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OXYGEN DELIVERY AND CONSUMPTION DURING HYPOTHERMIC OXYGENATED MACHINE PERFUSION AND THEIR IMPACT ON POST-LIVER TRANSPLANT OUTCOME

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ABSTRACT

Background

While authors recommend maintaining pO_2 >600 mmHg(80KPa) for sufficient oxygenation during liver hypothermic oxygenated machine perfusion (HOPE), recent studies have underscored the adverse relationship between increased carbon dioxide(CO_2) production and graft damage. This study aims to investigate the dynamic relationship between oxygen delivery(DO_2) and consumption(VO_2) during HOPE and assess their impact on post-transplant(LT) outcomes.

Methods

DHOPE cases performed at our Foundation were selected and divided according to perfusate pO_2 >600 mmHg(H-DO_2) and <600 mmHg(L-DO_2). PO2<600 mmHg was obtained by titration of post-liver pO2(>120 mmHg). DO2 and VO_2 were calculated using the modified Fick equation.

Results

Twentyseven transplanted livers underwent DHOPE, comprising 12 from brain-dead and 15 from cardiac-dead donors. Among these cases, 13(48.1%) were classified in the L-DO_2 group, while 14(51.9%) were in the H-DO_2 group. In L-DO_2 grafts, DO_2 measured 1.46±1.07ml/min(pO2 233±89 mmHg), with VO_2 at 0.82±0.44ml/min. In H-DO_2 grafts, DO_2 was >5.06±1.95ml/min(pO2>600 mmHg), and VO_2 was >0.56±1.14ml/min. The increase in DO_2 was directly correlated with VO_2(r=0.56; p=0.046) and both showed associations with portal flow(r=0.81, p=0.001;r=0.58;p=0.047), but portal flow was not different in the two study groups(p=0.214). Importantly, early allograft dysfunction was observed in grafts with a higher DO_2(p=0.021), but not VO_2(p=0.451). Grafts with steatosis ≤30% exhibited higher VO_2(0.933±0.216 ml/min) than those with steatosis >30%(0.594±0.233 ml/min) (p=0.038).

Conclusion

During DHOPE, elevated DO_2 may impact graft function post-LT, while steatosis may affect graft metabolic activation during HOPE. Consequently, titrating pO_2 to achieve lower DO_2, especially in grafts with high portal flow, should be considered.

Introduzione

Sebbene sia comunemente raccomandato mantenere una pO_2 >600 mmHg (80 kPa) per garantire un'adeguata ossigenazione durante la perfusione ipotermica epatica ossigenata con macchina (HOPE), studi recenti hanno evidenziato come l'aumento della produzione di anidride carbonica (CO_2) sia associato a un maggior danno del graft. Obiettivo di questo studio è analizzare la relazione dinamica tra apporto (DO_2) e consumo di ossigeno (VO_2) durante HOPE e valutarne l'impatto sugli esiti dopo trapianto di fegato (LT).

Metodi

Sono stati analizzati i casi di DHOPE eseguiti presso la nostra Fondazione, suddividendoli in due gruppi in base alla pO_2 del perfusato: >600 mmHg (H-DO_2) e <600 mmHg (L-DO_2). La pO_2 <600 mmHg è stata ottenuta mediante titolazione della pO_2 post-epatica (>120 mmHg). DO_2 e VO_2 sono stati calcolati utilizzando l'equazione di Fick modificata.

Risultati

Ventisette fegati trapiantati sono stati sottoposti a DHOPE, di cui 12 provenienti da donatori a cuore battente e 15 da donatori a cuore non battente. Tredici casi (48,1%) sono stati inclusi nel gruppo L-DO_2 e 14 (51,9%) nel gruppo H-DO_2. Nei graft L-DO_2, il DO_2 era pari a 1,46±1,07 ml/min, con una pO_2 di 233±89 mmHg, mentre il VO_2 risultava di 0,82±0,44 ml/min. Nei graft H-DO_2, il DO_2 era >5,06±1,95 ml/min (pO_2 >600 mmHg) e il VO_2 >0,56±1,14 ml/min. L'aumento del DO_2 mostrava una correlazione diretta con il VO_2 (r=0,56; p=0,046); entrambi risultavano inoltre associati al flusso portale (DO_2: r=0,81, p=0,001; VO_2: r=0,58, p=0,047), sebbene il flusso portale non differisse tra i due gruppi (p=0,214). È stata osservata una maggiore incidenza di disfunzione precoce dell'allotrapianto nei graft con DO_2 più elevato (p=0,021), mentre tale associazione non era presente per il VO_2 (p=0,451). I graft con steatosi ≤30% presentavano un VO_2 significativamente più elevato (0,933±0,216 ml/min) rispetto a quelli con steatosi >30% (0,594±0,233 ml/min; p=0,038).

Conclusioni

Durante la DHOPE, un apporto di ossigeno elevato (DO_2) può influenzare negativamente la funzione del graft nel periodo post-trapianto, mentre il grado di steatosi sembra incidere sull'attivazione metabolica del graft durante HOPE. Pertanto, la titolazione della pO_2 al fine di ottenere un DO_2 più contenuto, in particolare nei graft con elevato flusso portale, dovrebbe essere presa in considerazione.

INTRODUCTION

Organ scarcity: the challenge of ex-vivo machine perfusion

Liver disease is a major cause of mortality worldwide. According to OPTN 2019[1] the overall pretransplant mortality rate was 12.3 per 100 waiting list-years (IQR 10.8-16.1), as reported in figure 1. Besides the implementation and adaptation of allocation mechanisms, scarcity of organs still plays a dominant role for outcomes of candidates on the waiting list. Patients remain longer on the waiting list, subsequently developing progressive liver disease, resulting in higher mortality rates while waitlisted. Available resources and social circumstances have led to different ways of implementing liver transplantation (LT) around the world. The discrepancy between the number of patients waiting for a liver allograft and the available number of deceased donors forced transplant teams to explore new options to increase access to LT and decrease waitlist mortality.

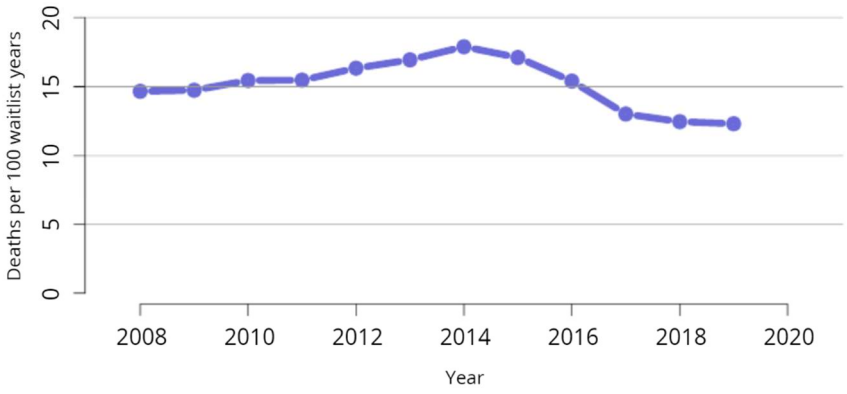

Figure 1 Overall pretransplant mortality rates among adults wait-listed for liver transplant

