Studies on bile duct Injury and the protective role of oxygenated machine perfusion in liver transplantation
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CHAPTER 6
Normothermic Machine Perfusion Reduces Bile Duct Injury and Improves Biliary Epithelial Function in Rat Donor Livers


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Chapter 6

ABSTRACT

Bile duct injury may occur during liver procurement and transplantation, especially in livers donated after circulatory death (DCD). Normothermic machine perfusion (NMP) has been shown to reduce hepatic injury, compared to static cold storage (SCS). However, it is unknown whether NMP provides better preservation of bile ducts. The aim of this study was to determine the impact of NMP on bile duct preservation in both DCD and non-DCD livers. DCD and non-DCD livers obtained from Lewis rats were preserved for 3h using either SCS or NMP, followed by 2h ex-vivo reperfusion. Biomarkers of bile duct injury (γ-GT and LDH in bile) were lower in NMP preserved livers, compared to SCS preserved livers. Biliary bicarbonate concentration, reflecting biliary epithelial function, was 2-fold higher in NMP preserved livers (p<0.01). In parallel with this, pH of bile was significantly higher in NMP preserved livers (7.63±0.02 and 7.74±0.05, for non-DCD and DCD livers, respectively), compared with SCS (7.46±0.02 and 7.49±0.04, for non-DCD and DCD livers, respectively). Scanning and transmission electron microscopy of donor extrahepatic bile ducts demonstrated significantly decreased injury of the biliary epithelium of NMP preserved donor livers (including the loss of lateral interdigitations and mitochondrial injury). Differences between NMP and SCS were most prominent in DCD livers. Compared to conventional SCS, NMP provides superior preservation of bile duct epithelial cell function and morphology, especially in DCD donor livers. By reducing biliary injury, NMP could have an important impact on the utilization of DCD livers and outcome after transplantation.
INTRODUCTION

Nonanastomotic bile duct strictures are a major cause of morbidity after liver transplantation (1-3). These biliary strictures occur more frequently in livers donated after circulatory death (DCD; 20.5-33.3%), compared to livers donated after brain death (DBD; 0-12.5%) and are notoriously therapy resistant (4-7). In an effort to expand the donor pool, DCD donors are increasingly used for transplantation. Indeed, the percentage of DCD donors in the US increased from 1.1% in 1995 to 11.2% in 2010 (8). DCD grafts, procured after the donors circulation ceases, are subject to a period of warm ischemia in addition to the cold storage period between procurement and implantation. The combination of subsequent warm and cold ischemia is thought to lead to increased biliary injury, which can explain the increased risk of biliary strictures in DCD donor livers (9). Biliary epithelial cells have shown to be more susceptible to ischemic injury than hepatocytes, which may explain the high rate of non-anastomotic biliary strictures following otherwise successful DCD liver transplantation (10,11). As the discrepancy between available donor organs and the number of patients waiting for transplantation increases, more DCD grafts will be used, necessitating the development of better preservation methods to minimize bile duct injury and the subsequent risk for nonanastomotic strictures.

Normothermic machine perfusion (NMP) of donor livers offers potential to meet the requirements for DCD graft preservation. An important advantage of NMP over conventional static cold storage (SCS) is the delivery of oxygen and nutrients at 37°C, providing full metabolic support. NMP can potentially minimize or even eliminate cold ischemia during preservation. Animal studies comparing NMP with SCS have demonstrated increased bile production, lower levels of hepatocellular enzymes and decreased parenchymal necrosis after reperfusion of NMP preserved livers (12-14). Transplantation of NMP preserved livers in animal models has been associated with improved survival(14,15). So far, studies on NMP preservation have focused on hepatocellular injury and general viability outcome parameters. It is still unknown whether NMP is protective for the bile ducts and whether it results in better preservation of biliary epithelial function than SCS.

We hypothesized that NMP provides better preservation of the bile ducts, when compared to conventional SCS. To test this hypothesis, we studied the impact of NMP on the preservation of biliary epithelium in both DCD and non-DCD rat donor livers.

