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Novel oxygenation technique for hypothermic machine perfusion of liver grafts: Validation in porcine Donation after Cardiac Death (DCD) liver model

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ABSTRACT

Background: Hypothermic oxygenated machine perfusion improves outcomes in Liver Transplantation, but application is limited as O_2 is supplied by a stationary circuit. A novel technique of O_2 "pre-charge" in a portable pump would broaden use and further mitigate ischemia damage from organ transport.
Methods: Porcine DCD livers were randomized to static cold storage (SCS, n = 8) or hypothermic machine perfusion (HMP). HMP was stratified into HMP-O_2 (n = 5), non-O_2 open to air HMP-RA (n = 5), and non-O_2 with sealed lids or no air HMP-NA (n = 5). HMP-O_2 was "pre-charged" using 100% O_2 delivered at 10 L/min over 15 min. Perfusate and tissue O_2 tension (pO_2), liver biopsies, and fluid chemistries were analyzed.
Results: "Pre-charge" achieves sustained tissue and perfusate pO_2 vs others. HMP-O_2 results in decreased markers of hepatocyte injury: ALT ($p < 0.05$) and LDH ($p < 0.05$), lower expression of CRP and higher expression of SOD1 vs SCS. This suggests decreased inflammation and improved ROS scavenging.
Conclusions: "Pre-charge" is an effective technique, which allows portability and transport without an O_2 source and improves graft parameters.

Table of contents summary: Hypothermic oxygenated machine perfusion is a dynamic method of *ex vivo* machine preservation of liver allografts. Oxygen "pre-charge" of hypothermic perfusate achieves sustained O_2 levels, allowing machine pump portability, and improved allograft preservation. Our results validate this novel technique and support its continued application.

Introduction

The success of liver transplantation (LT) as the gold standard treatment for end-stage liver disease and select liver malignancies has resulted in a persistent "organ gap" where the demand exceeds the supply of available donor organs. This has led to an increased reliance on extended criteria (ECD) and donation after circulatory death (DCD) livers. Such livers are particularly sensitive to Ischemia/Reperfusion injury (IRI) and related complications, including early allograft dysfunction and biliary complications. In addition, the recent transition of liver allocation to the Acuity Circles model is expected to result in longer travel distances and more frequent air travel to procure organs for transplant. Therefore, optimizing preservation techniques and mitigating ischemic damage during organ transport are key. Hypothermic machine perfusion is a dynamic preservation technique that has been successfully used for kidney transplants since the late 1960s. Over the last two decades, this technique has evolved and the feasibility of hypothermic *ex vivo* machine perfusion (HMP) and its application to LT

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are now showing promising results in improving post-operative outcomes, as well as "rehabilitating" marginal grafts, reducing IRI, and broadening access to LT[1–3].

Both HMP and cold storage maintain hypothermic conditions, resulting in a protective decrease in organ metabolic demands. Machine perfusion provides the additional benefit of dynamic flow, utilizing preservation solution with specialized additives (vasodilators and antioxidants) to effectively flush toxic metabolites from the cellular milieu.[4] Most recently, in efforts to mitigate ischemic conditions, groups have incorporated oxygenation into their perfusion circuits to augment the beneficial effects of HMP on liver allografts. Current perfusate oxygenation techniques provide end-ischemic (after a period of SCS) O_2 via a stationary continuous oxygenation circuit during recipient hepatectomy. While studies show this is sufficient to mitigate IRI ([3,5,6]), there is still a period of ischemic preservation injury which occurs during transport and static cold storage. Here we present a novel technique of oxygen "pre-charge" and hypothermic machine perfusion with pre-oxygenated solution using the LifePort® Liver Transporter 100 or LLT (Organ Recovery Systems, Itasca, IL) ([7]). LLT device design optimizes machine portability and obviates the need for a stationary oxygen source, allowing easier and safer transport. We propose this will broaden use of this preservation technique and mitigate ischemic damage sustained during organ transport. The present study evaluates the LifePort® Liver Transporter in oxygenated machine preservation and the effects on organ oxygenation and tissue ischemic damage utilizing porcine livers.

Methods

Procurement and study groups

Whole porcine livers were obtained from a local FDA-inspected slaughterhouse (Green Village Packing Company, NJ). Following animal slaughter, livers were dissected free of abdominal contents. The portal vein (PV) and hepatic artery (HA) were identified and cannulated. Livers were then serially flushed with 1 L heparinized (1000 IU) Lactated Ringers (LR) followed by 1 L LR containing 1 mg Tissue Plasminogen Activator (TPA) (250 ml via HA and 750 ml via PV for all). The hepatic artery was clamped for 10–15 min following TPA infusion to allow for TPA activity, after which all livers were flushed with 1 L of standard Static Preservation Solution (SPS-1®; Organ Recovery Systems, Itasca, IL). After discarding the flush, a second liter of SPS-1 was infused via the HA and PV, and the livers were placed on ice for transport to investigator's laboratory. Cholecystectomy and ligation of bile duct were performed at the time of procurement. Liver weights and time from animal slaughter to first flush were recorded. All interventions were performed on site, at the slaughterhouse, by designated, trained perfusion personnel.