The introduction of the Model for End-Stage Liver Disease (MELD) in 2002 in the USA and later in Europe improved access to LT for critically ill candidates and patients with hepatocellular carcinoma.[2,3] Furthermore, the need for liver allografts has pushed programs in North America and Europe to expand deceased donor selection criteria constantly. Most of these programs routinely consider extended-criteria donors (ECD) for LT in adult recipients, such as selected grafts from older brain-dead donors (> 70 years), as well as donors with steatosis (microsteatosis and/or macrosteatosis <30%),

hepatitis C infection, central nervous system malignancies, increased risk behaviors (e.g. intravenous drug use), or hepatitis B exposure (e.g. hepatitis B core antibody positivity).[4] Elderly donors, most often defined as originating from donors above the age of 60[5], run a risk of impaired metabolic function and cellular regeneration due to a life-long exposure to hepatotoxic agents and fibrotic remodeling[6]. With regard to hepatic steatosis, it is defined by the presence of triglyceride droplets in more than 5% of hepatocytes of either small (microvesicular) or large (macrovesicular) composition[7]. While grafts affected by mild macrovesicular steatosis (<30%) are considered suitable for transplantation[8], moderate to severe steatosis was revealed as independent prognostic factor for poor postoperative outcomes[9,10]. Although the pathophysiologic mechanisms are not completely understood, these fat deposits are often a result of genetic predisposition combined with high calories intake, alcohol abuse, or old age[11,12]. Hepatic steatosis and fibrotic remodeling of the liver are hypothesized to lead to decreased metabolic function, obstructed microvascular perfusion, and a greater susceptibility of ischemia-reperfusion injury (IRI) after transplantation[13,14].

To address organ shortage and reduce waitlist mortality, efforts have focused on expanding the donor pool. As a result, according to the Organ Procurement and Transplantation Network, 7,496 deceased donor LT were registered in 2016-a 70% increase over the 4,087 deceased donor LT performed in 1996.

One strategy to increase donor pool was donation after circulatory death (DCD). The number of DCD donors has doubled in the last decade (642 [8% of 8017 total donors] in 2006 versus 1684 [17% of 9971 total donors] in 2016); only 27% (518/1884) of the DCD livers were transplanted in 2017.[15] The reluctance to transplant DCD livers stems from several initial studies that published worse outcomes, mainly attributing to ischemic cholangiopathy (IC).[16–19] Because of initial worse outcomes with DCD liver transplantation (LT), the American Society of Transplant Surgeons (ASTS) recommended practice guidelines for DCD procurement and transplantation in 2009 that emphasized limiting the use of DCD donor livers with longer ischemia times or from older donors.[16–18] Variables such as older donor age, prolonged cold ischemia time (CIT), and donor warm ischemia time (WIT) have been identified as important risk factors associated with worse outcome, particularly for ischemic cholangiopathy.[16–20] However, application of these criteria has led to underuse of ECD DCD livers.[15] A

clear trend of transplanting DCD livers from younger donors was seen and only 13% of DCD LTs were from donors>50 years of age between 2005 and 2015.

Taken together, the use of marginal donor grafts is associated with an increased risk of clinically significant postoperative complications such as early allograft dysfunction (EAD), primary nonfunction (PNF), or ischemic cholangiopathy[21]. Historically, despite much progress in transplantation, static cold storage (SCS) imposed itself as the standard technique for graft preservation after procurement, as it reduces the sequelae of ischemic injury[22,23]. Its widespread use is explained by its simplicity, low cost, and logistical practicality. Nevertheless, as the demand for liver transplantation continues to rise and the availability of ideal donor organs remains limited, the shortcomings of SCS have become increasingly evident, since extended preservation times are associated with IRI, particularly in organs from extended criteria donors and donation after circulatory death.

Ex-vivo organ perfusion represents a valuable opportunity to improve the outcomes of marginal liver grafts that would otherwise be considered unsuitable for transplantation with static cold storage (SCS) preservation, thereby reducing waitlist mortality without compromising graft quality or long-term results. Amongst currently available perfusion techniques, hypothermic machine perfusion (HMP) is an easy and low-cost approach, and does not imply a change in organ procurement or graft implantation. Particularly based on a high oxygen concentration in the perfusate, cold organ perfusion achieves an organ protection through mitochondrial reprogramming and energy recharging before implantation. Subsequently, known features of IRI are reduced. In various experimental and clinical studies, this technique was associated with better outcomes, including less posttransplant complications and better graft survival, when compared to SCS alone.

Ischemia-reperfusion injury: pathophysiology, HOPE and the role of mitochondria

The IRI-cascade represents an accumulation of various processes, that start with tissue hypoxia during warm or cold ischemia and become visible when grafts undergo reperfusion under normothermic conditions[24] . While certain protective effects occur naturally due to the impact of organ cooling and lower oxygen requirements, the following detrimental metabolic effects were also identified.

During any type of ischemia, a shift towards an anaerobic metabolism occurs, which is based on mitochondrial dysfunction with a lack of Adenosine-trisphosphate (ATP), a calcium overload and the accumulation of certain metabolites in the cell and mitochondria[25]. The lack of oxygen puts the electron flow throughout the respiratory chain on hold with two main metabolic consequences, first the lack of ATP and secondly the accumulation of NADH at a non-functional complex I. Additionally, the impaired function of the Krebs cycle leads to an accumulation of Succinate together with a Complex II dysfunction[26,27]. Of note, such metabolic changes occur invisibly and progress throughout a prolonged hypoxia at all temperatures (Figure 2). When oxygen becomes reintroduced (reperfusion) at normothermic temperatures, mitochondria immediately aim to reestablish the interrupted electron flow, which is however initially undirected and retrograde, and causes the production and release of reactive oxygen species (ROS) mainly from Complex I. Another consequence of the anaerobic metabolism during ischemia is the impaired mitochondrial calcium (Ca^{2+}) buffering capacity, which leads to elevated cytosolic Ca^{2+} levels, resulting in a higher release of produced ROS through the mitochondrial permeability transition pore (MPTP) at reperfusion. ROS molecules in turn trigger the release of further pro-inflammatory molecules grouped as danger-associated molecular patterns (DAMPs), including mitochondrial DNA and many more[28]. The immediate downstream consequence of ROS and DAMPs release appears with activation of other residential liver cells, including Kupffer- and endothelial cells with subsequent release of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-alpha) and various interleukins (IL)[29,30]. This early critical status of the newly reperfused organ creates a proinflammatory environment with additional activation of circulating recipient immune cells (e.g., neutrophils) with intercellular crosstalk through chemotaxis. Importantly, these events escalate to an ongoing inflammation and organ dysfunction right after reintroduction of oxygen. Above-described mechanisms appear significantly less when mammalian tissues undergo reperfusion with oxygen at hypothermic temperatures below the Arrhenius breakpoint of 15 degrees Celsius (Figure 2)[31].

Each additional hour of CIT is associated with a 3.4% increase in the risk of graft loss[32] and a cold storage of more than 7 hours was shown to be linked to an increased odds of prolonged recipient length of stay[33]. Importantly, the negative effect of IRI becomes more evident in grafts from ECD, which include elderly donors, prolonged

CIT of more than 12 hours, moderate to severe macrosteatosis and DCDs[34] . The use of DCD livers with a CIT of more than 6 hours is associated with a longer post-transplant hospital stay, higher rates of primary non-function (PNF), and higher serum bilirubin six months after transplantation[35] . To revert or avoid hypoxia is the overall goal of all dynamic preservation methods. The early introduction of oxygen with recirculating donor blood after initial donor WIT is practiced with *in-situ* normothermic regional perfusion (NRP), the primary method to assess and procure DCD livers in various European countries, including Italy[36,37]. *Ex-situ* normothermic liver preservation clearly reduces the overall injury when applied instead of cold storage, rather than after SCS in the recipient centre[38,39]. This is in sharp contrast to hypothermic techniques, which enable the modulation of mitochondrial metabolisms before reperfusion at warm conditions during liver implantation. Hypothermic techniques were described, more than 50 years ago, and first in context of kidney transplantation and the required time for donor-recipient type and screening. Unit now more than 700 articles were published on perfusion techniques, frequently applied after organ transport to the recipient centre. Various experimental and clinical studies have demonstrated the protective effect in all solid human organs [29,40–43].

