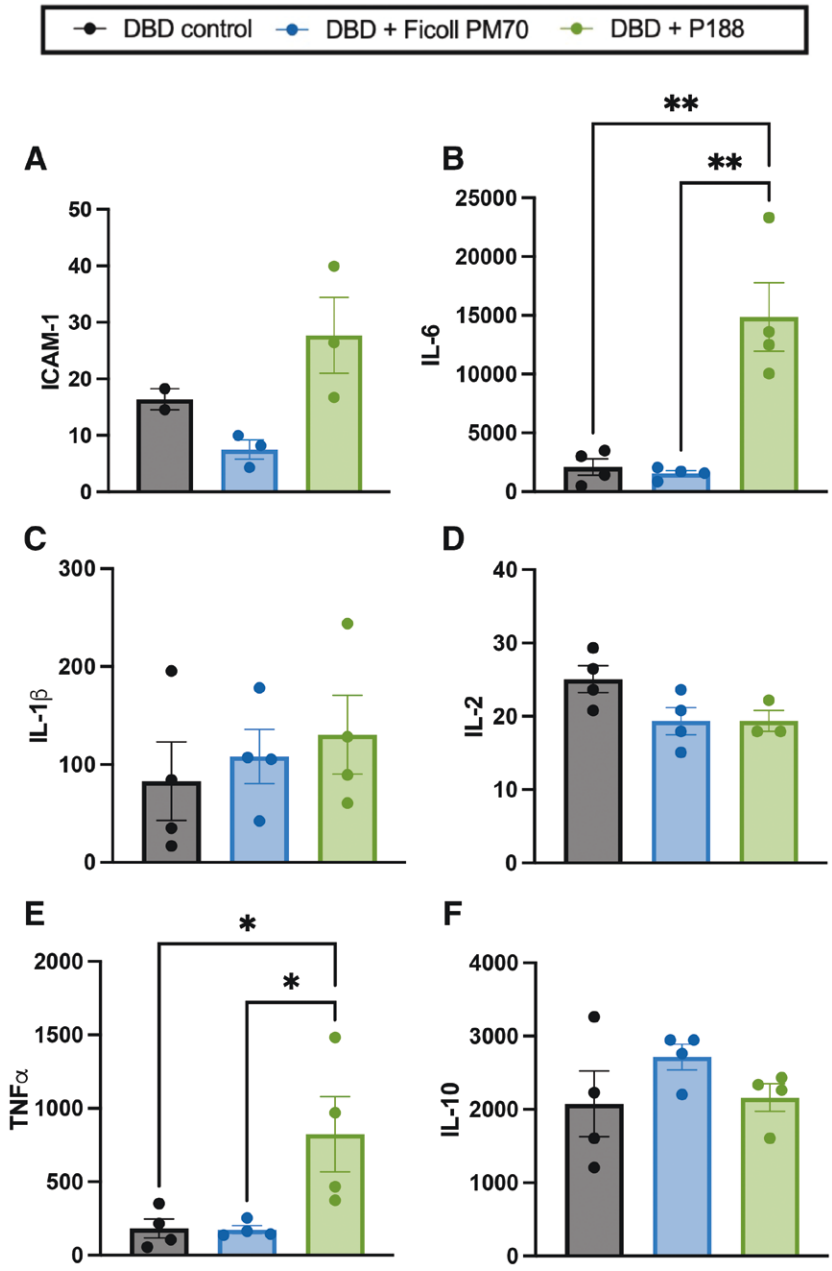


FIGURE 3. Pro- and anti-inflammatory cytokine profiles of DBD livers with Ficoll PM70 or Poloxamer 188 interventions during NMP. A, Levels of in-perfusate proinflammatory molecule ICAM-1. B–D, Proinflammatory cytokines IL-6, IL-1β, and IL-2. E, Proinflammatory chemical messenger TNF-α. F, Anti-inflammatory cytokine IL-10 (fold increase: T180/T0). Statistical significance is indicated as follows: $P < 0.05$ (*), $P < 0.01$ (**). Samples with values below the limit of detection were excluded from data set. DBD, donation after brain death; H&E, hematoxylin and eosin; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; NMP, normothermic machine perfusion; P188, Poloxamer 188; TNF, tumor necrosis factor. Images created with Biorender.

