ORIGINAL ARTICLE

Subnormothermic Ex Vivo Liver Perfusion Is a Safe Alternative to Cold Static Storage for Preserving Standard Criteria Grafts

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We developed a novel technique of subnormothermic ex vivo liver perfusion (SNEVLP) for the storage of liver grafts before transplantation. To test the safety of SNEVLP for the nonextended criteria grafts (standard grafts), we compared it to a control group with minimal cold static storage (CS) time. Heart-beating pig liver retrieval was performed. Grafts were either stored in cold unmodified University of Wisconsin solution (CS-1), in cold University of Wisconsin solution with ex vivo perfusion additives (CS-2), or preserved with a sequence of 3 hours CS and 3 hours SNEVLP (33°C), followed by orthotopic liver transplantation. Liver function tests and histology were investigated. Aspartate aminotransferase (AST) levels during SNEVLP remained stable (54.3 ± 12.6 U/L at 1 hour to 47.0 ± 31.9 U/L at 3 hours). Posttransplantation, SNEVLP versus CS-1 livers had decreased AST levels (peak at day 1, 1081.9 ± 788.5 versus 1546.7 ± 509.3 U/L; $P = 0.14$; at day 2, 316.7 ± 188.1 versus 948.2 ± 740.9 U/L; $P = 0.04$) and alkaline phosphatase levels (peak at day 1, 150.4 ± 19.3 versus 203.7 ± 33.6 U/L; $P = 0.003$). Bilirubin levels were constantly within the physiological range in the SNEVLP group, whereas the CS-1 group presented a large standard deviation, including pathologically increased values. Hyaluronic acid as a marker of endothelial cell (EC) function was markedly improved by SNEVLP during the early posttransplant phase (5 hours posttransplant, 1172.75 ± 598.5 versus 5540.5 ± 2755.4 ng/mL). Peak international normalized ratio was similar between SNEVLP and CS-1 groups after transplantation. Immunohistochemistry for cleaved caspase 3 demonstrated more apoptotic sinusoidal cells in the CS-1 group when compared to SNEVLP grafts 2 hours after reperfusion (19.4 ± 19.5 versus 133.2 ± 48.8 cells/high-power field; $P = 0.002$). Adding normothermic CS-2 had no impact on liver injury or function after transplantation when compared to CS-1. In conclusion, SNEVLP is safe to use for standard donor grafts and is associated with improved EC and bile duct injury even in grafts with minimal CS time. ***Liver Transpl 22:111-119, 2016***.
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Abbreviations: ALP, alkaline phosphatase; AST, aspartate aminotransferase; CIT, cold ischemia time; CS, cold static storage; CS-1, cold unmodified University of Wisconsin solution; CS-2, cold University of Wisconsin solution with ex vivo perfusion additives; DCD, donation after cardiac death; EC, endothelial cell; H&E, hematoxylin-eosin; HA, hyaluronic acid; HBD, heart-beating donor; HPF, high-power field; INR, international normalized ratio; SNEVLP, subnormothermic ex vivo liver perfusion; UW, University of Wisconsin.

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During the past decade, normothermic and subnormothermic ex vivo liver perfusion (SNEVLP) has been developed as an alternative to cold static storage (CS) for liver transplantation.[1,2] Previous research has focused mostly on improving liver preservation of marginal grafts. We and others have demonstrated that (sub)normothermic ex vivo liver perfusion reduces liver and bile duct injury in grafts harvested by donation after cardiac death (DCD) and fatty livers.[3-11] Improving organ preservation in marginal grafts has the potential to increase the donor pool and improve graft function after liver transplantation.

The potential for (sub)normothermic ex vivo liver perfusion exceeds the expansion of the donor pool using marginal livers. In addition, it offers for the first time the possibility for graft assessment and repair. (Sub)normothermic liver perfusion has the potential to be a platform to study liver metabolism during the preservation period,[12-14] alter the graft response to hepatitis C infection,[15] decrease graft immune response,[16] apply stem cell therapy,[17] and more. Furthermore, clinical trials will likely use standard criteria donors in the initial phase. Therefore, it is possible that in the near future, standard criteria grafts with minimal preservation injury will be exposed to warm ex vivo liver perfusion for graft assessment and modification. Large animal studies using good-quality grafts are important for the safety assessment before the start of a clinical trial.

