Strategies to Improve Outcome after Transplantation of Extended Criteria Donor Livers

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Oxygenated Hypothermic Machine Perfusion after Static Cold Storage Improves Hepatobiliary Function of Extended Criteria Donor Livers

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Abstract

**Background:** The mechanism through which oxygenated hypothermic machine perfusion (HMP) improves viability of human extended criteria donor (ECD) livers is not well known. Aim of this study was to examine the benefits of oxygenated HMP after static cold storage (SCS).

**Methods:** Eighteen ECD livers that were declined for transplantation underwent ex situ viability testing using normothermic (37°C) machine perfusion (NMP) after traditional SCS (0-4°C) for 7-9 hours. In the intervention group (n = 6), livers underwent 2 hours of oxygenated HMP (at 12°C) after SCS and before NMP. Twelve control livers underwent NMP without oxygenated HMP after SCS.

**Results:** During HMP, hepatic ATP content increased >15-fold and levels remained significantly higher during the first 4 hours of NMP in the HMP group, compared to controls. Cumulative bile production, as well as biliary secretion of bilirubin and bicarbonate, was significantly higher after HMP, compared to controls. In addition, the levels of lactate and glucose were less elevated after HMP compared to SCS preservation alone. In contrast, there were no differences in levels of hepatobiliary injury markers such as AST, ALT, LDH, and gamma-GT after 6 hours of NMP. Hepatic histology at baseline and after 6 hours of NMP revealed no differences in the amount of ischemic necrosis between both groups.

**Conclusion:** Two hours of oxygenated HMP after traditional SCS restores hepatic ATP levels and improves hepatobiliary function, but does not reduce (pre-existing) hepatobiliary injury in ECD livers.
Introduction

One of the greatest challenges in liver transplantation is the shortage of suitable donor livers. Today, many patients are waiting in vain for a liver transplant, which is often the only successful treatment of their liver failure (1). One way to minimize the shortage of liver donor grafts for transplantation is the use of extended criteria donor (ECD) livers. However, ECD livers are in general associated with a higher postoperative risk of early graft failure, compared to livers from optimal or reference donors (2). Because of this, many ECD livers are declined for transplantation and an important question is how these livers can be functionally improved and made suitable for transplantation.

During the last decade, machine perfusion (MP) has been receiving increasing attention as a promising method to reduce preservation injury, compared to the traditional method of static cold storage (SCS) (3-5). A short period of MP after SCS (end-ischemic MP) has been suggested to reduce the risk of early graft dysfunction after transplantation (6). In particular ECD livers, which have a higher risk of early graft failure due to the pre-existing hepatocellular injury and dysfunction, could potentially benefit from end-ischemic MP during organ preservation (6).

End-ischemic MP can be performed at different temperatures. Hypothermic machine perfusion (HMP; perfusion at 4-12 °C), has recently made a first step into clinical practice. Guerrera et al. (7,8) and Dutkowski et al. (9) have described successful application of end-ischemic MP of ECD livers in human liver transplantation. In these studies, livers that first underwent end-ischemic HMP displayed better graft function compared to matched control livers that did not undergo end-ischemic HMP. In addition, the incidence of ischemic cholangiopathy, also known as non-anastomotic biliary strictures (NAS), was lower in the group with end-ischemic HMP treatment (8,9). Although these results are very promising, knowledge about the mechanisms through which end-ischemic HMP may improve liver and biliary function of human ECD liver grafts is limited. The aim of the current study was therefore to assess whether end-ischemic dual (combined arterial and portal) oxygenated hypothermic machine perfusion (HMP) improves hepatobiliary function and reduces preservation injury of human ECD livers, compared to traditional SCS preservation alone. In the current study, hepatobiliary viability after traditional SCS preservation or after SCS followed by 2 hours of HMP was assessed during ex situ normothermic machine perfusion (NMP) for 6 hours.
**Materials and methods**

**Donor Livers**
Between August 2012 and August 2014 eighteen consecutive full size human donor livers were included in this study. All livers were declined for transplantation within the Eurotransplant region. The two main reasons for declining were: 1) the combination of donation after circulatory death (DCD) with advanced donor age; and 2) the combination of DCD or donation after brain death (DBD) and a high body mass index (suspicion of graft steatosis). Informed consent for research was obtained from the donor families. The study protocol was approved by the medical ethical committee of the University Medical Center Groningen (METc 2012.068) and the competent authority for organ donation in the Netherlands, the Dutch Transplantation Foundation (NTS). The Eurotransplant donor risk index (ET-DRI) was calculated for all livers using the method described by Braat et al. (10). During the study period, the first 12 ECD livers were included in the SCS only group, which consisted of NMP viability assessment after traditional SCS preservation. Subsequently, 6 other ECD livers were included in the HMP group. These liver grafts were exposed to 2 hours of HMP after the period of traditional SCS preservation. After HMP these livers underwent 6 hours of NMP for viability assessment (Figure 1 and 2).

