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Keywords

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OPEN

A Comparative Study of Single and Dual Perfusion During End-ischemic Subnormothermic Liver Machine Preservation

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Background. It remains controversial if arterial perfusion in addition to portal vein perfusion during machine preservation improves liver graft quality. Comparative studies using both techniques are lacking. We studied the impact of using single or dual machine perfusion of donation after circulatory death rat livers. In addition, we analyzed the effect of pulsatile versus continuous arterial flow. **Methods.** Donation after circulatory death rat livers (n = 18) were preserved by 6 hours cold storage, followed by 1 hour subnormothermic machine perfusion (20°C, pressure of 40/5 mm Hg) and 2 hours ex vivo warm reperfusion (37°C, pressure of 80/11 mm Hg, 9% whole blood). Machine preservation was either through single portal vein perfusion (SP), dual pulsatile (DPP), or dual continuous perfusion (DCP) of the portal vein and hepatic artery. Hydrodynamics, liver function tests, histopathology, and expression of endothelial specific genes were assessed during 2 hours warm reperfusion. **Results.** At the end of reperfusion, arterial flow in DPP livers tended to be higher compared to DCP and SP grafts. However, this difference was not significant nor was better flow associated with better outcome. No differences in bile production or alanine aminotransferase levels were observed. SP livers had significantly lower lactate compared to DCP, but not DPP livers. Levels of Caspase-3 and tumor necrosis factor- α were similar between the groups. Expression of endothelial genes Krüppel-like-factor 2 and endothelial nitric oxide synthase tended to be higher in dual perfused livers, but no histological evidence of better preservation of the biliary endothelium or vasculature of the hepatic artery was observed. **Conclusions.** This study shows comparable outcomes after using a dual or single perfusion approach during end-ischemic subnormothermic liver machine preservation.

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The current disparity between supply of and demand for liver grafts has increased the use of grafts from donation after circulatory death (DCD) donors. In the United States, numbers increased from 0.95% in 2000 to 8% in 2017.¹ DCD organs are associated with increased reperfusion injury,

posttransplant morbidity and graft loss.² A variety of machine perfusion techniques have been developed to protect DCD liver grafts with promising results.³⁻⁷ However, there are still several questions that need to be answered.

The blood supply from the portal vein and hepatic artery allows different perfusion routes during machine perfusion

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I.M.A.B. participated in research design, performance of the experiments, data analysis, writing of the article. C.M. participated in performance of the experiments and

data analysis. P.M. participated in research design, performance of the experiments, data analysis. M.F.T. participated in the performance of the experiments, data analysis. X.E. participated in data analysis. H.G.D.L. participated in interpretation of the data, revising the article. P.M. participated in revising the article. X.W. participated in data analysis. T.F.K. participated in data analysis, revising the article. R.J.P. participated in research design, interpretation of the data, revising the article. P.N.M. participated in research design, interpretation of the data, revising the article.

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to be used. A dual blood supply resembles the physiological situation, but single perfusion (SP) adds simplicity and has shown to be effective.² Most rodent experiments with machine preservation used single portal vein perfusion, because of the potential danger to damage the artery during cannulation.⁸⁻¹⁰ In human studies, SP adds simplicity to liver machine perfusion while providing protection.¹¹ Even though the portal vein supplies nutrients to hepatocytes and ensures a greater blood flow to the liver than the hepatic artery, it is not the main physiological route for oxygen delivery. The hepatic artery supports almost exclusively the vascularization of the biliary tree, including the peribiliary vascular plexus (PVP), and this indicates an important role in bile formation.¹²⁻¹⁴ In addition, absence of arterial flow and intraluminal pressure on the endothelium will cause a decreased expression of various mechanosensitive genes that code for cytoprotective proteins. This process is mediated by the vasoprotective transcription factor Krüppel-like factor 2 (KLF2).¹⁵ The KLF2 is important for the expression of proteins that enhance the anticoagulant and anti-inflammatory function of the endothelium, such as thrombomodulin (TM) and endothelial nitric oxid synthase (eNOS).^{16,17}

Pulsatile flow has been shown to be much more effective in activating KLF2 than steady shear stress.¹⁸ Several reports from renal perfusion studies suggest pulsatile perfusion to be superior compared to continuous perfusion.¹⁸⁻²⁰ However, the effect of pulsatile versus continuous arterial flow has not yet been studied in a liver perfusion model.

It is still debatable if arterial perfusion adds protection against ischemia-reperfusion injury during machine liver preservation and there is no published data comparing a dual versus single subnormothermic machine perfusion (SNMP) approach. Also, the influence of pulsatile arterial flow remains unknown. Therefore, this study was designed to analyze the effect of different machine perfusion modalities on graft function after ex vivo warm reperfusion of DCD liver grafts.

