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MAIN TEXT

Oxygenated versus non-oxygenated flush out and storage of donor livers—An experimental study

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Abstract

Background: During donor organ procurement and subsequent static cold storage (SCS), hepatic adenosine triphosphate (ATP) levels are progressively depleted, which contributes to ischemia-reperfusion injury (IRI). We sought to investigate a simple approach to prevent ATP depletion and IRI using a porcine donation after circulatory death (DCD) liver reperfusion model.

Methods: After 30 min warm ischemia, porcine livers were flushed via the portal vein with cold (4°C) non-oxygenated University of Wisconsin (UW) preservation solution ($n = 6$, control group) or with oxygenated UW ($n = 6$, OxyFlush group). Livers were then subjected to 4 h SCS in non-oxygenated (control) or oxygenated (OxyFlush) UW, followed by 4 h normothermic reperfusion using whole blood. Hepatic ATP levels were compared, and hepatobiliary function and injury were assessed.

Results: At the end of SCS, ATP was higher in the OxyFlush group compared to controls (delta ATP of +0.26 vs. −0.68 μmol/g protein, $p = 0.04$). All livers produced bile and metabolized lactate, and there were no differences between the groups. Grafts in the OxyFlush group had lower blood glucose levels after reperfusion ($p = 0.04$). Biliary pH, glucose and bicarbonate were not different between the groups. Injury markers including liver transaminases, lactate dehydrogenase, malondialdehyde, cell-free DNA and flavin mononucleotide in the SCS solution and during reperfusion were also similar. Histological assessment of the parenchyma and bile ducts did not reveal differences between the groups.

Conclusion: Oxygenated flush out and storage of DCD porcine livers prevents ATP depletion during ischemia, but this does not seem sufficient to mitigate early signs of IRI.

KEYWORDS

ischemia-reperfusion injury, liver transplantation, organ preservation, static cold storage

1 | INTRODUCTION

The worldwide shortage of suitable donor organs for transplantation has led to increased use of grafts from donation after circulatory death (DCD) donors.[1] DCD, compared to donation after brain death (DBD) donors, undergo variable periods of hypotension and hypoxia during the agonal phase after withdrawal of life support. After circulatory arrest, a mandatory "no-touch" period follows ranging from 5–30 min according to national legislation.[2] To minimize the detrimental effects of warm ischemia, the abdominal organs are rapidly perfused with an ice-cold preservation solution to cool down core organ temperatures and minimize ischemic injury. Hereafter, grafts are typically subjected to a period of cold ischemia during static cold storage (SCS) for transportation to the recipient center.

As a result of donor warm ischemia, adenosine triphosphate (ATP) levels are progressively depleted.[3] Hepatic ATP content is further decreased during SCS, because even under hypothermic conditions (0–4°C) the organs' metabolism and oxygen demands remain around 10% of normothermia.[4,5] During ischemia, changes in the mitochondrial electron supply and ATP demand result in the accumulation of succinate, which is the primary driver of mitochondrial reactive oxygen species production and ischemia-reperfusion injury (IRI).[6] Preservation of the mitochondria is crucial to prevent IRI and the recovery of ATP correlates with mitochondrial functionality.[7,8]

In recent years, several techniques have been developed to minimize ischemic injury and/or preserve ATP of the donor organ prior to transplantation. Normothermic regional perfusion in the donor and ischemia-free liver transplantation are promising techniques to reduce IRI.[9,10] End-ischemic hypothermic oxygenated machine perfusion (HOPE) reduces cold ischemia time, improves mitochondrial function, and replenishes cellular ATP levels before transplantation by inducing a hypometabolic state whilst delivering oxygen.[8,11] However, machine perfusion is a relatively costly and time-consuming procedure requiring experienced personnel.

This study was designed to investigate a simple approach to prevent ATP depletion during liver preservation. We hypothesized that active oxygenation of the preservation fluid used for in situ donor cold flush out and SCS of donor livers prevents ATP depletion with improved hepatobiliary function after reperfusion, compared to non-oxygenated flush out and SCS preservation.

2 | MATERIALS AND METHODS

2.1 | Porcine donation after circulatory death liver procurement

An established DCD porcine liver reperfusion model was used for this study, as described previously.[12] In brief, livers from 5-month-old white female landrace pigs were retrieved after circulatory death. Pigs were sacrificed by a standardized procedure of electrocution followed by exsanguination. Two liters of autologous blood was collected in a beaker with 25 000 IU of heparin (heparin LEO 5000 IU/ml, LEO Pharmaceutical Products, Denmark). By using porcine livers from an abattoir, this study was exempt from institutional review board approval.

