Studies on injury and repair of donor bile ducts after liver transplantation
Sutton, Michael Edward

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2013

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after cardiac death.

S. op den Dries
M.E. Sutton
N. Karimian
M.T. de Boer
J. Wiersema-Buist
A.S.H. Gouw
H.G.D. Leuvenink
T. Lisman
R.J. Porte

 manuscipt submitted
ABSTRACT

Background
Livers derived from donation after cardiac death (DCD) are increasingly accepted for transplantation. However, DCD livers suffer additional donor warm ischemia, leading to biliary injury and more biliary complications after transplantation. It is unknown whether oxygenated machine perfusion results in better preservation of biliary epithelium and the peribiliary vasculature. We compared oxygenated hypothermic machine perfusion (HMP) with simple cold storage (SCS) in a porcine DCD model.

Methods
After 30 min of cardiac arrest, livers were perfused in situ with HTK solution (4°C) and preserved for 4 h by either SCS (n=9) or oxygenated HMP (10°C; n=9), using pressure-controlled arterial and portal venous perfusion. To simulate transplantation, livers were reperfused ex-vivo at 37°C with oxygenated autologous blood. Bile duct injury and function were determined by biochemical and molecular markers, and a systematic histological scoring system.

Results
After reperfusion, arterial flow was higher in the HMP group, compared to SCS. Release of hepatocellular enzymes was significantly higher in the SCS group. Markers of biliary epithelial injury (biliary LDH, gamma-GT) and function (biliary pH and bicarbonate, and biliary transporter expression) were similar in the two groups. However, histology of bile ducts revealed significantly less arteriolonecrosis of the peribiliary vascular plexus in HMP preserved livers.

Conclusions
Oxygenated HMP prevents arteriolonecrosis of the peribiliary vascular plexus of the bile ducts of DCD pig livers and results in higher arterial flow after reperfusion. Together this may contribute to better perfusion of the bile ducts, providing a potential advantage in the post-ischemic recovery of bile ducts.
INTRODUCTION

Livers from donation after cardiac death (DCD) donors are increasingly used for transplantation. Although this may help to increase the number of donor livers available for transplantation, DCD livers are associated with a higher risk of non-anastomotic biliary strictures (NAS), compared to livers donated after brain death (DBD) (1-4). NAS have been reported in 20-33% of patients receiving a DCD liver, compared to 0-13% in patients receiving a DBD liver graft, contributes to a higher rate of morbidity and graft failure after DCD liver transplantation (5-7). In contrast to DBD liver grafts, livers from DCD donors suffer from warm ischemia in the donor during the time period between cardiac arrest and in situ cold perfusion. This donor warm ischemia in combination with subsequent cold ischemia during organ preservation is believed to be a main cause of bile duct injury leading to NAS after transplantation (8). Biliary epithelial cells have been shown to be more susceptible to warm ischemic injury than hepatocytes, which may explain the high rate of NAS following otherwise successful DCD liver transplantation (9,10). In a recent clinical study, using a standardized histological evaluation of biopsies taken from the extrahepatic bile duct at the time of transplantation, arteriolonecrosis of the peribiliary vascular plexus was identified as the only independent histological parameter predictive for the development of NAS after transplantation (11).

Machine perfusion is increasingly discussed as a promising tool to optimize livers before transplantation. During machine preservation livers are perfused with an oxygenated or non-oxygenated perfusion fluid at either low temperature or normal body temperature (12-16). So far, most investigations have focused on hypothermic machine perfusion (HMP) and studies have suggested that HMP results in better preservation of the liver parenchyma, compared to the classical method of organ preservation, static cold storage (SCS) (14-16). Although machine perfusion may be particularly beneficial for preservation of hepatocellular energy status and viability of high risk liver grafts, it is still unclear whether it also provides better protection of the biliary epithelium and the peribiliary vascular plexus, especially in livers from DCD donors.

We hypothesized that oxygenated HMP leads to reduced injury of the biliary epithelium and the bile duct wall, compared to SCS. To test this hypothesis we compared oxygenated HMP and SCS in an established DCD model of liver donation in pigs. Outcome parameters were selected to assess biliary epithelial injury and function, as well as bile duct morphology after graft preservation followed by ex-vivo reperfusion using oxygenated autologous blood.
MATERIALS AND METHODS

Liver Procurement and Preservation

Experiments were performed in accordance with 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.