**Procurement and Preparation of Donor Liver Grafts**
All donor livers were procured by one of the regional multi-organ procurement teams in the Netherlands using the standard technique of in situ cooling and flush out with preservation fluid (University of Wisconsin [UW] or histidine–tryptophan–ketoglutarate solution [HTK]) at 0-4 degrees Celsius. In case of DBD, 25,000 units of heparin were administered intravenously before cross clamping of the supratruncal aorta. In DCD cases, the surgical procedure was not started until after 5 minutes “no touch” period following declaration of circulatory death. During

![Figure 1: Schematic overview of the study groups to examine the effects of end-ischemic oxygenated hypothermic machine perfusion (HMP) after static cold storage (SCS). The group that underwent end-ischemic HMP contained 6 livers. The SCS only group contained 12 livers. In both groups ex situ normothermic machine perfusion (NMP) was used for viability testing for 6 hours.](image-url)
Figure 2: Photos of the liver during machine perfusion (MP) experiments. Panel A: In a bowl with melting ice and preservation solution, the liver is prepared for oxygenated hypothermic machine perfusion (HMP) or normothermic machine perfusion (NMP). Panel B: Liver perfused with HMP. Panel C: Liver perfused for 30 minutes with NMP. Panel D: Liver perfused for 6 hours with NMP. During MP, the organ chamber is covered with a transparent lid to prevent heat loss and to maintain a moist environment.

the flush out procedure, the same dose of heparin (25,000 units of heparin) was added to the first bag of preservation solution. All livers were packed in ice-cold preservation solution (UW or HTK), stored on ice, and transported to our center. Upon arrival in our center, an experienced transplant surgeon performed the back table preparation. The portal vein, the supratruncal aorta, and the bile duct were cannulated and the graft was prepared for oxygenated machine perfusion as described previously (11-13).

Machine Perfusion
HMP and NMP were performed using a machine perfusion device that includes two perfusion circuits, each comprising a rotary perfusion pump, a membrane oxygenator with integrated heat exchanger and pressure sensor for perfusion of the portal vein and hepatic artery, respectively (Liver Assist, Organ Assist, Groningen, the Netherlands). Flow through the hepatic artery was pulsatile, whereas flow through the portal vein was continuous. The system was both pressure and temperature controlled. During HMP, portal pressure was set at 4 mmHg, mean arterial pressure at 25 mmHg, and temperature of the perfusion fluid was set at
10-12°C, as described previously (11-13). The solution was oxygenated with 100% oxygen (1 L/min) resulting in a pO₂ between 50-80 kPa (375-600 mmHg). During NMP (37°C), the portal pressure was 11 mmHg and mean arterial pressure was 70 mmHg. In contrast to HMP, we oxygenated during NMP with a carbogen mixture of 95% O₂ and 5% CO₂ (2 L/min), resulting in a pO₂ between 50-80 kPa (375-600 mmHg). During HMP, livers were perfused with 3 L of Belzer-UW Machine Perfusion solution (Bridge-to-Life, Ltd. Northbrook, IL, USA). After 2 hours of HMP the liver graft was disconnected from the perfusion device, placed in a bowl with cold (0-4°C) UW cold storage solution (Bridge-to-Life, Ltd. Northbrook, IL, USA), in order to change the perfusion fluid and prime the perfusion device for NMP. Before NMP, the liver was flushed with 1 L cold NaCl 0.9% solution, followed by 500 mL warm NaCl 0.9% solution. Livers were perfused normothermically (37°C) for 6 hours with a perfusion solution based on heparinized human plasma and red blood cells enriched with nutrients, trace elements and antibiotics, as described previously (11-13). Before NMP and every 30 minutes during NMP samples from the perfusion fluid were taken for analysis of the blood gas parameters (pO₂, pCO₂, sO₂, HCO₃⁻, and pH) using an ABL800 FLEX analyzer (Radiometer, Brønhøj, Denmark). If needed, small amounts of sodium bicarbonate (8.4% solution) were added to maintain a pH within the physiological range (7.35-7.45), as been described previously (11-13).