The HyperOxygenated Perfusion (HOPE) was found to reduce IRI-associated inflammation by protecting mitochondria. Cold, dynamic preservation with a high perfusate oxygen of >60 kPa induces metabolic changes in the mitochondrial respiratory chain with steady, slow and forward electron flow without significant ROS release (Figure 2). Based on this, the Krebs cycle returns to normal function and previously accumulated succinate is metabolized together with ATP reloading at complex-V[30]. Additional effects of a homogenous, pressure-regulated HOPE appear with the clearance of catabolites and toxic molecules [44,45]. Related to the initial level of organ injury (donor quality), this mitochondrial recovery requires 90-120 min of HOPE-treatment and results in significantly reduced ROS levels at later reperfusion under normothermic conditions with less downstream inflammation and posttransplant complications[29].

Such relevant metabolic changes prepare cells of all origins for the later reintroduction of oxygen under warm conditions with lower complications and better posttransplant outcomes[46–48]. Of importance is however a high perfusate oxygen concentration during

HMP. As shown by Lazeyras et al, a partial oxygen pressure of only 20kPa (as seen in free fluids) or even 50 kPa does not induce the best possible ATP reloading[49]. Results from this kidney study were paralleled by the Minor group in livers[50]. Another parameter of importance is the perfusion duration. Mitochondria were found to recover from the ischemic insult during a 2hr HOPE-treatment[51]. Spectroscopic quantification of NADH in perfusates demonstrated the reduction of previously accumulated NADH during warm and cold ischemia within 90-120 min of HOPE, the time needed for mitochondria to switch and being prepared to undergo normothermic reperfusion at implantation[52].

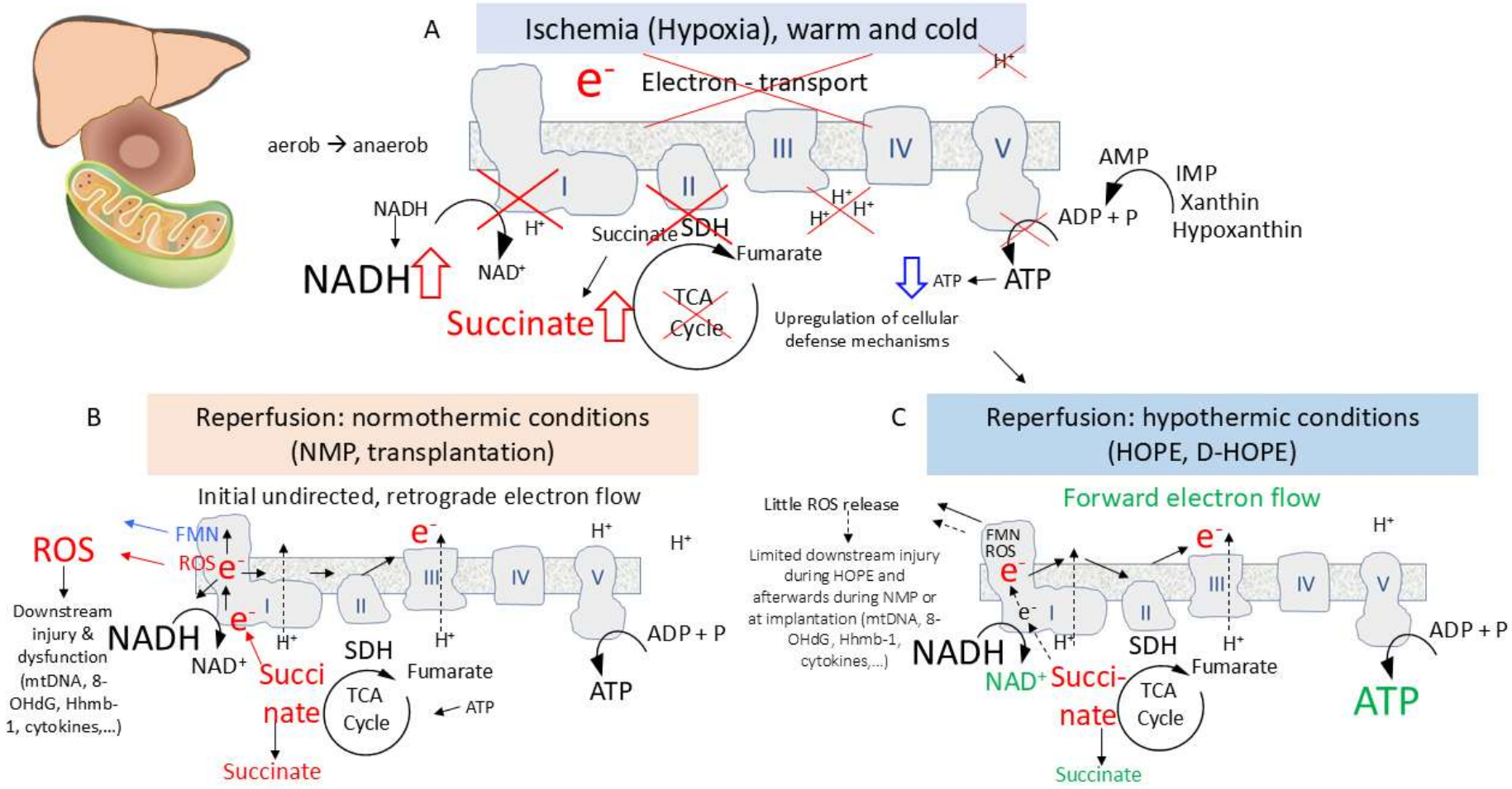

Figure 2. Mechanisms of Ischemia-Reperfusion-Injury and protection through hypothermic oxygenated perfusion of liver grafts

Beyond the immediate impact on graft quality and patient outcomes, the introduction of HMP carries broader implications for the field of transplantation. By enabling the utilization of grafts that would otherwise be discarded, such as those from elderly donors, steatotic livers, or grafts exposed to prolonged ischemia, HMP contributes to expanding the donor pool[53,54]. This is of particular importance in light of the growing discrepancy between organ demand and availability. Additionally, the reduction of post-transplant complications translates into shorter hospital stays, lower intensive care requirements, and ultimately improved cost-effectiveness. Although initial investment in perfusion devices and consumables is required, emerging studies suggest that the overall healthcare burden is mitigated by improved clinical outcomes[55].

DO2 and VO2

VO_2 and DO_2 are parameters originally derived from intensive care medicine, where they are routinely employed to assess tissue oxygen delivery and consumption. In this perspective, ex situ liver perfusion can be considered as a form of "intensive care for the organ," since the perfusion machines reproduce some principles of hemodynamic and metabolic support applied to critically ill patients. This conceptual overlap justifies the translation of intensivist concepts of tissue perfusion to the ex vivo liver setting, providing a rational framework for evaluating graft physiology and guiding optimization strategies.

