

Liver Preservation With Machine Perfusion and a Newly Developed Cell-Free Oxygen Carrier Solution Under Subnormothermic Conditions

P. Fontes^{1,2,3,4}, R. Lopez^{1,2}, A. van der Plaats⁵,
Y. Vodovotz⁴, M. Minervini⁶, V. Scott⁷,
K. Soltys^{1,2}, S. Shiva⁸, S. Paranjpe⁹,
D. Sadowsky⁴, D. Barclay⁴, R. Zamora⁴,
D. Stolz⁹, A. Demetris^{6,10}, G. Michalopoulos⁶
and J. W. Marsh^{1,2}

¹Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA

²Department of Surgery, Thomas E. Starzl Transplantation Institute, Pittsburgh, PA

³McGowan Institute of Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA

⁴Department of Surgery, University of Pittsburgh, Pittsburgh, PA

⁵Organ Assist, Groningen, the Netherlands

⁶Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA

⁷Department of Anesthesia, University of Pittsburgh, Pittsburgh, PA

⁸Vascular Medicine Institute, Department of Cardiothoracic Surgery, University of Pittsburgh, Pittsburgh, PA

⁹Department of Pathology, University of Pittsburgh, Pittsburgh, PA

¹⁰Thomas E. Starzl Transplantation Institute, Pittsburgh, PA

* Corresponding author: Paulo Fontes, fontesp@upmc.edu

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We describe a new preservation modality combining machine perfusion (MP) at subnormothermic conditions (21°C) with a new hemoglobin-based oxygen carrier (HBOC) solution. MP (n = 6) was compared to cold static preservation (CSP; n = 6) in porcine orthotopic liver transplants after 9 h of cold ischemia and 5-day follow-up. Recipients' peripheral blood, serial liver biopsies, preservation solutions and bile specimens were collected before, during and after liver preservation. Clinical laboratorial and histological analyses were performed in addition to mitochondrial functional assays, transcriptomic, metabolomic and inflamma-

tory mediator analyses. Compared with CSP, MP animals had: (1) significantly higher survival (100% vs. 33%; p < 0.05); (2) superior graft function (p < 0.05); (3) eight times higher hepatic O₂ delivery than O₂ consumption (0.78 mL O₂/g/h vs. 0.096 mL O₂/g/h) during MP; and (4) significantly greater bile production (MP = 378.5 ± 179.7; CS = 151.6 ± 116.85). MP down-regulated interferon (IFN)-α and IFN-γ in liver tissue. MP allografts cleared lactate, produced urea, sustained gluconeogenesis and produced hydrophilic bile after reperfusion. Enhanced oxygenation under subnormothermic conditions triggers regenerative and cell protective responses resulting in improved allograft function. MP at 21°C with the HBOC solution significantly improves liver preservation compared to CSP.

Abbreviations: BA, bile acid; BCAA, branched-chain amino acid; CSP, cold static preservation; DCDs, donors after cardiac death; ECDs, expanded criteria donors; GCDC, glycochenodeoxycholate; HA, hepatic artery; HBOC, hemoglobin-based oxygen carrier; IR, ischemia reperfusion; LT, liver transplantation; MP, machine perfusion; OCS, oxygen carrier solution; PCA, principal component analysis; PRS, postreperfusion syndrome; PTO₂, tissue oxygen tension; PV, portal vein; RBCs, red blood cells

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Introduction

Liver transplantation (LT) remains the ultimate therapy for end-stage liver disease and irreparable acute liver failure. Despite small increments in the number of deceased donors, LT remains severely limited by the number of suitable donor livers. Expanded criteria donors (ECDs) have increased significantly over time, while donors after cardiac death (DCDs) have risen even further (1). However, liver utilization remains suboptimal, with organ discard rates in the United States ranging from 20% to 40% overall and >50% for DCDs (2).

The inexorable growth of DCD offers has been offset by limitations imposed by cold static preservation (CSP). Organ storage under hypothermic (4°C) and anoxic conditions results in progressive decay of organ quality,

which exponentially increases the risk of using ECD livers in patients with the highest Model of End-Stage Liver Disease scores (3). Because of its potential negative impact on organ quality and function, CSP has downstream implications on recipient morbidity and mortality and is directly related to hospital length-of-stay, quality of life and cost (4). Enhanced machine perfusion (MP) devices and innovative preservation solutions are at the forefront of a revolution poised to eliminate the major limitations imposed by CSP. The first clinical application of MP in livers was performed with a system providing continuous pressures through both the portal vein (PV) and hepatic artery (HA) vascular beds using a nonoxygen carrier solution (non-OCS) under anoxic and hypothermic conditions for a short time (5). Subsequent clinical applications for MP employed a single roller pump device utilizing human red blood cells (RBCs) as the OCS under normothermic conditions (6). A third application was recently implemented by providing a brief period of hypothermic oxygenation, wherein continuous pressures were provided through the PV system only under subnormothermic conditions using a non-OCS (7).

Herein, we report the first application of a new preservation modality wherein liver allografts are fully oxygenated under dual pressures under subnormothermic conditions (21°C) with a new hemoglobin-based oxygen carrier (HBOC) solution specifically developed for *ex vivo* utilization.

Methods

Two groups of six animals (Landrace pigs, 60 kg) underwent orthotopic LT after a period of 9 h of cold ischemia time (CIT) and were followed for 5 days. The protocol was approved by the University of Pittsburgh Institutional Animal Care and Use Committee and conducted according to the NIH Guide for the Care and Use of Laboratory Animals. The allografts were recovered from animals under general anesthesia, procured according to standard surgical techniques and flushed simultaneously through the HA and PV with University of Wisconsin solution (total of 5 L) at 4°C immediately after cross clamp and exsanguination. A passive veno-venous bypass circuit was established during the anhepatic phase in the recipient's operation to assure stable hemodynamic conditions. The preservation solutions were rinsed from the allografts with a 1 L flush of cold lactated Ringer's solution immediately prior to implantation in both groups. All recipients received solumedrol (1 g) intraoperatively and tacrolimus (0.3 mg/kg) in the postoperative period.

The CSP allografts were preserved under standard hypothermic conditions with continuous temperature monitoring. The MP livers had both arterial and PV cannulas inserted before placement into the device. Baseline and postprocurement biopsies were performed in both groups. All allografts were weighed before and after preservation. Liver biopsies and perfusate samples were collected every 3 h. Bile and blood samples were obtained daily. All surviving animals underwent end-study necropsy after being euthanized on the fifth postoperative days (Table 1). Two Jackson-Pratt drains for ascites collection and one biliary drain for bile collection were placed and exteriorized prior to the completion of the operation.

Machine perfusion

An MP system (Liver Device) from Organ Assist (Groningen, the Netherlands) was utilized (8). The device was primed with 4 L of perfusate

to achieve an oxygen saturation $\text{SaO}_2 > 95\%$ and $\text{paO}_2 > 400$ mmHg within the target temperature (21°C) prior to liver allograft placement in the perfusion chamber. A closed perfusion circuit was established through the dual infusion ports (pulsatile HA flow at 60 bpm and continuous PV flow). The perfusate was oxygenated continuously ($\text{FiO}_2 = 60\%$ @ 800 mL/min, O_2 delivery = 14.1 mL O_2 /min) through microporous oxygenators. The common bile duct was cannulated for bile collection. The liver was kept fully immersed in the preservation solution in an upside down position, allowing free gravity drainage of the supra-hepatic veins into the inferior vena cava cuff. The following perfusate parameters were assessed every 15 min for 1 h and hourly thereafter: pH, pCO_2 , pO_2 , FiO_2 , HCO_3^- , K^+ , Na^+ , Ca^{2+} , lactate, aspartate transaminase (AST) and lactate dehydrogenase. MP was conducted under low flows (PV flow = 259 ± 40 mL/min; HA flow = 91 ± 36 mL/min), stable temperature (21°C) and low pressures (PV = 3.5 ± 0.5 mmHg, HA = 18 ± 2 mmHg) for an average time of 7 h and 28 min (448.66 ± 9.14 min).