**Assessment of Hepatobiliary Function and Injury**

During NMP, flow through the portal vein and hepatic artery as well as perfusion fluid temperature was recorded every 10 minutes. Vascular resistance of both vessels was expressed as mmHg/mL/min/kg liver.

Before and every 30 minutes during NMP, samples were taken from the perfusion fluid. Of each sample, one part was used for analysis for biochemical parameters (glucose and lactate) using an ABL800 FLEX analyzer (Radiometer, Brønhøj, Denmark). Another part of the perfusion fluid sample was centrifuged (2700g for 5 min at 4°C) and the supernatant was collected and stored at -80°C for determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and gamma-glutamyl transferase (gamma-GT), using standard biochemical methods.

Bile production was measured gravimetrically at 30-minute intervals by weighing Eppendorf tubes in which bile was collected from the biliary drain. The cumulative bile production was expressed as mL bile/kg liver. The density of bile was defined as 1 mg/mL. Based on the (cumulative) bile production during NMP, livers were classified as good or poor functioning using a modification of the method described by Sutton et al. (12). While Sutton et al. (12) expressed bile production as gram/hr, we expressed bile production as mL/hr/kg liver tissue. Livers were classified as good functioning if they met the following criteria: bile production of more than 2 mL/
hr/kg during 1.5 – 2.5 hours of NMP, and cumulative bile production of more than 5 mL/hr/kg after 2.5 hours of NMP. Biliary concentration of bicarbonate and pH were measured as markers of biliary epithelial cell (cholangiocyte) function (14). For this purpose, bile samples were collected under mineral oil and were analyzed immediately using an ABL800 FLEX analyzer (Radiometer, Brønhøj, Denmark). Biliary concentration of gamma-GT and LDH were measured as markers of biliary epithelial cell injury and biliary bilirubin concentration was measured as marker of hepatocellular secretory function (15), using standard biochemical methods.

Hepatic oxygen consumption was calculated every 30 minutes during NMP based on the difference between the arterial and venous oxygen content of the perfusion fluid, as described previously (12). In brief, samples were taken from the perfusion fluid before and after the liver. Prehepatic samples were taken from a side port before the perfusion cannula and posthepatic samples were taken directly from the suprahepatic vena cava.

Hepatic concentration of adenosine triphosphate (ATP) was used as an indicator of the energy status of grafts during HMP and NMP. For this purpose, liver tissue biopsies were immediately frozen in liquid nitrogen and were processed later for ATP measurement, as described previously (12).

**Histological Evaluation**

Biopsies were taken from the liver parenchyma at the end of SCS and after 6 hours of NMP. Samples were fixed in 10% formalin and paraffin embedded for further preparation for hematoxylin and eosin (H&E) staining. Histological assessment was performed by an expert liver pathologist (ASHG), who was not aware of the assigned study groups. To assess preservation injury, the degree of ischemic necrosis was semi-quantified based on the percentage of necrotic hepatocytes and categorized as: low (< 10%), mild (10 – 30%), moderate (30– 60%) or severe (> 60%).

**Statistical Analysis**

Continuous variables were presented as median with range or interquartile range (IQR). Categorical variables were presented as number and percentage. Continuous variables were compared between groups using the Mann-Whitney U test. Categorical variables were compared with the Pearson chi-square or Fischer’s exact test where appropriate. The level of significance was set at a p-value of < 0.05. All statistical analyses were performed using SPSS software version 22.0 for Windows (SPSS, Inc., Chicago, IL, USA).
Results

Donor Characteristics

A comparison of donor characteristics of livers that received 2 hours of HMP after SCS and livers that did not undergo HMP after SCS (SCS only) is provided in Table 1. There were no significant differences among the donor variables such as donor age, type of donor graft, donor warm ischemia time (in case of DCD), type

Table 1. Comparison of Donor Characteristics of Livers that Underwent Static Cold Storage Preservation (SCS) Alone and Livers that Underwent Oxygenated Hypothermic Machine Perfusion (HMP) after SCS