MATERIALS AND METHODS

Animals

Male Lewis rats (LEW/Han®Hsd), weighing 303±4 g (mean±SEM) were obtained from Harlan Laboratories (Boxmeer, Netherlands). Animals received care according to the Dutch Law on Animal Experiments and the study protocol was approved by the Institutional Animal Care and Use Committee of the University of Groningen (IACUC-RuG).
Experimental Design
Thirty-eight rat livers were divided into 4 experimental groups and a control group (Table 1). Livers were procured from DCD and non-DCD donors and in each group livers were either preserved by NMP or SCS (n=7-9 for each group). In the control group, rats (n=6) were used for in vivo collection of bile during 30 minutes (anesthesia as described below). Control rats were supported with mechanical ventilation to maintain a stable arterial blood gas (pH 7.35-7.45). After bile collection, the liver and extrahepatic bile duct were excised and tissue samples were processed immediately for further analyses.

Procurement of DCD and Non-DCD Donor Livers
Inhalation anesthesia with isoflurane and oxygen was used before and during the procurement (2-3% isoflurane). The extrahepatic bile duct was cannulated and 1 ml 0.9% NaCl with 500 IU of heparin was administered via the dorsal penile vein. In case of a DCD donation, cardiac arrest was induced by external compression of the heart (exogenous tamponade) until contractions ceased. Subsequently, the aorta and pulmonary artery were clamped close to the heart (to ensure a complete blood flow block) and the rat was kept at 37° C for 30 minutes (16). In case of non-DCD donation, livers were procured immediately after laparotomy. In all groups, the hepatectomy was performed by ligation of the splenic vein, mesenteric artery and mesenteric vein and cannulation of the celiac trunk. After clamping of the infra-hepatic inferior vena cava and the portal vein, the latter was cannulated. After immediate in situ perfusion of the liver with 5mL 0.9% NaCl (37°C) via the portal vein cannula, the supra-hepatic inferior vena cava was transected, followed by a cold flush out with 5 mL 0.9% NaCl (4°C) via the portal vein cannula. The liver was removed and flushed with an additional 20mL of cold (4°C) 0.9% NaCl via the portal vein and 5mL of cold (4°C) 0.9% NaCl via the hepatic artery (celiac trunk cannula) before preservation by either SCS or NMP.

Static Cold Storage and Normothermic Machine Perfusion
In the SCS groups, livers were stored in 0.9% NaCl at 4°C for 3h. In the NMP groups, livers were preserved by ex vivo perfusion for 3h, with a perfusion fluid consisting of 20mL human red blood cell concentrate (final hematocrit 15-20%), 59mL Williams Medium E solution, 20mL human albumin (200g/L Albuman®, Sanquin, Amsterdam, Netherlands), 1mL insulin (100 IE/mL Actrapid®, Novo Nordisk, Alphen aan den Rijn, Netherlands) and 0.1mL unfractionated heparin (5000 IE/mL), adding up to a total volume of 100mL. The same fluid was used for two hours of reperfusion. Previous studies have indicated that crystalloid fluids are

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sufficient for short-term cold preservation of rat donor liver parenchyma (17,18). In a pilot study we confirmed that rat donor liver preservation in either NaCl or HTK solution (n=6 in each group) does not result in a different degree of bile duct injury or function as measured by bilirubin, $\gamma$-GT, LDH, pH and HCO$_3^-$ in bile produced during reperfusion (data available on request). For both NMP and ex vivo reperfusion of rat donor livers we developed a liver machine perfusion system that enabled dual perfusion via both the hepatic artery and the portal vein in a closed circuit (Figure 1). Two roller pumps (Ismatec ISM404 + ISM719 and MS-2/6-160; IDEXX Health & Science, Wertheim-Mondfeld, Germany) provided pulsatile flow to the hepatic artery and continuous flow to the portal vein. The combination of elastic tubing, a special air chamber and a tubular membrane oxygenator between the roller pump and the portal vein severely reduced the pulses caused by the roller pump resulting in a continuous flow entering the portal vein. To maintain a pulsatile flow in the hepatic artery, we used minimal length of inelastic tubing between the arterial roller pump and the hepatic artery. Two tubular membrane oxygenators provided oxygenation of the perfusion solution and removal of CO$_2$. The system was pressure- and temperature-controlled by a computer algorithm; allowing auto regulation of blood flow through the liver, with constant pressure at variable flow rates. Flow, pressure and temperature were detected by inline sensors and data were analyzed by and displayed in real-time on a connected laptop (software kindly provided by Organ Assist, Groningen, Netherlands). Pressure was limited to a mean of 110 mmHg in the hepatic artery and 11 mmHg in the portal vein and temperature was set to 37°C. After each liver perfusion experiment, the system was thoroughly cleaned with biological soap based on active enzyme complexes (Biotex Groen, Unilever, Rotterdam, Netherlands), water, ethanol (70%) and subsequently dried with compressed air.