During HOPE, the oxygen plays a central role in this process because it helps to maintain the cellular integrity and to preserve organ vital functions during the perfusion period. In fact, it seems that a pO2 greater than 600 mmHg (80 kPa) during HOPE is associated with adequate oxygenation of hepatic tissue[56]. DO2 represents the transport and delivery of oxygen to the graft during the perfusion process with HOPE. Ensuring adequate DO2 is essential to secure that the liver has enough oxygen to support its metabolism and vital functions postoperatively.

VO2 represents the amount of oxygen consumed by the graft and can be indirectly estimated by assessing the difference between arterial and venous partial pressure of

oxygen and carbon dioxide. During the HOPE process, the liver is perfused with an oxygen-rich solution and regenerative agents to preserve its integrity and promote functional recovery[30]. Measuring VO2 during this process can provide valuable information about the graft's ability to utilize oxygen to support its metabolic and cellular survival functions. However, no data are reported on this issue.

Conversely, recent studies have suggested that increased CO2 production during HOPE may negatively correlate with graft damage and vitality. On the other hand, the use of hyperoxic perfusion solutions in normothermic liver machine perfusion has been associated with hemodynamic instability in recipients, with reported cases of refractory vasoplegia and post-reperfusion syndrome, likely due to the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS)[57–59].

In addition, there is a growing suspicion that excessive DO_2 during HOPE may induce “hyperoxic damage.” Indeed, the very high oxygen delivery could alter mitochondrial metabolism, favoring stress pathways over repair mechanisms, indicating that supraphysiological oxygen supplementation may not be harmless.

Although ischemia and oxygenation appear to play a crucial role in graft reconditioning[53,60], the role of DO2 and VO2, as well as the minimum and maximum amount of oxygen to be administered to ensure maximum benefit and to avoid the organ damage, has not been fully investigated.

AIM OF THE STUDY

The aim of this study is to understand the interaction between DO2 and VO2 during HOPE and evaluate their impact on liver transplantation (LT) outcomes to optimize the organ reconditioning process and improve clinical outcomes.

MATERIALS AND METHODS

Study design

The present research is a retrospective study based on a prospective collected database. Consecutive HOPE cases were enrolled at IRCCS Fondazione Ca' Granda Ospedale Maggiore Policlinico di Milano from October 2022 to August 2024 and divided according to perfusate in two study groups: pO2 >600 mmHg (H-DO2) and <600 mmHg (L-DO2).

Donors and liver procurement

The grafts were allocated to our center according to the North Italian Transplant program allocation policy. The surgical team of Policlinico of Milan procured all grafts. According to the Italian law, normothermic regional perfusion was applied after death declaration and before organ procurement in all DCD donors.
It consists of abdominal extracorporeal membrane oxygenation directly applied in the donors though the placement of femoral cannulas after death declaration.

Liver Machine Perfusion and oxygen dynamics

HOPE was conducted with LiverAssist® device though portal vein and hepatic artery after static cold storage (SCS) preservation and back-table preparation.

In parallel with the back table preparation, the LiverAssist® device was set up. It allows dual perfusion via the portal vein and hepatic artery using two centrifugal pumps, delivering continuous venous flow and pulsatile arterial flow at 60 bpm. The system is pressure-controlled, enabling autoregulation of liver perfusion. For this study, the disposable circuit was primed with 4 L of cold Belzer Machine Perfusion Perfusate. Perfusion pressures were set at 25 mmHg for the hepatic artery and 5 mmHg for the portal vein, lower than physiological values to reduce shear stress on the cold endothelium Before connection, the portal vein and aortic root were cannulated with 25 Fr cannulas. The perfusate was maintained at 10 °C and oxygenated with pure O_2 through each of the two membrane oxygenators, achieving pO_2 >600mmHg or <600 mmHg, that was obtained by titration of post-liver pO2 (>120 mmHg). Titration was achieved by setting the oxygen flow to 1 L/min and mixing oxygen with air using a 20 gauge needle inserted into the oxygen connector.

DO2 and VO2 were calculated according to the modified Fick equation. The rate of oxygen transport in arterial blood is determined by cardiac output and arterial oxygen content. In our scenario, cardiac output is replaced by the sum of the portal vein flow and the arterial flow.

$$\boldsymbol{DO_2} = CO \times (1.39 \times [Hb]) \times SaO_2 + (0.003 \times PaO_2)$$

CO (Cardiac output, litres per minute) → in this context corresponds to portal + arterial flow
[Hb] (Haemoglobin concentration, grams per litre)
1.39 ml per gram = Oxygen binding capacity of haemoglobin
SaO_2 (Haemoglobin oxygen saturation expressed as a fraction, e.g. 97% = 0.97)
0.003 × PaO_2 (Amount of dissolved oxygen in the blood, in ml): for every 1 mmHg of oxygen tension, 0.003 ml of oxygen gas is dissolved in 100 ml of blood

Perfusate samples were taken every 30 minutes till HOPE end. Pre-liver samples are defined as perfusate drawn after oxygenation and before liver metabolism, whereas post-liver samples are defined as perfusate drawn after liver perfusion and before membrane lung oxygenation.

The threshold of 600mmHg to split the two cohorts was identified according to the highest detectable level of the blood gas analyzer device.

All DCD grafts were procured with normothermic regional perfusion due to the compulsory 20 minutes of no touch period imposed by the italian government for declaration of death.

LiverAssist® device is depicted in figure 3.