Upon arrival to investigator laboratory, livers were randomized to Static Cold Storage (SCS) or to one of three Hypothermic Machine Perfusion (HMP) groups. Livers assigned to SCS remained in SPS-1 solution, on ice. Livers randomized to HMP underwent back table flush with 1 L of pre-mixed, chilled Vasosol® solution (Organ Recovery Systems, Itasca, IL), a proprietary preservation solution containing antioxidants and vasodilators that has been previously described.[8] Livers with aberrant anatomy, visibly narrowed vessels precluding adequate cannulation, and those showing visible parenchymal or vessel injury or tears were excluded from testing.

Transport time to investigator laboratory occurred in a median of 125 min. Upon arrival, livers were assigned to SCS (n = 8) or underwent HMP (n = 15): perfusion in a closed, no air (NA) system **HMP-NA**, n = 5 (to prevent equilibration with ambient room air); perfusion with cassette lids left open to room air (RA) **HMP-RA**, n = 5 (thereby allowing likely diffusion of environmental O_2 with the perfusion agitated fluid); and the **HMP-O_2** group, n = 5. HMP-O_2 was "pre-charged" using 100% O_2 delivered at 10 L/minute over 15 min prior to liver placement on the pump. Liver procurement and allocation is graphically depicted in Image 1. Machine perfusate oxygenation, liver cannulation and liver placement in the perfusion device are shown in Image 2.

Perfusion technique

Three liters of chilled, pre-mixed Vasosol® were decanted into the LifePort Liver Transporter. Cannulated porcine livers were placed in the organ chamber, and the PV and HA were connected to the perfusion circuit. Continuous, non-pulsatile, flow-controlled perfusion was then initiated via the HA and PV (dual perfusion). Target flow was adjusted per individual liver weight, at a rate of 0.66 mL/g liver/min, as previously described.[9] Perfusion variables, including temperature, flow, and HA and PV pressures, were monitored and recorded throughout preservation. All livers randomized to HMP underwent 4 h of perfusion.

Oxygen tension monitoring in solution and liver parenchyma

Dissolved preservation fluid oxygen content (pO_2) and liver tissue oxygen tension ($ptiO_2$) were monitored throughout organ preservation. The PreSens® oxygen detection system (Precision Sensing, Regensburg, Germany) was utilized for pO_2 measurement in fluid. The PreSens® Microx-4 instrument and sensors were set up following manufacturer guidelines. Briefly, sensors were exposed to ambient air for 5 min to establish baseline values. Once calibration was complete, probes were either submerged in preservation fluid next to livers (SCS) or inserted through designated, pre-drilled holes in organ chamber of HMP device. Efforts were made to maintain consistent probe placement between test livers. Areas of cable egress were sealed with Teflon plumber's tape to

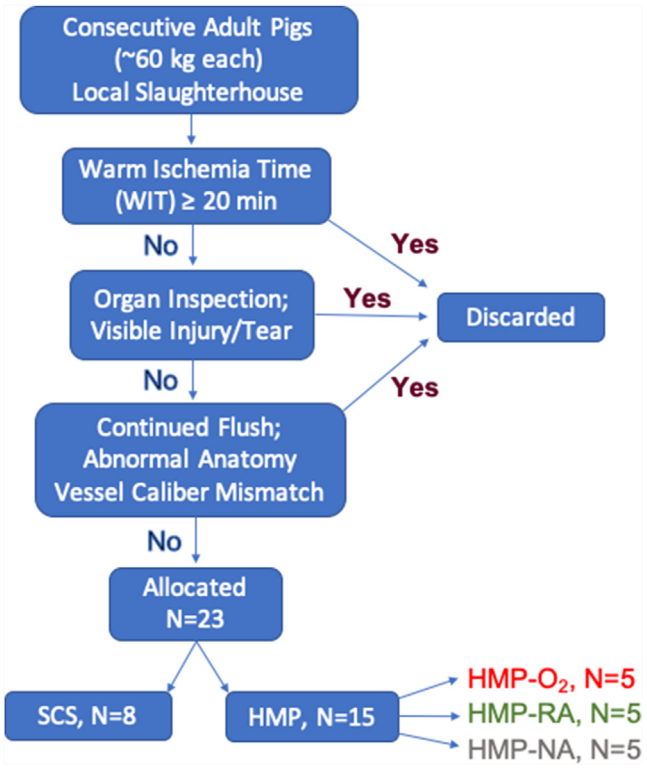

Fig. 7. Procurement and liver allocation scheme.