MATERIALS AND METHODS

Animals

To ensure humane treatment of laboratory animals, animal research was regulated by the United State Department of Agriculture (USDA)/Animal Care, and the Public Health Service/Office of Laboratory Animal Welfare. Animals received care according the Animal Center Committee of the University of Massachusetts Medical Center. The Institutional Review Board of the University of Massachusetts Medical Center approved this study.

Liver Procurement and Preservation

Eighteen Male Lewis rats, in the specified weight range of 280 to 320 g, were divided into 3 experimental groups. General anesthesia was induced with the inhalation of 2% to 3% isoflurane and oxygen before the procurement. The abdomen was opened through a mid-line incision after which the bile duct was cannulated. One milliliter of 0.9% NaCl with 500 IU of heparin was administered via the dorsal penile vein. A model of DCD donation was used as described by Op den Dries et al.²¹ The aorta and pulmonary artery were clamped close to the heart, after which a period of 30 minutes of warm ischemia was applied. The hepatectomy

was performed by ligation of the splenic vein, superior mesenteric artery and superior mesenteric vein and cannulation of the celiac trunk and portal vein. The liver was immediately flushed in situ with 5 mL of 0.9% NaCl at 37°C via de portal vein, followed by a cold flush of 5 mL histidine-tryptophan-ketoglutarate solution at 4°C. The liver was removed and flushed with an additional 6 mL of cold 0.9% NaCl via the portal vein and 3 mL of cold 0.9% NaCl via de celiac trunk. Livers were stored in Belzer University of Wisconsin solution at 4°C for 6 hours. After this period of static cold storage (SCS), the initial preservation fluid was washed out with 20 mL of 0.9% NaCl at room temperature before the liver was connected to the perfusion system.

Cannulation of the portal vein, celiac trunk, and bile duct was performed under the microscope (4-40× magnification). Cannulas were secured with 4-0 silk ties.

Subnormothermic Machine Perfusion

After 6-hour cold storage, livers were randomly assigned to one of the following groups: 1 hour of single portal machine perfusion (SP), 1 hour of dual pulsatile machine perfusion (DPP), or 1 hour of dual continuous machine perfusion (DCP) (n = 6 per group). DPP livers were perfused by a pulsatile flow through the hepatic artery and a continuous flow through the portal vein. The DCP livers were perfused by a continuous flow through both the hepatic artery and portal vein. Two roller pumps (Ismatec pump ISM 834C; Inacom Instruments, The Netherlands) enabled continuous flow to the portal vein and pulsatile or continuous flow to the hepatic artery. The usage of elastic tubing and a bubble trapper between the roller pump and portal vein made it possible to reduce pulses and create a continuous flow. Venous and arterial flows were continuously recorded by flow meters and displayed real time on a computer. Two tubular membrane oxygenators provided oxygenation of the perfusion solution with a 100% O₂. pO₂ during SNMP and warm reperfusion was 400 to 550 mm Hg on average as described previously.²² The system was pressure-controlled. Figure 1 shows a picture and a schematic representation of the system.

All livers were treated with end-ischemic SNMP (20°C) for 1 hour based on an earlier study from our group.²² The perfusion fluid consisted of 109 mL William's Medium E solution and 1 mL insulin (100 IE/mL Actrapid), adding up to a total volume of 110 mL. Sodium bicarbonate (8.4% solution) was added to the perfusion fluid to maintain a pH of 7.35-7.45. During SNMP pressure was limited to a mean arterial pressure of 40 mm Hg and mean portal pressure of 5 mm Hg. After 1 hour of SNMP, livers were left on a petri dish at room temperature for 30 minutes to mimic anastomosis time.

Normothermic Ex Vivo Reperfusion With Blood Added to Perfusate

Livers were reperfused ex vivo at 37°C for 2 hours with a perfusion fluid consisting of 10 mL rat whole blood, supplemented with 98.5 mL William's Medium E solution, 1 mL insulin (100 IE/mL) and 0.5 mL unfractionated heparin (1000 IE/mL), adding up to a total volume of 110 mL. Pressure was limited to a mean arterial pressure of 80 mm Hg and mean portal pressure of 11 mm Hg. During normothermic

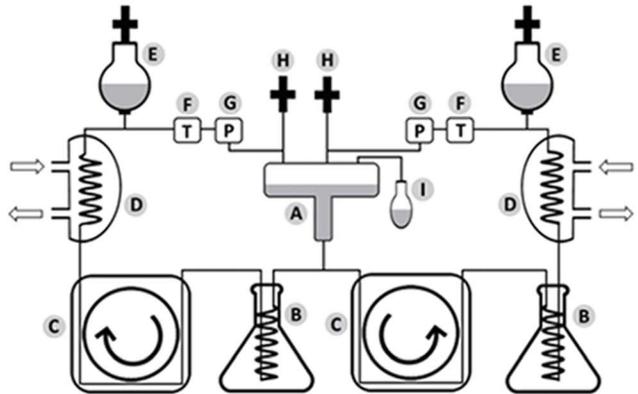


FIGURE 1. Left: photograph of the rat liver machine perfusion system. Right: schematic representation. A, organ chamber; B, tubular membrane oxygenator; C, roller pump; D, water bath; E, bubble trapper; F, temperature sensor; G, in-line flow and pressure sensor; H, 3-way connector bubble trap; I, bile collection tube.

reperfusion both the portal vein and hepatic artery were perfused.