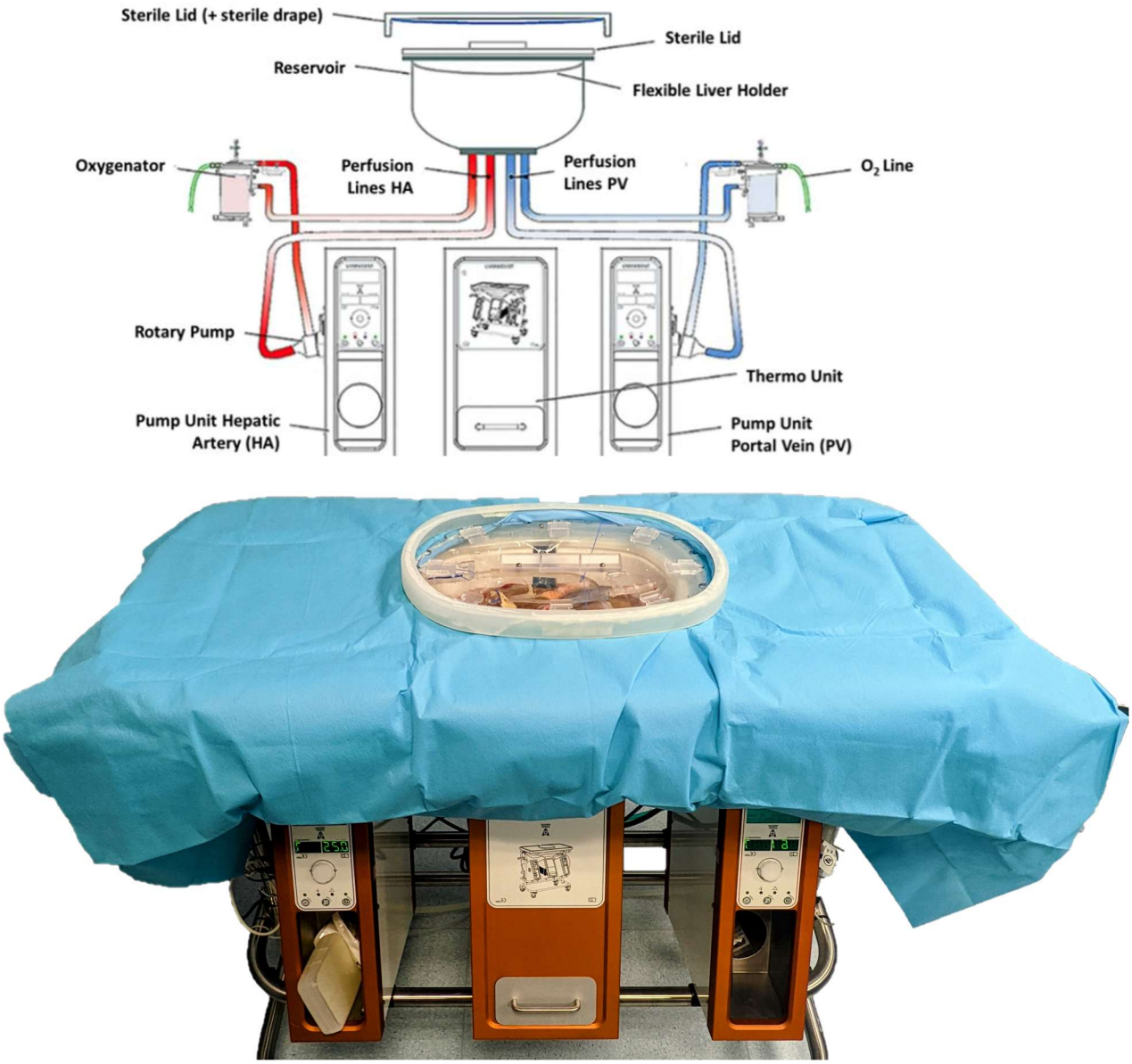

Figure 3 LiverAssist® Device for ex-vivo liver machine perfusion

VO_2 were calculated to assess metabolic activity of the liver graft. The oxygen content of the perfusate was determined both pre- and post-liver using the formula:

Content of Oxygen of pre-liver perfusate ($C_{PRE}O2$) = [1.34 x $Hb(g/dL)_{PRE\text{-}LIVER}$ x HbO2(%)+0.003 x $P_{PRE\text{-}LIVER}$O2(mmHg)

Content of Oxygen of post-liver perfusate ($C_{POST}O2$) = [1.34 x $Hb(g/dL)_{POST\text{-}LIVER}$ x HbO2(%)+0.003 x $P_{POST\text{-}LIVER}O2$(mmHg)

$\Delta_{PRE\text{-}POST}$ = $C_{PRE}O2 - C_{POST}O2$

$$VO_2 = \Delta_{PRE\text{-}POST} \text{ x Pimp flow (ml/min) x } 10$$

In this formula *Hb* is hemoglobin concentration (g/dL), $HbO_2\%$ is hemoglobin oxygen saturation, and pO_2 is the partial pressure of oxygen (mmHg). The difference between pre- and post-liver oxygen content (ΔCpre-post) multiplied by the pump flow provided VO_2, representing tissue oxygen uptake.

Perfusate Analyses

Perfusate samples were processed to obtain cell-free supernatants and donor-derived leukocyte pellets, which were analyzed to assess both biochemical and biomolecular parameters.

Standard assays included potassium (K^+), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), lactate (Lac), glucose (GLUC), and D-dimer.

In parallel, multiplex Luminex analyses were performed to quantify flavin mononucleotide (FMN), hyaluronic acid (HA), TOXILIGHT, angiopoietin-like 3 (ANGPTL3), protein C, hepatocyte growth factor (HGF), interleukin (IL)-6, and IL-10.

The ELLA system (ProteinSimple, Bio-techne, Minneapolis, MN, USA) is a microfluidics-based platform designed to perform fully automated enzyme-linked immunoassays. Operator involvement is limited to sample dilution and the loading of diluted samples together with the washing buffer into the cartridge. Each cartridge is pre-calibrated by the manufacturer, and the instrument retrieves calibration parameters via the barcode associated with the lot. Fluorescent signals are detected

within the ELLA device and quantified using master calibration curves. The complete assay requires approximately 72 minutes[61].

Additional cytokines and inflammatory mediators, including hepcidin, CXCL-9, IL-8, and tumor necrosis factor-α (TNF-α), were measured using the ELLA platform. Cell pellets were suspended in 0.25 mL 1X PBS solution (Sigma-Aldrich) and counted using an automated cell counter (Scepter, Millipore Corporation, Billerica, MA, USA). Free hemoglobin concentration was determined by applying the Allen correction to absorbance readings at 563, 577, and 600 nm with a Synergy HTX reader.

Transplantation and post-transplant care

All the grafts were flushed with 1000ml of Celsior® after disconnection and then moved to recipient table. All the perfused grafts were successfully transplanted.

Both transplantation and post-transplant care followed our clinical standard protocols. Histological samples of the liver were taken during the preservation phase and intraoperatively before abdominal closure. Patient and graft survival as well as complication rates and adverse events were measured. EAD is defined according to Olthoff criteria [62], on the presence of at least one of the following: total bilirubin≥10 mg/dL or international nationalized ratio (INR) ≥1.6 at post-operative day (POD) 7 or peak AST or ALT >2000 U/L at any time in the first seven days after transplantation.

Long-term outcomes were evaluated collecting data from all patients during their follow-up visits at one, three, six, nine and twelve months after transplantation.

Statistics

Continuous variables were reported as mean ± standard deviation and analyzed using the Student's t test or Mann-Whitney U test, according to distribution normality and variance equality. Categorical variables were presented as counts and percentages and analyzed using Fisher exact test. Effect sizes were calculated to better characterize the magnitude of differences between groups. For continuous variables, Cohen's d was computed using group means, standard deviations, and sample sizes, and interpreted as small (≈0.2), medium (≈0.5), or large (≥0.8) effects. For

categorical outcomes, effect size was assessed using Phi (Φ) coefficients, with values interpreted as small (≈0.1), medium (≈0.3), or large (≥0.5) associations. Odds ratios (OR) with 95% confidence intervals were also calculated to evaluate the relative likelihood of postoperative events between H-DO_2 and L-DO_2 groups. This approach was adopted to complement *p*-values and provide a more comprehensive assessment of both statistical and clinical relevance, consistent with current methodological recommendations in clinical research.. Data were analyzed with SPSS© version 23 (IBM Corp.). A probability value < 0.05 was considered significant.

RESULTS

Recipient, donor and graft characteristics

Twenty-seven recipients were enrolled. Recipient BMI was 26.8±5.8 with a MELD score at transplant of 14.7±8.2 and Child-Turcotte-Pugh of 7.2±1.9. Twelve livers were from deceased brain donors (DBD), whereas 15 were from death cardiac donors (DCD). Mean total CIT was 644 ± 122 minutes, whereas static cold storage (SCS) preservation before HOPE was 395±111 ml\min: functional warm ischemia time (fWIT) in DCD grafts were on average 63±15 minutes. Grafts were characterized by a macrosteatosis of 15±13%, with 4 grafts with ≥30% of macrosteatosis.

Graft during HOPE

During HOPE, no device malfunction or technical problem were recorded. All livers met the target hemodynamic parameters established, with a mean artery flow of 66±16 ml\min and portal flow of 236±84 ml\min. During dynamic preservation, temperature was always <8°C. HOPE duration was 225±39 min on average. Recipient, donor and grafts characteristics and performance during HOPE are reported in Table 1.