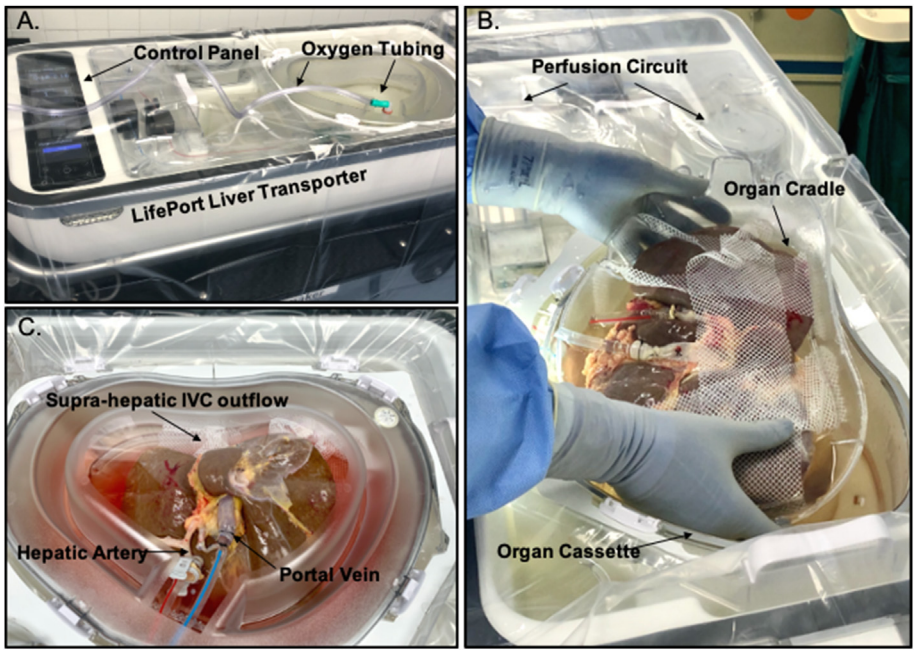

Fig. 8. The LifePort® Liver Transporter (ORS, Itasca, IL) with liver in the cassette and "on-pump." Panel A: Depicts the "pre-charge" oxygenation technique. Panel B: Depicts liver placement in organ cradle and the cassette/circuit. Panel C: Liver on pump, during perfusion. Corresponding parts of machine, control panel, circuit, and areas of cannulation are labeled.

approximate closed-system conditions. The OxyLite™ oxygen detection system (Oxford Optronix, Abingdon, UK) was utilized for liver tissue oxygen tension ($ptiO_2$) measurement. The OxyLite™ monitor and optode oxygen sensors were set up according to manufacturer guidelines. Briefly, probe sensors were exposed to ambient air for 5 min to establish baseline values. Once calibration was completed, probes were inserted into liver parenchyma. Areas of cable egress were similarly sealed to approximate closed-system conditions.

Solution chemistries

Preservation fluid samples were obtained at onset, 2 h (mid), and termination of preservation for further analysis. Perfusate levels of Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Lactic Acid Dehydrogenase (LDH) were measured in all livers, using enzyme activity assay kits, per manufacturer's instructions (Sigma-Aldrich kits MAK052, MAK055, MAK066). Soluble CD146 (sCD146) levels, a marker of endothelial damage and permeability, were measured in the first four consecutive liver perfusate samples from each treatment arm, using porcine MCAM/CD146 Sandwich ELISA (LifeSpan Biosciences, Inc., Seattle, WA), as per manufacturer instructions. Briefly, perfusate was added to a microtiter plate pre-coated with antibody to CD146. Plates were washed and the captured sCD146 was detected by adding biotin-conjugated detection antibody followed by avidin-horseradish peroxidase (HRP)-conjugated antibody. The color was developed by adding 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate and the optical density (OD) was measured at 450 nm.

RNA extraction, sequencing, and gene expression studies

Liver biopsies were obtained at baseline, mid, and post-preservation, and samples were divided for histologic and genetic analysis. Samples fixed in 10% Formalin were processed and sent for evaluation by a single, blinded expert pathologist at our institution. Biopsies stored in RNAlater™ (ThermoFisher Scientific, Waltham, MA) were used for gene expression analysis. Total RNA was extracted using the Qiagen RNeasy Mini Kit according to the manufacturer protocol (Qiagen Inc., Valencia, CA). RNA quality was assessed using ultraviolet absorption at 260 nm/280 nm (NanoDrop Technologies, Wilmington, DE) and agarose gel electrophoresis.

Two livers were selected from the SCS, hyperoxygenated (HMP-O_2), and no air (HMP-NA) groups, to represent standard of care and our two extremes of perfusate oxygen content. Samples were chosen based on availability and presence of sufficient extracted RNA of good quality. Gene microarray expression analysis was performed by an independent laboratory. Relative gene expression was normalized to house-keeping GAPDH. Briefly, 1 μg of total RNA was reverse transcribed to cDNA using the qScript™ cDNA SuperMix first-strand synthesis system kit (Quanta Biosciences, Gaithersburg, MD). The cDNA was added to SsoAdvanced Universal SYBR Green Supermix and overlaid onto custom 96-well PCR plates (Bio-Rad, Hercules, CA). qRT-PCR was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad).