HBOC solution

A new, cell-free HBOC solution was developed by combining a stable second-generation bovine-derived hemoglobin compound (9) (Hemopure™; OPK Biotech, Cambridge, MA) with a hetastarch-based colloid (Belzer Machine Perfusion Solution, Preservation Solutions, Inc., Elkhorn, WI). The HBOC component had an initial hemoglobin concentration of 13 g/dL, a half-life of 20 h and a clearance of 0.12 L/h. This solution can bind up to 1.36 mL O_2 per gram of hemoglobin when fully saturated (10,11). The combined solution (Vir1; Virtech Bio LLC, Wilmington, DE) had a final hemoglobin concentration of 3.5 g/dL and the following characteristics: pH 7.62, osmolality = 296 mOsm/kg, COP = 59.1 mmHg, $\text{Na}^+ = 105$ mmol/L, $\text{K}^+ = 17.3$ mmol/L, $\text{Cl}^- = 36$ mmol/L and $\text{Ca}^{++} = 0.24$ mmol/L (12).

Histological analysis and ischemia reperfusion scoring system

Tissue samples for the assessment of hepatocellular injury were collected before, during and after preservation, postreperfusion and at end-study necropsy. All liver samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned (5 μm) and stained with hematoxylin and eosin for histological analyses. The severity of liver ischemia reperfusion (IR) injuries was blindly graded by transplant pathologists initially using the International Banff Criteria (13). A modified Suzuki's criterion (14) was subsequently applied to quantify the IR injuries and correlate the clinical and histopathological findings. Complete histological features were assessed both in the portal tracts and the hepatic lobules. The scored number was further weighted (none = 0; mild 1–25% = 1; moderate 25–50% = 2; and significant >50% = 3); and then re-scored to reflect its contribution to the injury (grouped into four categories and subsequently expressed as mean \pm standard deviation [SD]).

Analysis of inflammatory mediators

Tissue and perfusate assays of interferon (IFN)- α , IFN- γ , IL-10, IL-12/IL-23 p40, IL-1 β , IL-4, IL-6, IL-8 and tumor necrosis factor (TNF)- α were carried out using a Luminex™ beadset from Affymetrix (Santa Clara, CA). GM-CSF, IL-1 α , IL-1ra, IL-2 and IL-18 were measured using a Luminex™ beadset from Millipore (Merck KGaA, Darmstadt, Germany) (15).

Transcriptomic profile-microarrays

The microarray analysis (probe labeling, hybridization and scanning) of 20 000 genes was performed using Affymetrix GeneChip Porcine Genome Array (16).

Metabolomics analysis

A total of 223 biochemical compounds of known identity in liver perfusate and 377 biochemicals in the bile that differed significantly between groups

Table 1: Study sample collection schedule

Test	Type	Frequency	Analysis
Clinical	Hemodynamics, ABG profile, methemoglobin, inputs and outputs, bile production, ascites, urine output, overall impression (mobility, ability to eat, mental status)	Hourly in the 1st 24 h and daily afterward	Medical team and veterinarians
Laboratorial (recipient's peripheral blood)	Total bilirubin, AST, ALT, GGTP, BUN, creatinine, CBC, electrolytes, Prograf levels, PLT, PT, INR, ABGs, albumin	Every 12 h in the 1st 24 h and daily afterward	University of Pittsburgh labs
Tissue studies	Histopathology	BL, PR, 3, 6, 9 h, PR, N	Blind analysis performed by staff pathologists
	EM	BL, PR, N	
	Microarray	BL, PR, N	Affymetrix Chips
	Oximetry	BL, 3, 6, 9 h, PR	Oxford Optronics probes
	Mitochondrial function	BL, 3, 6, 9 h	Oxygraph chamber
	Cytokine profile	BL, 3, 6, 9 h	Luminex
	Metabolomics	BL, 3, 6, 9 h	Metabolon
Perfusate	ABGs, electrolytes, AST, LDH, methemoglobin	BL, 3, 6, 9 h	University of Pittsburgh labs
	Cytokine profile	BL, 3, 6, 9 h	Luminex
	Metabolomics	BL, 3, 6, 9 h	Metabolon
Bile	Volume, metabolomics	PR, daily for 5 days, N	Metabolon

BL, baseline, N, necropsy, PR, postreperfusion.

Prospective sample collection schedule conducted throughout the study. Clinical data were reported by the clinicians and veterinarians directly involved in animal care. Peripheral blood samples were obtained from a previously placed central line (Broviac catheter) and processed by the University's clinical labs. Tissue studies were performed in liver biopsies' specimens and additional tissues (e.g. spleen, kidney, pancreas, lung, heart, lymph nodes) collected during the end-study necropsy. Perfusate samples were obtained from both groups. Extensive arterial blood gases' analysis was performed in the MP group. Bile samples were collected prospectively from a pediatric feeding tube placed into the common bile duct and exteriorized (external biliary drainage) after liver implantation. Additional analyses (e.g. microarray, cytokine and metabolomics) were performed on liver tissue, perfusate and bile.

were identified with t-tests or Wilcoxon rank sum tests (nonnormal data) for pair-wise comparisons (17). Overall process variability was determined by calculating the median relative SD. Principal component analysis (PCA) was used for data reduction; the original variables were replaced by independent linear combinations of the data whereby those linear combinations were ordered in terms of the percentage of variability encountered. Weights for the original variables reflected the degree to which a given variable contributes to a given principal component (i.e. to a given linear combination). PCA was subsequently performed to identify subsets of variables most strongly correlated with a given procedure (18).

Microbiological surveillance

Samples of preservation solutions from both groups were collected at baseline, 3, 6 and 9 h, homogenized and placed into sterile collection bottles for subsequent culture and sensitivity assays (19).

Additional details for above methods can be found in the online Supplemental Methods.

Statistical analysis

Continuous measures were expressed as the mean \pm SD within each group (e.g. MP and CSP). Two group comparisons of continuous measures between MP and CSP were conducted using the two-sample t-test at a given time point and Wilcoxon rank-sum test. For tests across multiple time points, a linear mixed model (with a random effect to account for multiple

measurements per subject) was used to test for an overall group difference. Differences in survival were evaluated using the log-rank test rather than the exact binomial test of final mortality counts. No adjustments were made for multiple comparisons as results were all in the same direction (with MP providing superior results) thus showing consistent evidence for the same conclusion. Time courses of inflammatory mediators were assessed across time and treatment groups using two-way analysis of variance. p-Values of less than 0.05 were considered statistically significant.

Results

Prereperfusion events

Baseline allograft weight increased in both groups but less so in the MP (MP = 63.37 ± 39.34 g and CSP = 107 ± 65.61 g, respectively).

The MP perfusate exhibited arterial oxygen saturations (SaO₂) between 90% and 100% in the HA port, and venous saturations (SvO₂) between 70% and 80% in the PV port. pO_{2a} (HA) was kept between 450 and 550 mmHg and pO_{2v} (PV) between 100 and 200 mmHg. The CO₂ content was assessed continuously from both the arterial and venous ports. Venous pCO₂ slightly increased during the

first 30 min of MP (17.5 ± 10 mmHg), but subsequently decreased to <10 mmHg within the first hour (Figure 1). Oxygen delivery (0.78 mL O_2 /g/h) was eightfold higher than oxygen consumption (0.096 mL O_2 /g/h) in the MP group at $21^\circ C$. The MP allografts maintained a physiologic pH during the *ex vivo* stage, and did not require additional $NaHCO_3$ infusions as previously seen with the use of non-OCSs with the same device. Furthermore, MP allografts were able to clear lactate while producing urea and bile (1 cc/h) (Figure 2). Methemoglobin values remained at normal levels both in the perfusate during MP and in the recipient's peripheral blood after allograft implantation (Figure 3).