It is well established that standard criteria grafts tolerate CS well with overall excellent graft function and survival. Little is known about warm perfusion of healthy liver grafts. So far, studies on ex vivo liver machine perfusion have focused on recovering marginal grafts (mainly DCD). The potential effect of (sub)normothermic ex vivo liver perfusion on standard criteria grafts is unknown.

We evaluated in a model of heart-beating donor (HBD) organ retrieval with minimal ischemic injury if SNEVLP provides equivalent outcomes compared to conventional CS in standard criteria liver grafts after pig liver transplantation.

MATERIALS AND METHODS

Study Design

CS and SNEVLP graft preservation was compared in a porcine transplant model (n = 8 all groups). Two different models of CS were used as control groups. First, cold unmodified University of Wisconsin solution (CS-1) as it is currently used in clinical practice, and second, cold University of Wisconsin solution with ex vivo perfusion additives (CS-2). This included 10,000 IU of heparin (Sandoz Canada, Quebec, QC, Canada), 24 mL of amino acid concentrate (Travasol 4.25% 50 mL; Baxter, Hamilton, ON, Canada), 50 U of insulin (NovoRapid, Novo Nordisk, Mississauga, ON, Canada), 1 g of cefazolin (Pharmaceutical Partners of Canada, Richmond Hill, ON, Canada), 500 mg of metronidazole (Baxter, Mississauga, ON, Canada), 1.25 mg of BQ-123 (AG Scientific, Kelowna, BC, Canada), alprostadil (Pfizer, Kirkland, BC, Canada), and 6 g of acetylcysteine (Sandoz, Quebec, QC, Canada).

HBD pig liver retrieval was performed and the liver grafts were either stored cold for 6 hours (minimal injury) or preserved with a sequence of 3 hours CS and 3 hours SNEVLP (33°C), followed by orthotopic liver transplantation. Pigs were killed 4 days after transplantation.

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 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

SNEVLP was performed for 3 hours as previously described by our group.[9,18] Briefly, the liver was perfused at 33°C with a continuous flow through the hepatic artery and portal vein. Perfusion through the hepatic artery was set at a pressure of 50 to 60 mm Hg resulting in a flow up to 400 mL/minute. The portal vein pressure was adjusted between 2 and 4 mm Hg corresponding to a flow of 900 to 1100 mL/minute (Fig. 1). Perfusate was composed of 1.5 L of Steen solution (XVIVO Perfusion, Gothenburg, Sweden) and washed pig erythrocytes to achieve a hematocrit of 15%. The erythrocytes were passed through a leukocyte filter and washed once in sterile saline solution to avoid contamination with leukocytes or plasma. The Steen solution is a buffered extracellular-type solution containing dextran and albumin to provide an optimized colloid osmotic pressure. The perfusate contained 10,000 IU of heparin (Sandoz Canada, Quebec, QC, Canada), amino acid concentrate (Travasol 4.25%, 50 mL Bolus plus 8 mL/hour; Baxter, Hamilton, ON, Canada), 2-5 mL/hour of D50W (Baxter, Mississauga, ON, Canada), and 125 IU/hour of insulin (NovoRapid, Novo Nordisk, Mississauga, ON, Canada). Also, 1 g of cefazolin (Pharmaceutical Partners of Canada, Richmond Hill, ON, Canada) and 500 mg of metronidazole (Baxter, Mississauga, ON, Canada) were added to prevent bacterial contamination. To improve flow properties by vasodilatation, a bolus of BQ-123[19] at both perfusion initiation and after 1.5 hours (1.25 mg, AG Scientific, Kelowna, BC, Canada) and a continuous infusion of 500 μg/3 hours of alprostadil[20] (Pfizer, Kirkland, QC, Canada) were used. Also, 6 g of acetylcysteine[21] (Sandoz, Quebec, QC, Canada) was added for its radical scavenging properties. A gas composition containing 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, 750 ppm of CO (Praxair, Burlington, ON, Canada) for its vasodilative and anti-