Recipient		**GRAFT**	
MELD	12.5±8	Macrosteatosis >30%	18% (n=4)
CTP	8±3	CIT (min)	610±140
BMI	25.6±7	SCS before HOPE (min) HOPE duration (min)	360±80 240±0
Donor		**Hemodynamics**	
DBD (n)	12	Hepatic artery flow (ml\min)	63±24
DCD (n)	15	Portal vein flow (ml\min)	225±95

Abbreviations: BMI: body mass index; CIT: cold ischemia time; CTP: Child–Turcotte–Pugh; DBD: donation after brain death; DCD: donation after circulatory death; HOPE: dual hypothermic oxygenated perfusion; MELD: Model for End-stage Liver Disease; SCS: static cold storage.

Table 1 Recipient and graft characteristics

Subgroup analysis

HOPE cases were divided into two groups according to perfusate pO2 >600 mmHg (H-DO2) and <600 mmHg (L-DO2). Of the 27 cases, 13 (48.1%) were included in the L- DO2 group and 14 (51.9%) in H-DO2. Recipient's CPT and graft macrosteatosis were statistically significant (Table 2).

	L-DO_2 (13)	**H-DO_2(14)**	**p**
Recipient			
MELD	15±7	9±7	0.06
CPT	8±1	7±2	**0.04**
BMI	25.6±4.6	25±13.6	0.09
GRAFT			
Macrosteatosis >30%, n	0	4	**0.02**
CIT (min)	592±104	635±180	0.25
SCS before HOPE (min)	355±111	360±80	0.32
Duration HOPE (min)	240±0	240±20	0.49

Table 2. Graft and recipient charasteristics between the two groups

Vascular flows

The two study groups showed comparable hemodynamic parameters in terms of vascular flows (Table 3). Hepatic artery flow was 59 ± 15 mL/min in the L-DO_2 group and 70 ± 24 mL/min in the H-DO_2 group (p = 0.49), while portal vein flow was 245 ± 84 mL/min and 210 ± 100 mL/min, respectively (p = 0.21). In L-DO2 grafts, DO2 was 1.46±1.07 ml/min (pO2 233±89 mmHg) and VO2 was 0.82±0.44 ml/min, while it was >5.06±1.95 ml/min (pO2> 600mmHg) and >0.56±1.14 ml/min in H- DO2.

	L-DO_2 (13)	**H-DO_2(14)**	***p***
Fluidodynamics			
Hepatic artery flow (ml\min)	59±15	70±24	0.49
Portal vein flow (ml\min)	245±84	210±100	0.21
DO_2(ml\min)	1.46±1.07	>5.06±1.95	**< 0.001**
VO_2 (ml\min)	0.82±0.44	>0.56±1.14	

Table 3. Hemodynamic parameters in the two groups

The increase in DO2 is directly correlated with that of VO2 (r=0.56; p=0.046) (Figure 4), and both are directly related to portal flow (r=0.81 p=0.001; r=0.58 p=0.047), but portal flow was not different in the two study groups (p=0.214).

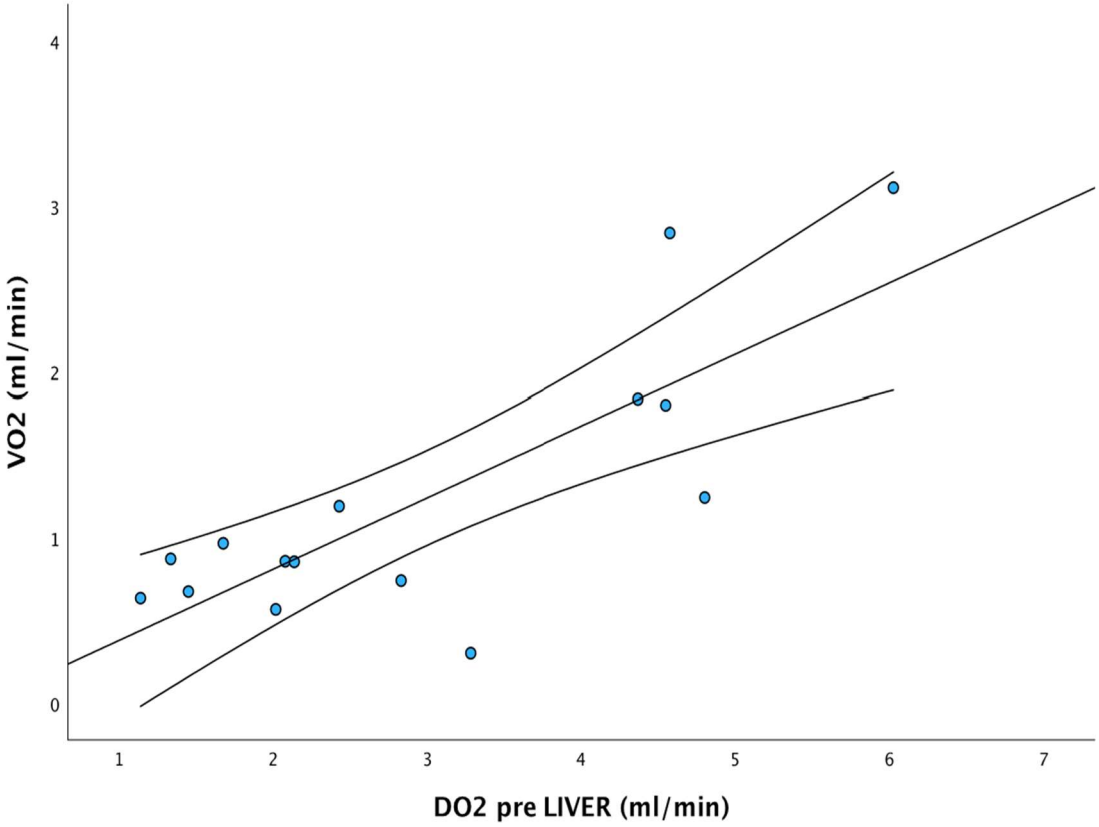

Figure 4 Correlation between DO2 and VO2 in perfused livers. A positive correlation was observed, indicating that higher oxygen delivery was associated with increased oxygen uptake by the graft

Post-transplant outcomes

No cases of primary non-function were registered. Six (22.2%) cases of EAD were reported. One (3,7%) case of hepatic artery thrombosis (HAT) occurred after 6 months form transplant, whereas no portal vein thrombosis were registered. Three (11,1%) recipients had non-anastomotic biliary stricture (NAS) and one (3,7%) case of bile leak were recorded. Five (18,5%) recipients had acute rejection within 12 month from transplant. Eight (29,6%) recipients had post-operative Clavien-Dindo (CD) grade >3a complication. One-year graft and patient survival was 100%, with no cases of retransplantation. No differences of the two groups in terms of post-LT CD >3a complications, 1-year graft and patient survival were recorded. All these data are resumed in table 4.

	Overall	**L-DO$_2$ (13)**	**H-DO$_2$(14)**	***OR***	***95% CI***	***Φ***	***p***
PNF	0	0	0	/	/	/	NS
EAD % (n)	**22.2 (6)**	**15.4 (2)**	**28.6 (4)**	**2.2**	**0.33-14.73**	**0.17**	**0.109**
PVT	0	0	0	/	/	/	NS
HAT % (n)	3.7 (1)	0	7.1 (1)	3	0.11-80-4	0.17	0.307
NAS % (n)	11.1 (3)	7.7 (1)	7.1 (1)	0.05	0.05-16.46	0.02	0.317
CD≥3a % (n)	29.6 (8)	38.5 (5)	35.7 (5)	0.19	0.19-4.24	0.04	0.615
1-year GS	100% (n=27)	100%	100%	/	/	/	NS

Table 4. Outcome of post-LT. EAD according to Olthoff criteria[62]

Abbreviations: CD ≥3a: Clavien-Dindo complication grade ≥3a; EAD: Early allograft dysfunction; GS: Graft survival; HAT: Hepatic artery thrombosis; NAS: Non-anastomotic stricture; PNF: Primary non-function; PVT: Portal vein thrombosis

Subgroup analysis showed that EAD was associated with the H-DO2 group (p=0.021), but not VO2 (p=0.45). Notably, EAD: Macrosteatosis≥30% vs <30%, p=0.95 (Table 5).

