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ORIGINAL ARTICLE

Subnormothermic Ex Vivo Liver Perfusion Reduces Endothelial Cell and Bile Duct Injury After Donation After Cardiac Death Pig Liver Transplantation

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An ischemic-type biliary stricture (ITBS) is a common feature after liver transplantation using donation after cardiac death (DCD) grafts. We compared sequential subnormothermic ex vivo liver perfusion (SNEVLP; 33°C) with cold storage (CS) for the prevention of ITBS in DCD liver grafts in pig liver transplantation (n = 5 for each group). Liver grafts were stored for 10 hours at 4°C (CS) or preserved with combined 7-hour CS and 3-hour SNEVLP. Parameters of hepatocyte [aspartate aminotransferase (AST), international normalized ratio (INR), factor V, and caspase 3 immunohistochemistry], endothelial cell (EC; CD31 immunohistochemistry and hyaluronic acid), and biliary injury and function [alkaline phosphatase (ALP), total bilirubin, and bile lactate dehydrogenase (LDH)] were determined. Long-term survival (7 days) after transplantation was similar between the SNEVLP and CS groups (60% versus 40%, $P = 0.13$). No difference was observed between SNEVLP- and CS-treated animals with respect to the peak of serum INR, factor V, or AST levels within 24 hours. CD31 staining 8 hours after transplantation demonstrated intact EC lining in SNEVLP-treated livers ($7.3 \times 10^{-4} \pm 2.6 \times 10^{-4}$ cells/μm^2) but not in CS-treated livers ($3.7 \times 10^{-4} \pm 1.3 \times 10^{-4}$ cells/μm^2, $P = 0.03$). Posttransplant SNEVLP animals had decreased serum ALP and serum bilirubin levels in comparison with CS animals. In addition, LDH in bile fluid was lower in SNEVLP pigs versus CS pigs (14 ± 10 versus 60 ± 18 μmol/L, $P = 0.02$). Bile duct histology revealed severe bile duct necrosis in 3 of 5 animals in the CS group but none in the SNEVLP group ($P = 0.03$). Sequential SNEVLP preservation of DCD grafts reduces bile duct and EC injury after liver transplantation. ***Liver Transpl 20:1296-1305, 2014***. © **2014 AASLD.**

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The demand for liver transplantation by far exceeds the availability of grafts. Approximately 20% to 30% of patients on the waiting list for liver transplantation die or are delisted for disease progression before they receive a transplant.[1,2] One possible strategy to increase the donor pool is the use of marginal grafts,

Abbreviations: 4-MU, 4-methylumbelliferone; ALP, alkaline phosphatase; AST, aspartate aminotransferase; CS, cold storage; DCD, donation after cardiac death; EC, endothelial cell; HA, hyaluronic acid; H&E, hematoxylin and eosin; INR, international normalized ratio; ITBS, ischemic-type biliary stricture; LDH, lactate dehydrogenase; MUG, 4-methylumbelliferyl-galactoside; PECAM, platelet endothelial cell adhesion molecule; SNEVLP, subnormothermic ex vivo liver perfusion; WI, warm ischemia.

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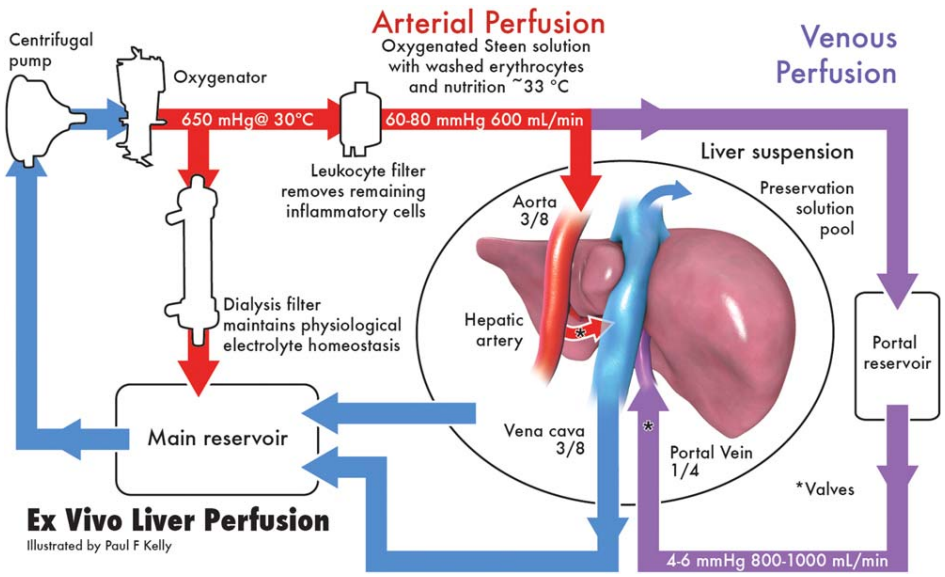

Figure 1. The circuit's perfusate is driven by a centrifugal pump at 1700 to 2000 rounds per minute. An oxygenator, including a heating-cooling unit, saturates the perfusate to an oxygen pressure of 650 mm Hg at 33°C. The perfusate passes through a leukocyte filter to remove inflammatory cells. The liver is perfused via the hepatic artery with a pressure of 60 to 70 mm Hg, and this results in an arterial flow of up to 500 mL/minute. The portal vein is perfused by gravity with a pressure of 4 to 8 mm Hg, and this results in a portal flow of 900 to 1100 mL/minute. The liver is placed on a heatable water bath detached by a sterile organ bag. The effluent returns in a closed system via the upper and lower vena cava back to the main reservoir.

such as steatotic grafts, grafts from older donors, or donation after cardiac death (DCD) livers. However, the current preservation technique of cold storage (CS) is poorly tolerated by marginal livers and results in severe reperfusion injury and graft dysfunction.[3-5] In addition, CS does not allow an assessment of liver function or injury and is unsuitable for the application of protective strategies during organ preservation.[6] Moreover, DCD grafts preserved by CS are associated with a 20% to 40% ischemic-type biliary stricture (ITBS) rate.[5,7-10] These shortcomings have triggered special interest in normothermic or subnormothermic perfused liver preservation, which avoids the negative effects of cooling and offers the opportunity to assess liver function at physiological or close-to-physiological temperatures. Furthermore, metabolically active grafts are likely more suitable for protective strategies.[11] Using diluted blood as a perfusate, other groups have demonstrated that graft preservation by exclusively normothermic ex vivo liver perfusion can reduce reperfusion injury in DCD grafts and improve animal survival.[12-14] However, the protective effect of warm-perfused preservation was lost when warm ischemia (WI) and cold ischemia were combined.[15,16] In addition, the posttransplant effects of warm-perfused liver preservation on bile duct injury in DCD grafts have not so far been investigated.

A blood-based perfusion solution contains inflammatory mediators such as leukocytes, platelets, and cytokines, which might lead to worse outcomes for grafts that undergo CS and then warm ex vivo perfusion. Here we have developed a novel technique of subnormothermic ex vivo liver perfusion (SNEVLP) using an albumin-based perfusate (Steen solution) plus leukocyte-depleted, washed erythrocytes. Here we compare CS with combined CS and SNEVLP for the preservation of DCD liver grafts in a model of pig liver transplantation. We investigate the effects of SNEVLP in DCD grafts on hepatocyte, sinusoidal endothelial cell (EC), and bile duct injury after transplantation.