Liver tissue oxygenation (PTO_2) was initially recorded at 80 mmHg during the donor ($FiO_2 = 100\%$) operation prior to organ removal and subsequently recorded as greater than 200 mmHg during MP. Mitochondrial function assessed by oxygraph chamber showed sustained ATP production and respiratory control ratio in both groups ($n=2$). Reactive oxygen species (H_2O_2 nmol/min/mg) were lower in the MP group after reperfusion compared to CSP.

Recipient operation and postreperfusion events

Operative times were 22% shorter ($p < 0.05$) in the MP group (MP = 269.17 ± 20.27 min; CSP = 337.2 ± 14.22 min) due to the lack of significant postreperfusion syndrome (PRS) mainly characterized by vasodilatation, hypotension, acidosis, coagulopathy and oliguria. Intra-operative administration of intravenous fluids was significantly higher (58.5% , $p < 0.05$) in the CSP group due to a pronounced period of vasodilatation (central venous pressure = 4.5 ± 1.5 mmHg, hemoglobin = 12 ± 1 g/dL, mean arterial pressure = 70 ± 15 mmHg). Liver allograft reperfusion was achieved after the completion of the PV anastomosis. The warm ischemia times were similar in both groups (MP = 35.83 ± 3.18 ; CSP = 30 ± 7.4). Arterial reperfusion occurred within 60 min of PV reperfusion (MP = 39.6 ± 14 ; CSP = 50.6 ± 19.9). The MP group had no to mild signs of PRS after implantation, while the CSP group had moderate to severe signs. The CSP animals experiencing moderate to severe PRS developed PNF subsequently and died from liver allograft failure (intractable lactic acidosis followed by respiratory failure). Postoperative alanine transaminase (ALT) and AST values were significantly lower ($p < 0.05$) in the MP group (ALT: MP = 27 ± 8.2 ; CSP = 84 ± 31.7 ; and AST: MP = 199 ± 136.9 ; CSP = 935 ± 512.5). Massive centrilobular necrosis was detected at necropsy in the CSP animals. These findings are corroborated by similar studies showing high mortality in this porcine model when CIT was >9 h ($20-22$).

All surviving animals were euthanized on the fifth day; the MP group had a significantly higher survival rate (100% vs. 33% , $p < 0.05$) (Figure 4). MP animals had significantly better ($p < 0.05$) graft function than the CSP animals when all data analyses were combined (mixed model). MP

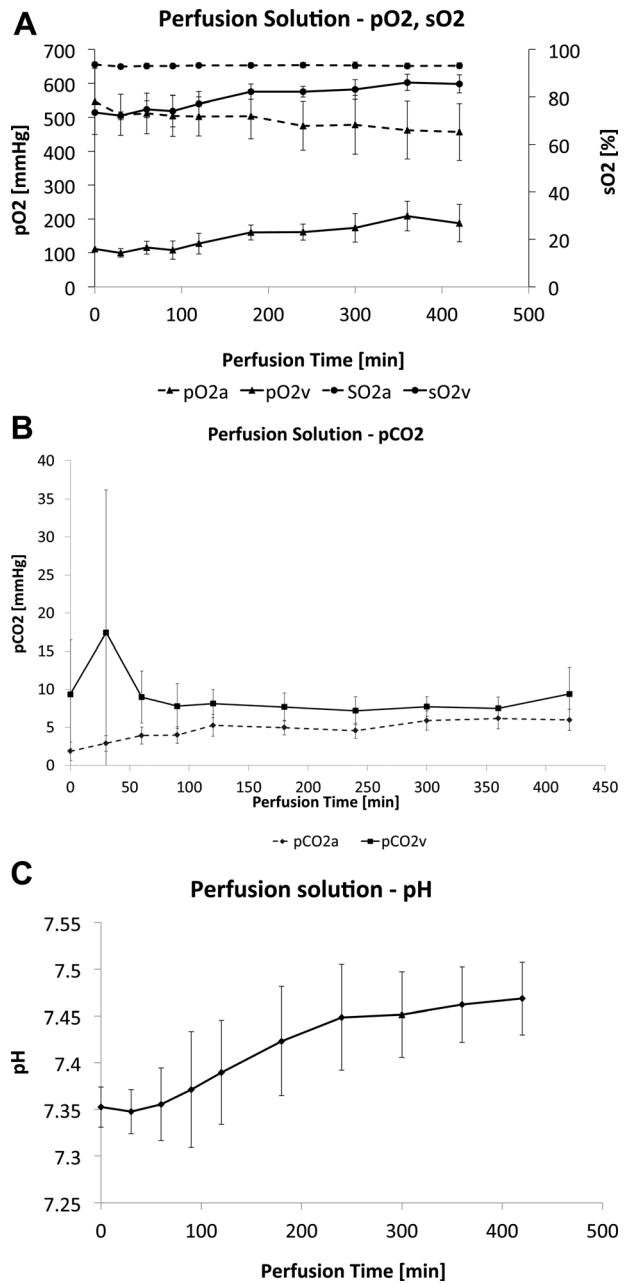


Figure 1: Arterial blood gases (ABGs) obtained from perfusate during machine perfusion protocol. (A) The y-axes show oxygen pressures (pO₂ mmHg) on the left and oxygen saturation (sO₂ %) on the right (mean ± SD). The x-axes show the perfusion time in minutes (min). The HA pO₂ is represented as pO_{2a}. The PV pO₂ is represented as pO_{2v}. The HA saturation is represented as sO_{2a}. The PV saturation is represented as sO_{2v}. The FiO₂ was constant at 60%. (B) Carbon dioxide (CO₂) pressures (mmHg) overtime (minutes) in the MP group (mean ± SD). (C) pH (mean ± SD) overtime (minutes) in the MP group.

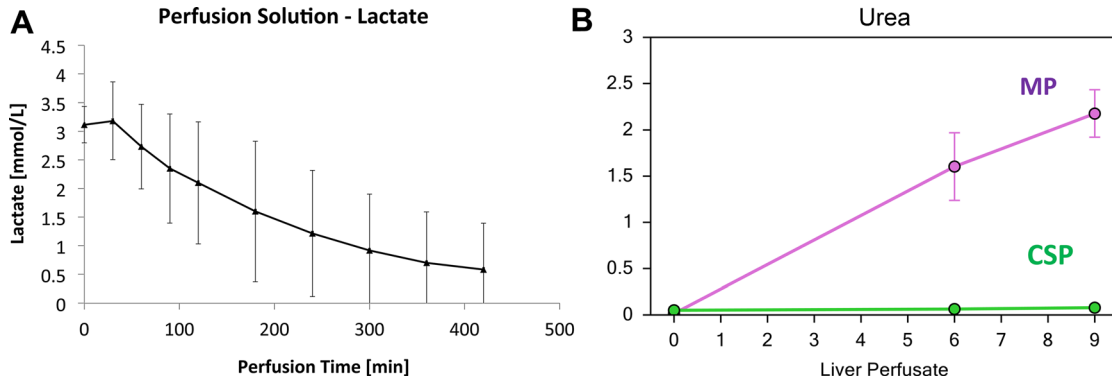


Figure 2: (A) Lactate concentration (mmol/L) values (mean ± SD) in the perfusate over time (minutes) during MP. (B) Urea concentration (mmol/L) values (mean ± SD) in the perfusate overtime (hours) during MP.