<table>
<thead>
<tr>
<th>Variables</th>
<th>SCS only (n = 12)</th>
<th>SCS + HMP (n = 6)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>61 (52-64)</td>
<td>64 (57-70)</td>
<td>0.21</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>0.49</td>
</tr>
<tr>
<td>- Male</td>
<td>8 (67%)</td>
<td>3 (50%)</td>
<td></td>
</tr>
<tr>
<td>- Female</td>
<td>4 (33%)</td>
<td>3 (50%)</td>
<td></td>
</tr>
<tr>
<td>Length (m)</td>
<td>1.77 (1.67-1.80)</td>
<td>1.75 (1.68-1.79)</td>
<td>0.75</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>88 (76-100)</td>
<td>73 (65-90)</td>
<td>0.08</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>27.3 (24.5-36.0)</td>
<td>24.6 (22.6-27.3)</td>
<td>0.10</td>
</tr>
<tr>
<td>Type of donor liver</td>
<td></td>
<td></td>
<td>0.18</td>
</tr>
<tr>
<td>- DCD</td>
<td>9 (75%)</td>
<td>6 (100%)</td>
<td></td>
</tr>
<tr>
<td>- DBD</td>
<td>3 (25%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cause of death</td>
<td></td>
<td></td>
<td>0.29</td>
</tr>
<tr>
<td>- Cardiovascular</td>
<td>2 (16%)</td>
<td>3 (50%)</td>
<td></td>
</tr>
<tr>
<td>- Post-anoxia</td>
<td>5 (42%)</td>
<td>2 (33%)</td>
<td></td>
</tr>
<tr>
<td>- Trauma</td>
<td>5 (42%)</td>
<td>1 (17%)</td>
<td></td>
</tr>
<tr>
<td>Reasons livers were declined for transplantation</td>
<td></td>
<td></td>
<td>0.29</td>
</tr>
<tr>
<td>- DCD + age &gt; 60 years</td>
<td>5 (42%)</td>
<td>5 (84%)</td>
<td></td>
</tr>
<tr>
<td>- DCD + high BMI</td>
<td>5 (42%)</td>
<td>1 (17%)</td>
<td></td>
</tr>
<tr>
<td>- DCD + high transaminases</td>
<td>2 (16%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Latest laboratory value gamma-GT (U/L)</td>
<td>83 (34-130)</td>
<td>30 (19-106)</td>
<td>0.30</td>
</tr>
<tr>
<td>Type of preservation solution</td>
<td></td>
<td></td>
<td>0.18</td>
</tr>
<tr>
<td>- UW solution</td>
<td>9 (75%)</td>
<td>6 (100%)</td>
<td></td>
</tr>
<tr>
<td>- HTK solution</td>
<td>3 (25%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Time between withdrawal of life support treatment and circulatory arrest (min)</td>
<td>20 (4-46)</td>
<td>14 (13-36)</td>
<td>0.61</td>
</tr>
<tr>
<td>Time between circulatory arrest and cold flush in situ (min)</td>
<td>18 (12-22)</td>
<td>15 (13-23)</td>
<td>0.96</td>
</tr>
<tr>
<td>Cold ischemia time (hr: min)</td>
<td>9:04 (7:01-11:14)</td>
<td>7:18 (6:10-8:32)</td>
<td>0.15</td>
</tr>
<tr>
<td>Total preservation time (hr:min)</td>
<td>9:04 (7:01-11:14)</td>
<td>11:37 (10:01-12:25)</td>
<td>0.10</td>
</tr>
<tr>
<td>Eurotransplant - Donor risk index</td>
<td>2.79 (2.24-3.21)</td>
<td>3.11 (2.77-3.39)</td>
<td>0.38</td>
</tr>
<tr>
<td>Weight of liver (kg)</td>
<td>2.11 (1.81-2.30)</td>
<td>1.83 (1.15-2.23)</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Data represent median with interquartile ranges for continuous variables or numbers (percentages) for categorical variables.