PATIENTS AND METHODS

Animals

Male Yorkshire pigs (30-35 kg) were used for this study. All animals received humane care in compliance with the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. The animal care committee of the Toronto General Research Institute approved all studies.

Subnormothermic Ex Vivo Perfusion Circuit

The perfusion circuit consisted of a hard-shell reservoir (Cardiotomy reservoir 2811), a centrifugal pump (Rotaflow centrifugal pump), a hollow-fiber oxygenator/heat exchanger plus a hard-shell reservoir (Quadrox-I Adult HMO70000 and VHK 2001; all from Maquet, Hirrlingen, Germany), a hollow-fiber dialyzer (NR16, Fresenius, Bad Homburg, Germany), and a leukocyte filter (LeukoGuard LG arterial filter, Pall Corp., Port Washington, NY; Fig. 1).

SNEVLP was performed with a 3-L Steen solution (XVIVO Perfusion, INC., Goteborg, Sweden) plus washed erythrocytes to achieve a hematocrit of 10%

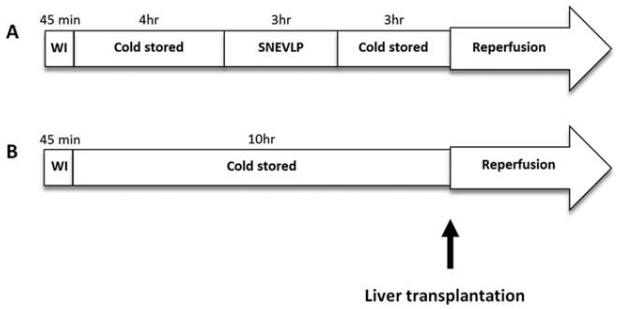

Figure 2. (A) Forty-five minutes of WI was applied to both groups as a model of DCD organ retrieval. Then, the treatment group was exposed to 4 hours of CS plus 3 hours of SNEVLP plus 3 hours of CS. The first CS period was used to simulate transport of the organ to the transplant center, and the second CS period was applied for cooling during recipient hepatectomy and implantation. (B) The control group grafts were conventionally cold-stored for 10 hours.

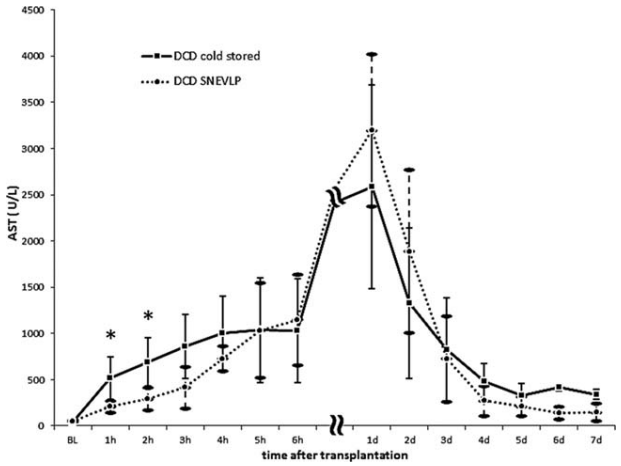

Figure 3. AST after DCD liver transplantation with CS- and SNEVLP-preserved grafts. AST levels were decreased early after transplantation in the SNEVLP group, but the 2 groups reached similar peak values within 24 hours after transplantation (n = 5 for each group, $*P < 0.05$).

to 12%. The erythrocytes were washed 3 times and passed through a leukocyte filter to avoid contamination with serum and to remove unwanted proinflammatory cells. The Steen solution is a buffered extracellular-type solution containing dextran and albumin to provide an optimized colloid osmotic pressure. The perfusate contained heparin (10,000 IU; Sandoz Canada, Boucherville, Canada) to prevent clot formation from residual coagulation factors. For metabolic supplies, the perfusate comprised an amino acid concentrate (50-mL bolus plus 8 mL/hour; 4.25% Travasol, Baxter, Hamilton, Canada), Ringers lactate in D5W (150 mL; Baxter), and insulin (40 IU/hour; Humulin R, Eli Lilly, Indianapolis, IN); cefazolin (1 g; Pharmaceutical Partners of Canada, Richmond Hill, Canada) and metronidazole (500 mg; Baxter, Toronto, Canada) were added to prevent bacterial contamination. To improve flow properties by vasodilatation, a bolus of BQ 123 (1.7 g; AG Scientific, Kelowna,

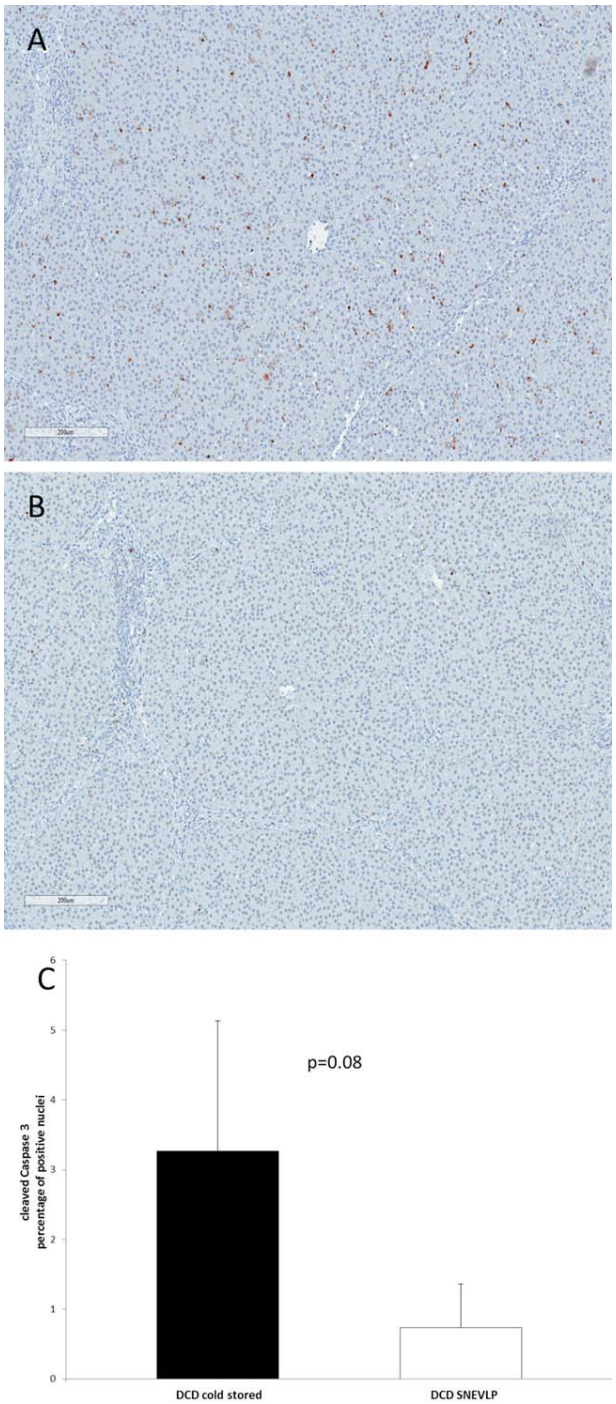

Figure 4. Cleaved caspase 3-positive cells were evaluated with image analysis software in scanned slides of liver tissue obtained 8 hours after reperfusion. (A) Immunohistochemistry for cleaved caspase 3 in the CS group. (B) Immunohistochemistry for cleaved caspase 3 in the SNEVLP group. (C) CS DCD grafts had a trend toward more caspase 3 staining than SNEVLP-treated liver grafts (n = 5 for each group, $P = 0.07$).