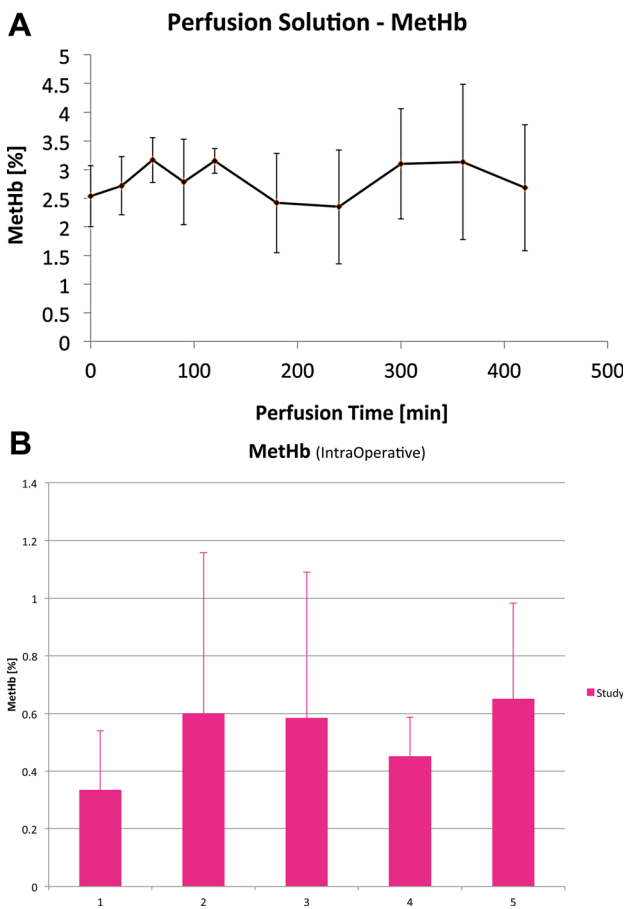


Figure 3: Methemoglobin levels during MP and after liver implantation. (A) Methemoglobin (MetHb) saturation (%) values (mean ± SD) in the perfusate over time (minutes) during MP. (B) MetHb saturation (%) values (mean ± SD) over time (hours) in the recipient's peripheral blood during liver transplantation in the MP group.

allograft biopsies showed no to mild IR injuries compared to moderate to severe in the CSP group. Based on IR scores, the MP group recovered significantly ($p < 0.05$) better from the histological point of view when compared to the CSP group (Figure 5).

Bile production (MP = 378.5 ± 179.7 ; CSP = 151.6 ± 116.85) was significantly higher ($p < 0.05$) during the first 24 h and throughout the study (5 days) in MP livers. The MP group had significantly ($p < 0.05$) higher urine output (MP = 3507.5 ± 840 mL; CSP = $840 \pm$

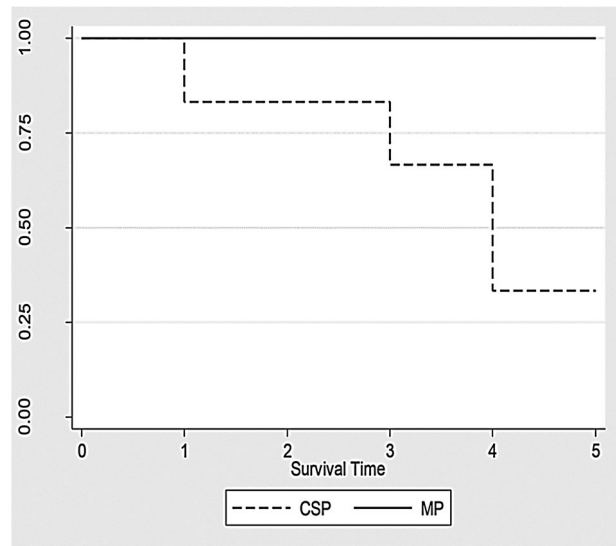


Figure 4: Survival curve for both groups CSP and MP during a 5-day period. MP had a 100% survival and CSP had a 33% survival ($p < 0.05$). Early deaths on the CSP group were due to primary non-function leading into irreversible liver allograft failure.

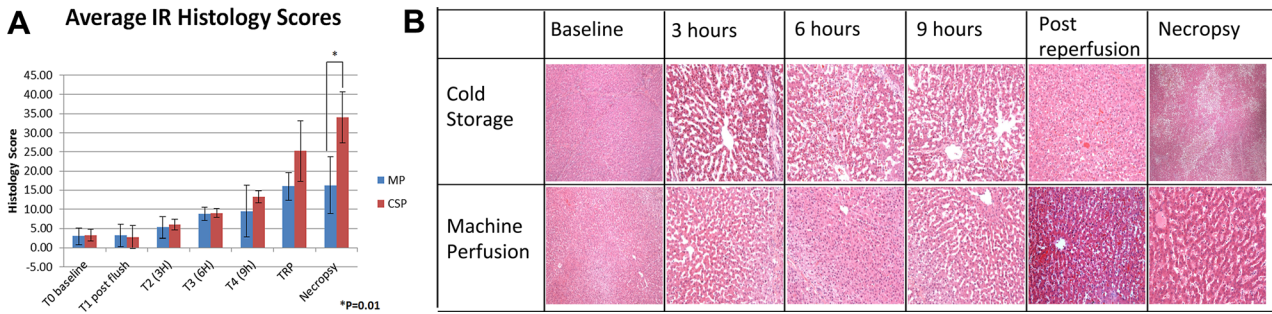


Figure 5: Histological analysis of ischemia reperfusion (IR). IR scores (Suzuki modified) were determined by serial analysis of inflammatory changes within the portal tracts and the hepatic lobules. (A) Comparison of average IR scores for cold static preservation (CSP) and machine perfusion (MP). The IR scores at necropsy were significantly lower in the MP group ($p < 0.05$). (B) Histological images hematoxylin and eosin of the liver biopsies obtained on both groups (CSP at the top and MP at the bottom) during preservation, after reperfusion and at the end-study necropsy.

577.1 mL) and lower ascites production (MP = 1409.3 ± 1057 mL; CSP = 2666.2 ± 1599.7 mL) during the same time period.

Transmission electron microscopy showed integrity of hepatocyte mitochondria after reperfusion and at necropsy in the MP group (Figure 6). The CSP group displayed significant mitochondrial damage at reperfusion and necropsy, characterized by progressive membrane damage leading to the formation of mitochondrial permeability transition pores. Furthermore, MP AST perfusate levels were significantly lower at 3 (55.67 ± 21.51 vs. 149.2 ± 53.49), 6 (59.66 ± 25.81 vs. 157 ± 57.74) and 9 h

(74.33 ± 29.21 vs. 186 ± 90.92) than those in the CSP livers ($p < 0.05$) (Figure 7).

Transcriptomic analysis

There was a striking increase in proliferation-associated genes (*Jun, Fos, ATP synthase F0 subunit 8, Apolipoprotein A-II, Metallothionein isoform, Acyl coenzyme A synthetase, Syndecan 2, Collagen Alpha 2, Prothymosin alpha*) in the MP group when compared to CSP. Many hepatocyte-differentiation genes were also up-regulated, including *albumin, apolipoproteins* and several *cytochrome P450* members.