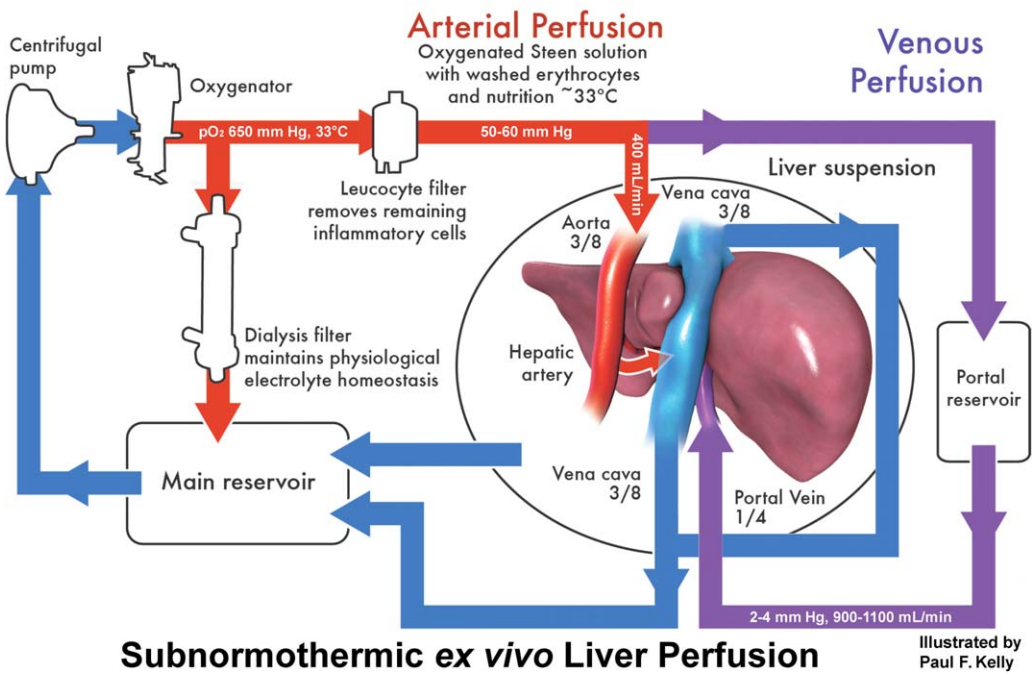

Figure 1. Subnormothermic ex vivo liver perfusion model. The circuit's perfusate is driven by a centrifugal pump at 1700-2000 rounds/minute. An oxygenator including a heating-cooling unit saturates the perfusate to an oxygen pressure of 650 mm Hg at 33°C. The perfusate passes through a leukocyte filter to remove inflammatory cells. The liver is perfused via the hepatic artery with a pressure of 50-60 mm Hg through a 3/8 in. tube resulting in an arterial flow up to 400 mL/minute. The portal vein is perfused by gravity with a pressure of 2-4 mm Hg through a 1/4 in. tube resulting in a portal flow of 900-1100 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 cavae through 3/8 in. tubes back to the main reservoir.

inflammatory properties[22-24] and 1% of sevoflurane (Abbott, Saint-Laurent, QC, Canada) for its protective properties on endothelial cells (ECs).[25]

Pig Liver Transplantation

A model of HBD liver procurement was used. The donor pigs received 30,000 IU of heparin 5 minutes before organ flush. The liver grafts were flushed through a dual vessel approach (aorta and portal vein) with altogether 3 L of cold University of Wisconsin (UW) solution (SPS-1, Organ Recovery Systems, Itasca, IL) and stored on ice. During liver flush, the donor pig's blood was collected through a central venous catheter. After the whole blood was passed through a leukocyte filter, erythrocytes were spun down, separated from the plasma fraction, and stored in SAG-M (Pack Pure WB, Fenwal, Lake Zurich, IL).

In the SNEVLP group, the liver was stored for 3 hours on ice (time frame designated to simulate the transport time from the donor hospital to the recipient hospital), followed by SNEVLP for 3 hours at 33°C. In the control group, the grafts were continuously stored on ice for 6 hours. At the end of the preservation time, orthotopic pig liver transplantation was performed using an active portojugular shunt (Rotaflow centrifugal pump, Maquet, Hirlingen, Germany). The animals were followed up for 4 days after transplantation.

In accordance to our animal use protocol, if predetermined animal suffering criteria were met (lethargy, failure to move coordinately, metabolic or respiratory decompensation, excessive bleeding) before the end of the intended survival period, pigs were killed under supervision of our veterinarian staff in order to avoid animal suffering. At autopsy, the patency of all anastomoses was confirmed. Pigs were exsanguinated under deep isoflurane anesthesia after central liver and bile duct (each right and left bile duct) specimens were obtained.