Canada) and a continuous infusion of alprostadil (250 μg/3 hours; Pfizer, Kirkland, Canada) were used. Acetylcysteine (6 g; Sandoz Canada) was added for its free radical–scavenging properties. Notably, none of

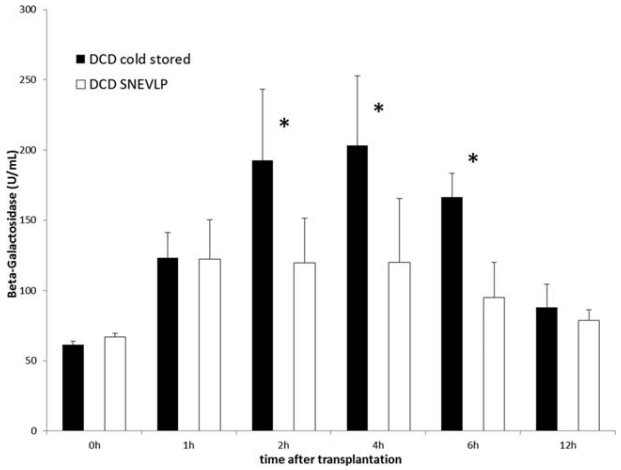

Figure 5. β-Galactosidase serum levels after DCD liver transplantation as a marker of Kupffer cell activation. β-Galactosidase levels were decreased in pigs receiving an SNEVLP graft versus CS DCD graft in the reperfusion phase between 2 and 6 hours (n = 5 for each group, $*P < 0.05$).

the perfusate components were renewed during perfusion. The perfusate did not contain any serum parts. A gas composition of 95% O_2 and 5% CO_2 was connected to the oxygenator at a sweep of 2 L/minute. Additionally, active gaseous components were added: CO (1000 ppm; Praxair, Burlington, Canada) for its vasodilatative and anti-inflammatory properties[17-19] and sevoflurane (1.5%; Abbott, Saint-Laurent, Canada) for its protective properties for ECs.[20] Both were also administered through the oxygenator. The liver was perfused at 33°C with a continuous flow. Dialysate was produced with a standard concentrate (D12188; Baxter), double-reverse-osmosis water, and sodium bicarbonate (Hospira, Montreal, Canada). Five hundred milliliters of the ready-to-use dialysate was perfused through the dialyzer per hour. The objective for the dialysis unit was to maintain the electrolyte concentration of the perfusate stably. The excreted dialysate was replaced 1:1 with dialysate fluid containing the desired electrolyte concentration. Perfusion through the hepatic artery was set at a pressure of 60 to 70 mm Hg, and this resulted in a flow of up to 500 mL/minute. The portal vein pressure was adjusted between 4 and 8 mm Hg, which corresponded to a flow of 900 to 1100 mL/minute. Ultrasonic flow probes (HT 110 flow meter, Transonic Systems, Ithaca, NY) were placed on the hepatic artery and portal vein circuit inflows for flow monitoring.

Study Design

A model of DCD livers was used via the induction of cardiac arrest by a potassium chloride injection (20 mEq; Hospira) at the end of the vascular dissection during the organ-recovery procedure. The donor pigs received in total 30,000 IU of heparin 5 minutes before cardiac arrest. After 45 minutes of cardiac arrest, the organs were flushed with 3 L of cold University of Wisconsin solution (SPS-1, Organ Recovery Systems, Itasca, IL) and stored on ice. During the donor WI time, the blood was collected. Erythrocytes

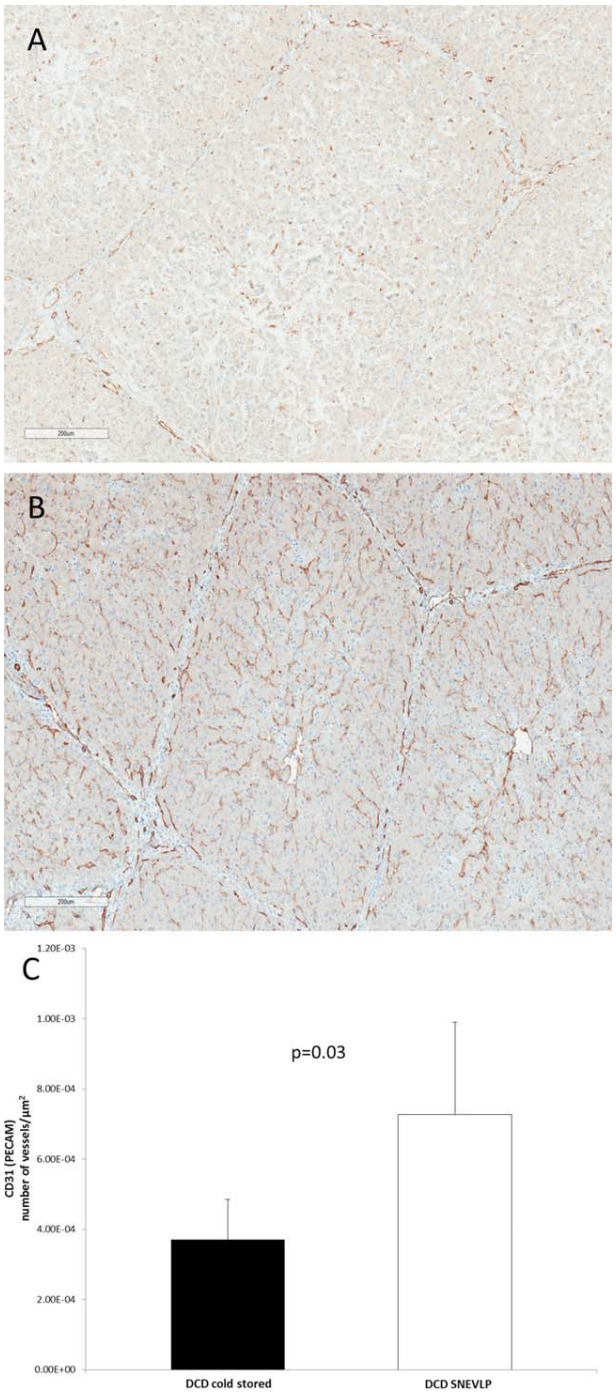

Figure 6. CD31 (PECAM) immunohistochemistry to assess sinusoidal EC injury. (A) DCD grafts in the CS group had severe injury of sinusoidal ECs 8 hours after transplantation. (B) SNEVLP-treated DCD grafts had preserved CD31 staining, which indicated reduced EC injury. (C) CS DCD grafts had a significantly lower vessel density as a marker of EC injury than SNEVLP grafts as assessed with imaging analysis software (n = 5 for each group, $P = 0.03$).