2.2 | Cold flush out of the liver

Livers were procured as described previously.[12] Thirty minutes after circulatory death, livers were flushed via the portal vein with 3 L of cold (4°C) University of Wisconsin (UW) cold storage solution (Bridge to Life, London, UK) supplemented with 25 000 IU of heparin. Flush out was performed either by gravity (from a height of 2 meters) using a non-oxygenated solution resembling current standard care ($n = 6$, control group) or with an oxygenated solution using a perfusion device (VitaSmart, Bridge to Life, London, UK) ($n = 6$, OxyFlush group). In the OxyFlush group, the solution was oxygenated with 100% oxygen at 1 L/min to achieve partial oxygen pressures of at least 70 kPa. The device was pressure-controlled with the pressure set to 5 mm Hg for flush out via the portal vein. The device had a flow limit of 250 ml/min, corresponding to a flush out time of 12 min for 3 L UW.

Noteworthy, the perfusion device was only used with the intention to enable the delivery of oxygen to the perfusate. If oxygenation of the perfusion fluid used for in situ cold flush improves hepatobiliary function, this method could be used clinically by adding an oxygenator between the flushing lines (for simplicity and low costs). To rule out any potential effect of the perfusion by the device in addition to oxygenation, 3 additional porcine livers were flushed using the perfusion device, but without active oxygenation of the perfusion solution (control 2).

The aorta was cannulated, and all side branches were clipped followed by cold arterial flush out with 250 ml UW solution using a syringe. All grafts underwent SCS for 4 h in non-oxygenated (control and control 2) or oxygenated (OxyFlush) UW solution.

2.3 | Normothermic reperfusion using whole blood

After SCS, livers were flushed by gravity with 1 L of cold (4°C) NaCl 0.9% solution (Baxter BV, Utrecht, The Netherlands) and 1 L of NaCl 0.9% at room temperature. Livers were then reperfused for 4 h at 37°C using 2L of heparinized autologous whole blood to simulate clinical reperfusion. Machine perfusion was performed using the Liver Assist device (Organ Assist, Groningen, and The Netherlands). Twenty milliliter of sodium bicarbonate (B. Braun Medical, Melsungen, Germany) was added before connecting the liver to reach a physiological pH. The blood was oxygenated with a carbogen mixture of 95% O_2 and 5% CO_2 at 1 L/min. The portal vein was perfused continuously with a pressure of 10 mm Hg and the hepatic artery was perfused in a pulsatile manner with a pressure of 60 mm Hg.

2.4 | Samples and biopsies

Liver parenchyma biopsies were obtained 30 min after circulatory death, after cold flush out, after SCS, and at the end of reperfusion. Bile duct biopsies were obtained 30 min after circulatory death and at the end of reperfusion. Biopsies were halved with one half being immediately frozen in liquid nitrogen and later stored in −80°, and the other half fixed in formalin and later embedded in paraffin. The first 20 ml of venous effluent during the first cold flush out was collected, as well as during the second flush out after SCS (before normothermic reperfusion). In addition, a sample from the SCS preservation fluid was collected after 4 h. During normothermic reperfusion, perfusate samples were collected prior to connecting the liver, 5 min after connection, and every hour thereafter. Arterial blood gas samples were taken prior to connecting the liver, 5 min after connection, and every hour thereafter. Partial oxygen pressure, base excess, bicarbonate, Hb, pH, glucose, lactate, sodium, and potassium levels were measured using the i-STAT clinical analyzer (Abbot Point of Care Inc, Princeton, NJ). Bile production was measured gravimetrically throughout normothermic reperfusion. Every hour, bile samples were collected and biliary pH, bicarbonate and glucose levels were analyzed using the i-STAT analyzer. Values are expressed as deltas or ratio between bile and arterial blood samples according to the literature.[13]

2.5 | Analytical procedures

Hepatic ATP content was measured as described previously.[14] Levels of ATP were expressed as micromole per gram protein corrected for baseline values (biopsy obtained after 30 min warm ischemia).