Serum Markers of Liver and EC Injury

Serum aspartate aminotransferase (AST) levels were measured hourly after blood reperfusion as a marker of hepatocyte injury. Total serum bilirubin and alkaline phosphatase (ALP) were also measured daily as markers of bile duct damage. Creatinine values were measured to evaluate kidney function and the general clinical condition of the pig after transplantation. A hyaluronic acid (HA) enzyme-linked immunosorbent assay was used to assess the EC injury[26,27] at 1, 3, and 5 hours after reperfusion (quantitative sandwich enzyme immunoassay technique, R&D Systems, Minneapolis, MN). International normalized ratio (INR) was used as a liver function marker.

Histological Evaluation

All histological specimens were stored in 10% formalin for 24 hours and then transferred to 70% ethanol until paraffin embedding. Sections of 5-μm thickness were cut and processed according to protocols of the Pathology Research Program, University Health Network Toronto.

Core liver biopsies obtained 2 hours after reperfusion were stained with cleaved caspase 3 antibody immunohistochemistry (Cell Signaling Technology, Danvers, MA) as a marker of apoptosis.[28] Histological slides were evaluated at 20× objective magnification. Staining positive cells were counted and averaged for 10 random high-power fields (HPFs).

Liver parenchyma and bile duct necrosis was assessed by H&E staining from specimens obtained at the end of animal survival. Bile duct slides were scored according to the Hansen criteria[29] for severity of bile duct necrosis (score 0, no necrosis; score 1, ≤25% of the bile duct wall necrotic; score 2, >25 and ≤50% of the bile duct wall necrotic; score 3, >50 and ≤75% of the bile duct wall necrotic; score 4, >75% of the bile duct wall necrotic). In this study, Hansen et al.[29] defined "severe necrosis" if >50% of the bile duct wall is necrotic. Sinusoidal endothelial integrity was assessed by CD31 immunohistochemistry[30] (PECAM, Santa Cruz Biotechnology, Dallas, TX) from core biopsies obtained 2 hours after reperfusion and at the end of animal survival. CD31 slides were scored for integrity of the sinusoidal cell lining (score 0, no CD31 staining with no viable EC visible; score 1, scattered CD31 staining without obvious lobule architecture; score 2, reduced CD32 staining throughout lobule but intact architecture; score 3, reduced CD31 staining in zone 3 only; score 4, physiological EC lining). All samples were evaluated by a blind investigator.

Statistical Analysis

The data were analyzed with the SPSS 22 statistical package (IBM, Chicago, IL). A Mann-Whitney U test was used for the comparison of continuous variables, whereas a chi-square test was applied for categorical outcome. The results are presented as mean ± standard deviation and were considered significant at the level of $P \leq 0.05$.

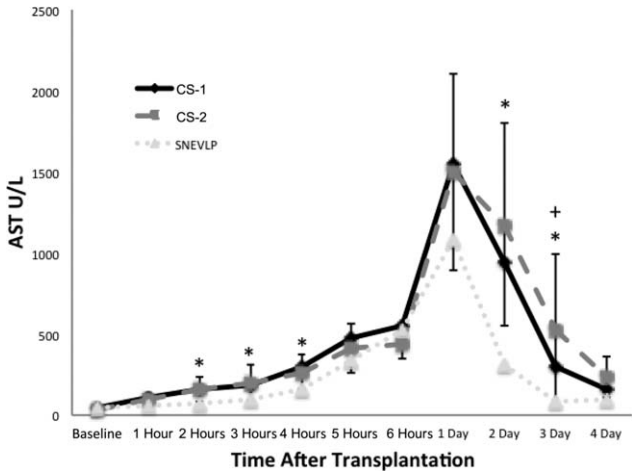

Figure 2. AST levels after pig liver transplantation. AST after liver transplantation with CS-1 versus CS-2 versus SNEVLP-preserved grafts. AST levels were decreased after transplantation in the SNEVLP group versus CS-1 group with significant differences for several time points (*$P < 0.05$, significant difference).

RESULTS

Liver Injury in SNEVLP Versus Cold-Stored DCD Liver Grafts After Transplantation

Perfusate AST levels remained stable and within normal range (54.3 ± 12.6 U/L at 1 hour to 47.0 ± 31.9 U/L at 3 hours) throughout the SNEVLP. Following pig liver transplantation, serum AST levels were significantly lower in the SNEVLP versus CS-1 group at 2 hours and 4 hours as well as at day 2 and day 4 (Fig. 2) after transplantation. For the remaining observation period up to 4 days after transplant, serum AST levels were also lower in the SNEVLP group but without reaching statistical difference.