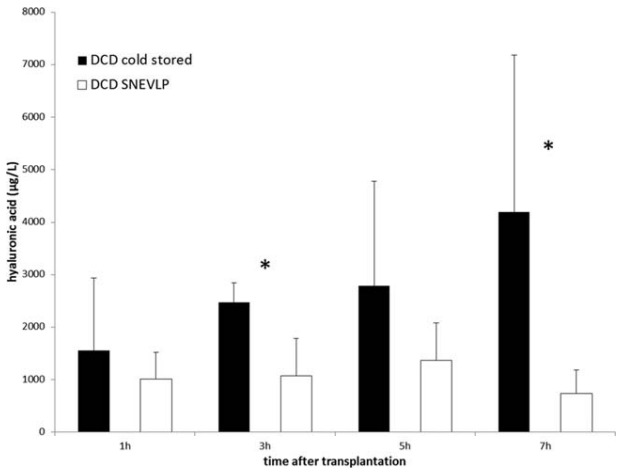

Figure 7. HA serum levels were evaluated after transplantation in CS- and SNEVLP-treated groups as a marker of HA clearance by ECs. Animals receiving a CS DCD liver graft had increased HA levels in comparison with pigs receiving a DCD graft preserved with the SNEVLP protocol (n = 5 for each group, $*P < 0.05$).

were isolated by soft spinning and were stored in citrate phosphate dextrose adenine (500-mL bag; Terumo, Somerset, NJ).

In the SNEVLP group, the liver was stored for 4 hours on ice (the time frame was designated to simulate the transport time from the donor hospital to the recipient hospital), and this was followed by SNEVLP for 3 hours at 33°C and then CS for 3 hours (10-hour total preservation time). In the control group, the grafts were continuously stored for 10 hours on ice (Fig. 2). At the end of the preservation time, orthotopic pig liver transplantation was performed with an active portojugular shunt (Rotaflow centrifugal pump, Maquet). Two different sets of experiments were performed. First, we performed a nonsurvival study (n = 5 per group). In the nonsurvival experiments, the animals were kept alive under anesthesia for 8 hours. Biopsies were obtained during this period, and bile production was measured by cannulation of the common bile duct. The animals were sacrificed at the end of the nonsurvival experiment. The second set of experiments was performed as a survival study (n = 5 per group). For the survival study, the animals were kept alive for 7 days. To prevent animal suffering, pigs were sacrificed before the end of the intended survival period in accordance with our animal use protocol and under the supervision of our veterinarian staff if predetermined animal-suffering criteria were met (lethargy, failure to move coordinately, metabolic or respiratory decompensation, and excessive bleeding). At autopsy, the patency of all anastomoses was confirmed. Pigs were exsanguinated while under deep isoflurane anesthesia after central liver and bile duct specimens (each right and left bile duct) had been obtained.

β-Galactosidase Assay

β-Galactosidase is a lysosomal enzyme that is rapidly released from Kupffer cells during hepatic reperfusion

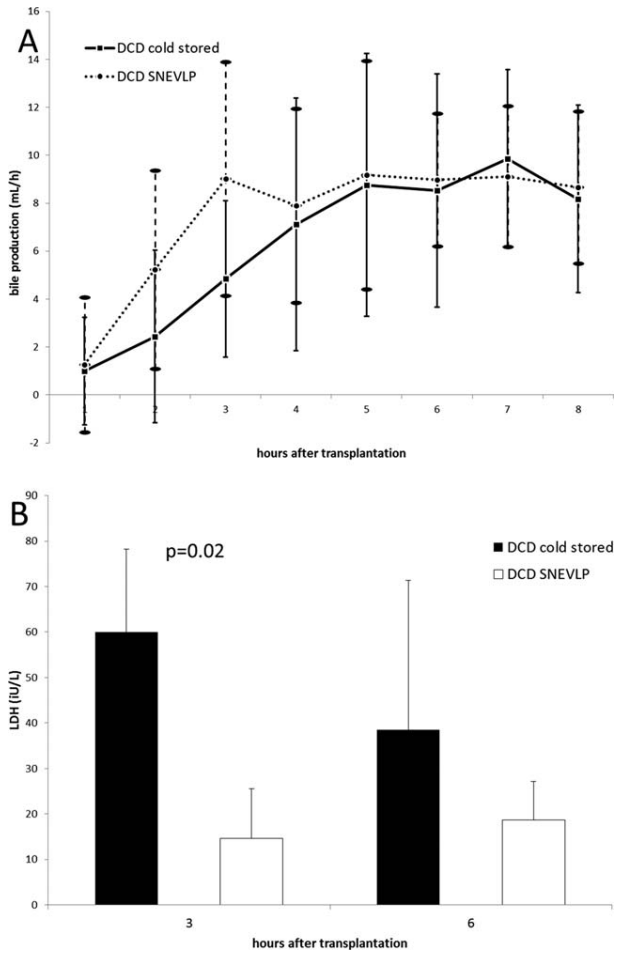

Figure 8. (A) CS-preserved and SNEVLP-treated liver grafts demonstrated similar bile production 4 hours after reperfusion (n = 5 for each group). (B) The bile LDH content at 3 hours after reperfusion as a marker of biliary injury was significantly lower in the SNEVLP grafts versus the CS grafts (n = 5 for each group, $P = 0.02$).

and is, therefore, considered an early marker of Kupffer cell activation.[21] We fluorometrically measured β-galactosidase serum levels hourly as described earlier by McGuire et al.[22] β-Galactosidase catalyzes the reaction of the substrate 4-methylumbelliferyl-galactoside (MUG) into 4-methylumbelliferone (4-MU), which can be detected fluorometrically by a microplate reader with an excitation wavelength of 340 nm and an emission wavelength of 465 nm. For each well, 10 µL of a 4× diluted serum sample was added to 80 µL of a solution of MUG substrate in a citrate-phosphate buffer (substrate concentration = 3.33 mmol/L, pH 4.5). Then, the microplate was incubated for 30 minutes at 37°C. The reaction was terminated by the addition of a glycine-NaOH buffer, and this raised the pH above 10. One unit of β-galactosidase is equivalent to 1 nmol of the substrate converted to the product in 1 hour at 37°C. Fluorometric values were compared with a 4-MU standard curve for each reading.