Hepatocellular injury was assessed by lactate, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) levels, using a standardized laboratory method. As an indicator of oxygen-free radical-induced tissue injury, thiobarbituric acid-reactive substances (TBARS) were measured in the perfusate and plasma with the OxiSelect TBARS assay kit (Cell Biolabs, USA). Flavin mononucleotide (FMN), a suggested marker of mitochondrial complex 1 injury, was measured in the SCS solution using fluorometric analysis.[15]

2.6 | Histological analyses

Paraffin-embedded biopsies were sectioned into slices and stained with hematoxylin and eosin (HE) and periodic acid Schiff (PAS) staining. Biliary injury was assessed using a modified scoring system as described by Op den Dries et al[16] Damage to the liver parenchyma was assessed using the Suzuki Score.[17] Scoring was conducted in a blinded fashion by two investigators (IMAB and WSvdP).

2.7 | Statistical analysis

All values are reported as mean ± standard error of the mean. Differences between the groups were tested using the Mann-Whitney U test. A p value of < 0.05 was considered statistically significant. Analyses were performed using SPSS Statistics version 25.0 (IBM, Corporation, Armonk, NY, USA) and GraphPad Prism version 9.0.1 (GraphPad Software, San Diego, CA, USA).

3 | RESULTS

3.1 | Influence of oxygenation of the flush out and SCS solution on ATP preservation

In the OxyFlush group, ATP content was higher after SCS compared to the control group (Figure 1). At the end of SCS, change in ATP content was +0.26 ± 0.28 μmol/g protein in the OxyFlush group versus −0.68 ± 0.20 μmol/g protein in the control group ($p = 0.04$). After normothermic reperfusion, ATP levels were similar in both groups.

There were no differences in ATP content between the standard control group and the additional control group 2.

3.2 | Influence of the perfusion device in addition to oxygenation

Besides equal ATP preservation, hepatobiliary function and injury markers were similar in the standard control group versus control group 2 (Figure S1A–D). From these results it may be suggested that, in addition to oxygenation, there is no effect of the perfusion device itself on outcome. The remaining part of the results will therefore be focused on the standard control group, resembling current clinical practice, versus the OxyFlush group.

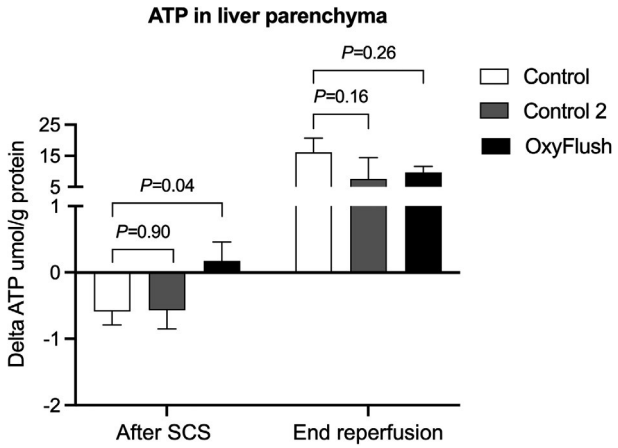

FIGURE 1 Hepatic ATP content. Levels of ATP at the end of SCS and after reperfusion corrected for baseline. Hepatic ATP was significantly higher in the OxyFlush group compared to the control group at the end of SCS. There were no differences between the standard control group and the additional control group 2 (Mann Whitney *U* test). ATP, adenosine triphosphate; SCS, static cold storage

3.3 | Hepatocellular function during normothermic reperfusion

Portal venous and arterial flows steadily increased in all livers during the first hour of normothermic reperfusion and remained stable thereafter (Figure 2A). Mean portal flow after 4 h of reperfusion was 1463 ± 75 ml/min in the OxyFlush group and 1366 ± 143 ml/min in the control group ($p = 0.28$). Mean arterial flow after 4 h of reperfusion was 417 ± 77 ml/min in the OxyFlush group and 386 ± 49 ml/min in the control group ($p = 0.31$). During normothermic reperfusion, blood pH dropped in the first hour and remained low, but stable, thereafter without further bicarbonate suppletion in all livers (Figure 2B). All livers cleared lactate and there were no differences between the groups (Figure 2C). All livers produced bile and there was no difference in cumulative bile production between the groups ($p = 0.15$) (Figure 2D). Both the peak glucose concentration after reperfusion and the total area under the curve during 4 h reperfusion were lower in the OxyFlush group compared to controls ($p = 0.04$) (Figure 2E).