Secretion of tumor necrosis factor-alpha (TNF-α) was significantly higher in the DBD + P188 group than in the DBD control ($P = 0.0386$) and DBD + Ficoll PM70 ($P = 0.0366$) groups (Figure 3E). The anti-inflammatory cytokine IL-10 showed comparable levels across all 3 groups, suggesting that neither P188 nor Ficoll PM70 significantly influenced IL-10 secretion under non-ischemic conditions (Figure 3F).

P188 and Ficoll PM70 Reduce Hemolysis in Warm Ischemic Livers During NMP

Hemoglobin-based oxygen carriers have been shown to be advantageous in mitigating IRI, especially at normothermic temperature.[32] However, it is also known to contribute significantly to the level of freely circulating hemoglobin because of hemolysis.

Warm ischemic livers treated with both Ficoll PM70 and P188 showed significantly lower levels of free hemoglobin in the perfusate than in untreated livers ($P < 0.05$), as indicated by the absorbance values in Figure 4A. No statistical differences were observed in vascular resistance (Figure 4B) between the warm ischemic groups. Interestingly, polymer-treated warm ischemic livers experienced lower oxygen uptake rates than untreated livers during the 3-h perfusion duration, although the difference was not statistically significant ($P > 0.05$, Figure 4C). Similar results were observed for AST and ALT levels, which were not statistically different and showed an upward trend (Figure 4D and E). Both polymer-treated and non-polymer-treated warm ischemic livers showed no statistically significant difference in lactate levels over the 3-h perfusion period (Figure 4F). No bile production was observed in either group (Figure 4G). Blinded pathological analysis of the warm ischemic model revealed focal disruptions in the liver lobule architecture in both groups (Figure 4H), likely because of ischemic injury. However, polymer-treated livers exhibited improved sinusoidal endothelial cell integrity and reduced vascular congestion compared with nontreated livers (Figure 4I and J). TUNEL staining indicated that liver hepatocytes remained viable in both groups after perfusion (Figure 4K and L).

DISCUSSION

This study investigated the effects of P188 and Ficoll PM70 on liver preservation during NMP, focusing on their ability to mitigate hemolysis, preserve sinusoidal endothelial integrity, and modulate immune responses in DBD and warm ischemic livers. Given that hemolysis remains a major challenge in NMP, leading to oxidative stress and endothelial dysfunction, incorporating membrane-stabilizing agents such as P188 and Ficoll PM70 may offer a promising strategy for improving organ preservation and transplantation outcomes.