1The total of both time periods can be defined as the total donor warm ischemia time during DCD donation.
2Time period between cold flush out of the liver in the donor and start of machine perfusion (either HMP or NMP).
3Time period between cold flush out of the liver in the donor and start of NMP.
of preservation solution, and the ET-DRI. The median ET-DRI was 3.11 in the HMP group and 2.79 in the SCS only group, illustrating the poor baseline quality of these donor livers. Moreover, the total preservation time (including 2 hours of HMP) was not significantly different between livers that underwent HMP (11:37 hr:min, IQR 10:01 – 12:25 hr:min) and livers that only underwent SCS preservation (9:04 hr:min, IQR 7:01 – 11:44 hr:min) prior to viability assessment using NMP.

**Vascular Resistance**

Changes in vascular resistance of the portal vein and hepatic artery during NMP are presented in Figure 3A and B. Although the vascular resistance was lower in livers that first underwent HMP, compared to only SCS preservation, this difference did not reach statistical significance.

![Figure 3: Vascular resistance patterns in the portal vein and the hepatic artery during 6 hours of normothermic machine perfusion (NMP). Panel A and panel B: During NMP, the vascular resistance in the portal vein and hepatic artery were lower in livers that first underwent oxygenated hypothermic machine perfusion (HMP) versus livers that underwent static cold storage (SCS) only; however these differences were not statistically significant. Data are represented as medians with IQR. The error bar of SCS only group illustrates the range from the median (50th percentile) till the 75 percentile; the error bar of the HMP group represents the range from the 25 percentile till the median.](image)
**Evaluation of Hepatobiliary Function**

Bile production was used as a marker of hepatocellular function and viability (12,16). During 6 hours of NMP, bile production was significantly higher in donor livers that first underwent HMP, compared to livers that underwent SCS alone (Figure 4A). Moreover, based on the (cumulative) bile production during NMP, all livers in the HMP group met the criteria for good functioning livers, whereas only 42% of the livers that underwent SCS alone met these criteria (Table 2). In both groups, there was no association between the ET-DRI and bile production during NMP.

The cumulative total biliary bilirubin concentration in the last 30 minutes of NMP was significantly higher in the group that underwent HMP, compared to livers that underwent only SCS preservation (Figure 4B). This finding is compatible with a better hepatocellular secretory function of livers in the HMP group. Similarly, HMP significantly improved the cumulative biliary bicarbonate secretion in the last 30 minutes of NMP, compared to only SCS preservation (Figure 4C). Moreover, median biliary pH after 6 hours of NMP was higher in the HMP group compared to livers that underwent SCS preservation only, but this difference did not reach statistical significance (Figure 4D).

Concentrations of glucose and lactate in the perfusion fluid during HMP and NMP are presented in Figure 5. During HMP, a small increase in the levels of glucose and lactate was observed. During subsequent NMP, glucose and lactate levels were significantly higher in the SCS group compared to HMP preserved livers. With respect to glucose levels, these levels normalized in the HMP group (median 11.6 mmol/L), whereas glucose levels in the SCS group remained elevated after 6 hours of NMP (median 27.5 mmol/L) ($p = 0.009$) (Figure 5A). In addition, in the SCS only group a significantly increase in the concentration of lactate was noticed in the first hour of NMP (median peak value of 10.1 mmol/L), which was not observed in the HMP group (median 2.3 mmol/L) ($p = 0.002$) (Figure 5B). Moreover, to maintain a physiological pH, significantly more sodium bicarbonate solution had to be administered to the perfusion fluid during NMP in the group of livers that underwent SCS only, compared to livers that first underwent 2 hours of HMP (Table 3).
Table 2. Bile Production During Ex Situ Viability Testing Using Normothermic Machine Perfusion of Livers that Underwent Static Cold Storage (SCS) Preservation Alone or SCS Followed by Oxygenated Hypothermic Machine Perfusion (HMP)