3.4 | Hepatocellular injury during preservation and after reperfusion

To investigate whether the lower glucose peak in the OxyFlush group could be attributed to reduced anaerobic glycolysis, lactate concentrations were determined in the SCS solution and in the venous effluent from the backtable flush after SCS. Lactate at the end of SCS was 0.32 ± 0.08 mmol/L in the control group versus 0.28 ± 0.03 mmol/L in the

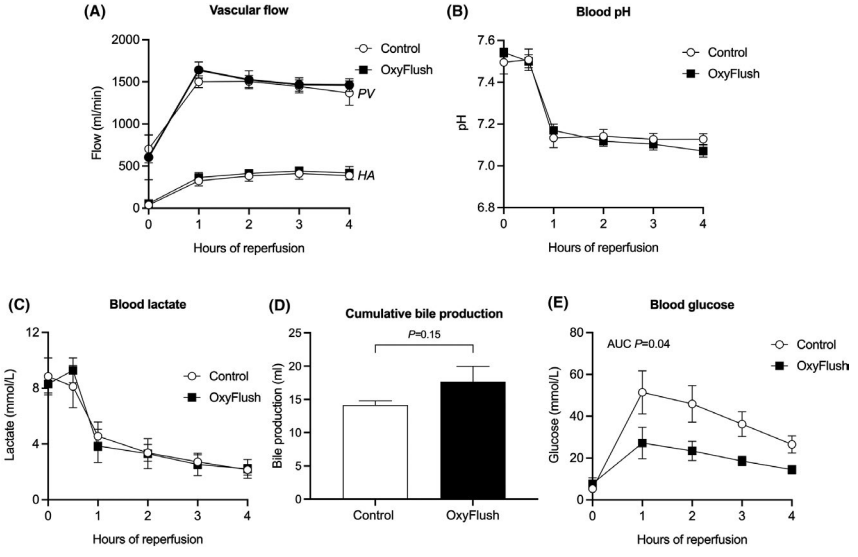

FIGURE 2 Hepatocellular function during 4 h of normothermic reperfusion. (A) flow in the portal vein and hepatic artery, (B) blood pH, (C) lactate concentration, (D) cumulative bile production, and (E) glucose levels. Glucose levels were lower in the OxyFlush group compared to controls during 4 h normothermic reperfusion. There were no significant differences in the other parameters shown (*T*-test or Mann Whitney *U* test for $n = 6$ animals per group). AUC, area under the curve; HA, hepatic artery; PV, portal vein

OxyFlush group ($p = 0.63$) (Figure 3A). Lactate in the venous effluent was significantly lower in the OxyFlush group compared to controls (0.36 ± 0.02 mmol/L vs. 0.56 ± 0.02 mmol/L, respectively, $p < 0.001$). Additionally, PAS staining was performed to assess tissue glycogen content. In both groups, PAS staining revealed extensive glycogen loss (>80% of hepatocytes) in 4/6 livers already prior to SCS (after 30 min warm ischemia) (Figure 3B).

Liver transaminases and LDH were similar in both groups at the end of SCS, in the effluent from the second backtable flush (after SCS), and at the end of reperfusion (Figure 3C–E). Levels of MDA (Figure 3F), cell-free DNA (Figure 3G) and FMN (Figure 3H) were similar between the groups at all timepoints.

3.5 | Cholangiocellular function and injury after reperfusion

Bile composition was used as a marker for cholangiocellular function. The delta between bile and arterial pH was not different between the groups at all timepoints during normothermic reperfusion (Figure 4A). Livers in the OxyFlush group had a significantly higher delta bicarbonate 2 h after reperfusion ($p = 0.03$), but this difference was not sustained thereafter (Figure 4B). The ratio between biliary glucose and arterial glucose was not different between the groups (Figure 4C). As a marker for bile duct injury, no differences in biliary LDH were observed. At the end of reperfusion, LDH was 1552 ± 623 U/L in the control group versus 1144 ± 628 U/L in the OxyFlush group, $p = 0.20$ (Figure 4D).