Dutch Landrace pigs (n= 18; weight 90-110 kg) were premedicated with atropine, tiletamine/zolazepam, and Finadyne. General anaesthesia was induced with a 4% sevoflurane mixture with air/oxygen and maintained after intubation by mechanical ventilation using 2% sevoflurane. After administration of 20,000 U heparin and 4 mg pancuronium iv mechanical ventilation was stopped and cardiac arrest was awaited. Animals were subsequently left untouched for 30 min, resulting in a mean time interval between cessation of ventilation and in situ cooling of 48 min. A midline laparotomy was performed and the distal aorta and inferior vena cava were cannulated for rapid in situ flush out with ice-cold histidine-tryptophan-ketoglutarate (HTK) preservation solution. The abdominal cavity was filled with slush ice for additional topical cooling. The first 2L of blood, drained from the inferior vena cava, was collected in a polyethylene bag with 20.000 IU of heparin.

On the backtable, liver grafts were perfused with additional 1L of cold HTK via the portal vein, the cystic duct was ligated. After careful flushing of the bile duct with preservation solution, an 8 Fr silicon catheter was inserted and secured in the distal extrahepatic bile duct for collection of bile.

Livers were randomly assigned to one of the following two groups: SCS for 4 h, followed by 2 h of ex-vivo reperfusion with autologous blood (n= 9) or oxygenated HMP for 4 h (n= 9), followed by 2 h of ex-vivo reperfusion with autologous blood at 37°C.

Hypothermic Oxygenated Machine Perfusion

HMP was initiated immediately after procurement, using a CE marked device that enables dual perfusion via both the hepatic artery and the portal vein in a closed circuit (Liver Assist®, Organ Assist, Groningen, Netherlands). Livers were perfused for 4 h with Belzer machine perfusion fluid (Bridge-to-Life, Ltd., Northbrook, IL), oxygenated with 100% O₂. Two rotary pumps provided pulsatile flow to the hepatic artery and continuous flow to the portal vein. Two hollow fiber membrane oxygenators provided oxygenation of the perfusion solution and removal of CO₂. The system was both pressure and temperature (10°C) controlled, allowing
auto-regulation of the blood flow through the liver. Pressure was limited to a mean of 30 mmHg in the hepatic artery and 5 mmHg in the portal vein, respectively. Livers that were preserved by SCS were packed in sterile organ bags with ice-cold HTK preservation fluid on melting ice.

**Normothermic Reperfusion**

To simulate transplantation, liver grafts were reperfused with pig blood, using the same perfusion machine as used for HMP. Temperature was kept at 37°C and mean arterial and portal pressure were limited to 60 mmHg and 11 mmHg, respectively. Flow was monitored continuously. Sodium bicarbonate (8.4% solution) was added to the perfusion fluid to maintain a physiological pH.

**Assessment of Hepatocellular Function and Injury**

During graft reperfusion, samples were taken from the perfusion fluid every 30 min and analyzed immediately for blood gas parameters (pO₂, pCO₂, sO₂, HCO₃⁻ and pH) using an ABL800 FLEX analyzer (Radiometer, Brønshøj, Denmark). In addition, plasma from the perfusion fluid was collected, frozen and stored at −80°C for determination of alkaline phosphatase, gamma-glutamyl transferase (gamma-GT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and total bilirubin, using standard biochemical methods. Total biliary bile salt concentration was measured spectrophotometrically using 3-hydroxysteroid dehydrogenase (27). Biliary phospholipid concentration was analyzed using a commercially available enzymatic method; the Phospholipids FS Kit (DiaSys Diagnostic Systems, Holzheim, Germany).

**Assessment of Biliary Epithelial Cell Function and Injury**

After reperfusion, bile production was measured gravimetrically at 30 min intervals. Biliary epithelial cell function was assessed by measuring pH, bicarbonate and glucose concentration in bile. For this purpose, bile samples were collected under mineral oil and analyzed immediately using an ABL800 FLEX analyzer. In addition, biliary concentrations of gamma-GT, alkaline phosphatase, and LDH were measured as biomarkers of biliary epithelial injury (28). Thiobarbituric acid reactive substances (TBARS) were measured in bile samples as a marker for oxidative stress in bile ducts (see Supplemental Data).