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver (number)</th>
<th>Eurotransplant donor risk index (ET-DRI)</th>
<th>Bile output between 1.5 and 2.5 hrs NMP (mL/kg liver)</th>
<th>Cumulative bile output at 2.5 hrs NMP (mL/kg liver)</th>
<th>Meets both criteria for classification of good functioning liver during NMP viability testing?</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCS only</td>
<td>1</td>
<td>2.5</td>
<td>0.17</td>
<td>0.84</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.8</td>
<td>0.95</td>
<td>2.02</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.3</td>
<td>1.26</td>
<td>3.27</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.4</td>
<td>1.30</td>
<td>3.57</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.2</td>
<td>1.43</td>
<td>3.86</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.4</td>
<td>1.48</td>
<td>5.00</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2.2</td>
<td>1.50</td>
<td>5.18</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2.0</td>
<td>2.33</td>
<td>5.91</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>2.5</td>
<td>2.37</td>
<td>7.86</td>
<td>yes</td>
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<tr>
<td></td>
<td>10</td>
<td>3.1</td>
<td>4.86</td>
<td>8.52</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>3.2</td>
<td>5.53</td>
<td>10.20</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2.8</td>
<td>7.44</td>
<td>15.84</td>
<td>yes</td>
</tr>
<tr>
<td>SCS + HMP</td>
<td>1</td>
<td>2.4</td>
<td>2.67</td>
<td>6.70</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.4</td>
<td>4.53</td>
<td>13.76</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.9</td>
<td>5.41</td>
<td>15.15</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.5</td>
<td>6.09</td>
<td>17.41</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.2</td>
<td>21.38</td>
<td>49.05</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>6*</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations used: HMP: hypothermic machine perfusion, SCS: static cold storage, NMP: normothermic machine perfusion.

1 Livers were classified as good functioning according to the following criteria: bile production of more than 2 mL/kg liver between 1.5 – 2.5 hours of NMP, and cumulative bile production of more than 5 mL/kg liver after 2.5 hours of NMP.

* The collected bile was mixed with blood due to hemobilia. Therefore, this liver was excluded from the assessment of (cumulative) bile production.

**Evaluation of Hepatobiliary Injury**

Release of injury markers during NMP was used to assess the degree of hepatobiliary injury. To correct for a wash-out effect of HMP, the relative increase in perfusate levels of AST, ALT, LDH, and gamma-GT during the first 30 minutes and after 6 hours were determined (Table 3). Except for gamma-GT, during the first 30 minutes of NMP, the relative increase in perfusate levels of AST, ALT, and LDH were significantly lower in the HMP group compared to the SCS only group. However, the relative increase in levels of AST, ALT, and LDH between the first 30 minutes and 6 hours of NMP were not significantly different between the two groups, reflecting a similar level of hepatobiliary injury after 6 hours of NMP (Table 3). Likewise, there were no significant differences in the biliary concentrations of LDH and gamma-GT after 6 hours of NMP, indicating that there was no significant difference in cholangiocyte injury (Table 3).
Figure 5: Perfusion fluid concentrations of glucose and lactate during 2 hours of oxygenated hypothermic machine perfusion (HMP) and 6 hours of normothermic machine perfusion (NMP). Panel A and B: Almost during the entire period of NMP, levels of glucose and lactate were significantly lower in the group of livers that first underwent 2 hours of HMP, compared to the group with only SCS preservation (*p < 0.05). Data are represented as medians with IQR, except for the levels of lactate in the SCS only group, the error bars in this group illustrates the range between the 50th and 75th percentile.

**Evaluation of Hepatic Energy Status and Oxygen Consumption**

Hepatic ATP concentration was used as an indicator of the cellular energy status. Hepatic ATP content was measured at the end of SCS, during and at the end of HMP and before, during and at the end of NMP, respectively (Figure 6A). In both groups, all livers were ATP-depleted at baseline after SCS. The median ATP concentration was 5 μmol/g protein in the HMP group versus 6.5 μmol/g protein in the SCS only group. During HMP, median ATP concentration increased >15-fold to 76 μmol/g protein (IQR 48-122 μmol/g protein). After 2 hours of HMP, while the perfusion fluid was changed, ATP concentration dropped; however, before start of NMP ATP concentrations were still significantly higher in the HMP group, compared to the SCS only group (15 μmol/g protein in the HMP group versus 6.5 μmol/g protein in the SCS only group). Moreover, hepatic ATP content increased more rapidly during the first hour of NMP in livers that first underwent HMP, compared to livers with SCS preservation alone. In addition, ATP levels in livers that underwent HMP
remained significantly higher compared to SCS only livers during the first 4 hours of NMP. Although not statistically significant, at the end of 6 hours of NMP, the median hepatic ATP content in the HMP group was 88 μmol/g protein (IQR: 12-118 μmol/g protein), while it was only 24 μmol/g protein (IQR: 14-51 μmol/g protein) in the group with only SCS preservation.

In parallel with changes in ATP concentration, the peak value of the oxygen consumption during NMP was significantly higher in livers that first underwent HMP (2.9.10⁻³ mL O₂/min/gr liver) compared to livers with only SCS preservation (1.4.10⁻³ mL O₂/min/gr liver) (Figure 6B).