At 2 hours after pig liver transplantation, SNEVLP versus cold-preserved grafts (CS-1) had a reduced cleaved caspase 3 staining (19.4 ± 19.5 positive cells/HPF versus 133.2 ± 48.8 positive cells/HPF; $P = 0.002$; Fig. 3), indicating decreased early apoptosis. The

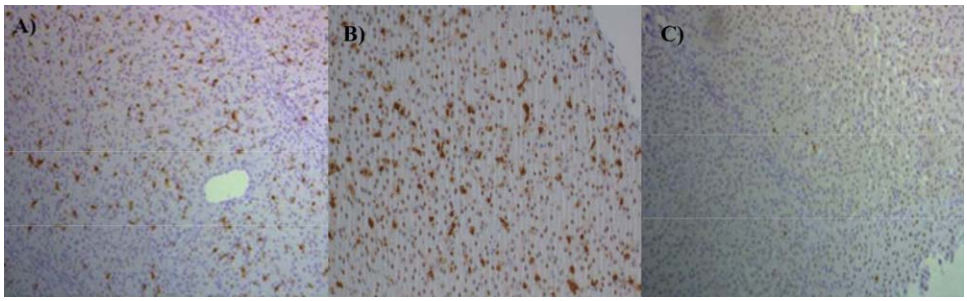

Figure 3. Histology imaging showing cleaved caspase 3 staining 2 hours after reperfusion. Immunohistochemistry for cleaved caspase 3 at 2 hours after reperfusion as a marker of apoptosis was significantly higher in the cold-stored groups (A) CS-1 and (B) CS-2, when compared to the (C) SNEVLP group (133 ± 49 and 122 ± 53 versus 19 ± 20 positive cells/HPF; $P = 0.004$). The positively stained cells were predominantly sinusoidal ECs and not hepatocytes.

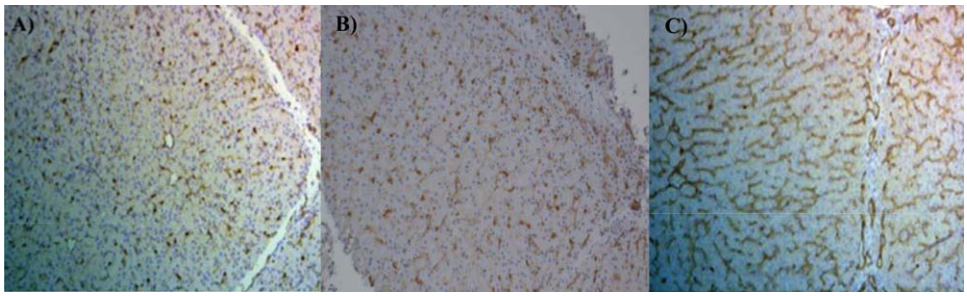

Figure 4. CD31 staining in liver biopsies 2 hours after reperfusion and at animal death. CD31 immunohistochemistry was assessed in liver samples obtained 2 hours after reperfusion and at animal death. CD31 slides were scored for integrity of the sinusoidal cell lining (score 0, no CD31 staining with no viable EC visible; score 1, scattered CD31 staining without obvious lobule architecture; score 2, reduced CD31 staining throughout lobule but intact architecture; score 3, reduced CD31 staining in zone 3 only; score 4, physiological EC lining). Endothelial damage after reperfusion was more severe in the (A) CS-1 and (B) CS-2 group, when compared to (C) SNEVLP.

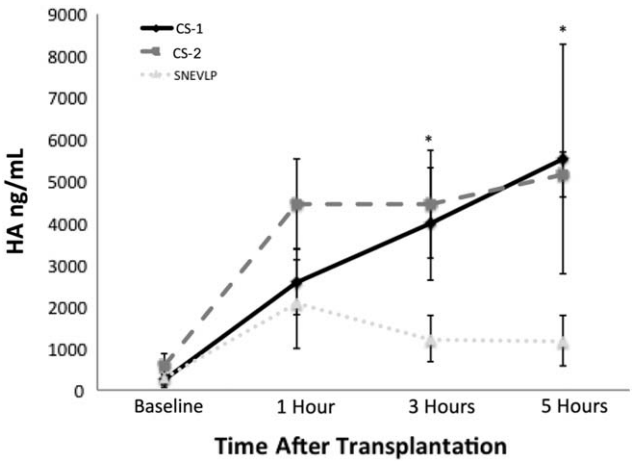

Figure 5. HA levels after transplantation. HA serum levels were evaluated following transplantation in the CS-1 group, CS-2 group, and SNEVLP treated group as a marker of EC function. Animals receiving a cold-stored liver graft (CS-1 and CS-2) had increased levels when compared to pigs receiving a graft preserved by the SNEVLP protocol, suggesting improved EC preservation in the SNEVLP group (* significant difference).

positively stained cells were predominantly sinusoidal ECs and not hepatocytes.