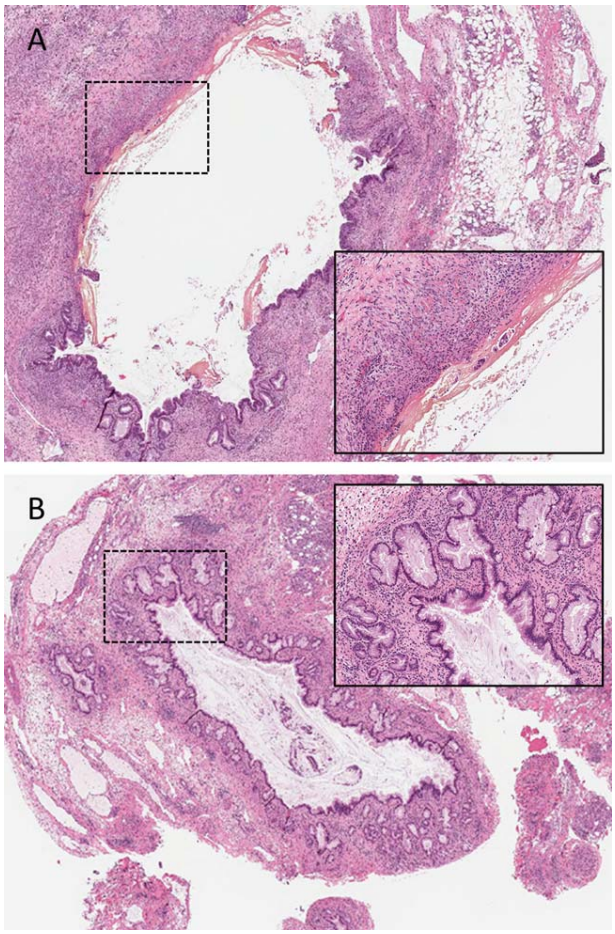

Figure 9. H&E staining of bile ducts in the CS and SNEVLP groups. (A) Massive necrosis in the CS livers. (B) The bile duct mucosa was completely preserved in all SNEVLP DCD grafts.

Hyaluronic Acid Assay

Under normal conditions, hyaluronic acid (HA) is metabolized by ECs. We tested its levels to assess EC function.[23] HA was measured with a commercially available enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). In brief, this assay uses a quantitative sandwich enzyme immunoassay technique. Recombinant human aggrecan, which is precoated in the plate well, binds any hyaluronan of serum and standard samples. After any unbound substances are washed away, enzyme-linked aggrecan is added to the wells. After a wash to remove any unbound aggrecan-enzyme reagent, a substrate solution is added to the wells, and color develops in proportion to the amount of hyaluronan bound in the initial step. The color development is stopped, and the intensity of the color is measured.

Parameters of Hepatocyte and Bile Duct Injury

Aspartate aminotransferase (AST) was measured every hour after blood reperfusion as a marker of hepatocyte injury with a modular bench-top chemistry system (Vitros DT60 II, Ortho Clinical Diagnostics, Rochester, NY). Liver necrosis was assessed by hematoxylin and eosin (H&E) staining at 8 hours after reperfusion. Cleaved caspase-3 staining was used as a marker of apoptosis. The international normalized ratio (INR) and factor V were used as markers of liver function.

Bile production was monitored hourly in the nonsurvival study. Bile fluid was analyzed for lactate dehydrogenase (LDH) content as a marker of bile duct injury. Total serum bilirubin and alkaline phosphatase (ALP) were measured daily in the survival model as a marker of bile duct damage. Bile duct necrosis was investigated by H&E staining at the end of animal survival.

Histology

At 8 hours after reperfusion in the nonsurvival group and at the end of animal survival at day 7 in the survival group, central liver and bile duct biopsies were taken and stored in 10% formalin overnight and then exchanged for 70% ethanol until paraffin embedding. Sections of 5-μm thickness were cut. Cleaved caspase 3 and CD31 [platelet endothelial cell adhesion molecule (PECAM)] staining were performed via immunohistochemistry.[24] Histology was evaluated by a blinded investigator (H&E) and with image analysis software (cleaved caspase 3, CD31).

For morphometric analysis, stained cells were identified with Spectrum 10.2.2.2317 (Aperio Technologies, Vista, CA). Briefly, slides stained immunohistochemically with cleaved caspase 3 (Cell Signaling Technology, Danvers, MA) and CD31 antibodies (Santa Cruz Biotechnology, Dallas, TX) were scanned at ×20 and were qualitatively analyzed with a nuclear (for caspase 3) and cytoplasmic (CD31) positive-pixel-count algorithm.

Statistical Analysis

The data were analyzed with the SPSS 22 statistical package (IBM, Chicago, IL). The Mann-Whitney test was used for the comparison of continuous variables, and the chi-square test was applied for categorical outcomes. The results are presented as means and standard deviations and are considered significant at $P \leq 0.05$.

RESULTS

Liver Injury in SNEVLP and CS DCD Liver Grafts After Transplantation

First, we determined whether SNEVLP alone induced injury in DCD liver grafts. The liver grafts were exposed to 45 minutes of WI plus 4 hours of CS before SNEVLP. AST as a marker of hepatocyte injury did not increase during 3 hours of SNEVLP with a mean perfusate AST level of 152 ± 23 U/L after 1 hour and 146 ± 25 U/L after 3 hours ($P = 0.8$). H&E staining of liver tissues at the end of SNEVLP

TABLE 1. ALP and Bilirubin as Markers of Bile Duct Injury in Pigs With and Without Biliary Necrosis

	Pigs With Bile Duct Necrosis (n = 3)	Pigs Without Bile Duct Necrosis (n = 7)	*P* Value
Total bilirubin peak (μmol/L)	32 ± 30	15 ± 7	0.58
ALP peak (U/L)	369 ± 45	203 ± 80	0.03

preservation showed minimal liver necrosis (<5%). This minimal necrosis during ex vivo perfusion demonstrated that our perfusion system did not induce liver damage by itself.

Next, we compared liver injury in SNEVLP-preserved and cold-preserved livers after orthotopic liver transplantation. Serum AST levels at 2 hours after transplantation were significantly lower in SNEVLP grafts versus CS grafts (261 ± 175 versus 691 ± 261 U/L, $P = 0.008$; Fig. 3). However, peak AST levels within 24 hours after reperfusion were similar between SNEVLP and CS grafts (3198 ± 826 versus 2585 ± 1102 U/L, $P = 0.46$). After 8 hours of reperfusion, SNEVLP-preserved grafts versus cold-preserved DCD grafts had a trend toward reduced cleaved caspase 3 staining (0.7% ± 0.6% versus 3.3% ± 1.9% positive cells, $P = 0.07$) as a marker of apoptosis (Fig. 4).

β-Galactosidase, a marker of Kupffer cell activation, was determined after transplantation. As shown in Fig. 5, serum levels of β-galactosidase were significantly lower in SNEVLP grafts versus CS grafts between 2 and 6 hours after reperfusion, and this indicated reduced Kupffer cell activation with SNEVLP preservation.

SNEVLP Instead of CS Reduces EC Injury in DCD Grafts

To assess EC viability, we stained liver tissue via CD31 immunohistochemistry at 8 hours after liver transplantation. Slides were analyzed with image analysis software. SNEVLP-preserved DCD livers had intact sinusoidal EC lining and minimal EC injury ($7.3 \times 10^{-4} \pm 2.6 \times 10^{-4}$ cells/μm^2). In contrast, CS DCD grafts had lost the sinusoidal EC lining, with only clumps of EC remaining; this indicated severe EC injury ($3.7 \times 10^{-4} \pm 1.3 \times 10^{-4}$ cells/μm^2, $P = 0.03$; Fig. 6).