Biochemical Markers of Function and Injury

Flow and temperature were registered every 15 minutes during machine perfusion. Bile production was measured at the end of SNMP and reperfusion. During reperfusion, blood gas analysis was performed every 30 minutes using the i-STAT clinical analyzer (Abbott Point of Care Inc., Princeton, NJ). Perfusion fluid samples were collected every 30 minutes. Samples were centrifuged (2700 rpm for 5 minutes at 4°C), and plasma was collected, frozen and stored at -80°C for determination of alanine aminotransferase (ALT) and glucose.

Histological Evaluation of Injury of the Liver Parenchyma, Hepatic Artery and Bile Ducts

At the end of reperfusion, liver parenchyma, hepatic artery, and bile duct samples were collected. Samples were fixed in formalin and embedded in paraffin and prepared for microscopic assessment using hematoxylin and eosin staining. Bile duct biopsies were stored in glutaraldehyde for electromicroscopy (EM) assessment. Injury of the bile ducts was assessed using a scoring system as previously described by Hansen et al.²³ and modified by Op den Dries et al.²⁴ Injury of the liver parenchyma was analyzed using Suzuki scores.²⁵ Histological evaluation of endothelial injury of the hepatic artery was scored blindly by a pathologist (X.W.) using a scoring system developed by Burlage et al.²⁶

Gene Expression

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to determine gene expression of endothelial specific proteins and proteins related to inflammation and apoptosis. Liver parenchyma biopsies were obtained after 2 hours reperfusion and stored in -80°C until analyzed. Total RNA was extracted from snap-frozen liver biopsies using the miRNeasy Micro Kit (Qiagen, Valencia, CA). The RNA concentration was determined using NanoDrop 2000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Equal amounts of RNA were converted to complementary DNA using SuperScript IV VILOMaster Mix (Invitrogen, Carlsbad, CA). Complementary DNA levels were measured in triplets using ViiA 7 Real-Time PCR System (Applied

Biosystems). Relative expression of the mRNA of interest was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are presented as relative quantification (RQ) to GAPDH. Primer sequences, sense and antisense are shown in Table 1.

Statistical Analysis

Continuous data are presented as median and interquartile range. Kruskal-Wallis test was used to compare more than 2 groups of continuous variables and comparisons between 2 groups were made using the Mann-Whitney U test. Categorical data were compared using Pearson χ^2 test. A *P* value less than 0.05 was considered to be significant. Analyses were performed using IBM SPSS software version 23 for windows.

RESULTS

Flows During SNMP and Subsequent Warm Reperfusion

Arterial and portal flows during SNMP and reperfusion are shown in Figure 2. Arterial flows during SNMP were comparable between DPP and DCP. Portal flows remained constant during SNMP, and no significant differences between the groups were observed. During reperfusion, arterial flow remained stable in all groups. The DPP livers seem to have highest

TABLE 1.

qRT-PCR primers of the housekeeping gene (GAPDH), CD31, KLF2, TM, eNOS, VEGF- α , and caspase-3 sense and antisense

Primers	Sense	Antisense
GAPDH	GTATGACTCTACCCACGGCAAGTT	GATGGGTTTCCC GTTGATGA
CD31	AAAGGGCTCATTGCAGTCGTT	CAGGAAGTAGTATTTGGCTGCAACT
KLF2	GGTGAGAAGCCTTATCATTGCAAC	CCTGTGACCCGTGTGCTTG
TM	TGTTTGCCATTTGTAGTCCAA	CCCAGACTCTTTCCCAAGTT
eNOS	AGTCTCACCGCCTTTTCCA	GCACGGGTGAACCTCC
VEGF- α	CCTTGTGTGATCAGACCATTGAAA	CTTGGGGTCTCTGCC
Caspase-3	GGTATTGAGACAGACAGTGG	CATGGGATCTGTTCTTTGC
TNF- α	GCCGCTCCTACCCGAACAAG	CACAGGGCAATGATCCCAAAG

VEGF- α , vascular endothelial growth factor α ; TNF- α , tumor necrosis factor- α .