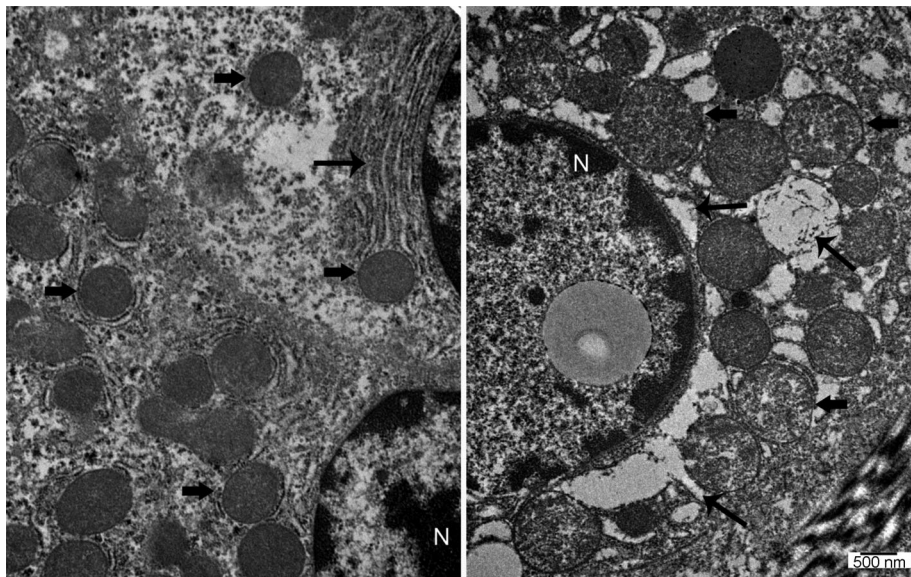


Figure 6: Transmission electron microscopy analysis. The thick arrows point to the mitochondria. Machine perfusion (MP) mitochondria in the left panel have normal features. Cold static preservation (CSP) mitochondria in the right panel are swollen, which is usually an indication of mitochondrial dysfunction. The thinner arrows point to the endoplasmic reticulum (ER). MP in the left panel has normal anatomical features. CSP in the right panel show signs of ER stress. The nuclei (N) are shown as they indicate that these cells are alive and not necrotic.

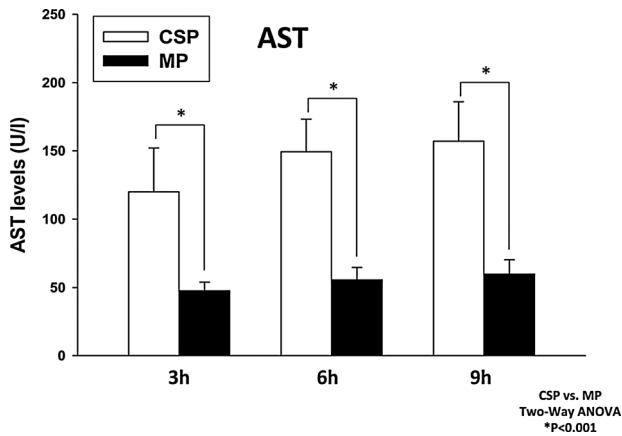


Figure 7: AST (U/l) levels in the perfusate of both groups (CSP white; MP black) during liver preservation. MP provided effective oxygenation and significantly lower ($p < 0.05$) scores of hepatocellular damage when compared to CSP over a period of 9 h.

The 100 most affected genes in the MP group showed over-expression of general metabolic, anti-inflammatory and regenerative functions, as well as protective mechanisms against free radicals. MP also resulted in increased expression of several genes associated with entry of hepatocytes into the G1 cell cycle phase. Genes associated with hepatocyte differentiation were also up-regulated. Enrichment by biological process networks following MP suggested a marked up-regulation of genes related to metabolic processes (34%), cellular processes (25%) and cell communication (17%). Additional Biological Process Network Analysis (INGENUITY[®]; QIAGEN Silicon Valley, Redwood City, CA) (23) showed a significant ($p < 0.05$) difference in gene expression related to hepatic system development and function, cellular growth and proliferation, cellular development and cell cycle when MP was compared to CSP. Metabolic Network Analysis (INGENUITY[®]) showed significant up-regulation of genes related to drug, amino acid, vitamin, mineral and carbohydrate metabolism in addition to free radical scavenging and energy production in the MP group. The MP group displayed significantly higher serum albumin levels ($p < 0.05$) in the immediate postoperative period, which appeared to be prompted by a significant up-regulation of albumin-related genes during MP. Thus, MP with full oxygenation enhanced signaling pathways for HGF, EGF, TGF- β , ErbB and PI3K/AKT among others, and triggered proliferative and regenerative transcriptional pathways compared to CSP (24) (Figure 8).

Metabolomic analysis

Over 600 metabolites were analyzed when the two groups were initially compared. Ascorbate and threonate levels were significantly higher in the MP group at 6 and 9 h. Evidence of lipoxygenase activity differed by preservation method. 15-HETE was specifically elevated in MP perfusate, whereas 12-HETE was elevated in CSP across all time

points. Signs of energy stress and purine nucleotide breakdown were noted in the CSP livers, as indicated by the significantly higher levels of adenosine monophosphate and hypoxanthine. Glucose, amino acid cycle and branched-chain amino acid (BCAA) mobilization were markedly elevated in MP samples. Glucose levels were higher in the MP perfusate throughout the study. BCAA oxidation showed a strong differential increase in MP perfusate at 6 and 9 h as demonstrated by valine, isoleucine and leucine levels and their respective deamination products.

Five bile acid (BA) conjugates (e.g. taurocholate and glycochenodeoxycholate [GCDC]) were significantly higher in CSP perfusate during preservation. Similarly, taurine and its immediate precursor, hypotaurine, were relatively abundant in CSP perfusate but significantly lower in the MP group. Immediately following transplantation, the bile sterol precursors lathosterol and cholesterol were similar in both groups; however, lathosterol levels became significantly higher in the bile of the MP group, showing an early recovering in biliary phospholipid secretion. The same pathway was seen with campesterol levels, which fell significantly in the CSP when compared to MP group (Figure 9). CSP livers secreted a relatively higher bile salt/phospholipid ratio after transplant when compared to MP.

MP-treated livers did not release GCDC during perfusion; however, its secretion resumed almost immediately following reperfusion. BA secretion was decreased in both groups immediately after reperfusion, but was restored subsequently in MP livers within the first 24 h. Furthermore, the levels of a few sulfated BAs (e.g. glycocholate sulfate) were significantly elevated in the CSP group.

The insulin resistance biomarker alpha-hydroxybutyrate was initially low in both groups but its levels in CSP bile increased during subsequent follow up. Insulin resistance is likely a sign of impaired functional recovery following transplant (25). Signs of oxidative and inflammatory stress differed with a greater level of hydrogen peroxide quenching by ascorbate in MP perfusate and greater pro-inflammatory 12-lipoxygenase product 12-HETE in CSP perfusate (26).

MP livers experienced a sustainable level of metabolic activity at 21°C, which resulted in a more seamless resumption of hepatic functions, such as regulating the supply of glucose, lipids and amino acids for catabolic and anabolic metabolism after transplantation. MP bile samples displayed significantly higher ($p < 0.05$) levels of cholesterol, lathosterol and campesterol, in the initial postoperative period revealing a sustained ability to extract lipophilic nutrients from the gut.