	EAD	***No EAD***	***p***	***d***
VO2 (ml\min)	1.3±1.2	1.2±0.7	0.45	0.12
DO2 (ml\min)	4.2±2.1	2.4±1.9	***0.021***	***0.93***
SCS before HOPE (min)	391±130	396±108	0.93	- 0.04
CIT (min)	639±114	645±132	0.92	- 0.05
Hepatic artery flow(ml\min)	67±15	65±17	0.79	0.12
Portal vein flow(ml\min)	230±32	227±94	0.92	0.04
Lowest MAP during LT (mmHg)	53±7	48±8	0.17	0.64

Table 5. Early allograft dysfunction

Abbreviations: CIT: Cold ischemia time; HOPE: Hypothermic oxygenated perfusion; EAD: Early allograft dysfunction; MAP: Mean arterial pressure; LT: liver transplantation; SCS: Static cold storage

Grafts with steatosis <30% had a higher VO2 than grafts with steatosis≥30% (0.933±0.216 ml/min vs 0.594±0.233 ml/min; p=0.038) (Table 6).

	Macrosteatosis<30 %	***Macrosteatosis≥30%***	***p***
VO2 (ml\min)	0.933±0.216	0.594±0.233	**0.04**
pre-DO2 (ml\min)	2.7±1.3	1.9±0.96	0.29
Hepatic artery flow(ml\min)	64±17	58±15	0.58
Portal vein flow(ml\min)	281±105	193±38	0.15

Table 6. Graft Macrosteatosis

Perfusate analysis

Perfusate analysis performed with the Luminex platform highlighted significant differences in two key hepatoprotective molecules: hepcidin and hepatocyte growth factor (HGF). Both markers were found at lower concentrations in the high oxygen delivery (H-DO_2) group compared with the low oxygen delivery (L-DO_2) group. Specifically, hepcidin levels were significantly reduced in the H-DO_2 group ($p = 0.011$), while HGF also showed a marked decrease ($p = 0.027$). Since these molecules play a protective role in liver physiology—HGF promoting hepatocyte proliferation and repair, and hepcidin modulating iron homeostasis—their lower expression under high oxygenation suggests a potential link between oxygen transport conditions and the modulation of protective biomolecular pathways in the graft (Figure 5).

Heatmap for perfusate biomarker concentrations in L-DO_2 and H-DO_2 grafts is depicted in Figure 6.

Perfusate biomarkers concentrations

	H-DO2 vs L-DO2 p
K+	0.835
AST	0.134
ALT	0.211
LDH	0.346
Lac	0.119
GLUC	0.459
D-Dimer	0.220
FMN	0.152
HA	0.603
TOXILIGHT	0.091
ANGPTL3	0.296
Protein C	0.343
HGF	**0.027***
IL-6	0.933
IL-10	0.087
Hepcidin	**0.011***
CXCL-9	0.684
IL-8	0.384
TNF-a	0.113

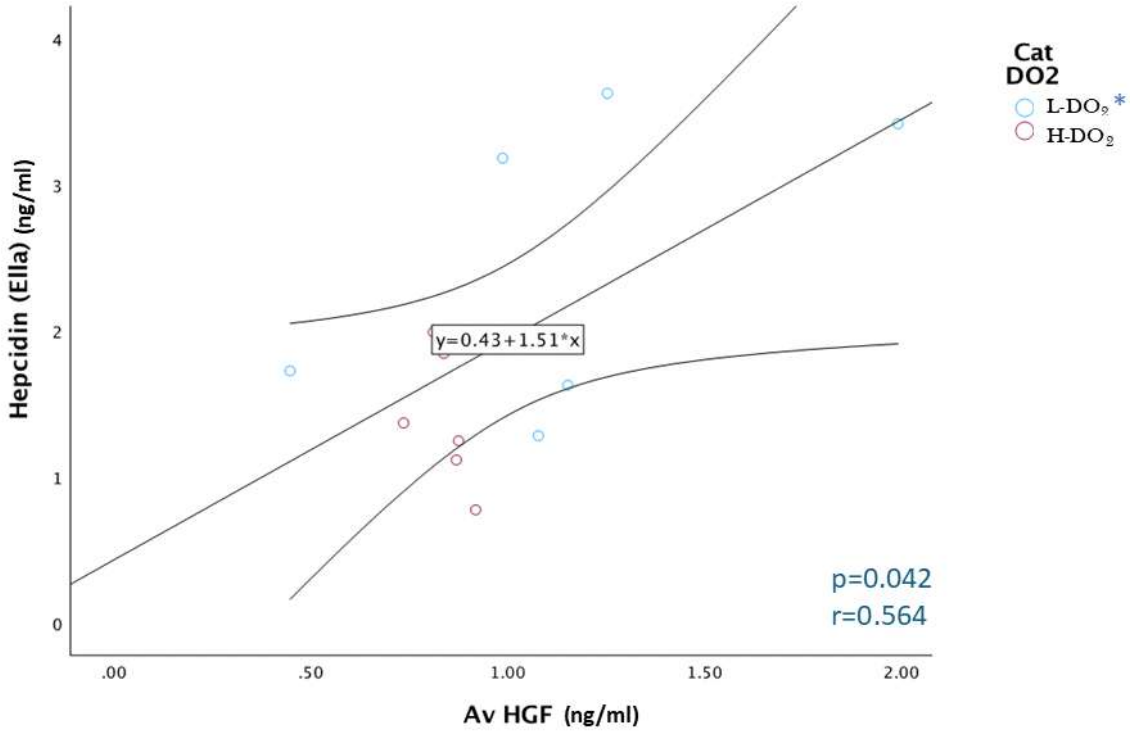

Figure 5. Perfusate biomarker analysis. Comparison between H-DO_2 and L-DO_2 groups revealed significantly lower levels of hepatocyte growth factor (HGF, p=0.027) and hepcidin (p=0.011) in the H-DO_2 group. A positive correlation between average HGF and hepcidin concentrations was observed (r = 0.564, p = 0.042).