Liver necrosis was determined at the end of the survival period by hematoxylin-eosin (H & E) staining. Minimal necrosis (<5%) was present in both groups without evidence of significant ischemic injury.

SNEVLP Versus CS Reduces EC Injury in HBD Grafts

To assess EC viability, liver tissue was stained with CD31 immunohistochemistry 2 hours after transplantation. All SNEVLP-preserved liver grafts had an intact sinusoidal EC lining and minimal EC injury. In contrast, more than 50% of the cold-stored grafts (CS-1) showed signs of severe EC injury ($P = 0.05$; Fig. 4A-C). Notably, significantly higher numbers of sinusoidal cells were inducing apoptosis at this time point in the CS-1 compared to the SNEVLP group, as demonstrated by cleaved caspase 3 staining (Fig. 3). At the end of the survival period, sinusoidal ECs recovered in both groups.

In a second approach, we assessed sinusoidal EC function by measuring HA clearance. HA is removed by EC from the circulation and increased serum HA levels correlates with decreased EC function. Although serum HA levels increased continuously after transplantation in the CS-1 grafts, HA levels remained stable in the SNEVLP group. At 3 hours and 5 hours posttransplantation, serum HA levels were significantly reduced in SNEVLP versus CS-1 livers indicating improved EC function in SNEVLP-preserved HBD grafts (Fig. 5).

Bile Duct Injury and Biliary Function After HBD Liver Transplantation

Bile duct necrosis was investigated by H & E staining at 4 days after transplantation or at the end of the survival period using the Hansen duct necrosis score.[29] No bile duct necrosis >50% was seen in the SNEVLP group. Severe bile duct necrosis was seen in only 1 animal in the CS-1 group (Table 1).

Serum ALP and total serum bilirubin were measured as markers of bile duct injury and function at various time points after liver transplantation in both groups. ALP levels were significantly lower in pigs receiving HBD grafts preserved with SNEVLP versus CS-1 at days 1 and 3 after transplantation (Fig. 6A). Similarly, serum bilirubin levels were lower in animals receiving a SNEVLP versus cold-stored graft, without reaching a statistically significant difference (Fig. 6B). Notably, in the SNEVLP group, bilirubin levels were in physiological range in all animals at all time points. In contrast, in the CS-1 group, 2 out of 8 animals had increased bilirubin levels (33 μmol/L and 14 μmol/L) after transplantation.

Animal Survival After Liver Transplantation and Liver Function

Animal survival was considered permanent 4 days after pig liver transplantation. Out of 8 pigs, 3 animals with cold-stored grafts (CS-1) were killed before the intended survival due to metabolic decompensation

TABLE 1. Hansen Score for Assessment of Bile Duct Necrosis

	Hansen Score*				
	0	1	2	3	4
CS-1, %	12.5	0	75	0	12.5
SNEVLP, %	25	37.5	37.5	0	0

NOTE: Scores >2 were considered as "severe" necrosis. No bile duct necrosis >50% was seen in the SNEVLP group. Severe bile duct necrosis was seen in only 1 animal in the CS-1 group, and not in the CS-2 group. χ^2, $P = 0.15$.

*Bile duct slides were scored according to the Hansen criteria for severity of bile duct necrosis: score 0, no necrosis; score 1, $\leq 25\%$ of the bile duct wall necrotic; score 2, >25 and $\leq 50\%$ of the bile duct wall necrotic; score 3, >50 and $\leq 75\%$ of the bile duct wall necrotic; score 4, $>75\%$ of the bile duct wall necrotic.

(acidosis, hyperkalemia, hypoglycemia; 2 pigs after 12 hours and 1 at day 2 after liver transplantation). In comparison, only 2 animals had to be killed before the intended survival in the SNEVLP group ($P = 0.62$). Of those, 1 died of asphyxia (laryngeal spasm) after extubation 5 hours after reperfusion, whereas the second animal was killed after 36 hours due to bowel perforation with excellent liver function.