**Biochemical Markers of Function and Injury**

During ex vivo reperfusion, flow and temperature were registered every 15 minutes. Before reperfusion and 1h after reperfusion, samples were taken from the perfusion fluid. Samples were centrifuged (2700 rpm for 5 min at 4°C) and the supernatant of the perfusion fluid (referred to as “plasma”) was collected, frozen and stored at -80°C for determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and albumin, using standard biochemical methods.

Bile production was measured at 30-minute intervals by weighing Eppendorf tubes in which bile was collected from the biliary drain. Biliary epithelial cell function was assessed by measuring pH and bicarbonate concentration in bile. For this purpose, bile samples were collected under mineral oil and analyzed immediately using an ABL800 FLEX analyzer (Radiometer, Brnshj, Denmark). Concentration of gamma-glutamyl transferase ($\gamma$-GT) and LDH in bile were measured as biomarkers of biliary epithelial cell injury (19), and bilirubin concentration in bile was measured as biomarker of hepatocellular secretory function, using standard biochemical methods.

**Immunohistochemistry for Activated Caspase-3 of the Extrahepatic Bile Ducts**

Immunohistochemistry for activated caspase-3 was performed to detect apoptotic cell death in extrahepatic bile ducts. After 2h of reperfusion, a segment of the extrahepatic bile duct
Figure 1. *Ex vivo* rat liver machine perfusion system. Two roller pumps provided a pulsatile flow to the hepatic artery (A) and a continuous flow to the portal vein (B), after eliminating pulses with an air chamber (C). Two tubular membrane oxygenators provided oxygenation of the perfusion solution, as well as removal of CO₂ (D). The system was both pressure and temperature controlled. Flow (ϕ), pressure (P) and temperature (T) were detected by inline sensors and data were analyzed by and displayed in real-time on a connected laptop (E). Heat exchangers (F) and a plexiglass box encapsulating the perfusion system (G), ensured temperature control at 37 °C. The rat liver was placed into an organ chamber (H), protected with a transparent cover to maintain a moist and warm environment. Bile was collected in Eppendorf tubes (I). Several three-way connectors were used as bubble traps (J).
proximal from the tip of the biliary catheter (and therefore not mechanically injured) was dissected free and stored in 10% formaldehyde for inclusion in paraffin. Slides were prepared for immunohistochemical detection of activated caspase-3 (Asp175, Cell Signaling #9661; 1:100 dilution). Antigen retrieval was performed with 1mM EDTA (pH 8.0) and microwave (15 min, 400W). GaRpo (1:50 dilution; DAKO p0448) and RaGpo (1:50 dilution; DAKO P0449) were used as secondary and tertiary antibodies. Slides were counterstained with hematoxylin.

**Electron Microscopy of the Extrahepatic Bile Duct Epithelium**

Scanning and transmission electron microscopy (EM) were used for ultrastructural assessment of morphology of biliary epithelial lining of extrahepatic bile ducts. For this purpose, in at least three livers per group, a segment of the extrahepatic bile duct proximal from the tip of the biliary catheter was excised after 2 hours of reperfusion and samples were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate, postfixed with osmium tetroxide, dehydrated through ethanol and critical point dried for scanning EM or embedded in EPON before sectioning for transmission EM. Scanning EM (Jeol6301F; 2kV) was used for qualitative assessment of the apical surface of the biliary epithelial layer. Transmission EM (FEI Cm100; 80kV) was used to assess ultrastructural changes, focusing on cellular attachments (including lateral interdigitations, tight-junctions, and cell-basement membrane contact), microvilli, and mitochondria. Characteristic ultrastructural mitochondrial changes were assessed, using a semi-quantitative scoring system modified from Crouser et al. (20). Ultrastructural morphology of mitochondria was graded as normal (grade 0), minimal-moderate swelling (grade 1) and severe swelling and/or flocculent condensation (grade 2). Two investigators (SodD and NK) independently examined all mitochondria in at least ten epithelial cells per bile duct, resulting in 20-40 biliary epithelial cells per group. Both investigators were blinded to the treatment allocation. Mean total number of mitochondria per cell and relative distribution of the injury grades were calculated per group.