PCA was carried out on the metabolomic profile of the perfusates of both groups (Figure 10) to elucidate principal drivers of the metabolic changes across the three time points. This analysis suggested that the principal dynamic

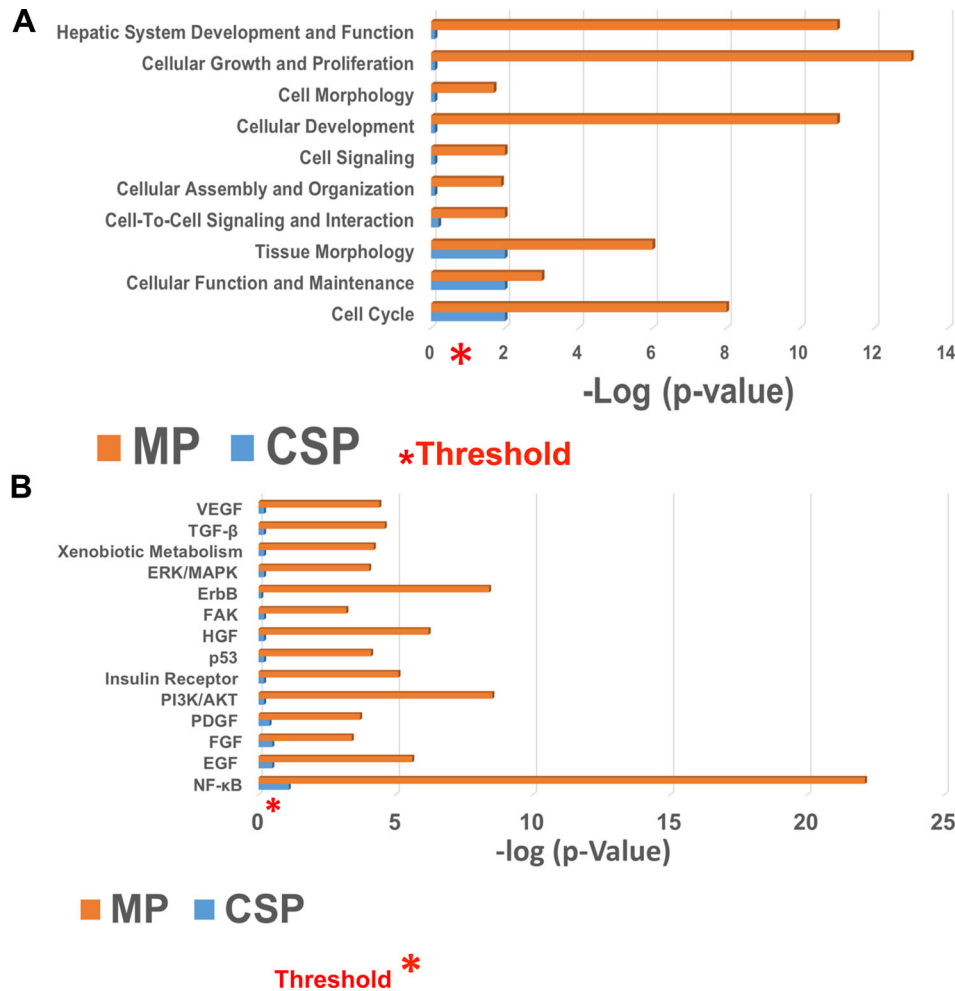


Figure 8: Comparison of biological processes and signaling pathways for machine perfusion (MP) and cold static preservation (CSP). (A) Biological process network analysis. Differential regulation of biological process networks during liver preservation was analyzed by comparing machine perfusion (MP) to cold storage preservation (CSP) in liver tissues after allograft reperfusion (both portal and arterial) using INGENUITY[®]. The biological processes that were found to be significantly affected are displayed along the y-axis. The x-axis displays the $-\log$ of p-value (threshold) and was calculated using Fisher’s exact test right-tailed. The most significant up-regulation was observed in genes associated with hepatic system development and cellular proliferation. (B) Signaling pathway analysis comparing machine perfusion (MP) and CSP after the full implantation of the liver allograft. Growth factor signaling pathways that are critical for liver function and growth were generated using INGENUITY[®]. The x-axis displays the $-\log$ of p-value (threshold) and was calculated by Fisher’s exact test right-tailed. It was observed that signaling pathways that are critical for liver growth were significantly up-regulated in MP liver samples compared to the CSP group. NF-κB is fully activated in hepatocyte regeneration and undergoes significant up-regulation after partial hepatectomy.

characteristics in MP livers were carbohydrate metabolism (ribulose, ribose, glycolate) and antioxidant defenses (oxidized homo-glutathione). In CSP livers, PCA suggested the characteristics were predominantly due to ethanolamine, suggesting a role for fatty acid metabolism (27).

Inflammatory mediator analyses

MP was associated with down-regulation of both type I (IFN-α) and type II (IFN-γ) interferons, consistent with prior studies that showed elevated inflammation and apoptosis subsequent to IR due to the IRF-1 pathway in CSP (28,29).

MP was associated with down-regulated TNF-α levels in liver tissue when compared to CSP. This detrimental TNF-α activation pathway seen in the CSP group was further corroborated by higher levels of additional Kupffer cell mediators IL-1β and IL-12/IL-23 p40 found on liver tissues (30,31) (Figure 11).

Microbiological surveillance

There were no microorganisms isolated from any samples from either group.

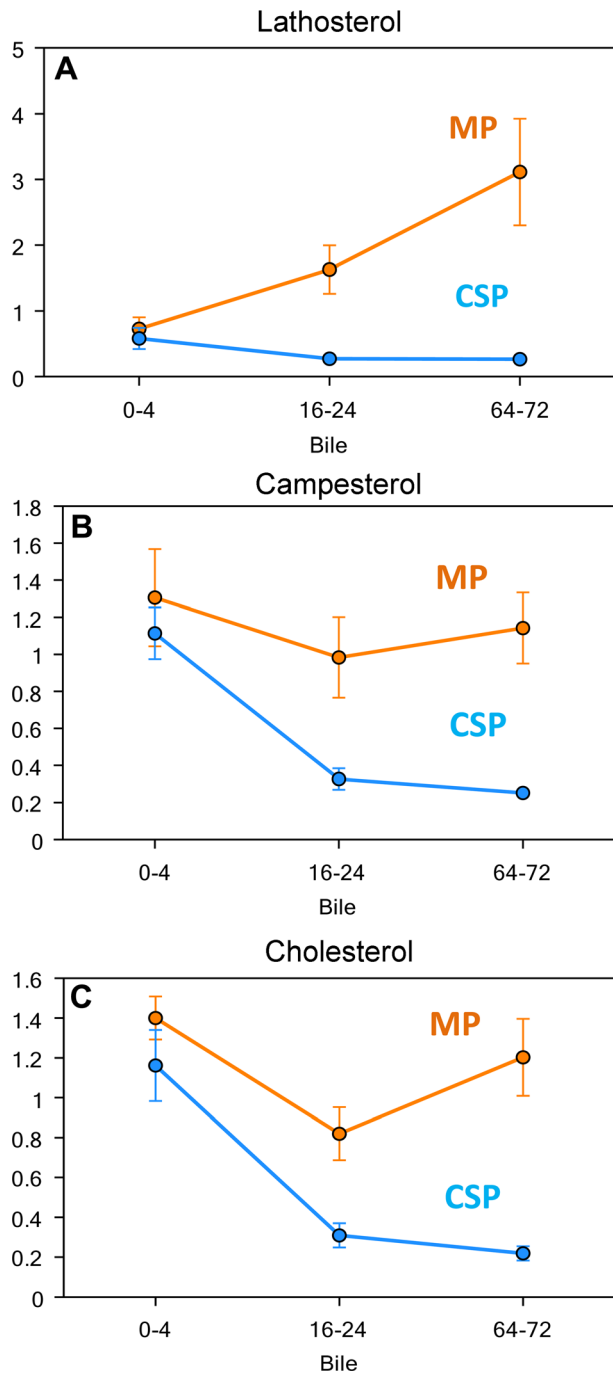


Figure 9: Comparison of lipid concentration in bile of MP and CSP groups performed by metabolomics' analysis (Metabolon[®], Durham, NC). The MP is brown and the CSP is blue. Bile samples were sequentially obtained through a catheter previously placed in the common bile duct (external biliary drainage). (A) Lathosterol concentration (μM) performed by gas chromatography/mass spectrometry (GC/MS) in the recipient's bile for the first 72 h after liver transplantation. (B) Campesterol concentration (μM) performed by GC/MS in the recipient's bile for the first 72 h after liver transplantation. (C) Cholesterol concentration (μM) performed by GC/MS in the recipient's bile for the first 72 h after liver transplantation.