HA serum levels were assessed after liver transplantation as a marker of EC function. HA is cleared by ECs, and increased HA levels correspond to decreased EC function. HA serum levels increased continuously after transplantation in CS grafts, whereas HA levels remained stable in SNEVLP DCD grafts. At 3 hours after transplantation, HA serum levels were significantly reduced in SNEVLP livers versus CS livers (1077 ± 711 versus 2476 ± 364 ng/mL, $P = 0.01$), and this indicated improved EC function in SNEVLP DCD grafts (Fig. 7).

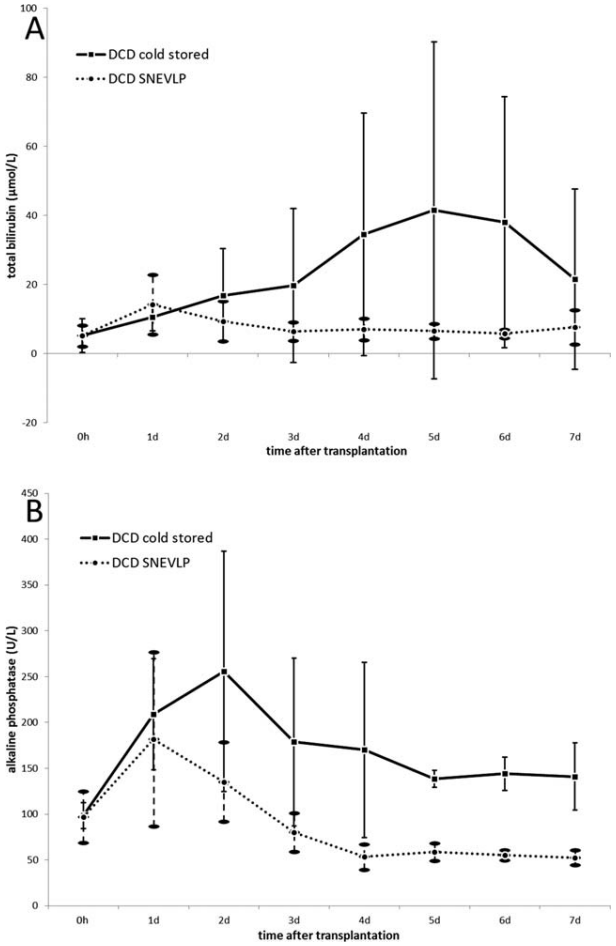

Figure 10. (A) Total bilirubin levels after DCD liver transplantation. Pigs in the SNEVLP group had uniformly low bilirubin levels in a physiological range after transplantation; bilirubin levels in pigs receiving a CS DCD liver graft were higher and more heterogeneous, but there was no significant difference from the SNEVLP group (n = 5 for each group). (B) ALP after DCD liver transplantation. Pigs in the SNEVLP group had lower ALP levels after transplantation in comparison with pigs receiving a CS DCD liver graft (n = 5 for each group, $P < 0.05$).

SNEVLP Instead of CS Reduces Bile Duct Injury and Improves Biliary Function After DCD Liver Transplantation

Hourly bile flow during the first 8 hours after transplantation was similar in SNEVLP and CS livers (Fig. 8A). LDH was measured in bile fluid as a marker of biliary epithelial injury. SNEVLP versus CS livers had significantly lower bile LDH levels 3 hours after transplantation (14 ± 10 versus 60 ± 18 μmol/L, $P = 0.02$; Fig. 8B).

Bile duct necrosis was investigated via H&E staining 7 days after transplantation or at the end of animal survival. Severe bile duct necrosis was present in 3 of 5 CS grafts. In contrast, no bile duct necrosis was

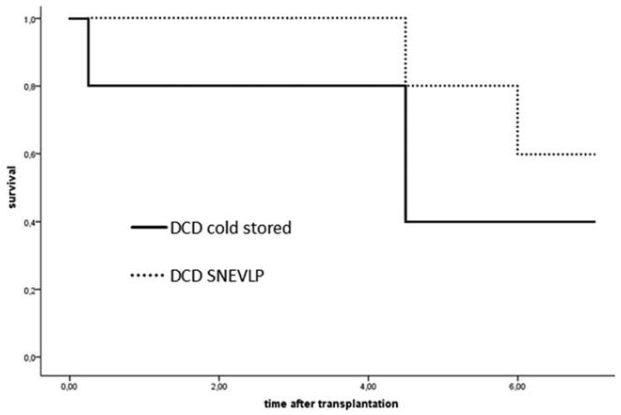

Figure 11. Survival curve after DCD liver transplantation in the CS and SNEVLP groups. Pigs receiving a CS graft had lower but not significantly different survival in comparison with pigs treated receiving an SNEVLP graft (n = 5 for each group, $P = 0.13$).

observed in SNEVLP DCD grafts ($P = 0.03$; Fig. 9). Bile duct necrosis was associated with increased ALP levels after transplantation (Table 1).

Serum bilirubin and ALP levels were measured at various time points after liver transplantation as markers of bile duct injury. As shown in Fig. 10A, serum bilirubin levels were lower in animals receiving an SNEVLP graft versus a CS graft, but the probability was not significant. Similarly, serum ALP levels were lower in pigs receiving DCD grafts preserved with SNEVLP versus CS; in this case, there was significant probability for postoperative days 3 and 4 (Fig. 10B).

Liver Function and Animal Survival After Liver Transplantation

Animal survival was considered permanent after 7 days after transplantation. Three animals with CS grafts died before the end of the survival period: 1 after 12 hours and 2 on day 5 after liver transplantation. In contrast, 2 deaths were registered in the SNEVLP group: the first on day 5 after transplantation and the second on day 7 (log-rank test, $P = 0.13$; Fig. 11). No significant difference between the 2 groups was observed in the coagulation parameters (INR ratio or factor V levels; Fig. 12). INR was normalized within 48 hours of transplantation in both groups.

DISCUSSION

This study demonstrates that SNEVLP protects DCD liver grafts against sinusoidal EC injury and decreases bile duct necrosis after liver transplantation. DCD liver grafts represent a large donor pool, which could significantly improve the current donor shortage. Unfortunately, DCD liver transplantation is associated with a high risk (20%-40% of cases) for ischemic-type bile duct injury.[5,7-10] This has resulted in strict selection criteria for DCD grafts, and as a