**Statistical Analyses**

Continuous data are presented as mean ± SEM. Student T-test was used to compare two groups of continuous variables. Kruskal-Wallis Test was used for statistical comparison of >2 groups. Categorical data are expressed as numbers and percentage and groups were compared using Pearson chi-square test or Fischers exact test as appropriate. The level of significance was set at p-value of 0.05. Analyses were performed using SPSS software version 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

**RESULTS**

**NMP Provides Better Protection of Hepatic Parenchyma**

In a pressure-controlled perfusion system, blood flow through the liver can be used as an indicator of intrahepatic vascular resistance. The average arterial flow at 1h after perfusion was 5.3±0.4 mL/min and the portal flow was 22.6±0.8 mL/min. Changes in portal flow during
reperfusion are presented in Figure 2. The lowest initial portal flow was noted in livers preserved by SCS (both DCD and non-DCD) and the highest flow in non-DCD livers preserved by NMP. This resulted in a significant difference in portal flow between the four groups during the first 1.5h of reperfusion (p=0.001 to 0.045 for the various time points). No significant differences in arterial flow were observed between the groups (data not shown).

The relative increase of cellular enzymes AST, ALT and LDH in the perfusion fluid after reperfusion was used as a biochemical marker of hepatocellular injury. In both non-DCD and DCD donor livers the release of hepatocellular enzymes was significantly higher after SCS preservation, compared to NMP preservation (Figure 3A-C). The highest increase in plasma levels of AST, ALT and LDH was noted in the DCD+SCS group and lowest increase in the non-DCD+NMP group.

Bile production (an important parameter of hepatocyte function) was significantly higher after reperfusion of NMP preserved DCD livers, compared to SCS preserved DCD livers (Figure 3D). This beneficial effect of NMP was less pronounced in non-DCD donor livers. In all four groups, bile flow after ex vivo reperfusion remained lower than in vivo bile flow in control rats. In NMP preserved livers from both DCD and non-DCD donors, biliary bilirubin concentration was not significantly different from that in bile collected in vivo from normal controls. However, bilirubin concentration in bile produced by SCS preserved livers (both DCD and non-DCD) were about 4-times lower, compared with NMP preserved livers (p<0.05) (Figure 3E).

Figure 2. Markers of vascular resistance during reperfusion
DCD and non-DCD rat livers were preserved by normothermic machine perfusion (NMP) or static cold storage (SCS) for 3h and subsequently reperfused ex vivo. Portal vein flow during reperfusion in the four groups. A significant difference in portal flow between the four groups was observed during the first 1.5h of reperfusion. Data are expressed as mean±SEM; * p <0.05.
NMP Provides Better Protection Against Bile Duct Epithelial Cell Injury
Concentrations of $\gamma$-GT and LDH in bile were used as biomarkers of biliary epithelial injury (21). The highest biliary concentrations of enzymes at 1h after reperfusion were found in DCD livers preserved by SCS (Figure 4A and B). In DCD livers preserved by NMP, the concentration of $\gamma$-GT in bile was significantly lower compared to SCS. In fact, biliary $\gamma$-GT concentration in the DCD+NMP group was not different from values in the non-DCD groups or in vivo normal controls (Figure 4A). A similar pattern was seen for biliary LDH concentration. Although the highest biliary concentration of LDH was again found in the DCD+SCS group, the differences between the experimental groups did not reach statistical significance (Figure 4B).

NMP Provides Better Preservation of Bile Duct Epithelial Cell Function
Biliary epithelial cells contribute to bile flow and composition by active secretion of bicarbonate ($\text{HCO}_3^-$) into bile, resulting in an alkalotic pH. Biliary pH and bicarbonate concentration were used as markers of epithelial function. Bile collected at 1h of reperfusion of NMP preserved livers contained 2-times higher concentration of bicarbonate compared to SCS preserved liv-

![Graphs and Images]