Discussion

LT volumes have been relatively stagnant for the past several years in the United States. As indications for the procedure continue to expand, the volume of available livers is decreasing, resulting in an unacceptable number of deaths on the waiting list (32). Therefore, it is imperative that we improve the viability and usability of currently available but discarded organs.

The combination of an HBOC with hetastarch-based colloids has been previously shown to be effective in sustaining adequate oxygenation and flow through the microcirculation while minimizing adverse effects seen with HBOC alone when utilized *in vivo* under physiologic conditions (33). This dilutional environment (hemoglobin = 3.5 g/dL) at lower temperatures was able to sustain low methemoglobin levels while providing reliable oxygen delivery and effective CO₂ removal. Additional advantages of this solution include no risk of hemolytic reactions, low viscosity, unlimited availability and long storage life. Furthermore, the HBOC solution is universally compatible and carries an insignificant risk of disease transmission (34,35).

The high mortality observed in the CSP animals was expected based on previous reports (19–22) from swine models utilizing CIT > 9 h. Volume infusion and vaso-pressors were required to sustain hemodynamic stability after reperfusion. There were no technical complications in either experimental group. An additional CSP control group (n = 6) was performed using HTK, Custodiol[®] (Essential Pharmaceuticals, Ewing, NJ), and the results were similar to the University of Wisconsin animals (mortality = 67% in 5 days; data not shown).

Previous studies outlining the use of RBCs in extra-corporeal devices have shown unavoidable hemolysis followed by the progressive deterioration of cell integrity documented on both roller and centrifugal pumps. Hemolysis remains a biological limitation for the prolonged utilization of RBCs in MP systems (36).

Among the options available for OCSs, HBOCs have shown superiority compared to perfluoro carbon and other non-hemoglobin based products over the last two decades (37). The solution described herein is a second-generation HBOC, in which bovine hemoglobin molecules are successfully extracted, purified and chemically cross-linked (polymerized) for stability. We have tested another non-HBOC solution (Lifor; Lifeblood Medical, Adelphia, NJ) (38–40) with the same device at 21°C in three additional animals. Massive centrilobular necrosis was observed after 9 h on MP, in spite of bile production and acceptable pH values during preservation (data not shown). Additional experiments were also conducted with the same MP device utilizing BMPS (Preservation Solutions, Elkhorn, WI) as the perfusate at 12°C (data not shown). The recipients

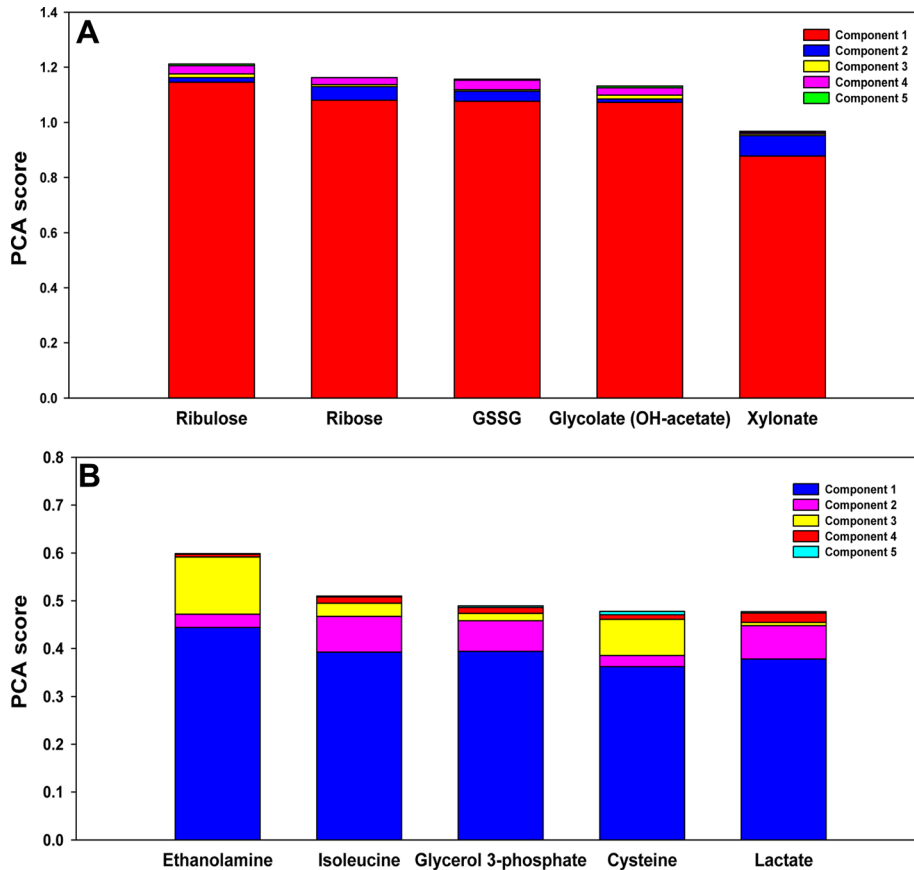


Figure 10: Principal component analysis (PCA) of perfusate metabolomic profile. (A) PCA suggests importance of carbohydrate metabolism and antioxidant defenses in MP livers. PCA was carried out on the metabolomic profile of perfusate at three time points (3, 6 and 9 h). Variables are ordered by the sum of their contribution to all components, with contributions to individual components represented by different colored sections of the bars. In MP livers, variables representing carbohydrate metabolism (ribulose, ribose, glycolate) and antioxidant defenses (oxidized homo-glutathione–GSSG) are principal drivers of metabolic changes. (B) PCA suggests importance of fatty acid metabolism in CSP livers. PCA was carried out on the metabolomic profile of perfusate at three time points (3, 6 and 9 h). Variables are ordered by the sum of their contribution to all components, with contributions to individual components represented by different colored sections of the bars. In CSP livers, PCA showed ethanolamine to be the principal driver of metabolic changes, suggesting a role for fatty acid metabolism.

developed moderate to severe PRS leading to allograft failure and subsequent death after 9 h of MP.

The debate over the role of pulsatile pressures in MP continues (41). We believe pulsatile pressure is important to assure effective perfusion through the high-resistance microvasculature within the arterial-biliary plexus as well as sustain sinusoidal system patency in the absence of the transient negative pressures supplied by diaphragmatic excursions in our physiological *in vivo* environment.

Adequate liver function has historically been associated with sustainable bile production, and these experiments reinforce this principle (42). MP allografts were able to produce up to 1 cc/h of bile despite being kept at 21°C for an

extended time period. Sequential bile analysis showed lower cholesterol and lipid amounts within the bile of the CSP allografts, which translates to a deficiency in bile conjugation after implantation (43).