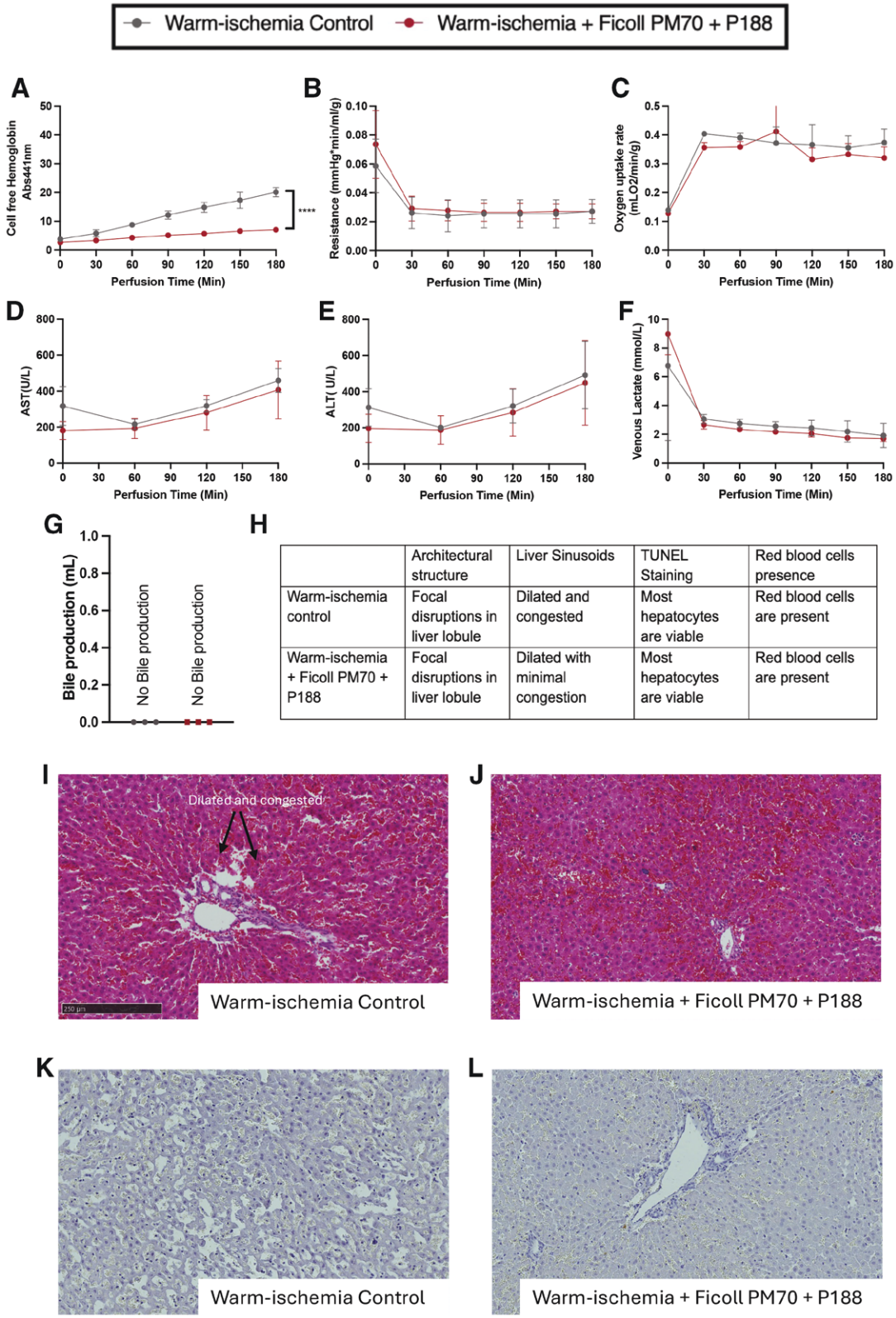
The presence of circulating free hemoglobin in the perfusate was significantly reduced following treatment with either Ficoll PM70 or P188 in the DBD group (Figure 2A). This reduction in free hemoglobin suggests that these polymers help maintain RBC integrity by preventing excessive hemolysis, thereby improving perfusate quality. As hemolysis can exacerbate IRI through the release of free heme, which can drive oxidative stress and endothelial dysfunction, reducing free hemoglobin levels could be important for graft preservation.

Histological analysis further demonstrated improved liver sinusoidal endothelial cell (LSECs) integrity and reduced congestion in the polymer-treated groups, reinforcing the role of Ficoll PM70 and P188 in vascular protection during NMP. LSECs have been shown to play a crucial role in maintaining hepatic microcirculation in response to shear stress and in regulating blood flow through the release of vasodilators such as nitric oxide, carbon monoxide (CO), and prostacyclin.[33] The observed reduction in congestion may be attributed to the ability of P188 to reduce viscosity,[34] thereby promoting better sinusoidal blood flow within the liver vessels. Similarly, Ficoll PM70 likely contributes to preserving microcirculatory integrity owing to its oncotic properties, which help to maintain endothelial integrity that could otherwise impact LSECs function.