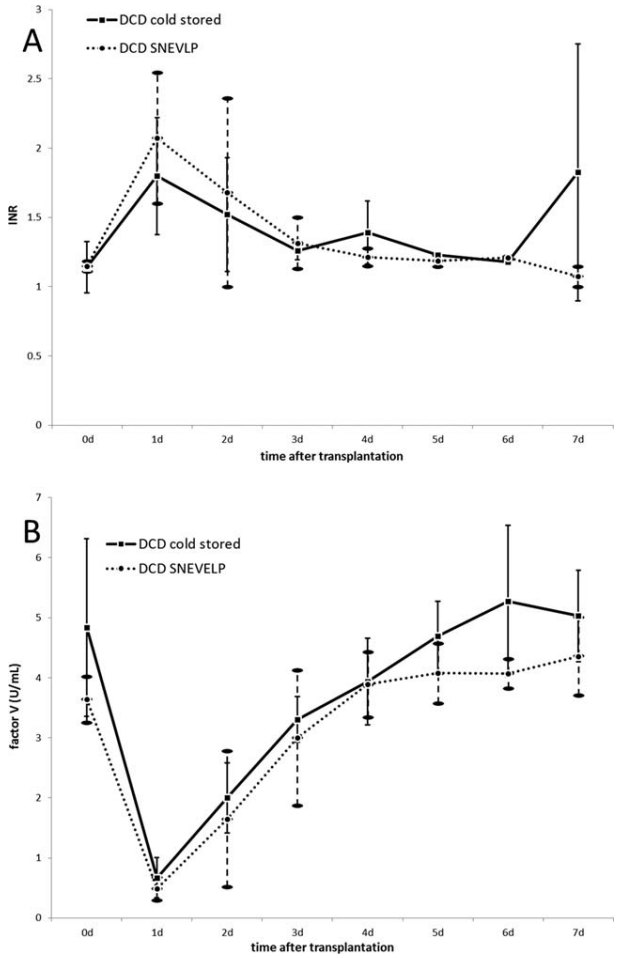

Figure 12. (A) INR and (B) factor V as parameters of liver function at the baseline and after DCD liver transplantation in pigs receiving either a CS graft or a graft treated with SNEVLP. Liver function was similar in the 2 groups (n = 5 for each group, P = not significant for all time points).

result, they are often declined on the basis of donor age or warm and cold ischemia times. A better preservation technique is required to protect bile ducts in DCD liver grafts and to make this donor group more broadly available for liver transplantation.[3-10]

Normothermic ex vivo liver perfusion or SNEVLP has been previously used to protect DCD liver grafts against preservation injury. Brockmann et al.[13] compared normothermic ex vivo liver perfusion with CS in DCD liver grafts. Using diluted blood as a perfusate, they reported a significant reduction of liver injury and improved survival with warm perfused storage. In their model, the authors used only 1 hour of CS before warm perfusion and did not investigate bile duct injury. The same group determined that the protective effect was lost when the length of CS before warm-perfusion preservation was prolonged.[15,16] Similarly, Schön et al.[12] demonstrated that normothermic ex vivo liver perfusion versus CS improved survival after liver transplantation with DCD grafts. Similarly to Brockmann et al.,[13] this group did not use CS

before their normothermic perfusion model. Fondevila et al.[14] used combined ECMO in vivo plus normothermic ex vivo perfusion in pig DCD liver grafts. The authors demonstrated that normothermic perfusion improves the outcome of DCD liver transplantation if CS can be avoided. Using ECMO during DCD organ retrieval is an interesting approach and could supplement normothermic ex vivo perfusion by minimizing organ cooling before normothermic perfusion of DCD grafts.

Cold nonoxygenated or oxygenated perfusion systems have been used by others.[25-28] Cold perfusion at 4°C offers the advantage for the liver of minimal oxygen requirement and, therefore, prevents reperfusion injury during the ex vivo perfusion. Also, failure of the perfusion system during transport would not automatically result in a loss of the liver graft. Unfortunately, the effects of cold perfusion on bile duct injury have not been investigated so far in a large animal model. Schlegel et al.[28] demonstrated a protective effect on biliary tissue in a rodent transplant model without arterial reconstruction. However, the role of hepatic arterialization in biliary injury is considered to be of superior importance in the occurrence of ITBS.[29] In addition, the low metabolism at cold temperatures precludes an assessment of graft function and makes the application of protective interventions more difficult. De Rougemont et al.[26] used 1 hour of oxygenated cold perfusion before DCD liver transplantation. Although no difference in transaminase release was observed after transplantation, the mean animal survival was slightly increased from 5 hours to 9 hours after transplantation.

Currently, transporting grafts from the donor hospital to the transplant center requires static cooling of the liver grafts for several hours. Portable perfusion devices that allow avoidance of any cooling period are attractive solutions for preventing cold ischemic injury. Such devices are in development but have not yet reached routine clinical practice. Meanwhile, transportation of the graft to the transplant center with CS followed by warm perfusion would simplify the logistics of the process. Hence, our study was designed to mimic a clinical scenario in which the grafts were to be transported from the donor hospital to the transplant center in CS before SNEVLP. Therefore, we included 4 hours of CS before the start of SNEVLP in our protocol. Because warm perfusion is technically challenging and a failure of perfusion after skin incision in the recipient would seriously jeopardize the patient's life, we added an additional 3 hours of CS after SNEVLP for the time of the recipient's preparation and hepatectomy.

Previously published studies have used blood or diluted blood as a perfusate for warm ex vivo perfusion. Blood-derived perfusate solutions have several disadvantages. Blood contains mediators of reperfusion injury, such as leukocytes, platelets, and cytokines. Thus, warm ex vivo perfusion after a period of CS might induce reperfusion injury, which could explain the inferior outcome for combined CS and warm ex vivo perfusion as mentioned previously.

In previous experiments, we used a completely acellular normothermic perfusion solution without any blood components for normothermically perfused organ preservation.[30] Using whole-blood ex vivo reperfusion as a model for transplantation, we found decreased bile duct injury in DCD grafts compared with CS livers. However, acellular perfusion was associated with decreased liver function and inferior long-term survival after pig liver transplantation. Therefore, in our current study, we changed our perfusion technique by using leukocyte-depleted, washed erythrocytes as oxygen carriers. In contrast to other solutions, our ex vivo perfusion solution was designed to minimize contamination with leukocytes, platelets, and serum during the ex vivo perfusion. Our albumin-based perfusate (Steen solution) with its osmotic properties replaced the cytokine-rich plasma fraction. Along with the addition of other active substances, the solution was able to decrease the activation of the inflammatory cascade substantially. Adding washed, leukocyte-depleted, and serum-free erythrocytes as oxygen carriers to the perfusate improved liver function after transplantation with similar protective effects on bile ducts in comparison with the acellular perfusate. A second important finding in this study is the protection of ECs by SNEVLP preservation. It is possible that decreased EC injury improves arterial perfusion of the biliary tree, which could result in protection of bile ducts against ischemic injury.

Hepatocyte injury was similar in the CS and SNEVLP groups. Although AST levels were decreased early after transplantation in pigs with SNEVLP versus CS, peak AST levels were similar at day 1, with a similar pattern of decline afterward. This indicates that hepatocyte injury was delayed, but not decreased, by SNEVLP preservation. However, primary nonfunction is rare in clinical DCD liver transplantation, and the high incidence of ITBS is the major obstacle for the extensive use of DCD liver grafts. Our finding that bile duct injury is reduced in SNEVLP versus CS DCD grafts could, therefore, represent an important advantage of warm-perfusion preservation of DCD grafts in comparison with CS.

This study has several limitations. First, mechanisms of protection were not investigated in detail. Further studies are needed to investigate the impact of warm-perfusion preservation on the bile duct blood supply and injury after transplantation. Furthermore, the protective effect was limited to bile duct protection without reducing hepatocyte death and without improving graft function. The experimental design with 45 minutes of WI and 10 hours of preservation, accounting for only limited hepatocyte injury, allowed graft recovery and pig survival in both the SNEVLP and CS groups. Longer ischemia times might be required to investigate whether SNEVLP has an effect on hepatocyte injury and function as well as graft survival. We chose 4 hours of CS before SNEVLP and 3 hours of CS after SNEVLP in our model. In clinical practice, these CS times could often be reduced, and prolonged CS before warm perfusion might not be

required in a large proportion of cases. Finally, we cannot exclude additional mechanisms present in human grafts in comparison with the porcine model.