Porcine C-Reactive Protein (CRP) and Superoxide Dismutase-1 (SOD1) were chosen for further analysis based on transcripts demonstrating variability across groups. All available samples of good quality from SCS and HMP treatment arms were analyzed and compared at baseline and at termination of preservation (SCS = 4, HMP = 15, 5 from each perfusion technique). Human 18S ribosomal RNA (which is highly conserved in porcine 18s ribosomal RNA) was used as an endogenous control. Total RNA (1000 ng) from porcine liver samples was used to generate cDNA. Real-time PCR was then performed with TaqMan Gene expression kits (SOD1, Ss03373476; CRP, Ss03390889; 18s ribosomal RNA, 4310893E) and using TaqMan Universal PCR Master Mix kit. Analyses were conducted on the AB 7500 Real-Time PCR System as per the manufacturer's protocols (Applied Biosystems, Foster City, CA). All reactions were performed in triplicate and run on consecutive days. Runs from different days were included as discrete data points to account for inter-experimental variability. The Livak method for gene expression calculation was utilized.[10]

Statistical analysis

Statistical analysis was performed using SPSS v26 and GraphPad Prism v8.3.0. After proving the assumption of normality and equal variance across groups, differences were tested using Analysis of Variance (ANOVA), the two-tailed Student's t-test, and the One Sample *t*-test, unless otherwise specified. $P < 0.05$ was considered significant. For statistical analysis of outliers, ROUT and Grubbs' statistical test methods were used, utilizing a False Discovery Rate of 1%. The tests were selected to assess the probability that an aberrant value of certain magnitude (a potential outlier) would occur in the sampled population. In our analysis, we set the cut-off to an adjusted p-value of <0.01 (i.e. a p-value adjusted for selected false discovery rate). This analysis was performed for all data sets included in the study. Two outliers were identified when performing sCD146 analysis, which were excluded from final analysis.

Results

Oxygen tension in solution and liver parenchyma

Twenty-three porcine DCD livers were procured and assigned to four tested organ preservation interventions: SCS (current clinical standard of care), HMP-NA (sealed, no air), HMP-RA (room air), and HMP-O_2 (exogenous oxygen) (Image 1). Liver weights, warm ischemia times (time from slaughter to first cold flush), and cold ischemia times (time from first cold flush to termination of preservation) were similar between groups. Hypothermia was maintained throughout the 4 h of machine preservation with temperature ranges from 1.6 to 5.5 °C. Study group characteristics are summarized in Table 1. Perfusate oxygen tension was persistently and significantly higher in the "pre-charge" group (HMP-O_2) for at least 2 h of preservation compared with the three other study groups (Fig. 1, panel A). Liver tissue O_2 levels showed a similar pattern – with values significantly higher in the HMP-O_2 group compared with SCS and the HMP-RA and -NA groups, for the first 2 h of perfusion (Fig. 1, panel B). Perfusate pre-oxygenation resulted in higher tissue oxygen content at onset of preservation, and this persisted beyond 90 min of preservation without the need for O_2 supplementation. After 2 h, planned biopsy and perfusate fluid collection occurred, which intermittently disrupted the experimental systems. Sensors showed spikes in oxygen content correlating with additional sample collection, making data unreliable after this time point. Therefore, oxygenation data beyond 2 h was not analyzed.

Solution chemistries

To assess liver injury, preservation fluid was collected at different time points and evaluated for AST, ALT, LDH and soluble CD146, markers associated with hepatic and endothelial injury, as well as inflammation. ALT measured in SCS solutions vs HMP (HMP-all) showed statistically significant overall decrease within the aggregate pumped cohort ($p < 0.01$). At 4 hrs (termination of preservation), perfusate from the pre-oxygenated group showed a statistically significant drop in ALT vs SCS ($p < 0.05$, Fig. 2). When stratified by HMP intervention, HMP-O_2 resulted in the lowest ALT release throughout perfusion (data not shown). There was also a significant decrease of LDH released at termination of preservation in the HMP-all aggregate cohort vs the SCS group ($p < 0.01$). Compared to SCS, HMP-O_2 showed a significant decrease in both peak and termination of perfusion LDH release, $p < 0.05$ (Fig. 3). When compared between HMP-groups, HMP-O_2 resulted in consistently lower values throughout preservation (data not shown).

Soluble CD146 (sCD146) was compared between SCS and all HMP-preserved livers at termination of preservation; inter-pump group variability was assessed over time. Two livers (one from HMP-O_2 group and one from HMP-RA group) were ultimately excluded from final sCD146 analysis. These two organs showed values of one order of magnitude (a factor of 10) higher than all other test livers, at baseline and throughout preservation (Fig. 4A and B). These two organs also displayed the highest onset perfusion pressures within their cohorts, suggesting a baseline injury (likely during procurement) unrelated to preservation technique. After performing statistical analysis of outliers, these values were removed from final analysis. Machine perfusion resulted in significantly lower release of sCD146 vs cold storage ($p < 0.01$, Fig. 4C). When comparing HMP-O_2 with SCS directly, sCD146 levels were significantly lower at termination of perfusion, favoring the oxygenated group, $p < 0.05$ (Fig. 4, C).

RNA extraction, sequencing, and gene expression studies

Pathologic evaluation of biopsy samples obtained from all four cohorts showed normal hepatic architecture. To elucidate the genetic alterations associated with the protective effects of machine perfusion observed above, gene expression studies were performed. Microarray analysis showed variability between groups in expression of genes responsible for inflammation, cell turnover/apoptosis, as well as general and lipid metabolism (Fig. 5). C-Reactive Protein (CRP), an acute phase reactant, and Superoxide Dismutase 1 (SOD1), a reactive oxygen species (ROS) scavenger, were selected for further evaluation via the more sensitive RT-PCR technique. At termination of preservation, the machine perfused livers exposed to oxygen (both the hyperoxygenated O_2 and the room air groups) showed persistent gene downregulation of CRP relative to SCS but did not reach significance (data not shown). Relative expression of SOD1 showed an opposite pattern of upregulation. At termination of preservation, all HMP groups showed

Table 1
Study groups characteristics.