INR as a marker of liver function normalized faster in SNEVLP-preserved liver grafts than CS-1 grafts. INR values were significantly lower for SNVELP versus CS-1 grafts at the second and third day after transplantation (day 2, 1.06 ± 0.05 versus 1.22 ± 0.23; $P = 0.04$; day 3, 0.99 ± 0.15 versus 1.13 ± 0.10; $P = 0.05$). Creatinine levels after transplantation were comparable in both groups and were within physiological range for all surviving pigs.

Impact of Subnormothermic Perfusion Additives on CS

In the next set of experiments, we investigated if the additives of the subnormothermic ex vivo perfusion solution by itself have protective effects during CS. UW was supplemented with all additives of the SNEVLP solution (CS-2). Following 6 hours CS in either CS-1 or CS-2, pig liver transplant was performed. No significant difference was observed between both groups regarding AST as a marker of hepatocyte injury (Fig. 2) or bile duct injury (bilirubin, ALP, histology; Fig. 6A,B). Similarly, peak INR as a marker of graft function was similar between both groups (CS-1, 1.7 ± 0.64; CS-2, 2.2 ± 1.4; $P = 0.2$). EC injury and function as determined by CD31 staining and HA levels were identical (Figs. 4 and 5). Three animals died in both groups (CS-1 and CS-2) after transplantation.

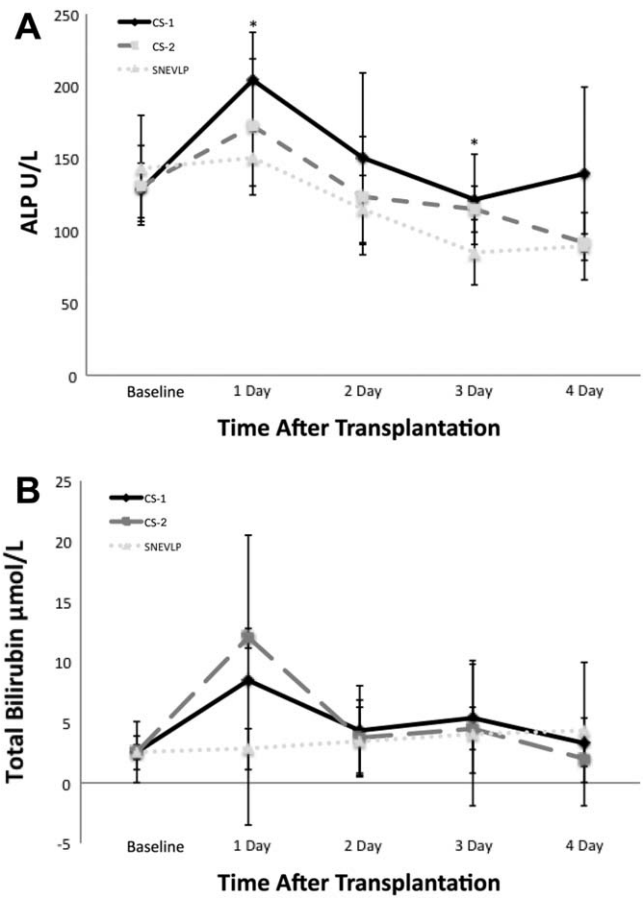

Figure 6. ALP and total bilirubin levels after transplantation. (A) ALP levels were significantly lower in pigs receiving HBD grafts preserved with SNEVLP versus CS-1 at day 1 and 3 after transplantation. Comparable ALP levels were observed between groups CS-1 and CS-2. (B) Serum bilirubin levels were lower in animals receiving a SNEVLP versus CS-1 and CS-2 grafts, without reaching a statistically significant difference.