This new MP/HBOC system has a unique ability to provide effective oxygenation to the liver tissue. Perfused livers appear to sustain lower oxygen extraction under subnormothermic conditions (44), which reinforces the discrepancy between oxygen delivery and oxygen consumption witnessed in our *ex vivo* system with low hemoglobin levels (3.5 g/dL). MP animals experienced a markedly uncomplicated course compared with the CSP group. The initial bile production seen during the MP stage further increased during implantation as the allografts were

MP at 21°C With an Oxygen Carrier Solution

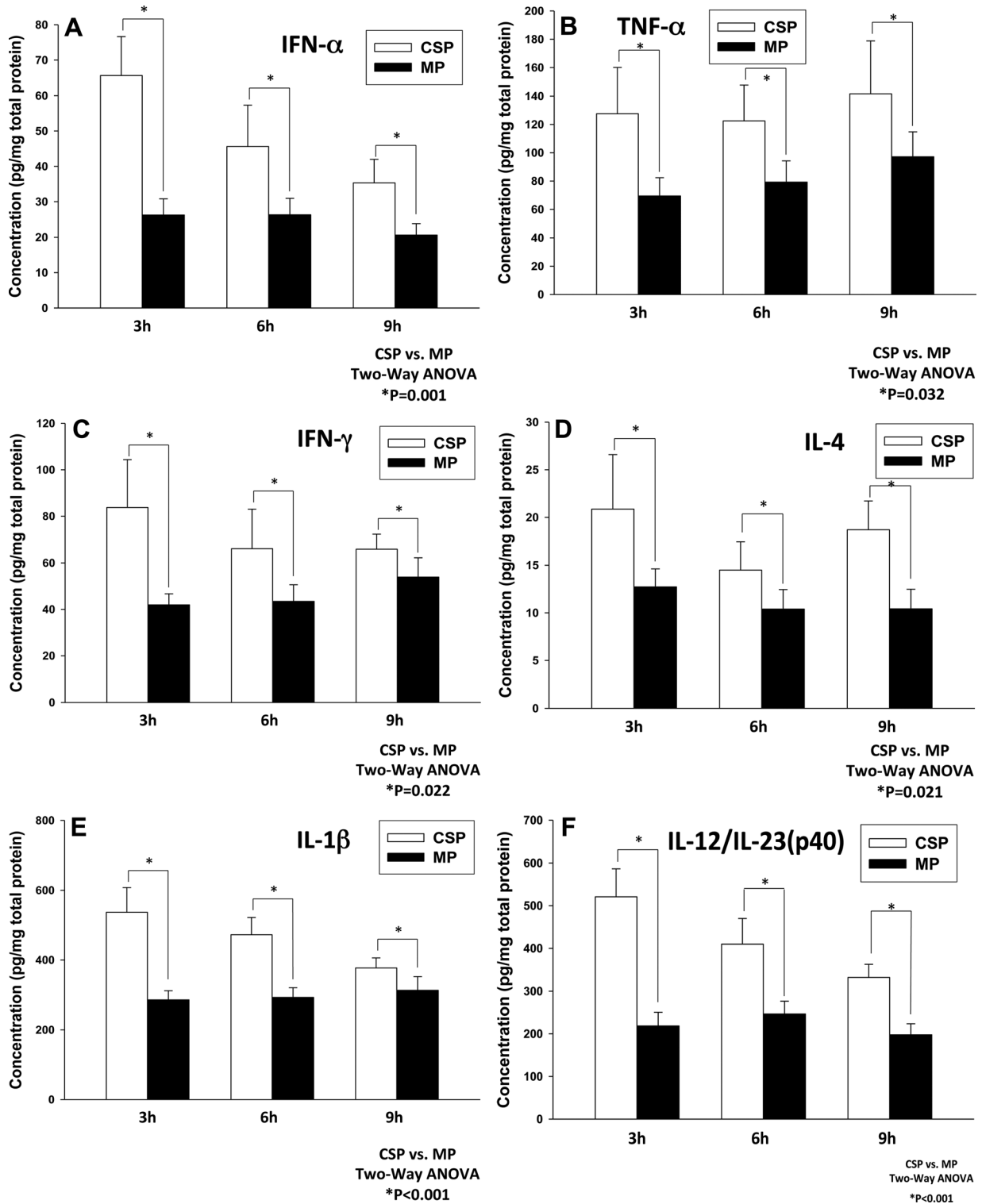


Figure 11: Comparison of the cytokine profile for machine perfusion (MP) and cold static preservation (CSP) groups obtained at 3, 6 and 9 h during liver preservation. MP suppresses TNF- α driven pro-inflammatory cytokines secreted by T cells and NKT (IFN- γ), Kupffer cells (IL-1b) and hepatocytes (IL-12/IL-23) when compared to CSP. (A) MP suppresses IFN- α secretion. (B) MP suppresses TNF- α secretion. (C) MP suppresses IFN- γ secretion. (D) MP suppresses IL-4 secretion. (E) MP suppresses IL-1 β secretion. (F) MP suppresses IL-12/IL-23 secretion.

rewarmed. The MP group showed signs of a prompt recovery from mild IR injuries in addition to an anatomically intact biliary system.

Our comprehensive data analysis combining transcriptomic, metabolomic and protein-level inflammatory mediators highlights the notable benefits of MP when compared to CSP. Gene expression was clearly driven toward reanimation and resuscitation pathways while most gene expression related to metabolic, cellular and cell communication processes experienced significant up-regulation (45) compared to the anoxic and hypothermic conditions imposed by CSP.

From the clinical standpoint, the recipients of liver allografts preserved by this combination of MP with an HBOC solution experienced significant benefits when modulation in gene expression (46) and inflammatory pathways were translated into graft function. A clear drive toward the induction of liver regeneration was detected by transcriptomic analysis. Inflammatory mediator analysis revealed a down-regulation of TNF- α in the MP group. Metabolomics' analysis revealed sustained metabolic activity (e.g. gluconeogenesis, albumin secretion, BCAA secretion, urea production and reactive oxygen species scavenging) in the MP group. Bile analysis over a 5-day period suggested that hydrophilic bile was being secreted by the MP group in contrast to signs of hydrophobic bile documented in the CSP group. In spite of these encouraging findings, we acknowledge that this was a small study, and further work is required to fully explore the mechanisms initially outlined by transcriptomics and metabolomics' analysis.

The sustained increase in BCAA in the perfusate seen in our porcine model has been previously described in healthy fasted rats under MP while being adequately oxygenated (47).

The detergent action of hydrophobic bile salts (e.g. GCDC) plays a significant role in biliary epithelial cell membrane damage and seems to promote apoptosis (48). Conversely, hydrophilic bile salts can protect cholangiocytes from hydrophobic bile salt-induced injury (49). When analyzing BA synthesis and subsequent bile secretion, MP livers secreted hydrophilic bile (e.g. significantly lower levels of taurodeoxycholate), which is by itself an important protective factor for biliary epithelial cells after reperfusion compared to the hydrophobic bile (e.g. significantly higher levels of glycocholate sulfate) secreted in CSP.

In conclusion, MP at 21°C with an HBOC solution achieved a 100% survival rate with excellent graft function in these pre-clinical large animal experiments. The modified HBOC solution provided consistent oxygen delivery to the tissues and was not shown to cause additional oxidative stress damage after reperfusion. Porcine liver allografts under MP produced urea, cleared lactate, secreted bile and showed

no signs of secondary infections while being sustained at a low metabolic rate at 21°C over a 7:30 h period. MP livers appeared to secrete protective hydrophilic bile after reperfusion while showing no signs of subsequent epithelial biliary cell damage. MP with a cell-free OCS can be safely and effectively conducted at 21°C under dual pressures with low flows. This approach has shown significant advantages compared to the current standard-of-care (CSP) and might allow the expansion of this technology into the recovery and amelioration of grafts obtained from ECDs.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Supplemental Methods