**Gene Expression of Hepatobiliary Transporter Proteins**

Hepatic mRNA expression of relevant hepatocellular and cholangiocyte transporter proteins involved in bile secretion was determined by quantitative real-time PCR as described previously (29). Sense and antisense porcine-specific primers used for real-time PCR as well as a detailed description of the methods can been found in the Supplemental Data.
Adenosine-5'-triphosphate (ATP) Extraction and Measurement

Hepatic concentration of ATP was used as an indicator of the energy status of grafts. Liver samples were immediately frozen in liquid nitrogen and processed for quantification of ATP as described in the Supplemental Data.

Histological Evaluation of Liver Parenchyma and Bile Ducts

Before and after SCS or HMP, biopsies were taken from liver parenchyma and distal extrahepatic bile ducts. After reperfusion, additional biopsies were taken from liver parenchyma, the extrahepatic bile duct (proximal from the tip of the biliary catheter), and from the intrahepatic bile ducts beyond the level of the first and second bifurcation. Bile ducts were gently grasped with fine forceps, taking care not to touch the mucosa and specimens were excised using fine Metzenbaum scissors or a scalpel. Biopsies were divided into two sections: one preserved in 10% formaldehyde for inclusion in paraffin and one snap-frozen in liquid nitrogen and stored at -80°C for RNA extraction. Paraffin-embedded slides were prepared for hematoxylin and eosin (H&E) staining. Additional slides were prepared for immunohistochemical detection of activated caspase-3 (Asp175, Cell Signaling #9661; 1:100), a marker for apoptosis. Injury of bile ducts was semiquantified using a systematic scoring system described by Hansen et al. (11). All sections were examined in a blinded fashion by two independent observers. In case of discordant results slides were examined by a third investigator.

Statistical Analysis

Continuous variables are presented as mean ± standard error (SE). Categorical variables are presented as number and percentage. Continuous variables were compared between groups using the Student-T test and within groups by a paired T-test. Categorical variables were compared with the Pearson chi-square or Fisher’s exact test. The level of significance was set at a p-value of 0.05. All statistical analyses were performed using SPSS software version 16.0 for Windows (SPSS, Inc., Chicago, IL).

RESULTS

Reperfusion Characteristics

After 4 h of either HMP or SCS livers were reperfused ex-vivo for 2 h at 37°C using oxygenated autologous blood. During the entire reperfusion period hepatic artery flow was higher in the HMP group, which was statistically significant at 1 h after reperfusion (Figure.
Portal vein flow was similar in the two groups (Figure 7.1B).

Cellular energy status as assessed by hepatic ATP content decreased significantly during SCS, but increased during oxygenated HMP. In both groups hepatic ATP content increased after normothermic reperfusion (Figure 7.1C).

Mean weight of livers before and after HMP+reperfusion was 1427±208 g and 1381±154 g, respectively. Liver weight before and after SCS+reperfusion was 1301±207 g and 1610±161 g, respectively. Changes in weight were not significantly different between the two groups.
Impact of HMP on Hepatocellular Injury and Function

Release of liver enzymes after reperfusion was used to assess the degree of hepatocellular injury. To correct for a wash out effect of machine perfusion, the relative increase in plasma levels of AST and LDH during and after the first 15 min of reperfusion was determined. For both time intervals increase in serum enzymes was lower in the HMP group, which was significant for the first time period (Figure 7.1).

During reperfusion, increasing bile production was observed in both groups, without significant differences between the groups (Figure provided as Supplemental data). There were no significant differences in biliary concentrations of bile salts and phospholipids, as well as their ratio at 2 h after reperfusion or in gene expression levels of the transporter proteins BSEP and MDR3, responsible for the biliary secretion of bile salts and phospholipids, respectively (Supplemental data).