Despite improvements in hemolysis, perfusion parameters did not differ significantly between the treated and untreated groups. This is likely because of the inherent limitations of the model and assessment method, including short (3-h) perfusion duration and the absence of transplantation to assess long-term graft function. Notably, venous lactate levels were lowest in the untreated group, although the difference was not statistically significant. The increase in lactate production may reflect unintended alterations in liver metabolism caused by P188 and Ficoll PM70 in the hepatic microcirculation. Although these findings do not indicate hemodynamic benefits, the overall stability of the perfusion parameters suggests that the addition of these polymers does not compromise liver function during NMP.

In this study, cytokine analysis revealed complex immune responses following polymer treatment. In DBD livers, TNF-α and IL-6 levels were significantly elevated in the P188-treated group, indicating possible proinflammatory response. P188 has been shown to integrate into disrupted bilayer, binding and exposing the hydrophobic domains, promoting membrane sealing and may be removed from the cell once it has repaired its injuries.[35] Although these membrane-stabilizing effects can be cytoprotective, it has also been shown to alter lipid microdomains and adhesion receptors in endothelial and myeloid cells. In vitro treatment of P188 showed restored endothelial cell membranes in traumatic brain injury model while influencing downstream signaling and oxidative pathways, suggesting that membrane repair can simultaneously trigger protective intracellular signaling,[36] which may also contribute to the observed increase in ICAM, IL-6, and TNFα expression. Other studies have reported similar cytokine responses, highlighting the dual and context-dependent roles of IL-6 and TNF-α in the injured liver. For example, a study demonstrated that IL-6 protects against injury, whereby IL-6 deficient mice exhibited more extensive hepatocellular necrosis and poorer survival, and administration of recombinant IL-6 reduced injury and promoted hepatocyte proliferation.[37] Additional work has demonstrated that IL-6 can support regeneration and metabolic recovery through classic signaling pathways,[38] and transient IL-6 elevation during hepatic ischemia has been associated with improved hepatocyte integrity, reduced oxidative stress, and increased expression of antioxidant enzymes.[39] Because this study observed improved LSEC and sinusoidal morphology in histological analysis, this cytokine elevation, such as observed in P188-treated livers, may reflect an adaptive response to ischemic injury, although further investigation is required.

Treatment with a combination of P188 and Ficoll PM70 in warm ischemic livers resulted in a significant reduction in circulating free hemoglobin compared with the untreated group, which showed an increase in free hemoglobin over the



	Architectural structure	Liver Sinusoids	TUNEL Staining	Red blood cells presence
Warm-ischemia control	Focal disruptions in liver lobule	Dilated and congested	Most hepatocytes are viable	Red blood cells are present
Warm-ischemia + Ficoll PM70 + P188	Focal disruptions in liver lobule	Dilated with minimal congestion	Most hepatocytes are viable	Red blood cells are present

FIGURE 4. Evaluation of hemolysis, histological changes, and cell death and perfusion parameters in warm ischemia liver perfusion groups treated with combination of Ficoll 70 and P188. A, Quantification of red blood cell hemolysis in perfusate of warm ischemia livers with and without polymer intervention. B, Vascular resistance (mm Hg/mL/min/g) for warm ischemia group: control and Ficoll 70 and P188 (Ficoll PM70 + F68) treated livers. C, Oxygen uptake rate (mL O_2/min/g) for warm ischemia liver groups. D and E, Levels of AST and ALT (U/L) over time in warm ischemia liver groups. F, Venous lactate levels (mmol/L) during perfusion for warm ischemia liver groups. G, Bile production (μL) for warm ischemia liver groups. H, Summary table of histological findings for warm ischemia groups. I and J, Representative H&E images for warm ischemia liver groups with and without polymer intervention. K and L, Representative TUNEL images illustrating apoptotic cell death in warm ischemia liver groups. Hemolysis levels at each time point were normalized to baseline measurements taken before perfusion .Statistical significance is indicated as follows: $P < 0.0001$ (****). Scale bar = 250 μm. ALT, alanine transaminase; AST, aspartate transaminase; DBD, donation after brain death; H&E, hematoxylin and eosin; P188, Poloxamer 188; TUNEL, terminal deoxynucleotidyl transferase dUTP Nick end labeling. Images created with Biorender.