**Histological Assessment**

Figure 7 provides histological evaluation of the hepatic biopsies at the end of the regular period of SCS and after 6 hours NMP viability testing. At baseline,
there were no statistical differences in the degree of ischemic necrosis between both groups. The majority of livers had signs of ischemic necrosis grade 1 or grade 2 (Figure 7A). In addition, there was no major increase in the amount of ischemic necrosis after NMP in both groups (Figure 7B). Examples of normal liver parenchyma and different grades of ischemic necrosis are presented in Figure 7, panels C-H. In addition, no major differences between the groups were noticed in the degree of intrahepatic bile duct injury at the end of 6 hours NMP (Figure 8).
Figure 7: Comparison of the degree of ischemic necrosis between livers that underwent 2 hours of oxygenated hypothermic machine perfusion (HMP) after static cold storage (SCS) and those that underwent SCS preservation alone, and examples of different grades of ischemic necrosis. Panel A and B: There were no statistical differences in the degree of ischemic necrosis at the end of regular SCS preservation and after 6 hours of normothermic machine perfusion between the two groups. Panel C and D: Examples of liver parenchyma without ischemic necrosis. Panel D is a detailed picture of Panel C. Panel E and F: Grade 1 ischemic necrosis (0-30% of hepatocytes involved). Panel F is a detailed picture of Panel E. Panel G and H: Ischemic necrosis grade 2 (30-60% of hepatocytes involved). Panel H is a detailed picture of Panel G. Scale bars in Panels D, F, and H indicate 30 μm. Scale bars in Panels C, E, and G indicate 300 μm.
Figure 8: Histological comparison of intrahepatic bile duct injury at the end of 6 hours normothermic machine perfusion (NMP). There were no differences in the degree of intrahepatic biliary injury between livers that underwent oxygenated hypothermic perfusion (HMP) after static cold storage (SCS) preservation and livers that underwent SCS alone. Panel A: example of intrahepatic bile duct in a liver of the SCS only group. Panel B: example of intrahepatic bile duct in a liver of the HMP group. White arrows point at the intrahepatic bile ducts. Scale bars indicate 20 μm.

Discussion

The aim of this study was to assess whether 2 hours of end-ischemic oxygenated HMP after the regular period of SCS restores hepatobiliary function and reduces injury in human ECD livers, as assessed during 6 hours of ex situ NMP viability testing. Our study demonstrated that 2 hours of HMP after traditional SCS significantly improves hepatobiliary function compared to SCS preservation alone. These improvements in hepatobiliary function are likely explained by a significant increase in hepatic ATP content during HMP. Although livers that underwent HMP after SCS displayed better function during NMP, compared to livers that underwent SCS alone, we did not observe significant differences in the amount of hepatobiliary injury.

The results of this study suggest that the benefit of oxygenated end-ischemic HMP is a result of a restoration of the oxygen debt and recovery of mitochondrial function prior to warm reoxygenation. By providing oxygen, mitochondria are able to generate ATP even if the donor liver is kept at a low temperature. Our study results support previous experimental studies using animal models that oxygenated end-ischemic HMP is able to enhance the energy status of the cold-stored graft and subsequently improved function after warm reoxygenation (17,18). In our study, HMP enhanced the hepatic ATP content more than 15-fold compared to values immediately after SCS. Moreover, the ATP levels remained significantly higher during the first 4 hours of NMP in the group of livers that underwent HMP compared to livers that were only preserved by SCS. In parallel with the enhanced ATP levels in livers that underwent HMP, these livers displayed a higher oxygen consumption and produced significantly more bile, compared to livers that did not undergo HMP. The link between increased bile production and higher ATP levels has also been described in porcine livers (16). Bile production is an energy
consuming process and sufficient hepatocellular levels of ATP are needed for the active secretion of bile acids, bile salts, bilirubin, cholesterol, and other organic molecules into bile. Similarly, higher levels of biliary bilirubin were noticed in livers that first underwent HMP, reflecting better hepatocellular function of these livers.

Other evidence of improved hepatobiliary function after end-ischemic HMP was provided by stable concentrations of glucose and lactate in the perfusion fluid during NMP. Notably, lactate, which can be seen as a marker of anaerobic metabolism, did not increase during NMP of livers that first underwent HMP after SCS, while a clear peak in the lactate concentration was observed in the SCS alone group.