	SCS	HMP-O2	HMP-RA	HMP-NA
Test livers per group (N)	N=8	N=5	N=5	N=5
Liver weight (g)	1203 ± 122	1110 ± 207	1204 ± 113	1299 ± 60
Cold Ischemic Time (CIT, hrs)	9.8 ± 3.0	8.1 ± 0.7	8.2 ± 1.0	7.5 ± 0.5
Warm Ischemic Time (WIT, min)	15.4 ± 2.6	16.8 ± 1.3	14.8 ± 2.8	16.2 ± 1.5
Flow Rate (L/min)	NA	0.73 ± 0.14	0.79 ± 0.08	0.81 ± 0.07
HA Pressure (mmHg)	NA	35–37	37–42	36–42
PV Pressure (mmHg)	NA	10–12	12–21	14–17
Temperature (°C)				
Initial	2.9 ± 2.3	4.3 ± 0.9	4 ± 1.2	3.2 ± 0.4
2hrs	1.6 ± 1.5	5.4 ± 0.6	5.2 ± 0.6	4.5 ± 0.4
4hrs	1.6 ± 2.1	5.5 ± 0.7	4.9 ± 0.8	4.6 ± 0.4

HA, hepatic artery; PV, portal vein; SCS, static cold storage; HMP-O_2, hypothermic machine perfusion with exogenous oxygen; HMP-RA, hypothermic machine perfusion open to room air; HMP-NA, hypothermic machine perfusion with no air (sealed).

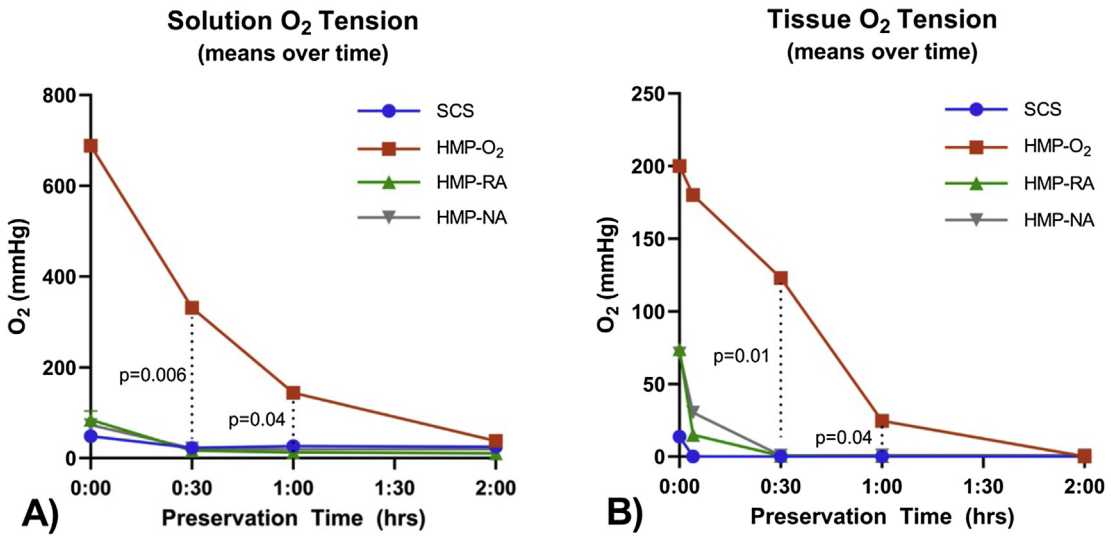

Fig. 1. Oxygen tension presented over time. Panel A: Oxygen tension (pO_2) in preservation fluid. Panel B: Porcine liver tissue oxygen tension (ptiO2). Results depicted as means. Statistical comparison between SCS and HMP-O_2 cohorts at 30- and 60-min of preservation. Student's t-test performed; $p < 0.05$ was considered significant.

consistent upregulation of SOD1 gene expression relative to SCS. These all reached statistical significance, with $p < 0.001$ for the aggregate all-pump cohort vs SCS (Fig. 6).

Discussion

Liver transplantation offers the only cure for end stage liver disease. As the number of patients awaiting transplant continues to grow, the need for suitable grafts is more pressing. In an effort to meet these demands, utilization of DCD and ECD organs is on the rise. However, these "marginal" grafts are uniquely vulnerable to preservation injury, resulting in higher rates of delayed graft function and primary non-function, as well as higher rates of biliary complications – a source of major post-transplant morbidity. *Ex vivo* machine perfusion techniques are undergoing active clinical study in an effort to optimize organ preservation. Currently, three paradigms of organ perfusion exist: normothermic, sub-normothermic, and hypothermic machine perfusion. Hypothermic machine perfusion decreases organ metabolic demands, has the added benefit of static cold storage as a failsafe in cases of machine failure, and does not require blood products or additional nutrients. Previous studies evaluating this technique have shown consistent benefits in mitigating preservation injury to liver grafts, including marginal organs, and in lowering biliary complications.[4,5,11–13] The addition of oxygen to HMP has evolved