**Figure 7.1.** Perfusion characteristics during and changes in serum concentrations of AST and LDH after 2 h of normothermic ex-vivo sanguineous reperfusion of DCD livers that were preserved by either 4 h of oxygenated HMP or SCS. Panel A: Blood flow through the hepatic artery. Panel B: Blood flow through the portal vein. Panel C: Changes in hepatic energy content as reflected by hepatic ATP content. Panel D and E: Relative increase of liver enzymes was significantly greater for SCS preserved livers, compared to livers preserved by oxygenated HMP. * p-value < 0.05.
Impact of HMP on Biliary Epithelial Injury and Function

Biliary concentrations of LDH, alkaline phosphatase and gamma-GT at 2 h after reperfusion were similar in the two groups (Figure. 7.2A-C). In parallel with this, there was no difference in concentration of TBARS in bile, a marker of oxidative stress in the bile ducts (Figure. 7.2D). Moreover, there were no significant differences in biliary pH and concentrations of bicarbonate and glucose in bile at 2 h after reperfusion or in gene expression of the biliary epithelial transporters involved in the secretion of bicarbonate into the bile, CFTR and AE2 between the two groups (Supplemental data).

Impact of HMP on Preservation of Hepatocellular and Biliary Morphology

H&E staining and activated caspase-3 immunohistochemistry of liver parenchyma revealed less signs of ischemia-reperfusion injury in the HMP group, compared to SCS preserved livers (Figure. 7.3). H&E staining of bile duct samples was used for semiquantitative analysis of bile duct injury, as described by Hansen et al (11). The results of this morphological analysis of extrahepatic bile ducts are summarized in Table 1. In this model of DCD livers, we observed extensive loss of biliary epithelial lining immediately after organ procurement. The underlying mural stroma and peribiliary vascular plexus, however, appeared relatively normal (Figure. 7.4A). After preservation and reperfusion, bile ducts of both HMP and SCS preserved livers
displayed signs of increased mural necrosis with loss of cell nuclei in the subepithelial stroma (Figure 7.4B). Immunohistochemistry revealed very few activated caspase-3 positive cells, suggesting that cells died from necrosis rather than apoptosis (Figure 7.4C). In both groups, the degree of bile duct injury was less severe in the more proximal (intrahepatic) biliary branches (data not shown). Interestingly, the degree of arteriolonecrosis of the peribiliary vascular plexus was significantly lower in the HMP group, compared to the SCS group (Table 7.1 and Figure 7.4D).
Figure 7.4. Representative examples of H&E histology of bile ducts of DCD liver grafts preserved by either 4 h of oxygenated HMP or 4 h of SCS followed by 2 h by normothermic ex-vivo sanguineous perfusion. Panel A: extrahepatic bile duct immediately after procurement (magnification 200x). The insert represents a higher magnification of 400x. Panel B: extrahepatic bile duct after preservation and reperfusion (magnification 280x). Panel C: Immunohistochemistry for activated caspase-3 of the same bile ducts as presented in panel B (brown color indicates immunopositivity; counterstaining with hematoxylin). Very few caspase-3 positive cells were detected in the bile duct wall stroma. Remnant biliary epithelial cells (i.e. in the peribiliary glands) were not positive for activated caspase-3. Panel D: higher magnification (400x) of extrahepatic bile ducts focusing on the peribiliary plexus. Arrows indicate peribiliary arterioles. Bile ducts of livers preserved by oxygenated HMP displayed significantly less signs of arteriolonecrosis, compared to livers preserved by SCS (see also Table 7.1).
DISCUSSION

Although several authors have proposed machine perfusion as an attractive alternative method for preservation of DCD livers, it remains to be established whether this technique will reduce the amount of bile duct injury and contributes to a reduction of biliary complications. We here report the first study on the impact of oxygenated HMP on the preservation of the bile ducts in a porcine DCD model. The most striking histological difference between oxygenated HMP and SCS was a lower degree of arteriolonecrosis of the peribiliary plexus in the extrahepatic bile ducts of grafts preserved by HMP. In parallel with this we observed a higher arterial blood flow after reperfusion of HMP preserved livers, compared to SCS. Although HMP did not result in a lower amount of injury of the biliary epithelial lining or underlying stroma, the observed difference in preservation of the peribiliary vascular plexus may be clinically relevant as the presence of arteriolonecrosis in extrahepatic bile duct biopsies at the time of transplantation was recently identified as the only independent histological parameter predictive for the

Table 7.1 Histological analysis of bile duct injury according to Hansen et al. (11)