duration of perfusion, indicating ongoing hemolysis and damage to RBCs. Histological analysis showed that LSECs treated with both polymers experienced minimal congestion compared with the untreated group. LSECs have been shown to be particularly vulnerable to reperfusion injury, which negatively impacts microcirculation.[32,36] The addition of polymers may mitigate this effect and alleviate the congestion upon reperfusion. Additionally, oxygen consumption was lower in polymer-treated livers, although not statistically significant. Although it is possible this is because of the combined effects of Ficoll PM70 and P188, further evidence is required to understand if these perfusate modifications have any direct role in metabolic alterations.

In contrast to the DBD livers, treatment with the polymer combination in warm ischemia livers appeared to reduce inflammatory responses, although the differences were not statistically significant. Many cytokine levels in the warm ischemia samples were below the detection limit of the assay, which may reflect a lower level of inflammation than sensitivity. This is consistent with previous studies suggesting that prolonged ischemia may have less active proinflammatory signaling[37-39] compared with DBD organs, likely because of differing inflammatory dynamics, nature of injury, and immune activation between the 2 models.

The findings of this study highlight the potential of Ficoll PM70 and P188 as additives in NMP for liver transplantation. By mitigating hemolysis, preserving histological integrity, and modulating immune responses, these polymers may address the challenges in NMP preservation. Although perfusion parameters remain unchanged, the impact on metabolic function, inflammatory modulation, and the direct effects on LSECs requires further investigation. Future studies should investigate the mechanistic effects of elevated TNF-α and IL-6 levels in P188-treated livers, optimize polymer dosages, explore interactions with other perfusion strategies, and assess long-term effects, including potential clinical relevance for LSEC function.

LIMITATIONS

This study has several limitations. Because of sensitivity in Luminex assay, not all data points were usable across all experimental groups, particularly for cytokine and endothelial marker analyses, as several measurements were below the limit of detection. The relatively small sample size (3–4 per group) may also limit statistical power and the generalizability of the findings. Our model of warm ischemic injury while previously validated in our laboratory as transplantable after 1 h of ischemia, may not fully reflect clinical DCD protocols. Although the anti-inflammatory properties of P188 have been widely reported across multiple injury models, including myocardial infarction, lung injury, microglia activation and IRI,[24,29,36] the findings of this study suggest a tissue-specific response in the liver, emphasizing the need for future studies that include additional endothelial biomarkers to better assess graft function during preservation. Proinflammatory markers such as IL-6 and TNF-α have diverse, context-dependent roles, and in some cases can exert protective effects, therefore the cytokine elevations observed in this study may represent a combination of protective and injurious signaling. In addition, donor packed RBCs were not litter-matched to the liver grafts. However, all animals were sourced from Charles River Laboratories, providing a consistent genetic background, and were age- and weight-matched to reduce biological variability. Additionally, both male and female animals were included following standard practice. Although this approach does not entirely eliminate the possibility of subtle immunological differences between grafts and donor blood, the use of standardized animals minimizes this risk as a confounding factor. Finally, although both additives have shown safety in other organ systems, further preclinical studies will be needed to confirm their safety and efficacy in liver perfusion and eventual clinical use. Despite these limitations, the observed reduction in hemolysis may represent a benefit, even in the absence of changes in liver function markers, although additional studies are required to assess longer-term and clinical relevance.

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