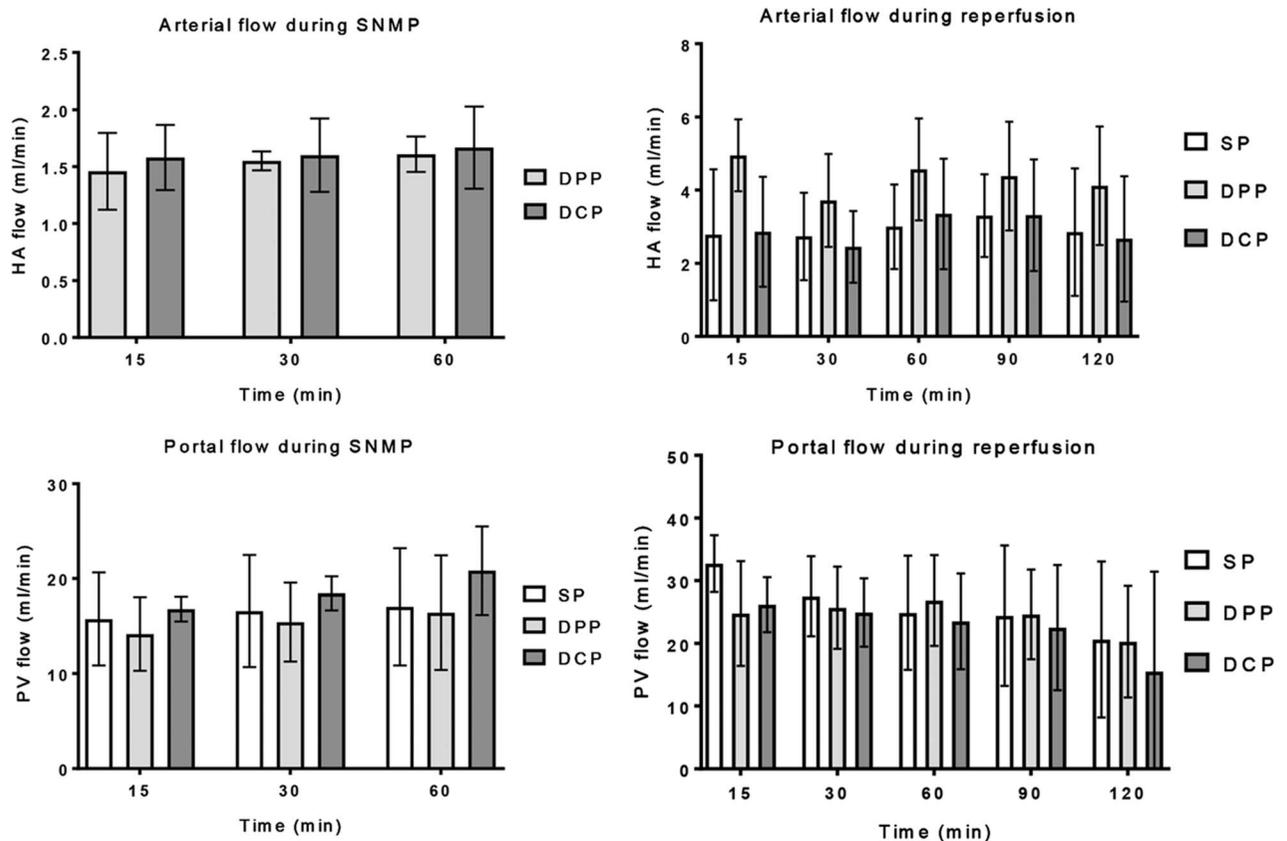


FIGURE 2. Arterial and portal flows during SNMP and subsequent reperfusion using SP, DPP, or DCP.

arterial flows during total reperfusion time. Shortly after reperfusion, DPP livers had a median arterial flow of 4.80 mL/min (3.80-4.80) compared with 2.75 mL/min (1.08-4.13) in DCP and 1.85 mL/min (1.12-3.48) in SP. The difference was significant when DPP was compared with DCP ($P = 0.048$). Portal flows during reperfusion were similar between the groups. In 2 DCP livers portal flow reached 0 after 120 minutes reperfusion, probably due to gradually increasing edema.

Liver Function

Figure 3 shows cumulative bile production at the end of SNMP and reperfusion, lactate during reperfusion, and ALT levels at the end of reperfusion. All livers produced bile during reperfusion, but no difference between the groups was observed. At the end of reperfusion, SP livers had significantly lower lactate compared to DCP livers (3.71 [2.76-4.31] u/L vs 4.84 [4.40-4.95] u/L; $P = 0.03$), but not compared to DPP livers (3.90 [3.51-4.41] u/L; $P = 0.47$). No differences in ALT level were observed.