In summary, this study demonstrates that organ preservation with combined CS and subsequent SNEVLP protects DCD liver grafts against ischemic-type bile duct injury and reduces EC death. Reducing the incidence of ITBS could allow us to use DCD liver grafts better and to increase the donor pool for liver transplantation.

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REFERENCES

1. US Department of Health and Human Services. Organ Procurement and Transplantation Network. http://optn.transplant.hrsa.gov/. Accessed May 2014.
2. Qiu J, Ozawa M, Terasaki PI. Liver transplantation in the United States. Clin Transpl 2005:17-28.
3. Le Dinh H, de Roover A, Kaba A, Lauwick S, Joris J, Delwaide J, et al. Donation after cardio-circulatory death liver transplantation. World J Gastroenterol 2012;18:4491-4506.
4. Rhee J, Kern B, Cooper J, Freeman RB. Organ donation. Semin Liver Dis 2009;29:19-39.
5. Cursio R, Gugenheim J. Ischemia-reperfusion injury and ischemic-type biliary lesions following liver transplantation. J Transplant 2012;2012:164329.
6. Rubbini M. Perfusion machines for liver transplantation: technology and multifunctionality. Updates Surg 2014;66:101-108.
7. Seehofer D, Eurich D, Veltzke-Schlieker W, Neuhaus P. Biliary complications after liver transplantation: old problems and new challenges. Am J Transplant 2013;13:253-265.
8. Maheshwari A, Maley W, Li Z, Thuluvath PJ. Biliary complications and outcomes of liver transplantation from donors after cardiac death. Liver Transpl 2007;13:1645-1653.
9. Reich DJ, Hong JC. Current status of donation after cardiac death liver transplantation. Curr Opin Organ Transplant 2010;15:316-321.
10. Heidenhain C, Pratschke J, Puhl G, Neumann U, Pascher A, Veltzke-Schlieker W, Neuhaus P. Incidence of and risk factors for ischemic-type biliary lesions following orthotopic liver transplantation. Transpl Int 2010;23:14-22.
11. Hessheimer AJ, Fondevila C, Garcia-Valdecasas JC. Extracorporeal machine liver perfusion: are we warming up? Curr Opin Organ Transplant 2012;17:143-147.
12. Schön MR, Kollmar O, Wolf S, Schrem H, Matthes M, Akkoc N, et al. Liver transplantation after organ preservation with normothermic extracorporeal perfusion. Ann Surg 2001;233:114-123.
13. Brockmann J, Reddy S, Coussios C, Pigott D, Guirriero D, Hughes D, et al. Normothermic perfusion: a new paradigm for organ preservation. Ann Surg 2009;250:1-6.
14. Fondevila C, Hessheimer AJ, Maathuis MH, Muñoz J, Taura P, Calatayud D, et al. Superior preservation of DCD livers with continuous normothermic perfusion. Ann Surg 2011;254:1000-1007.
15. Reddy S, Greenwood J, Maniakin N, Bhattacharjya S, Zilvetti M, Brockmann J, et al. Non-heart-beating donor porcine livers: the adverse effect of cooling. Liver Transpl 2005;11:35-38.
16. Reddy SP, Bhattacharjya S, Maniakin N, Greenwood J, Guerreiro D, Hughes D, et al. Preservation of porcine non-heart-beating donor livers by sequential cold storage and warm perfusion. Transplantation 2004;77:1328-1332.
17. Vollmar B, Menger MD. The hepatic microcirculation: mechanistic contributions and therapeutic targets in liver injury and repair. Physiol Rev 2009;89:1269-1339.
18. Tomiyama K, Ikeda A, Ueki S, Nakao A, Stolz DB, Koike Y, et al. Inhibition of Kupffer cell-mediated early proinflammatory response with carbon monoxide in transplant-induced hepatic ischemia/reperfusion injury in rats. Hepatology 2008;48:1608-1620.
19. Lee LY, Kaizu T, Toyokawa H, Zhang M, Ross M, Stolz DB, et al. Carbon monoxide induces hypothermia tolerance in Kupffer cells and attenuates liver ischemia/reperfusion injury in rats. Liver Transpl 2011;17:1457-1466.
20. Annecke T, Rehm M, Bruegger D, Kubitz JC, Kemming GI, Stoeckelhuber M, et al. Ischemia-reperfusion-induced unmeasured anion generation and glycocalyx shedding: sevoflurane versus propofol anesthesia. J Invest Surg 2012;25:162-168.
21. St Peter SD, Imber CJ, Lopez I, McGuire J, James T, Taylor R, et al. Beta-galactosidase as a novel marker of ischaemic injury and a mechanism for viability assessment in liver transplantation. Transplant Proc 2001;33:3753-3755.
22. McGuire JB, James TJ, Imber CJ, St. Peter SD, Friend PJ, Taylor RP. Optimisation of an enzymatic method for beta-galactosidase. Clin Chim Acta 2002;326:123-129.
23. Soejima Y, Yanaga K, Wakiyama S, Nishizaki T, Yoshizumi T, Sugimachi K. Serum hyaluronic acid as a reliable parameter of allograft viability in porcine liver transplantation. Hepatogastroenterology 1996;43:590-595.
24. Guo JY, Yang T, Sun XG, Zhou NY, Li FS, Long D, et al. Ischemic postconditioning attenuates liver warm ischemia-reperfusion injury through Akt-eNOS-NO-HIF pathway. J Biomed Sci 2011;18:79.
25. Guarrera JV, Estevez J, Boykin J, Boyce R, Rashid J, Sun S, Arrington B. Hypothermic machine perfusion of liver grafts for transplantation: technical development in human discard and miniature swine models. Transplant Proc 2005;37:323-325.
26. de Rougemont O, Breitenstein S, Leskosek B, Weber A, Graf R, Clavien PA, Dutkowski P. One hour hypothermic oxygenated perfusion (HOPE) protects nonviable liver allografts donated after cardiac death. Ann Surg 2009;250:674-683.
27. van der Plaats A, Maathuis MH, 'T Hart NA, Bellekom AA, Hofker HS, van der Houwen EB, et al. The Groningen hypothermic liver perfusion pump: functional evaluation of a new machine perfusion system. Ann Biomed Eng 2006;34:1924-1934.
28. Schlegel A, Graf R, Clavien PA, Dutkowski P. Hypothermic oxygenated perfusion (HOPE) protects from biliary injury in a rodent model of DCD liver transplantation. J Hepatol 2013;59:984-991.
29. Hashimoto K, Eghtesad B, Gunasekaran G, Fujiki M, Uso TD, Quintini C, et al. Use of tissue plasminogen activator in liver transplantation from donation after cardiac death donors. Am J Transplant 2010;10:2665-2672.
30. Boehnert MU, Yeung JC, Bazerbachi F, Knaak JM, Selzner N, McGilvray ID, et al. Normothermic acellular ex vivo liver perfusion reduces liver and bile duct injury of pig livers retrieved after cardiac death. Am J Transplant 2013;13:1441-1449.