<table>
<thead>
<tr>
<th></th>
<th>Oxygenated HMP group (n=8)*</th>
<th>Static Cold Storage Group (n=9)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mucosal loss</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score 0 (no BEC loss)</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>Score 1 (≤50% BEC loss)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Score 2 (&gt;50% BEC loss)</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td><strong>Mural necrosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score 0 (no necrosis)</td>
<td>0</td>
<td>0</td>
<td>0.92</td>
</tr>
<tr>
<td>Score 1 (≤25% necrosis)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Arteriolonecrosis</strong></td>
<td></td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>Score 0 (no arteriolonecrosis)</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Score 1 (≤50% arteriolonecrosis)</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Score 2 (&gt;50% arteriolonecrosis)</td>
<td>1</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td><strong>Bleeding</strong></td>
<td></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>Score 0 (no bleeding)</td>
<td>8</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Score 1 (≤50% of the bile duct)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Score 2 (&gt;50% of the bile duct)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Thrombosis</strong></td>
<td></td>
<td></td>
<td>0.60</td>
</tr>
<tr>
<td>Score 0 (no thrombi)</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Score 1 (occurrence of thrombi)</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

* Bile duct histology was missing for one pig in the HMP group. Abbreviations used: BEC, biliary epithelial cells; NA, not assessable.
development of NAS after human liver transplantation (11). Machine perfusion of liver grafts may enable better preservation of the arterial vasculature of the biliary tree by providing a continuous supply of oxygen to the endothelium, better distribution of the preservation fluid, and wash out of waste products. Adequate blood supply to the bile ducts is known to be of critical importance in maintaining viability. Insufficient arterial perfusion of liver grafts, either due to hepatic artery thrombosis or stenosis, leads to ischemic cholangiopathy, characterized by loss of biliary epithelium, necrosis of the bile duct wall and subsequent narrowing of the bile duct lumen due to sclerosing fibrosis (9, 17). Therefore, better preservation of the peribiliary plexus and higher arterial flow after reperfusion as observed in the current study is an important finding.

Apart from the differences in arterial flow after reperfusion and better preservation of the peribiliary vascular plexus, we found no histological differences in amount of biliary epithelial cell loss or bile duct stroma. In parallel with this, biochemical parameters of biliary epithelial cell function (biliary pH and bicarbonate secretion) or biliary injury (concentrations LDH, AF, and gamma-GT in bile) did not differ between the two groups. Nevertheless, better preservation and perfusion of the peribiliary vascular plexus as observed in the HMP group may result in faster and more efficient regeneration of the biliary epithelium. Two recent clinical studies have demonstrated that biliary epithelial cell loss can be found in more than 80% of all human liver grafts before transplantation (11, 18). This interesting new finding has changed the perspective from which to view the pathogenesis of biliary strictures after transplantation as it suggests that the critical factor that determines whether a graft will develop biliary strictures or not is insufficient biliary regeneration rather than the initial biliary injury (19).

In accordance with previous studies (12, 16, 20, 21) we have observed a reduction in biochemical histological markers of hepatocellular injury and histological injury of the parenchyma. Release of ALT and LDH after graft reperfusion was lower in the HMP group, compared to SCS preserved livers. A difference persisted when we corrected for a possible wash out effect in HMP preserved livers by calculating the relative change in concentration of these enzymes after the first 15 min of reperfusion. Although preservation of the liver parenchyma was not the primary focus of the current study, these findings confirm previous studies suggesting that HMP provides better preservation of the liver parenchyma than SCS.

Although great progress has been made in the development of machine perfusion as an alternative to SCS for preservation of donor organs, it remains to be established which method of machine perfusion is most effective. Several variations of machine perfusion have been explored, including hypothermic oxygenated or non-oxygenated, subnormothermic
and normothermic perfusion (12, 13, 21-24). It also remains unclear whether or not machine perfusion should replace SCS completely, or whether it is sufficient to provide a short period of machine perfusion at the end of cold storage before transplantation (16). The most basic type of machine preservation is non-oxygenated hypothermic perfusion, a technique that was first applied in a clinical setting by Guarrera et al (12). The advantage of this technique is its relative simplicity and safety, but there is doubt whether this method is sufficient to protect high risk grafts such as those from DCD donors (20, 25). Optimal preservation and protection of the biliary epithelium and bile duct wall may require a more advanced technique in which liver grafts are perfused with oxygenated perfusion fluid and at body temperature. Using a porcine model of DCD livers, Boehnert et al recently reported favourable effects of normothermic acellular machine perfusion of bile duct preservation in pig DCD livers (26). Our group previously demonstrated that normothermic, oxygenated perfusion of human donor livers is technically feasible; however, more research in this area is needed to determine whether this will also result in a reduction of biliary complications after transplantation (13).