Histological Analysis of Biliary Glands

At the end of reperfusion, 2 SP and 3 DPP livers showed loss of biliary epithelium. None of the DCP livers revealed loss of biliary epithelium. Vascular thrombosis was present in 1 SP liver, 2 DPP, and 2 DCP livers. No injury was observed in the PVP or on other bile duct wall components (data not shown). Light microscopy and EM analysis of bile duct biopsies did not reveal significant differences between dual or single perfused livers (Figure 4).

Histological Analysis of Liver Parenchyma

Table 2 shows Suzuki scores of liver ischemia-reperfusion injury after 2 hours reperfusion. The DCP grafts tend to score best on all components, but no significant differences were observed.

Histological Analysis of the Hepatic Artery

There were no differences in vascular injury scores of hepatic artery histology between the groups: SP, 6.50 (6.00-8.50); DPP, 6.00 (6.00-7.50); and DCP, 6.00 (6.00-7.00) (Figure 5). All arteries showed presence of endothelial cells lining the vasculature and endothelial walls. One SP and 1 DCP liver revealed pyknotic endothelial cells. Two SP, 1 DCP and 1 DPP liver showed lifting of endothelial cells lining the vasculature. Swollen vessel walls were found in 2 SP, 1 DPP, and 1 DCP livers. Necrosis of the vessel wall was absent in all groups.

Expression of Endothelial Specific Genes

Gene transcription of endothelial specific proteins measured in liver parenchyma biopsies is shown in Figure 6. Dual perfused livers had a tendency towards higher expression of KLF2 compared to single perfused grafts, but the difference was not statistically significant. In DCP livers gene expression of KLF2 was highest with 1.44 (1.05-1.55) compared to 0.81 (0.60-1.03) in SP and 0.94 (0.82-0.99) in DPP ($P = 0.30$). Dual perfused livers tended to show more expression of eNOS, but expression of TM was comparable between the groups. The expression of vascular endothelial growth factor α was not significantly different between the perfusion modalities.

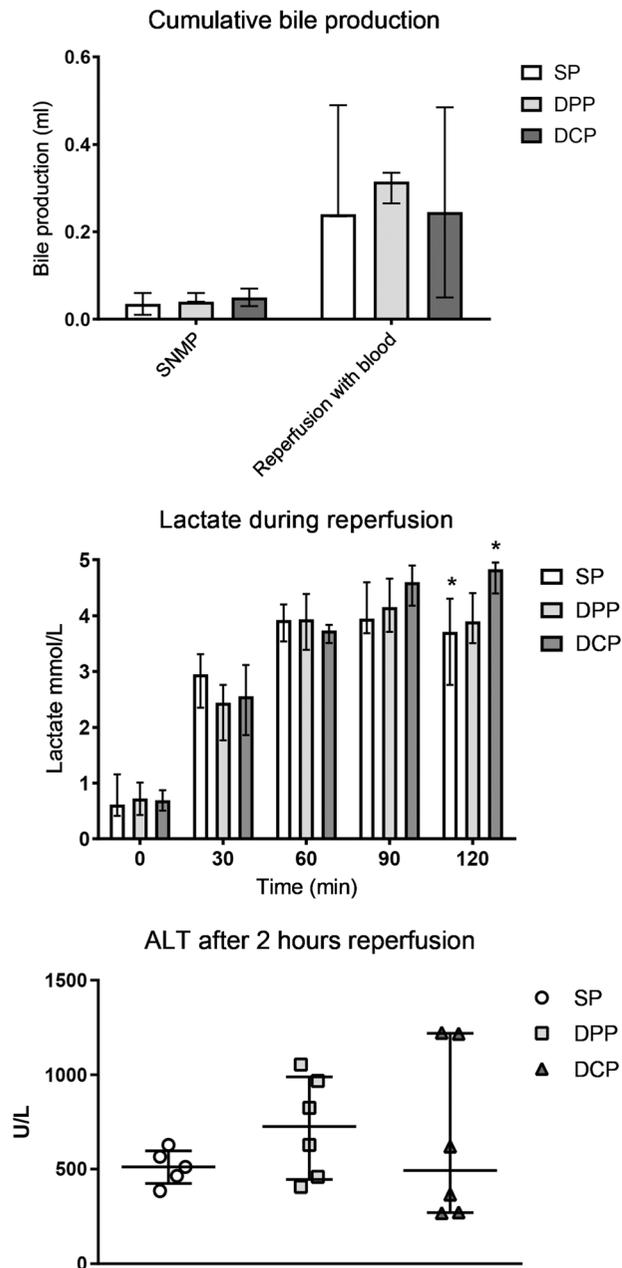


FIGURE 3. Bile production at the end of SNMP and reperfusion. Lactate and ALT of the perfusion solution using SP, DPP, or DCP. * $P < 0.05$.

Expression of Genes Related to Inflammation and Apoptosis

Gene expression of tumor necrosis factor- α , a marker of Kupffer cell activation was not significantly different between the groups. The expression of caspase-3, a marker of apoptosis, was also similar (Figure 6).