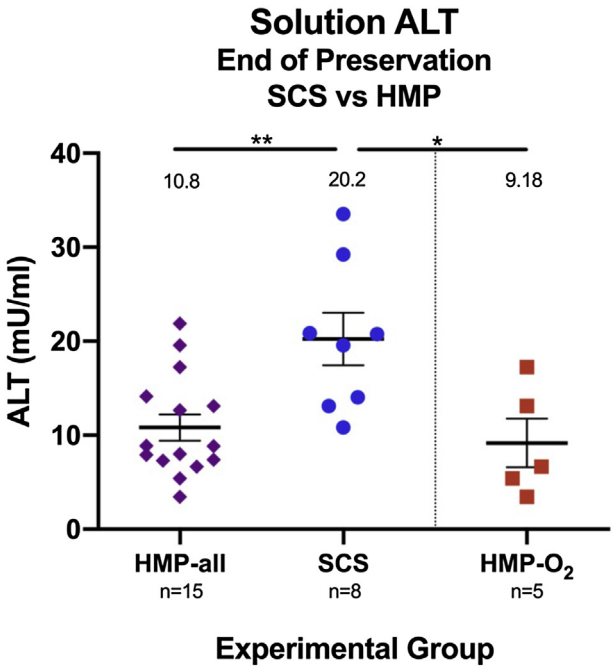

Fig. 2. Preservation solution ALT. SCS compared to HMP-all and HMP-O_2. Means are represented numerically. Bars indicate standard error. Student's t-test performed; $p < 0.05$ was considered significant. *$p < 0.05$, **$p < 0.01$,***$p < 0.001$.

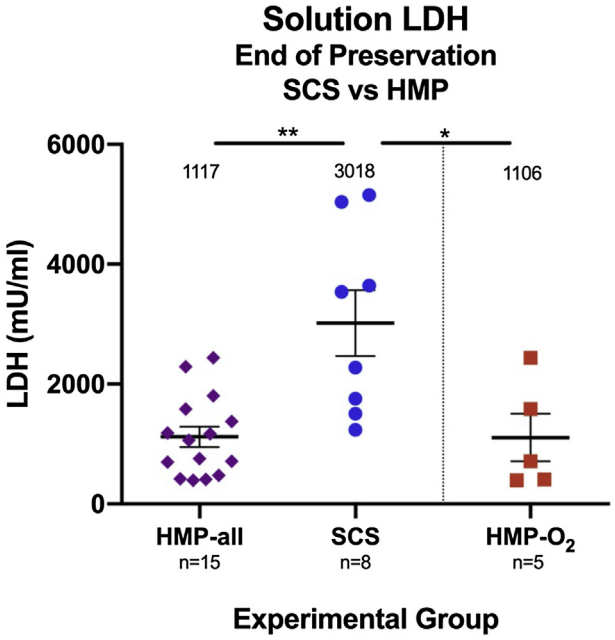

Fig. 3. Preservation solution LDH. SCS compared to HMP-all and HMP-O_2. Means are represented numerically. Bars indicate standard error. Student's t-test performed; $p < 0.05$ was considered significant. *$p < 0.05$, **$p < 0.01$,***$p < 0.001$.