DISCUSSION

In this study, we investigated the safety and benefits of SNEVLP in standard criteria liver grafts. HBD grafts preserved with SNEVLP were compared to a control group with CS-1 preservation. SNEVLP reduced sinusoidal EC and biliary injury even when compared to CS-1 grafts with rather short preservation times. Survival rates were similar in both groups. Even though standard criteria grafts were used, recipient deaths occurred in both groups. These results are comparable to survival rates reported in the literature.[31-33]

So far, 2 other groups have studied the effects of warm liver perfusion in large animal HBD liver transplantation models with a survival period. Both groups used the HBD as control groups for a DCD model and did not investigate in detail possible risks or benefits of warm perfusion in healthy liver grafts. Schön et al.[3] compared in a model of pig liver transplantation HBD grafts with a cold ischemia time (CIT) of 4 hours to livers preserved with 4 hours of normothermic perfusion. In this study, histological examination demonstrated increased sinusoidal EC and hepatocyte injury

in perfused versus cold-stored grafts after transplantation. The authors further found more mitochondrial swelling in grafts preserved with normothermic perfusion versus CS. Serum bilirubin, ALP, and bile duct histology were not investigated after liver transplantation.

Brockmann et al.[4] compared 5 hours normothermic liver perfusion with 5 hours CS for liver transplantation with HBD grafts. At the end of the preservation and reperfusion period, warm-perfused versus cold-stored grafts had increased hemorrhage and necrosis on histological examination, although the clinical relevance of these findings were not further investigated. Because the study was focused on DCD liver grafts, it did not report markers of liver, EC, or bile duct injury (AST, bilirubin, ALP) after transplantation. Bile duct histology was not investigated.

EC death plays an important role in hepatic reperfusion injury[34-36] because these cells are more susceptible to CIT than hepatocytes.[37,38] Endothelial injury promotes the activation of proinflammatory and prothrombotic cascades leading to microcirculatory dysfunction and subsequent liver injury.[39] As demonstrated by our study, endothelial injury occurs after a very short period of CS. In our model with a short preservation time and using healthy grafts, ECs were able to regenerate within 3 to 4 days after transplantation and EC damage was repaired at the end of the survival period. The clinical relevance of EC damages are important in the setting of marginal grafts, such as older grafts or livers with underlying parenchymal changes, such as steatosis or warm ischemic injury. We recently demonstrated in a model of DCD pig liver transplantation that SNEVLP versus CS results in decreased EC and bile duct injury.[8,9] It is possible that more severe EC injury contributes to decreased arterial perfusion of the bile ducts, resulting in ischemic-type bile duct injury.[40] Bile duct necrosis was observed in our current study as well. Biliary injury after liver transplantation is common, also in standard criteria grafts. Hansen et al.[29] determined the presence of bile duct injury in human liver grafts just after reperfusion. In this publication, the authors described an epithelial damage >50% as "severe." In our study population, only 1 pig presented a "severe" damage of the bile duct at the end of the survival period. Sutton et al.[41] demonstrated that Ki-47 (+) stem cells in the peribiliary glands are responsible for bile duct regeneration. It is likely that these stem cells are not extinguished during reperfusion and promote bile duct regeneration. The extent of bile duct injury in our study population appears to be a normal early stage of biliary reperfusion injury and regeneration. Nevertheless, in our current study, we also observed improved markers of bile duct injury (bilirubin, ALP) in SNEVLP versus cold-stored grafts. As expected in a model with HBD grafts, the difference between the SNEVLP and CS group was less distinct than in the DCD model.

Adding additives of the SNEVLP group to CS solution (CS-2) did not improve outcome after transplantation. In particular, no effects on EC injury or function after transplantation were observed. It is possible that the perfusion additives need active metabolism to provide protective effects. Alternatively, the perfusion temperature might be more important than the additives for the beneficial effects on graft injury and function.

SNEVLP offers a unique opportunity as a protective strategy against preservation/reperfusion injury before liver transplantation. The advantages of warm machine perfusion over conventional CS have been reviewed extensively before.[2,4,11,42,43] Future protective strategies might include deliveries of radical scavenging molecules (eg, *N*-acetylcysteine or tocopherol), or protective enzymes like hemoxigenase-1, or H_2S during SNEVLP.[22,44] However, the options for graft pretreatment during SNEVLP are not limited to protection against preservation injury. It could also be extended to immunomodulation therapy to reduce rejection after transplantation, or prevention of disease recurrence such as HCV or hepatocellular carcinoma. Complex immunomodulation therapies might include vector gene delivery,[16] antisense nucleotides,[15,45] or stem cell therapy.[17,46] These strategies are not limited to marginal grafts but are actually more practical in standard quality grafts.

In conclusion, SNEVLP can be performed safely in standard criteria liver grafts. Even in the setting of short preservation times and good-quality grafts, SNEVLP reduces EC and bile duct injury. SNEVLP offers the opportunity for graft modification beyond the reduction of preservation injury.

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