In conclusion, this study suggests that oxygenated HMP of liver grafts from DCD donors provides protection against ischemic injury of the peribiliary vascular plexus, as reflected by a lower degree of arteriolonecrosis in the bile duct wall. Moreover, it associated with higher arterial flow rates early after reperfusion, compared to SCS. Although, we observed no differences in the degree of biliary epithelial cell loss or bile duct necrosis between livers preserved by HMP or SCS, the combination of better preservation of the peribiliary plexus and higher arterial flow may contribute to a faster recovery of the post ischemic bile ducts. Whether this results in a reduction of the rate of biliary complications after transplantation of DCD livers should be answered in clinical trials.

ACKNOWLEDGEMENTS

This research work was financially supported by grants provided by Innovatief Actieprogramma Groningen (IAG-3) and the Jan Kornelis de Cock Stichting in The Netherlands. We are grateful to Arjan van der Plaats and Martin Kuizenga (Organ Assist, Groningen, Netherlands) for their technical support and assistance during the perfusion experiments and to Martha op den Dries for her surgical assistance during the liver procurement procedures.
SUPPLEMENTAL DATA

Measurement of thiobarbituric acid reactive substances (TBARS)

TBARS were measured in bile samples as a marker for oxidative stress in bile ducts (see Supplemental Digital Content). Twenty microliters of bile (1:10 diluted with H2O) was mixed with 90mL sodium dodecyl sulfate, 10mL 0.05M butylated hydroxytoluene, 400mL 0.1 N HCL, 50mL 10% phosphotungstic acid and 200mL 0.7% 2-thiobarbituric acid. Samples were kept at 92-97°C for exactly 30 min and the content was mixed with 800mL 1-butanol. After centrifugation for 10 min (3,000 rpm/960g), samples from the butanol layer were transferred to a 96-well microtitre plate and fluorescence was detected at an excitation wavelength of 530nm and emission wavelength of 590nm. TBARS concentrations were calculated from a calibration curve of malondialdehyde constructed on the same plate.

Gene Expression of Hepatobiliary Transporter Proteins

Hepatic mRNA expression of relevant hepatocellular and cholangiocyte transporter proteins involved in bile secretion was determined by quantitative real-time PCR. Total RNA was isolated from frozen liver tissue using TRIzol (Gibco Life Technologies, Grand Island, NY), and M-MLV reverse transcriptase (Invitrogen, Basel, Switzerland) was used to convert RNA into cDNA. For quantitative real-time detection, sense and antisense porcine-specific primers (Invitrogen, Paisly, Scotland) were designed for the following genes, using Primer Express software (Applied Biosystems, Foster City, CA): the hepatocellular transporters BSEP (bile salt export pump; Abcb11) and MDR3 (the phospholipid translocator multidrug resistant protein 3; Abcb4), as well as the biliary epithelial transporters involved in biliary bicarbonate secretion CFTR (cystic fibrosis transmembrane conductance regulator; Abc35) and AE2 (anion exchanger 2; Slc4a2), CK19 (cytokeratin 19; used as internal standard for biliary epithelium) and 18s rRNA (internal standard). Amplification and detection were performed with the ABI Prism 7900HT Sequence Detection System using emission from SYBR® Green (Applied Biosystems). Copy numbers of CK19 and transporter gene mRNA were normalized for 18s rRNA and additionally for CK19 mRNA if appropriate.