DISCUSSION

Many different perfusion settings have been used in the constantly evolving field of machine liver preservation.^{3,4} One of the debates has been on using a single (portal vein) or dual (portal vein and hepatic artery) approach during machine perfusion preservation.^{11,27} Although SP adds simplicity, the effect of additional arterial perfusion has not been

investigated. In this study we report outcomes after using a single or dual approach in a DCD rat ex vivo reperfusion model. Dual perfusion was further subdivided into 2 groups using a continuous or pulsatile flow through the hepatic artery.

Overall, this study demonstrates comparable outcomes for dual versus SP during SNMP of DCD rat liver grafts. In addition, pulsatile or continuous perfusion through the hepatic artery did not result in different outcomes. The role of pulsatility in the proper function of the human body remains unclear and there is no published data on the role of pulsatile arterial perfusion during liver machine perfusion. Pulsatile, compared to continuous, perfusion improved urine production and creatinine clearance in a renal perfusion study.¹⁸ In our model, bile production was similar, but lactate clearance was higher in DPP compared to DCP livers. Our results were consistent with studies on flow devices for mechanical circulatory support showing that short-term continuous arterial flow does not have disadvantages compared to pulsatile arterial flow.^{28,29} A short period of continuous arterial flow during machine preservation might therefore also not be disadvantageous. Histological damage in DCP liver parenchyma even seemed to be lower compared to DPP livers and no relevant differences in arterial wall histopathology were observed between pulsatile or continuous arterial flows.

No significant histological differences between bile ducts from dual or single perfused livers were observed. Biliary epithelial loss was evident in SP and DPP livers, but not in DCP grafts. None of the livers showed injury of the periluminal peribiliary glands, mural stroma or PVP. There is a theoretical advantage of providing hepatic artery oxygenated perfusion during machine preservation. However, we were not able to demonstrate this in our model. It may be possible that during hypothermic and subnormothermic settings SP may be sufficient, while hepatic artery perfusion is more important during normothermic machine perfusion. At physiologic temperatures the metabolic oxygen demand of bile ducts may not be sufficiently supplied by portal vein perfusion alone. The hepatic artery has been considered mainly responsible for the biliary blood supply and preservation of the PVP. Insufficient arterial perfusion of liver grafts, for example, because of hepatic artery thrombosis can lead to ischemic

TABLE 2.

Suzuki scores for ischemia-reperfusion injury in liver parenchyma

	Injury score			P
	SP (n = 6)	DPP (n = 6)	DCP (n = 6)	
Congestion				0.34
Minimal	2	0	2	
Mild	1	3	3	
Moderate	3	3	1	
Vacuolization				0.19
None	0	0	1	
Minimal	2	1	4	
Mild	3	5	1	
Moderate	1	0	0	
Necrosis				0.21
None	2	3	5	
Single-cell necrosis	4	3	1	

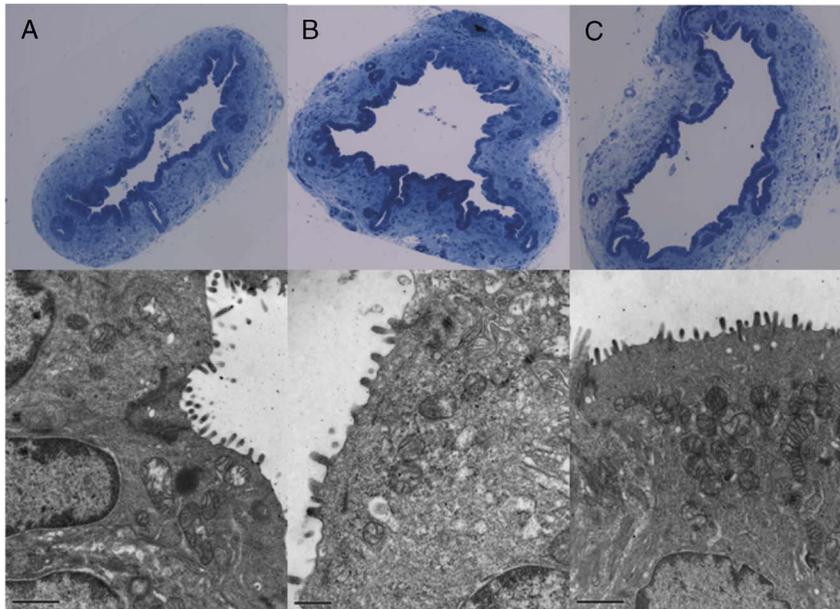


FIGURE 4. Light microscopy (top) and EM (bottom) of bile ducts after 2 hours ex situ reperfusion. Representative example of a bile duct from SP (A), DPP (B), and DCP (C).