3.6 | Liver parenchyma and bile duct histology

Histological assessment of the bile duct and liver parenchyma after HE staining did not reveal major differences between the groups. At baseline (30 min warm ischemia), all bile ducts showed >50% epithelial loss (Figure 5A). At the end of reperfusion, there was extensive damage of

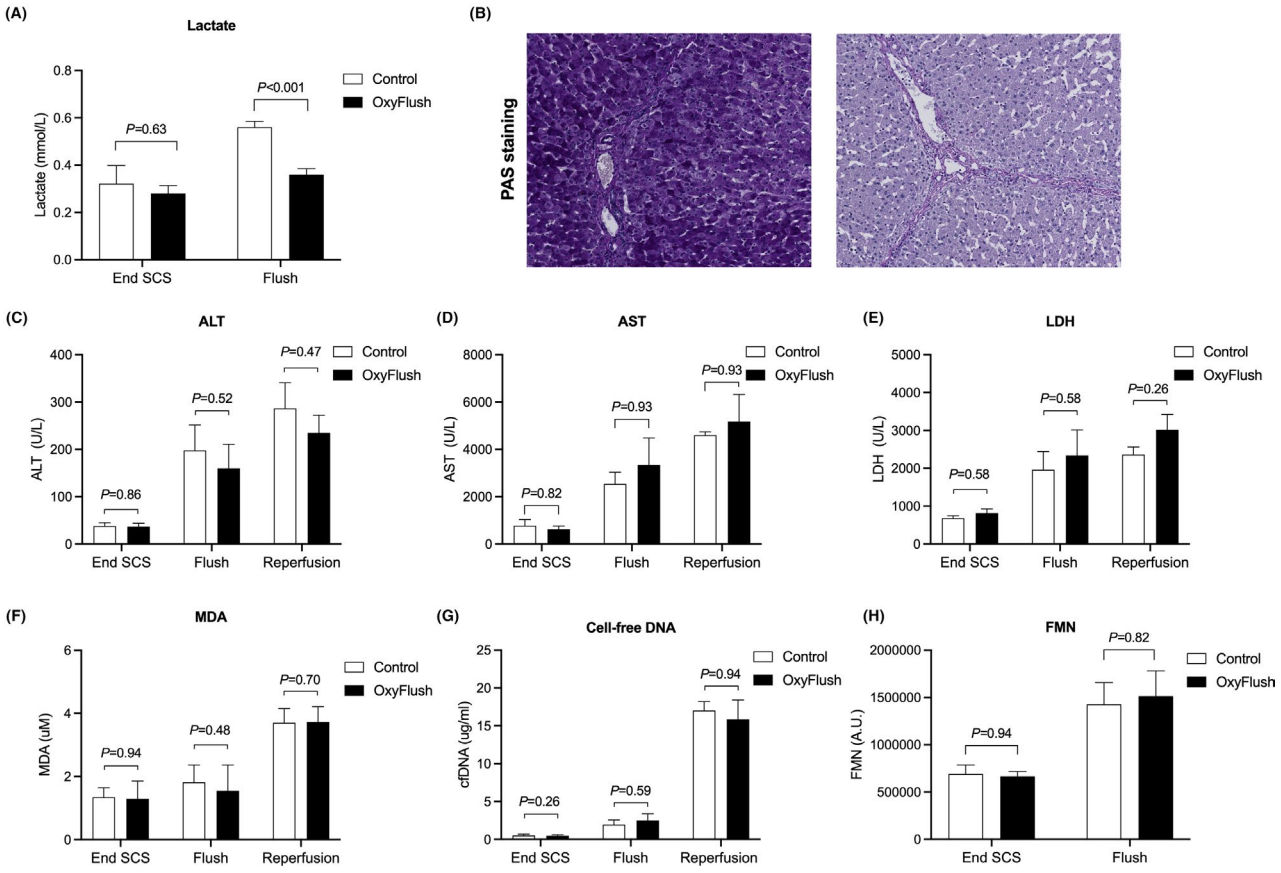

FIGURE 3 Markers for anaerobic glycolysis and cellular injury. (A) Lactate concentration in the preservation solution at the end of SCS and in the venous effluent from the second backtable flush. (B) PAS staining with glycogen deposits stained purple, (C) ALT, (D) AST, (E) LDH, (F) MDA, (G) cell-free DNA, and (H) FMN at the end of SCS, in the venous effluent from the second backtable flush, and at the end of reperfusion. There were no significant differences between the groups (Mann Whitney *U* test for $n = 6$ animals per group). ALT, alanine aminotransferase; AST, aspartate aminotransferase; FMN, flavin mononucleotide; LDH, lactate dehydrogenase; MDA, malondialdehyde