Figure 3. Biochemical markers of hepatocyte injury and function, measured in the perfusion fluid
DCD and non-DCD rat livers were preserved by normothermic machine perfusion (NMP) or static cold storage (SCS) for 3h and subsequently reperfused ex vivo. Panel A-C: Relative change in “plasma” concentration of AST, ALT and LDH into the perfusion fluid during the first hour of reperfusion. Panel D and E: Bile production and bilirubin concentration in bile at 1 hour after reperfusion. Data are expressed as mean±SEM; * p <0.05.
ers, in both DCD and non-DCD groups (p<0.01). The concentration of biliary bicarbonate, however, was significantly lower in all experimental groups than in bile collected from in vivo controls (Figure 4C). In both DCD and non-DCD livers, pH of bile was significantly higher in NMP preserved livers (pH 7.63±0.02 and 7.74±0.02, respectively), compared with SCS (pH 7.46±0.02 and 7.49±0.04, respectively). In fact, the biliary pH of NMP-preserved livers was normal and not significantly different from values obtained in in vivo controls (Figure 4D).

NMP Provides Better Preservation of Bile Duct Epithelial Cell Morphology

Light microscopy (H&E) did not show obvious injury to the bile duct epithelium. To determine whether biliary epithelial cell were apoptotic, immunohistochemistry for activated caspase-3 was compared among the groups. In general, there were hardly any caspase-3 positive cells

Figure 4. Biochemical markers of biliary epithelia cell injury and function, detected in bile

Panel A and B: Markers of biliary epithelial cell injury: LDH and γGT in bile at 1h after reperfusion. Panel C and D: Markers of cholangiocyte function: HCO$_3$ concentration in bile and pH of bile at 1h after reperfusion. Data are expressed as mean±SEM; * p <0.05.
detected (Figure 5). Only in the group of DCD livers preserved by SCS, caspase-3 positive biliary epithelial cells were sporadically noted (Figure 5D).

Ultrastructural changes in the morphology of extrahepatic bile ducts were assessed by scanning and transmission EM. Although the degree of morphological changes varied along the length of extrahepatic bile ducts, there were clear differences between the groups (Figures 6 and 7). Scanning EM revealed no or only minor changes in bile ducts of non-DCD livers preserved by either SCS or NMP (Figure 6). The biliary epithelial layer was intact and most cells had normal appearing microvilli and cilia on their luminal membrane, except for the non-DCD+SCS group where some cells displayed reduced density of microvilli. Scanning EM of bile ducts of DCD livers preserved by NMP displayed well-preserved apical surface of the epithelial layer (Figure 7B). In contrast with this, bile ducts of DCD livers preserved by SCS exhibited signs of severe loss of lateral cell-cell contact, loss of microvilli, and sporadic epithelial cell apoptosis (Figure 7A).

Loosening of cell-cell contact between biliary epithelial cells of DCD livers preserved by SCS was confirmed by transmission EM. Lateral cell contacts at the level of tight junction appeared less firm and a loss of intercellular interdigitations was noted along the basolateral membrane of biliary epithelial cells in the DCD+SCS group (Figure 7A and 7B). Differences between SCS and NMP were much less pronounced for non-DCD livers (Figure 6B and 6C). In addition, transmission EM revealed a significantly reduced number of mitochondria per cell in SCS preserved DCD livers, when compared to the other three experimental groups as well as normal controls. The degree of mitochondrial injury, as assessed by a semi-quantitative scoring system, was significantly higher in SCS preserved DCD livers, compared to the other groups (Figure 7C).

**Static Cold Storage with HTK vs. NaCl**

Although previous studies have indicated that saline is a suitable preservation fluid for short-term cold preservation of rat donor livers, this may not reflect clinical practice. Therefore, we have added a group of 6 DCD rat livers, which are preserved in HTK solution instead of saline (3h SCS, followed by 2h of *ex vivo* reperfusion at 37 °C).

When comparing biliary functional and injury markers of livers preserved in HTK and livers preserved in saline we did not find statistically significant differences. This extra subgroup and additional analyses confirmed that a short cold preservation of rat livers in saline is feasible and does not lead to worse preservation of the rat bile ducts, compared to HTK.

**DISCUSSION**

Machine perfusion is receiving increasing attention as an attractive alternative for static cold storage of liver grafts before transplantation. Animal studies on NMP have shown decreased hepatocellular injury and better early posttransplant survival in NMP preserved livers, compared to SCS preserved livers (12-15). However, it remains unknown whether NMP also provides better protection against biliary injury. In the current study we have performed a de-
tailed analysis of bile duct epithelial injury and function in both DCD and non-DCD rat livers preserved by either NMP or SCS.