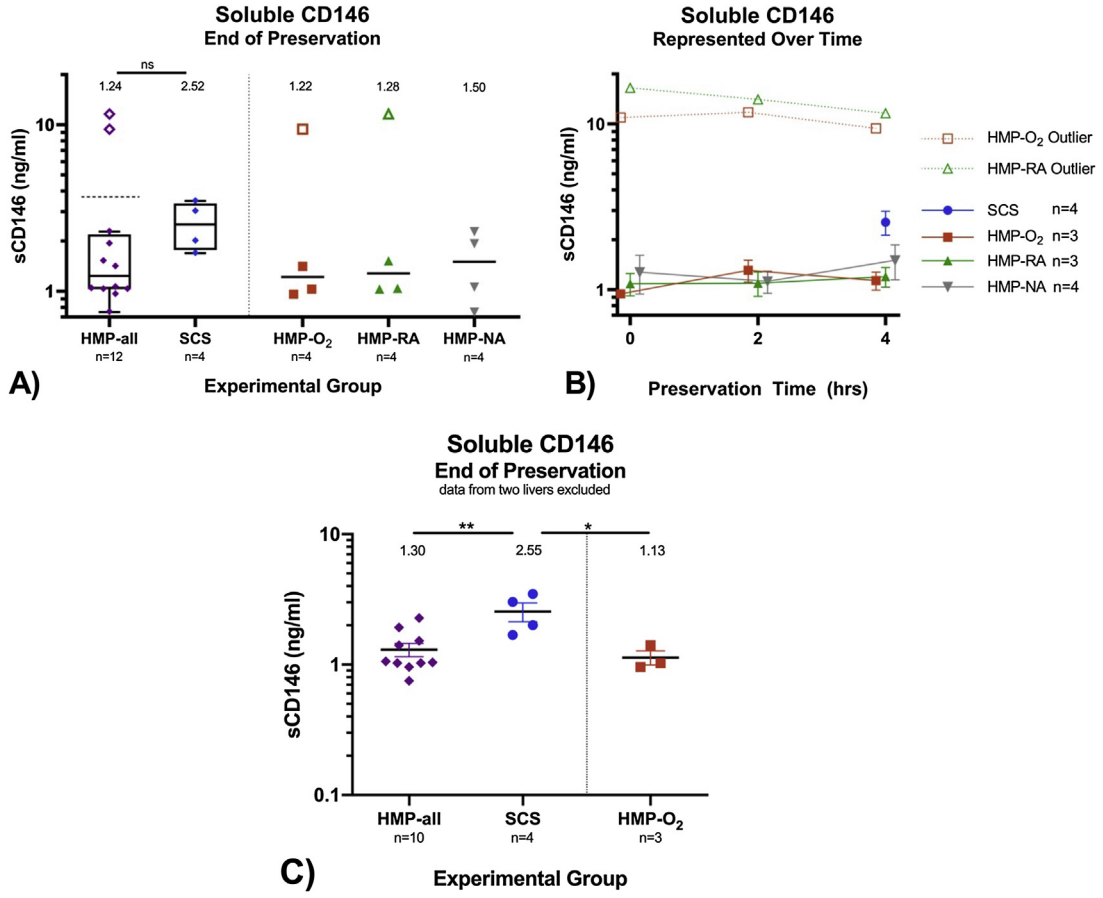

Fig. 4. Perfusate soluble CD146 level. Panel A: Tukey plot of sCD146 levels at termination of preservation: SCS vs HMP-all. Medians are represented numerically. Bars indicate inter quartile range (IQR). Horizontal dotted line represents outlier fence (1.5*IQR). Panel A: Scatter plot of inter-pump group variability. Medians are numerically represented. Panel B: sCD146 level over time, means, stratified by treatment. Panel C: Status post Prism ROUT analysis of outliers. SCS vs HMP-all vs HMP-O_2. Means are numerically represented. Bars indicate standard error. Student's t-test performed; $p < 0.05$ was considered significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

over the past decade, but the exact role of oxygenation, the optimal amount of O_2 needed, and the method of delivery are still under investigation. A portable O_2 source adds weight, complexity and regulatory hurdles to the device. Therefore, the aim of this study was to develop a simple and reproducible technique of perfusate oxygenation, without the need for a continuous, stationary oxygen source. This will allow portability and improve organ preservation

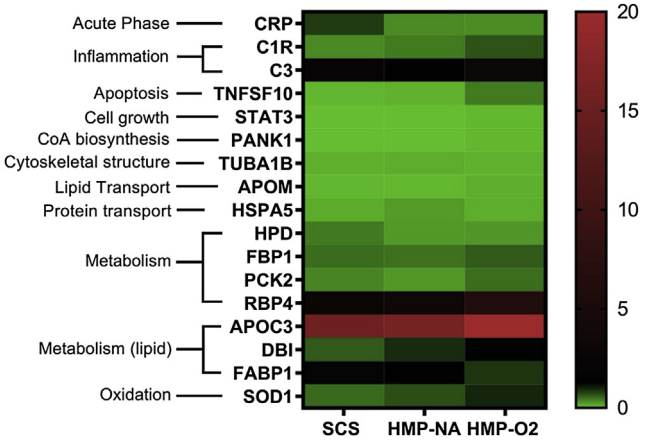

Fig. 5. Heatmap of mean gene expression relative to house-keeping GAPDH via Microarray. Two livers from each of three study groups analyzed, representing extremes of experimental conditions: SCS, No Air, and Oxygenated. Panels depict relative fold change with corresponding color scale change. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

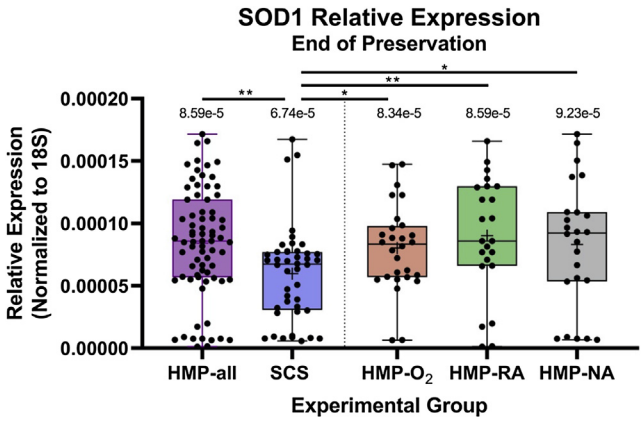

Fig. 6. RT-PCR study of Superoxide Dismutase 1 (SOD1) gene expression. Expression normalized to 18S. Data points are presented in aggregate from triplicate testing performed over several days. Treatment arms compared to SCS. Medians are numerically represented. Bars indicate interquartile range (IQR). Mann-Whitney and Kruskal-Wallis tests were performed, $p < 0.05$ was considered significant. *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$.

in transit, which is important in light of longer distances traveled to procure organs under the new liver allocation system.