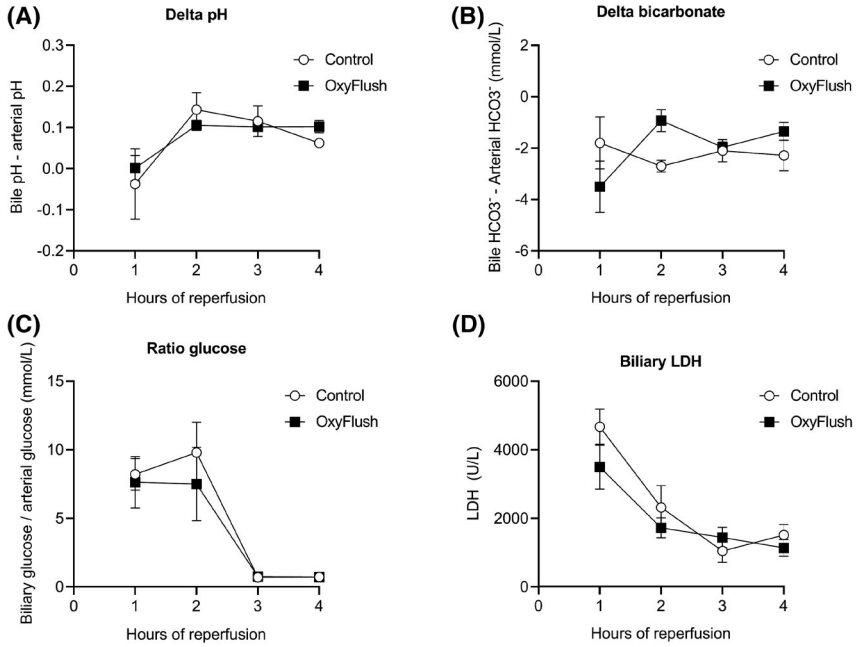

FIGURE 4 Cholangiocellular function during 4 h of normothermic reperfusion. (A) Delta pH, (B) delta bicarbonate, (C) glucose ratio, and (D) LDH in bile during normothermic reperfusion. There were no differences between the groups, except for a higher delta bicarbonate in the OxyFlush group 2 h after reperfusion ($p = 0.03$) (Mann Whitney U test for $n = 6$ animals per group). LDH, lactate dehydrogenase

the mural stroma and periluminal peribiliary glands in all bile duct biopsies (Figure 5B). Areas of necrosis of the liver parenchyma were increased after SCS compared to baseline in all livers (Figure 5C–F). In addition, congestion and vacuolization became more apparent at the end of reperfusion in both groups. There was no difference in Suzuki scores between the groups (4.4 ± 1.3 for controls vs. 4.2 ± 1.9 for OxyFlush, $p = 0.76$).

4 | DISCUSSION

In this study, we have demonstrated that oxygenation of the in situ donor cold flush out and SCS solution enhanced ATP preservation during SCS of DCD porcine livers. However, this did not improve hepatobiliary function nor reduced injury after reperfusion.

Rapid cooling at the onset of ischemia remains the cornerstone of liver preservation, and in situ flushing of donor organs with cold UW solution has become a widely used procedure. During ischemia, there is still continuous consumption of ATP for essential cell functions. In the absence of oxygen there is no ATP production by oxidative phosphorylation, but, instead, anaerobic glycolysis is essential to maintain a sufficient ATP:ADP ratio for cell viability.[3] Due to the absence of blood flow during ischemia, the glucose required to drive glycolysis cannot be provided from the circulation, but comes from the breakdown of hepatic glycogen stores.[18] Increased glycogenolysis during preservation and hyperclycemia after reperfusion have been observed when cold ischemia times are extended.[19,20] Moreover, lower post-reperfusion glucose levels have been observed after preservation by HOPE compared to SCS. In the present study, glucose was lower after reperfusion of livers that were flushed with and stored in an oxygenated solution, suggesting reduced anaerobic glycolysis in this group compared to non-oxygenated controls. Lactate, the primary end product of glycolysis, was also lower in the venous effluent from the backtable flush after SCS in the oxygenated group. In both groups, livers were already severely glycogen depleted prior to SCS, which prevented us from drawing any conclusions on the extend of glycogenolysis during cold storage.