Adenosine-5’-triphosphate (ATP) Extraction and Measurement

Hepatic concentration of ATP was used as an indicator of the energy status of grafts. Liver samples were immediately frozen in liquid nitrogen. Frozen tissue was cut into 20mm slices and a total amount of ±50mg was homogenized in 1 mL of SONOP (0.372g EDTA in 130mL H2O and NaOH (pH 10.9) + 370 mL 96% ethanol) and sonificated. Precipitate was removed by centrifugation (13,000 rcf for 10 min). Supernatant was diluted with SONOP to attain a protein
concentration of 200-300 mg/mL (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL) and mixed with 450mL of 100mM phosphate buffer (Merck; pH 7.6-8.0). Fifty microliters of phosphate buffered supernatant was used for ATP measurement using ATP Bioluminescence assay kit CLS II (Boehringer, Mannheim, Germany) and a luminometer (Victor3TM 1420 multilabel counter, PerkinElmer). ATP concentrations were calculated from a calibration curve constructed on the same plate, corrected for amount of protein, and values were expressed as μmol/g protein.

**Supplemental Table:** Sequences of primers used for real time RT PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Code</th>
<th>Primers</th>
<th>PCR Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSEP (Abcb11)</td>
<td>DQ530510</td>
<td>Forward 5'-GCC AAG AAA GGA GCC TAC TAC AAA C-3'</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>U20587</td>
<td>Reverse 5'-CTG TGC ATG AAG TTC TCA AGT CAG AT-3'</td>
<td></td>
</tr>
<tr>
<td>MDR3 (Abcb4)</td>
<td>EF067318</td>
<td>Forward 5'-CCA GGA AGC AAA GAA ACT CAA TG-3'</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5'-CTC CTC CAG GGT CAC AAT GC-3'</td>
<td></td>
</tr>
<tr>
<td>CFTR (ABc35)</td>
<td>NM_001104950.1</td>
<td>Forward 5'-AATGACTGTCAAGACCTCAGC-3'</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5'-TAATACGGCGTTCCCACCAT-3'</td>
<td></td>
</tr>
<tr>
<td>AE2 (Slc4a2)</td>
<td>XM_00360081.2</td>
<td>Forward 5'-GATTTTTCTGTCATAGGTTGCTCAC-3'</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5'-GCTCGTAGAAGCTGTTGTTCAA-3'</td>
<td></td>
</tr>
<tr>
<td>CK19</td>
<td>XM_003131437.1</td>
<td>Forward 5'-TGCCACCATTGAGAACTCCA-3'</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5'-CAGACGGCGTTGCTCA-3'</td>
<td></td>
</tr>
<tr>
<td>18s</td>
<td>NR_66261</td>
<td>Forward 5'-AGTCCCTGGCCTTTGTACAC-3'</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse 5'-AACCATCCATTCTCTAGGG-3'</td>
<td></td>
</tr>
</tbody>
</table>
Supplemental Figure: Parameters of hepatocellular secretory function of DCD livers that were preserved by either 4 h of oxygenated HMP or SCS and subsequently reperfused for 2 h by normothermic ex-vivo sanguineous perfusion. Panel A: Evolution of bile production in the HMP and SCS group. Panel B and C: Biliary concentration of bile salts and phospholipids, respectively. Panel D: Bile salt toxicity, as represented by the ratio of biliary bile salt and phospholipid concentrations. There were no statistically significant differences between the groups. Panel E-F: Relative mRNA expression of the main hepatocellular bile transporters BSEP (bile salt export pump; Abcb11) and MDR3 (multidrug resistance protein 3, Abcb4) after 2 h of ex-vivo sanguineous reperfusion of DCD livers that were preserved by either 4 h of oxygenated HMP or SCS. There were no significant differences between the two groups.

Supplemental Figure: Comparison of functional parameters of biliary epithelial cell function in DCD livers that were preserved by either 4 h of oxygenated HMP or SCS and subsequently reperfused for 2 h by normothermic ex-vivo sanguineous perfusion. There were no significant differences in biliary pH (A), biliary bicarbonate (B) and glucose concentration (C) at 2 h after reperfusion between the two groups. Relative mRNA expression of the main cholangiocyte transporter proteins involved in biliary bicarbonate secretion, CFTR (cystic fibrosis transmembrane conductance regulator; ABCC7) (D) and AE2 (anion exchanger 2; SLCA2) (E) after 2 h of ex-vivo sanguineous reperfusion of DCD livers that were preserved by either 4 h of oxygenated HMP or SCS. There were no significant differences between the two groups.
Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after cardiac death.

REFERENCES