cholangiopathy, characterized by loss of biliary epithelium, necrosis of the bile ducts wall and eventually narrowing of the bile duct lumen.^{30,31} Our group has shown in a clinical study that dual hypothermic oxygenated machine perfusion (dual HOPE) prevented arteriolenecrosis of the PVP of the bile ducts of DCD pig livers.³² Recently, Schlegel et al have described a single portal vein approach to be effective for HOPE of DCD liver grafts. In addition, no mural necrosis, vascular injury, or deep peribiliary gland injury was observed after using single HOPE.¹¹ However, previous studies have shown that rat liver bile ducts are more resistant to ischemic bile duct injury than human bile ducts.^{21,22} Therefore, we should be careful to say that dual and SP are equally effective in preservation of the biliary tract as shown in this model. ATP measurements in bile ducts tissue could further support the presence of oxygen for aerobic metabolism in biliary epithelium. Unfortunately, rat bile duct samples were too small to extract RNA from and a study in a larger animal model will be initiated to validate our findings. A longer follow-up period is required to assess biliary complications, but this can only be achieved in a transplantation study.

Our study demonstrates that KLF2 expression tends to be higher in dual perfused livers compared to single perfused grafts. Reduced biomechanical stimulation of epithelium, due to absence of arterial flow and pressure in the vessels, will cause decreased endothelial expression of mechanosensitive genes that code for cytoprotective proteins.³³ This process is mediated by the vasoprotective transcription factor KLF2. In the vasculature, KLF2 is endothelial specific and its expression confers endothelial protection against inflammation, thrombosis, and vasoconstriction.¹⁷ The main *in vivo* biomechanical stimulus able to induce KLF2 expression is blood-derived shear stress, and it has been reported that shear stress upregulates the KLF2 target eNOS in the liver endothelium.³⁴ In line with this, higher levels of KLF2 were accompanied by elevated expression of eNOS in dual perfused rat livers. KLF2 also potently induces gene expression of antithrombotic agents such as

TM.³⁵ In our model, however, higher expression of KLF2 was not accompanied by elevated TM. Thrombomodulin is a downstream target of KLF2 and therefore, 2 hours of reperfusion was probably too short to initially upregulate KLF2 and subsequently increase expression of TM. An *in vivo* model in human lungs demonstrated that endothelial KLF2 expression is induced in the presence of continuous flow as well as pulsatile flow.¹⁷ Renal perfusion studies, however, suggest that pulsatile endothelial stimulation is more effective in activating KLF2 and triggering the transcription of anti-inflammatory and antithrombotic genes.^{18,20} In the present study KLF2 expression tends to be higher in continuous perfused livers, but the reperfusion period might have been too short to induce more distinct differences. Higher expression of KLF2 and eNOS in dual perfused livers did not correlate with less injury in hepatic artery histology.

Bile production has generally been accepted as an early sign of liver function after transplantation.³⁶ In the present study, we did not observe a difference in bile production between dual or single perfused livers. Even though DPP livers were associated with higher arterial flows after reperfusion, we failed to observe a beneficial effect on bile production in this group. A study by Foley et al¹² showed that the unique distribution of the arterial blood supply to the biliary tree indicates an important role in bile formation under normothermic conditions. The same group has shown in a porcine transplant model that dual vessel extracorporeal porcine liver perfusion results in better bile production compared to single portal vein perfusion after 120 to 180 minutes.¹³ Therefore, the 2-hour reperfusion time in our model might have been too short to reveal differences in bile production between different perfusion settings.

Another limitation of this study is that we did not confirm our findings in a transplantation model to evaluate long-term outcomes. Also, preservation of the biliary tract could not be proven in this model because of the relatively high tolerance of rat bile ducts to ischemia and the inability to measure ATP content of intra and extrahepatic bile ducts. Future studies

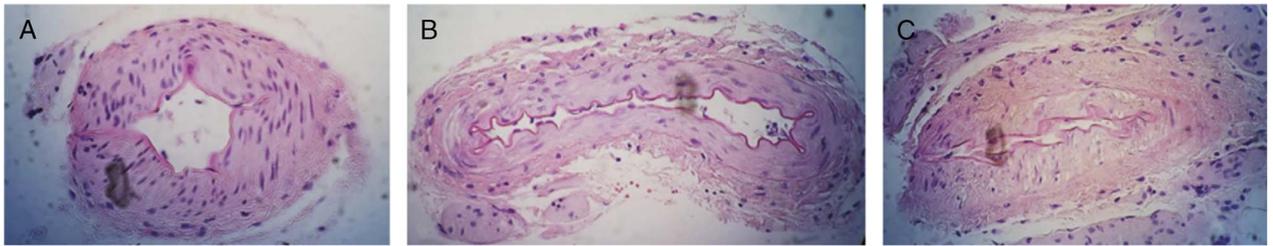


FIGURE 5. H&E staining of hepatic artery biopsies after 2 hours ex situ reperfusion. Representative example of histology of a hepatic artery from SP (A), DPP (B), and DCP (C).

are needed to study biliary complications in particular and a randomized controlled trial would be the ultimate tool to assess the effect of arterial perfusion on ischemic cholangiopathy.