Key to IRI is the buildup of succinate during ischemia, which is rapidly oxidized after reperfusion, driving reverse electron transport at mitochondrial complex I.[6] This can cause extensive damage to the mitochondria and lead to cell death. Based on the present study, oxygenation of the solution used for flush out and SCS does not seem sufficient to prevent mitochondrial damage during ischemia. Levels of FMN, a marker of mitochondrial complex 1 injury, were similar in both groups. In addition, none of the injury markers measured in this study (i.e., ALT, AST, cell-free DNA, MDA, and histological signs of necrosis) were reduced after oxygenated flush out and SCS.

Oxygenation of the in situ donor cold flush only marginally enhanced ATP levels compared to non-oxygenated controls, which could explain why we failed to observe a reduction in hepatobiliary injury after reperfusion. End-ischemic preservation by HOPE, on the other hand, is able to restore ATP content by multifold.[21] The limited effect of an oxygenated flush on ATP could partly be explained by the short duration of oxygenation (only during several minutes of in situ donor flush compared with 1–2 h HOPE). Theoretically, the flush out time can be prolonged to enhance the effect of

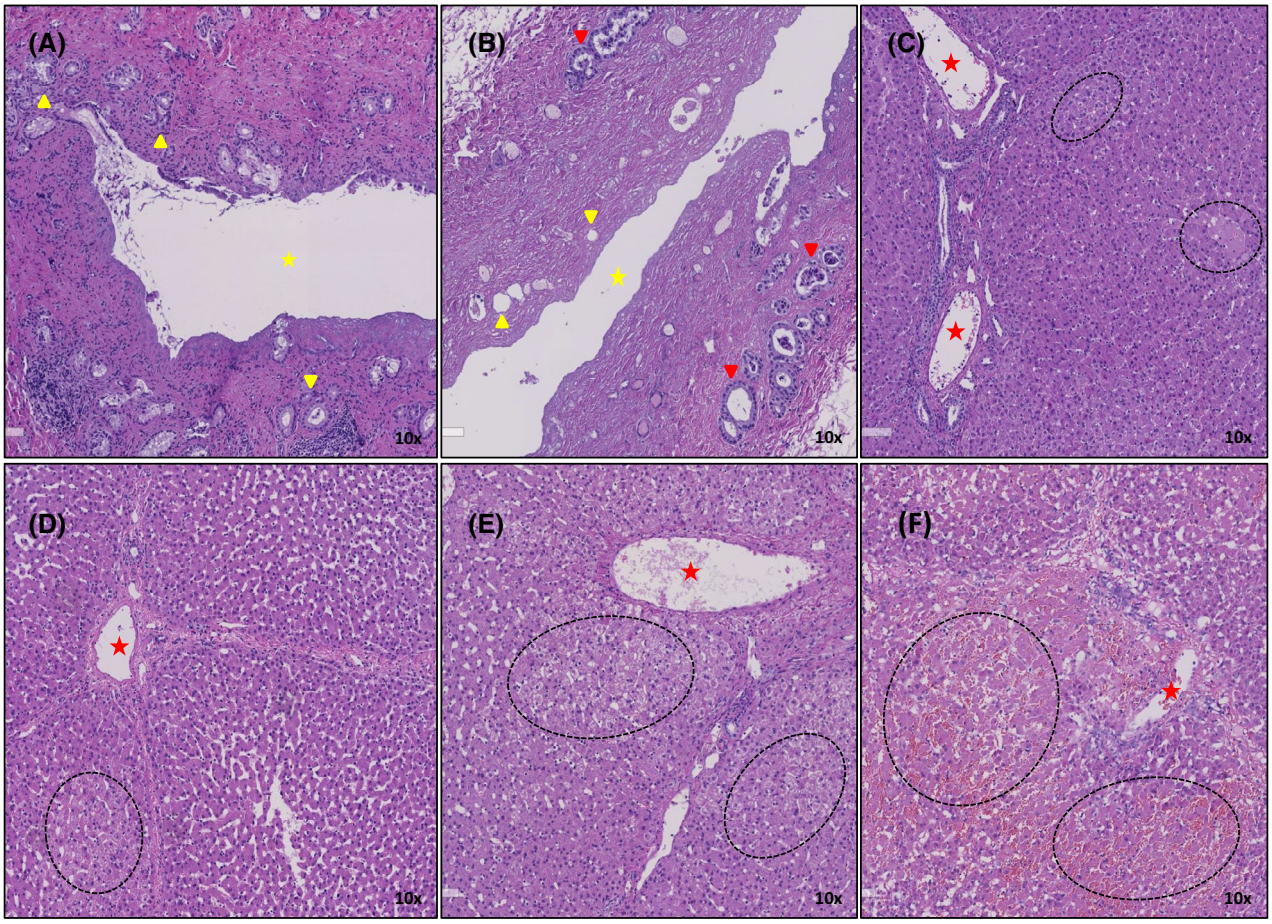

FIGURE 5 Bile duct and liver parenchyma histology. Representative example of a bile duct obtained (A) 30 min after circulatory arrest and (B) at the end of normothermic reperfusion. There were no differences between the groups regarding histological bile duct injury. Representative example of liver parenchyma obtained (C) 30 min after circulatory arrest, (D) after cold flush out, (E) at the end of static cold storage, and (F) at the end of normothermic reperfusion. There were no differences between the groups. Yellow asterisk: bile duct lumen, yellow arrowhead: luminal peribiliary glands, red arrowhead: deep peribiliary glands, red asterisk: portal vein, dashed circle: necrotic area

an oxygenated flush. However, because it is an open system without recirculation of the perfusate, several additional liters of preservation solution would be required, which raises costs.

Another, potentially more beneficial, approach could be to perform an oxygenated flush after SCS, prior to reperfusion. A study by Tamaki et al showed that an end-ischemic cold flush with molecular hydrogen added to the preservation solution protected liver grafts from IRI.[22] The authors also found that flushing through the hepatic artery was superior in reducing biliary damage compared to flushing through the portal vein.