The principal novel finding of our study is that liver graft preservation by NMP provided significantly better protection against biliary epithelial cell injury and dysfunction than conventional SCS. In addition, NMP resulted in better preservation of the cellular ultrastructural morphology of biliary epithelium, compared to SCS. Importantly, the beneficial effects of NMP were most prominent in DCD livers.

In both DCD and non-DCD livers, the post-reperfusion release of AST, ALT and LDH was significantly lower in NMP preserved livers, compared to SCS preserved livers. These findings confirm data from previous studies in rat and porcine livers, which have shown a protective effect of NMP against preservation injury of liver parenchyma (12-15). Based on a porcine model of normothermic perfusion, bile output has been suggested as the most relevant parameter of liver viability (12,13). In our study we observed both increased bile production and higher levels of bilirubin in bile in NMP preserved livers, suggesting improved preservation of

![Figure 5. Immunohistochemistry of activated caspase-3 as marker of apoptosis](image)

Biopsies were taken 2h after reperfusion of DCD and non-DCD rat livers preserved by either normothermic machine perfusion (NMP) or static cold storage (SCS). Immunoreactivity for caspase-3 (brown color) was only noted sporadically in DCD livers preserved by SCS. **Panel A**: non-DCD liver preserved by NMP. **Panel B**: non-DCD liver preserved by SCS. **Panel C**: DCD liver preserved by NMP. **Panel D**: DCD liver preserved by SCS. The insert presents a higher magnification with a caspase-3 positive biliary epithelial cell (arrow). Original magnification 200x. Counter staining was with hematoxylin (blue color).
the donor liver parenchyma by NMP.

The highest post-reperfusion concentrations of biliary \(\gamma\)-GT and LDH [established biomarkers of biliary epithelial injury (19)] were found in bile samples obtained from DCD livers preserved by SCS. On the contrary, biliary \(\gamma\)-GT concentrations in NMP preserved DCD livers were low, similar to those in bile from normal controls. This indicates that NMP provides important protection of biliary epithelium against ischemia-reperfusion injury in DCD livers.

In parallel with this, we observed a significantly better preservation of biliary epithelial cell function in NMP preserved livers, as was reflected by increased biliary secretion of bicarbonate and higher biliary pH. This functional beneficial effect of NMP was seen in both DCD and

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**Figure 6. Non-DCD rat livers: Images of scanning and transmission electron microscopy of biliary epithelium of extrahepatic bile ducts**

Biopsies were taken 2h after reperfusion of non-DCD rat livers preserved by either normothermic machine perfusion (NMP) or static cold storage (SCS). **Panel A**: Images of normal bile duct epithelium. **Panel B**: Bile duct epithelium of SCS preserved non-DCD livers. **Panel C**: Bile duct epithelium of NMP preserved non-DCD livers. The lumen of the bile duct is marked with an asterisk. Scale bars are 1 \(\mu m\).
non-DCD livers. Bicarbonate secretion by biliary epithelial cells contributes to bile flow and plays an important role in protection of these cells against bile salt toxicity (22). Secretion of bicarbonate results in elevation of the biliary pH, especially near the apical cell membrane, which is clinically relevant as this prevents bile salts from passively entering cells, causing apoptosis (21,22). Several studies have shown that bile salt toxicity contributes to bile duct injury and is associated with the occurrence of non-anastomotic biliary strictures after trans-