It has been shown that even a short period (1 hour) of end-ischemic machine perfusion after SCS improves graft and bile duct viability independent of the machine perfusion temperature.^{22,37-39} The present study uses a similar model of 1 hour SNMP after 6 hours SCS. We decided to study

the effect of end-ischemic machine preservation, because it provides easier logistics in the clinical setting. However, the field of machine preservation is rapidly evolving and, more recently, some machine perfusion devices became transportable. Continuous perfusion of the organ during transportation decreases SCS time and may provide further benefit. The effects of dual or SP might be more pronounced when the graft is perfused for a longer time period. We are

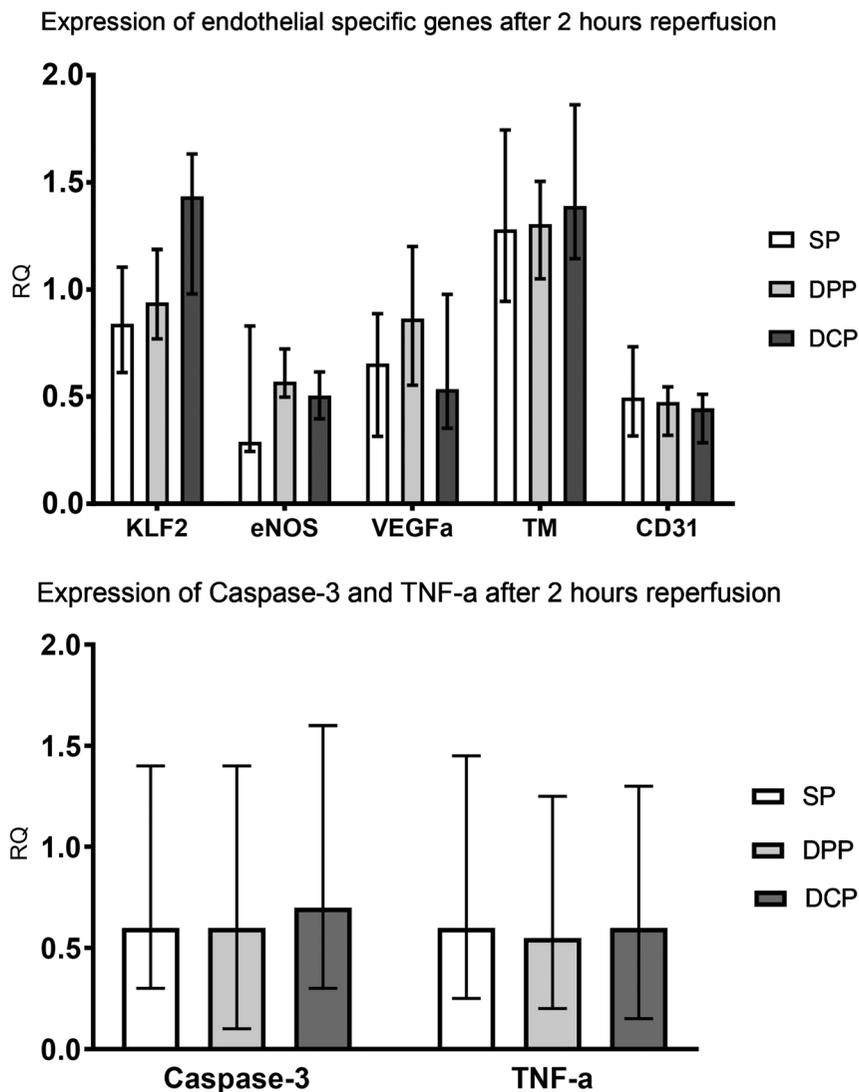


FIGURE 6. Top: Gene expression of endothelial specific genes in liver parenchyma after 2 hours reperfusion. Bottom: Gene expression of TNF- α and Caspase-3. Results are expressed as RQ to GAPDH.

planning to conduct future studies with longer machine perfusion times and longer reperfusion times to detect slight and later changes.

To conclude, our study in a rat *ex vivo* reperfusion model is the first to show comparable outcomes after using dual or SP during end-ischemic SNMP of DCD liver grafts. Arterial flow after reperfusion was higher in DPP livers, but this did not result in less histological damage nor in better liver function. Further research should investigate the effect of hepatic artery perfusion on preservation of the biliary tract by histological analysis and ATP content of the bile ducts.

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