Current clinical application of oxygenated hypothermic perfusion is limited to a stationary, continuous source, applied for one to 2 h during recipient hepatectomy.[5,6,11,12] The Hypothermic Oxygenated PErfusion (HOPE) system, developed by Organ Assist (Groningen, Netherlands), utilizes this paradigm, and maintains perfusate oxygen tension between 300 and 450 mmHg.[11,14] Our oxygen "pre-charge" technique results in solution O_2 tension levels of up to 700 mmHg at onset of preservation, with values as high as 150 mmHg at 90 min. Other groups have shown that liver oxygen consumption during HMP ceases after 90 min of perfusion.[3] Furthermore, 1 h of oxygenated perfusion is sufficient to restore cellular ATP and charge mitochondrial energy stores.[6] Based on these data, our technique would provide adequate levels of O_2. As this study was designed to validate this oxygenation technique, testing system integrity and exposure to ambient air were controlled. Therefore, timed hepatic venous system sampling was not performed. This limits our analysis as organ oxygen extraction rates could not be calculated. However, indwelling liver probes showed elevated tissue oxygen tension, with gradual decline over time, in our hyperoxygenated group (Fig. 1). This suggests ongoing oxygen extraction from solution and, therefore, likely utilization. As mitochondrial energy depletion in hypoxic conditions, leading to mitochondrial failure and production of toxic metabolites, has been implicated as a pivotal event in the ischemia/reperfusion cascade,[15–19] the oxygen "pre-charge" technique presents a valid alternative to continuous oxygenated perfusion.

In addition to sustained solution and tissue oxygen tension, HMP-O_2 treatment also resulted in a statistically significant decrease in liver markers of injury. Machine perfusion overall (HMP-all aggregate group) resulted in significantly lower ALT, LDH and sCD146 release in the pumped vs SCS cohort - all markers previously implicated in liver reperfusion injury.[3,17,20,21] Subgroup analysis within pumped cohort showed oxygen pre-charge by itself (HMP-O_2 vs SCS) significantly lowered these markers of injury (Figs. 2–4). Two livers (one from HMP-O_2 group and one from HMP-RA group) were initially excluded from final sCD146 data comparison due to unusually high baseline values, which persisted throughout preservation. Given an unusually high perfusion pressure noted for each of these livers, we felt that these outliers likely reflect baseline endothelial injury, which was possibly incurred at the time of procurement. Due to the nature of the procurement at the slaughterhouse, analysis and control of factors contributing to such injury were felt to be beyond the scope of this study. Statistical analysis of outliers was further performed (see Methods), which identified these two livers as outliers.

The above results suggest oxygenated machine perfusion results in less hepatocyte damage, decreased anaerobic metabolism, and preserved endothelial integrity. Even with livers showing high baseline values (outliers in sCD146 analysis), likely indicating insult prior to preservation, machine perfusion attenuated injury over time (Fig. 4B). These results are consistent with the previously cited studies of hypothermic machine perfusion and its effects on liver allograft preservation. At the genetic level, gene microarray analysis showed oxygenation results in changes in genes responsible for metabolism and cellular turnover, perhaps suggesting improved cellular homeostasis. When compared to SCS, HMP-O_2 resulted in decreased CRP and increased SOD1 gene expression. Both of these have been implicated as mediators of reperfusion injury[2,19] and perhaps differential upregulation and attenuation of their expression contributes to the benefits of HMP-O_2.

A major limitation of this study is that due to the experimental design, organ function could not be assessed post-reperfusion as this was not a transplant model. In addition, while our preservation fluid analysis yielded sufficient results to compare preservation techniques with respect to injury markers, dilutional corrections between SCS and HMP groups could not be applied as total fluid volumes differed. Our study was also limited by a small number of livers randomized to each group, which affected statistical power. Furthermore, while porcine livers are classically considered a good study surrogate for human liver allografts, there are likely confounding factors of injury which could not be accounted for due to variations in the DCD model. Finally, our results likely underestimate the degree of liver injury incurred during static cold storage, as fluid samples were obtained from the organ solution bath rather than after venous flush. This means toxic metabolites produced during storage were not sufficiently flushed and therefore available for detection. Despite this, statistically significant differences were still noted, favoring the pumped cohort.

In conclusion, oxygen "pre-charge" with 100% O_2 achieves sustained levels of oxygen tension in perfusion solution. This results in elevated tissue oxygen content, indicating likely oxygen extraction by the organ during preservation. Similar to results observed by others, we show that compared to static cold storage, hypothermic machine perfusion results in lower markers of hepatocyte injury (decreased ALT), lower markers of endothelial injury (decreased sCD146, suggesting decreased inflammatory changes and vessel permeability), and increased aerobic metabolism (lower LDH). These changes are associated with downregulated expression of acute phase inflammatory genes (CRP) and upregulated expression of ROS scavengers (SOD1), both markers of improved cellular homeostasis and priming to protect from ischemia/reperfusion injury. When comparing between different modes of hypothermic perfusion over time, these effects are most pronounced after HMP hyperoxygenation supporting the role for oxygen in addition to perfusion itself.

"Pre-charge" is an effective technique, which will allow machine pump portability and initiation of oxygenated perfusion at the donor hospital. As transplant centers become increasingly reliant on DCD and ECD grafts to meet the needs of their patients, mitigating preservation injury will be paramount. Utilizing this portable machine preservation technique will improve access to liver transplant and decrease damage otherwise sustained during organ transport.

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