In contrast to the regenerated hepatic ATP and improved functional recovery in livers that first underwent HMP, we did not observe a reduction in the amount of hepatobiliary injury. There were no significant differences in the concentration of hepatobiliary injury markers such as AST, ALT, and LDH in the perfusion fluid at the end of 6 hours NMP between the two study groups. In parallel with this, there were no significant differences in the histological degree of hepatic ischemic necrosis at the end of SCS and after 6 hours NMP. Almost 70% of livers in both groups displayed some degree of ischemic necrosis at baseline. This is not a surprise as all livers were declined for transplantation and derived from donors with a high ET-DRI. Moreover, 15 of the 18 livers included in this study were obtained from DCD donors and suffered a donor warm ischemia time of more than 30 min. The combination of suboptimal graft quality, substantial donor warm ischemia time, and the subsequent period of SCS (median 7-9 hours) can explain the observed hepatocellular injury at baseline. It is obvious that 2 hours of end-ischemic HMP does not restore pre-existing hepatocellular injury; however it does restore cellular function of the remaining vital parts of the liver.

Next to a better functional recovery of hepatocellular function, we also observed an improved cholangiocellular function in livers that first underwent HMP. An important aspect of cholangiocyte function is the secretion of bicarbonate into bile. Cholangiocytes actively secrete bicarbonate to increase biliary pH and protect themselves against the effects of toxic hydrophobic bile salts. This phenomenon has been described as the biliary “bicarbonate umbrella” (14,19). In an alkaline environment, bile salts are deprotonated, which is clinically relevant as protonated bile salts can passively enter cells by uncontrolled membrane permeation, causing apoptosis (14,19). Several experimental and clinical studies have shown that bile salt toxicity is associated with the development of NAS after liver transplantation (20-22). Therefore, the increased secretion of bicarbonate can be seen as an important and promising feature of end-ischemic HMP in human liver transplantation.
In the current study, end-ischemic HMP was performed using dual perfusion of both the portal vein and the hepatic artery. Bile ducts are largely dependent on arterial blood supply for the delivery of oxygen and nutrients and we believe that dual perfusion results in better preservation and functional recovery of the biliary epithelium than portal vein perfusion alone. It is well known that hepatic artery thrombosis after transplantation leads to insufficient oxygenation of the biliary epithelium and subsequent development of ischemic biliary strictures (23). Moreover, in a mouse model, we have previously shown that complete arterial deprivation results in a loss of bile secretory function and subsequent intrahepatic cholestasis (24). In the current study, cholangiocellular bicarbonate secretion was significantly higher in livers that first underwent HMP. We, therefore, propose that combined arterial and portal perfusion during HMP has a potential advantage in the post-ischemic recovery of the bile ducts in comparison with single perfusion through the portal vein alone. However, this still needs to be demonstrated in a separate study comparing single versus dual liver perfusion.

The aim of the current study was not to study whether HMP reduces clinical ischemia-reperfusion injury. *Ex situ* NMP was performed with perfusion fluid consisting of plasma, red blood cells and nutrients, but no leukocytes and platelets, with the aim to assess liver graft viability and function after either SCS or SCS followed by HMP. To examine whether HMP is not only associated with a restoration of hepatic ATP and hepatobiliary function prior to transplantation, but also reduces ischemia/reperfusion injury, a clinical trial is needed. Based on the favorable results of the current study, we recently initiated a clinical trial in which end-ischemic HMP is applied in DCD liver transplantation (Dutch Trial Register no. NTR 4493; www.trialregister.nl).

In conclusion, this study demonstrates that a short period of two hours of end-ischemic oxygenated HMP after traditional SCS of ECD livers results in a restoration of intrahepatic energy resources. The higher hepatocellular energy levels in livers that underwent HMP corresponded with a significantly better recovery of hepatobiliary function as assessed during *ex situ* NMP, when compared with livers that underwent only SCS preservation. Despite this better immediate hepatobiliary function, end-ischemic oxygenated HMP does not seem to reduce (pre-existing) hepatobiliary injury. The beneficial effects of end-ischemic oxygenated HMP in this preclinical study provide strong support for further testing end-ischemic oxygenated HMP in a clinical liver transplant trial.

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