Alternative strategies to prevent ATP depletion during SCS include the addition of oxygen carriers to the SCS solution or gaseous oxygen insufflation. For example, the addition of perfluorocarbon (a high-capacity oxygen-binding compound) improved ATP preservation and reduced the severity of ischemic tissue damage in DCD rat livers.[23] Similar results were obtained in pancreas preservation.[24,25] Studies in rat livers show that adding the biological oxygen carrier M101 to the SCS solution prevents ATP depletion and attenuates IRI compared with grafts preserved by SCS without an oxygen carrier.[26,27] Minor et al have extensively studied the effects of gaseous insufflation, which includes the delivery of oxygen via a catheter in the caval vein during several hours of SCS preservation.[28–30] After preclinical studies, results from a prospective clinical trial demonstrated a positive effect of gaseous insufflation on the development of early allograft dysfunction.[31]

Although this study provides novel insights on optimizing liver preservation techniques, there are several limitations. Firstly, the physiology of electrocution and exsanguination of animals at the abattoir is not completely equivalent to a donor with actual cardiac arrest. It is likely that these animals experience more acute stress and that the process causes extracellular damage or microcirculatory collapse. Secondly, we did not confirm our findings in a transplantation model, but in a previously

described ex situ 4-h reperfusion model using whole blood.[12] Noteworthy, clinical studies have shown that hepatobiliary viability assessment during 2.5 h NMP adequately predicts graft function after transplantation.[13] Lastly, the duration of SCS in our model was relatively short. However, previous studies have used similar SCS durations, because the pig liver is a very rigorous model of organ preservation with maximum successful SCS preservation times that are substantially shorter than those achieved in clinical practice.[12,32–34] Although histological analysis revealed significant injury, possible benefits of oxygenated cold flush could be more prominent in a setting with increased ischemic injury.

In this study, we investigated a simple approach to prevent ATP depletion of donor livers during preservation. The results show that oxygenation of the in situ donor cold flush and SCS solution marginally enhances ATP levels prior to reperfusion of DCD porcine livers, but without a reduction in early reperfusion injury.

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CONFLICT OF INTEREST

Nothing to declare.

AUTHOR CONTRIBUTIONS

Isabel M. A. Brüggenwirth, Otto B. van Leeuwen, Michel Rayar, Vincent E. de Meijer, Robert J. Porte designed the study; Isabel M. A. Brüggenwirth, Willemijn S. van der Plas, Otto B. van Leeuwen, Adam M. Thorne, Michel Rayar performed the experiments; Isabel M. A. Brüggenwirth and Willemijn S. van der Plas performed laboratory and data analyses; Isabel M. A. Brüggenwirth, Michel Rayar, Vincent E. de Meijer, Robert J. Porte interpreted the data; Isabel M. A. Brüggenwirth wrote the manuscript, Willemijn S. van der Plas, Otto B. van Leeuwen, Adam M. Thorne, Michel Rayar, Vincent E. de Meijer, Robert J. Porte critically revised the manuscript. All authors approved the final version of the manuscript.

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