Figure 7. DCD rat livers: images of scanning and transmission EM of biliary epithelium of extrahepatic bile ducts. Biopsies were taken 2 hours after reperfusion of DCD rat livers preserved by either NMP or SCS. (A) Bile duct epithelium of SCS-preserved DCD livers. (B) Bile duct epithelium of NMP-preserved DCD livers. The lumen of the bile duct is marked with an asterisk, and the arrows point at widening of cell-cell junctions (scanning EM) and loss of lateral interdigitations (transmission EM). White triangles indicate loss of microvilli at the apical membrane. An apoptotic bile duct epithelium cell is marked with a pound sign (#). (C) Summary of mitochondrial injury in biliary epithelial cells in all 4 groups, based on a modified semi quantitative grading system, as described by Crouser et al. (20). Scale bars are 1 um.
plantation (17,23,24). The observed increased bicarbonate concentrations in bile samples of NMP preserved livers in the current study could, therefore, be an important ancillary factor protecting these cells against bile salt-induced injury after transplantation. Although previous studies have suggested that apoptosis is a possible mechanism of biliary cell death due to ischemia (25), this is not confirmed by the current study. Morphological changes compatible with apoptosis were sporadically found. Paucity of apoptotic cells was confirmed by EM of the biliary epithelium. The most prominent ultrastructural change after reperfusion of SCS preserved DCD livers was loss of cell-cell contact and intracellular digitations between biliary epithelial cells. In addition, the number of mitochondria per cell was significantly reduced and mitochondria displayed prominent changes compatible with ischemia-induced injury (20). These ultrastructural changes were most severe in DCD livers preserved by SCS and absent in NMP preserved DCD livers. Intracellular depletion of adenosine triphosphate (ATP) in SCS preserved livers is a likely explanation for the observed morphological changes. In an in vitro model of cultured normal rat biliary epithelial cells, depletion of ATP has been shown to result in substantial morphological changes as detected by EM, including extensive loss of basolateral interdigitations and apical microvilli (26,27). Interestingly, several hours after restoration of ATP, viable cells still failed to display organized secondary membrane structures such as lateral interdigitations, which coincided with a protracted recovery of cellular functions. Necrotic or apoptotic cells were noted only occasionally (26,27). The morphological changes described in these in vitro cell culture studies are remarkably similar to those observed in SCS preserved livers in the current study. The use of 0.9% NaCl for SCS preservation is a potential limitation of this study, since it is not a commonly used preservation fluid for human livers. However, previous studies have demonstrated that crystalloid solutions such as NaCl 0.9% are sufficient preservation fluids for short-term cold preservation of rodent livers (17,18). In a pilot study, we compared all biliary outcome parameters (bilirubin, \( \gamma \)-GT, LDH, pH and \( \text{HCO}_3^- \) in bile produced during reperfusion) of 6 DCD livers statically preserved in HTK with 6 DCD livers preserved in NaCl 0.9%. No differences were observed. Apart from the protective effect of NMP on bile duct epithelium, NMP could provide additional clinical opportunities, such as ex vivo selection of donor livers prior to implantation (28,29). According to the United Network for Organ Sharing (UNOS) database, 58.2% of the DCD livers with consent for donation are currently not accepted due to the perceived high risk of complications after transplantation (8). NMP could not only provide better preservation of these DCD livers, but also allows pharmacological preconditioning and ex vivo testing of hepatic viability and function prior to transplantation. Our group has recently demonstrated that normothermic, oxygenated perfusion of human donor livers is technically feasible, which could contribute to a considerable expansion of the number of organs available for transplantation (30). The ex vivo reperfusion system as used in this study is a model for studying the bile duct integrity and function after reperfusion. The model differs from transplantation, as there are less immune cells and platelets in the perfusion fluid, therefore lacking several mediators of reperfusion injury. The advantage of ex vivo reperfusion was the ability to measure bile production and quality, and thereby obtaining information on the functionality of the bile duct epithelium as well as bile duct epithelium injury. On the other hand, this model did not allow us to deter-
mine the process of biliary epithelial cell injury more long-term after reperfusion; whether the observed injury is progressive with time or reversible.

Three recent clinical studies have demonstrated that biliary epithelial cell loss can be found in more than 80% of all human liver grafts before transplantation (31-33). In accordance with this, our group has recently observed extensive loss of biliary epithelial cells immediately after liver procurement in a DCD model of pig livers (34). Collectively, these findings suggest that detachment and loss of the biliary epithelium is a key event in ischemia-induced injury of donor livers. In the current study we observed epithelial cell injury and detachment, but no complete loss of the epithelial layer. Apparently, biliary epithelial cells of rat bile ducts are less susceptible to ischemia or the process of detachment is slower compared to human and porcine bile ducts. Alternatively, it could be that the differences between rats, pigs and humans are explained by the more hydrophobic, and therefore, more toxic bile salt composition in the latter two (35).

In conclusion, this study suggests that NMP provides superior preservation of the bile ducts of both DCD and non-DCD liver grafts, compared to conventional SCS. This beneficial effect of NMP is most pronounced in DCD livers. By reducing biliary injury, NMP could have an important impact on the utilization of DCD livers and may improve outcome after transplantation. These findings provide a strong stimulus for a clinical trial of NMP in human DCD liver transplantation.

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