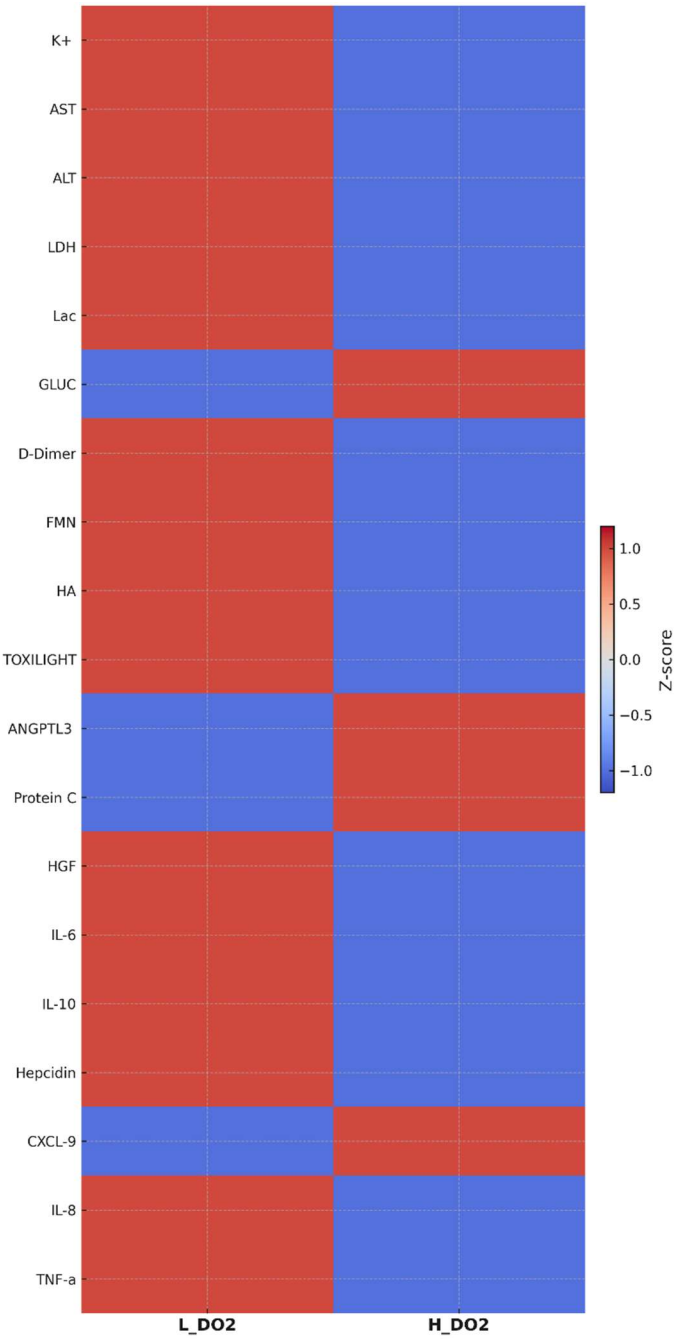

Figure 6. Heatmap for perfusate biomarker concentrations in L-DO_2 and H-DO_2 grafts. Values represent z-score–standardized mean concentrations, computed by normalizing each biomarker across the two groups. Colors reflect relative differences within each biomarker: warmer colors (red) indicate relatively higher standardized concentrations, whereas cooler colors (blue) indicate relatively lower concentrations compared with that biomarker's mean.Because each biomarker is standardized independently, the heatmap highlights directional shifts between H-DO_2 and L-DO_2, rather than absolute magnitude differences.

DISCUSSION

This study shows that moving from known DO_2 levels during HOPE, VO_2 can be calculated, providing additional quantitative parameters to assess graft metabolism. Importantly, titration of DO_2 based on perfusate pO_2 (>120 mmHg) did not significantly influence post-LT outcomes, indicating that absolute oxygen pressure alone is not a reliable target. Instead, the balance between flow and oxygen utilization appears more relevant.

We observed a positive correlation between DO_2 and VO_2, but excessive oxygen delivery did not prove universally beneficial. In particular, a high DO_2 (>2.1 mL/min) was associated with impaired early graft function, despite an increase in VO_2. This result may be due or cause a downregulation of repair mechanisms, as suggested by the reduced levels of Hep and HGF in the high-DO_2 group. Indeed, we could hypothesize that high DO_2 may lead to an increase in VO_2, resulting in an excessive metabolic activation of the graft and oxidative stress. This condition might be associated with consumption of the ATP accumulated during HOPE and a reduced activation of repair mechanisms, and the lower expression of Hep and HGF may be an expression of it. Further investigation will be conducted to solve this possible paradox.

VO_2 was also influenced by steatosis, with lower values observed in steatotic grafts, likely reflecting impaired mitochondrial activity. These findings are consistent with previous studies showing that machine perfusion improves outcomes compared with static cold storage, but excessive oxygen supplementation may exacerbate oxidative stress and reperfusion injury[63,64]. Our biomolecular results provide preliminary mechanistic support, linking hyperoxia to suppression of protective and regenerative pathways.

In this context, it is relevant to recall that the concept of DO_2 and VO_2 originates from intensive care medicine, where these parameters are widely used to monitor tissue perfusion and guide resuscitation strategies. In critically ill patients, VO_2 increases with DO_2 only up to a critical point, beyond which oxygen consumption becomes independent of delivery. Above this threshold, further oxygen supplementation does not improve tissue metabolism and may even be harmful, as

demonstrated in studies showing that targeting supranormal DO_2 failed to improve patient outcomes[65,66]. These parallels strengthen the rationale for exploring DO_2 and VO_2 in ex vivo perfusion, where the perfusion machine can be viewed as a form of intensive care for the organ.

Additional insights come from other organ perfusion models. In kidney machine perfusion, VO_2 has been correlated with post-transplant function and helps identify grafts with recovery potential[67]. In ex situ heart perfusion, myocardial oxygen consumption strongly predicts functional recovery and lactate clearance, proving superior to classical viability markers[68]. Similarly, in lung perfusion, supraphysiological oxygenation worsened oxidative stress, whereas controlled oxygen levels improved graft quality[69]. These data emphasize that oxygen metrics are not only measurable but also biologically meaningful across different organs, supporting their integration into liver perfusion research.

The study has several limitations, including its retrospective design, limited sample size, and the inherent challenge of titrating DO_2 during HOPE. Furthermore, gas analyzer machines with higher thresholds of pO_2 are currently not available, and it is therefore not possible to define a gradual scale beyond 600 mmHg. The only reference we have is the limit imposed by the analyzer itself, which was originally designed for measurements in human blood rather than in hyperoxygenated preservation solutions. The next technological step should therefore be the development of more accurate methods for analyzing hyperoxygenated perfusates, in order to avoid working with out-of-scale values and instead rely on absolute, precise measurements. This would allow a more conscious and effective modulation of the oxygen actually delivered to the graft. It should also be noted that the oxygenators used in commercially available devices are adapted from extracorporeal circulation (ECMO) systems for patients, and are thus largely oversized for a single organ, which has an effective metabolic activity of only about 3%.

Nonetheless, these data suggest that intermediate oxygen delivery levels may represent a safer and more physiologic target. The parallels with critical care and other organ perfusion systems reinforce the concept that DO_2 and VO_2 could evolve into valuable biomarkers of graft function and viability during ex vivo liver preservation. Future studies should aim to define optimal thresholds of DO_2,

integrate real-time analytic tools for perfusate oxygenation, and clarify the predictive role of these parameters in order to avoid hyperoxic damage and improve post-transplant outcomes.

CONCLUSION

This study highlights the potential of applying intensive care–derived parameters, such as DO_2 and VO_2, to the setting of ex situ liver perfusion. We observed that oxygen delivery and consumption can be reliably calculated during HOPE, providing novel insights into graft physiology. During HOPE, elevated DO_2 and the consequent increase in VO_2 may negatively impact graft function post-LT, possibly through excessive metabolic activation, ATP consumption, and reduced engagement of protective repair pathways such as those mediated by hepcidin and HGF. As a result, titrating pO_2 to achieve lower DO_2, particularly in grafts with high portal flow, should be carefully considered.

At present, DO_2 and VO_2 during HOPE remain poorly evaluated, and their predictive value for clinical outcomes requires further investigation. Future research should focus on defining the role of DO_2 and VO_2 as biomarkers of graft function and viability, while the development of new tools for titration and real-time analysis will be crucial to establish threshold values of DO_2, avoid hyperoxic damage, and refine perfusion strategies in clinical practice.

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