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ORIGINAL ARTICLE

Oxygenated hypothermic machine perfusion after static cold storage improves endothelial function of extended criteria donor livers

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Abstract

Background: Lack of oxygen and biomechanical stimulation during static cold storage (SCS) of donor livers compromises endothelial cell function. We investigated the effect of end-ischemic oxygenated hypothermic machine perfusion (HMP) on endothelial cell function of extended criteria donor (ECD) livers.

Methods: Eighteen livers, declined for transplantation, were transported to our center using static cold storage (SCS). After SCS, 6 livers underwent two hours of HMP, and subsequent normothermic machine perfusion (NMP) to assess viability. Twelve control livers underwent NMP immediately after SCS. mRNA expression of transcription factor Krüppel-like-factor 2 (KLF2), endothelial nitric oxide synthase (eNOS), and thrombomodulin (TM) was quantified by RT-PCR. Endothelial cell function and injury were assessed by nitric oxide (NO) production and release of TM into the perfusate.

Results: In HMP livers, mRNA expression of KLF2 ($p = 0.043$), eNOS ($p = 0.028$), and TM ($p = 0.028$) increased significantly during NMP. In parallel, NO levels increased during NMP in HMP livers but not in controls. At the end of NMP cumulative TM release was significantly lower HMP livers, compared to controls ($p = 0.028$).

Conclusion: A short period of two hours oxygenated HMP restores endothelial cell viability after SCS and subsequent normothermic reoxygenation of ECD livers.

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Introduction

The discrepancy between the demand for liver transplants and organs available for transplantation is a worldwide problem. In an endeavor to reduce organ scarcity and its associated waitlist mortality, livers of extended criteria donors (ECD) are being transplanted more often.[1] ECD livers are, however, of suboptimal quality and are less capable to withstand ischemia/reperfusion (I/R) injury.

I/R injury is generally described as a biphasic continuum of processes that cause injury to cells during preservation, and which is aggravated upon restoration of blood supply.[2,3] During cold preservation, hypothermia changes the structure of cell organelles and disrupts the cytoskeleton, whilst lack of oxygen results in depletion of cellular energy levels via mitochondrial dysfunction.[3] As a result, cells are unable to maintain a membrane electrical potential gradient via the energy-dependent Na/K-ATPase. Subsequently, swelling of both sinusoidal endothelial cells and Kupffer cells narrows sinusoids, thereby enhancing leukocyte entrapment and microcirculatory dysfunction.[4] During I/R, the microcirculation is also endangered by diminished production of nitric oxide (NO) via eNOS,[5,6] as NO is an important substance that promotes vasodilatation and inhibits platelet aggregation.[7]

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Upon re-oxygenation of the graft, mitochondrial dysfunction and Kupffer cell activation facilitate formation of reactive oxygen species (ROS). This oxidative environment induces apoptosis and necrosis of both hepatocytes and endothelial cells.[8,9] Apoptotic and swollen endothelial cells, together with leukocyte accumulation may lead to reperfusion no-reflow, which is associated with delayed graft function and primary non-function.[9]

To date, the high incidence of I/R injury associated complications in ECD liver transplantation, such as primary non-function, non-anastomotic biliary strictures and hepatic artery thrombosis after transplantation, still greatly compromise the success of ECD liver transplantation.[10–13] A promising technique to improve outcome of ECD liver transplantation is end-ischemic hypothermic machine perfusion (HMP).[14,15] Previous studies have shown that a short period of end-ischemic HMP increases hepatic ATP content and hepatobiliary excretory function upon re-oxygenation of the liver graft, compared to livers preserved by conventional static cold storage (SCS).[16] However, the effect of end-ischemic HMP on the endothelial cells lining the hepatic vasculature remains largely unexplored.

Endothelial cells are dynamic cells that actively play part in the regulation of vascular tone and hemostasis, in response to the hemodynamic forces of the blood flow.[17] Blood flow-induced shear stress on endothelial cells leads to up regulation of transcription factor Krüppel-like-factor 2 (KLF2).[18,19] KLF2 expression results in downstream expression of cytoprotective genes, such as the anti-thrombotic and anti-inflammatory molecule thrombomodulin (TM), as well as endothelial nitric oxide synthase (eNOS).[20,21] During conventional SCS, the vascular endothelium is deprived of both oxygen and biomechanical stimulation, which would greatly impair endothelial cell function via down regulation of transcription factor KLF2 and downstream TM and eNOS. Moreover, KLF2 induction down regulates expression of vasoconstrictor endothelin-1 (ET-1).[22] Thus, while NO production via eNOS is already decreased as a consequence of I/R injury, absence of biomechanical stimulation will aggravate vasoconstriction even more.

Lack of oxygen triggers endothelial cells to quickly induce the transcription of hypoxia inducible factors (HIF-2α). One of the downstream effects of induced HIF-2α is an increase of vascular endothelial growth factor A (VEGF-A). Via regulation of the vascular tone, VEGF-A up regulation minimizes the harmful effects caused by low oxygen levels.[23] Moreover, during oxidative stress, cells rely on the quick up regulation of heme oxygenase-1 (HO-1), which negatively affects the catabolism of free radicals via free heme, thereby preventing induction of programmed cell death.[24]

Since end-ischemic oxygenated HMP of donor livers provides endothelial cells with both oxygen and biomechanical stimulation, valuable stimuli that are absent during SCS, we hypothesized a beneficial effect of end-ischemic HMP on endothelial cell function. The aim of this study was to investigate the effect of two hours of end-ischemic oxygenated HMP on endothelial cell function of ECD livers.

Materials and methods

Study design

This study is designed as an experimental substudy of a project previously published by Westerkamp *et al.*[16]

Donor livers

In the period from 2012 until 2014, a total of 18 human livers that were declined for transplantation, were assigned to our institute for machine perfusion research after informed consent was obtained from the donor's relatives. Approval of the study protocol was provided by the medical ethical committee of the University Medical Center Groningen (METc 2012.068) and the Dutch Transplantation Foundation (NTS), the competent authority for organ donation in the Netherlands. Livers were procured by regional multi-organ procurement teams using topical iced cooling *in situ* and aortic flush out with preservation solution (University of Wisconsin [UW] or histidine–tryptophan–ketoglutarate solution [HTK]).

Machine perfusion

HMP and NMP were performed using a pressure- and temperature-controlled machine perfusion device (Liver Assist, Organ Assist, Groningen, The Netherlands) as described previously.[16] Dual perfusion was established through a continuous flow via the portal vein and pulsatile flow via the hepatic artery.

HMP was established using oxygenated Belzer-UW Machine Perfusion solution (Bridge-to-Life, Ltd. Northbrook, IL, USA). The temperature of the perfusion fluid was 10 °C and portal and mean arterial pressure were set at 5 and 25 ± 5 mmHg, respectively. To oxygenate the cold perfusion fluid 100% oxygen (1 L/min) was used, resulting in an oxygen pressure of 50–80 kPa (375–600 mmHg).

After two hours of HMP, the same machine perfusion device was used for NMP. For this reason, the liver had to be disconnected from the machine. While the perfusion device was being primed for NMP, the liver was preserved in a bowl of ice-cold UW cold storage solution (Bridge-to-Life, Ltd. Northbrook, IL, USA) for a maximum of 90 min. Prior to the start of NMP, the liver was flushed with 1 L of cold, and subsequently 0.5 L of warm, NaCl 0.9% solution.[16,26]

NMP was performed at physiological temperature (37 °C) with portal and mean arterial pressure set at 11 and 70 ± 14 mmHg, respectively.[25] During NMP, oxygenated of the perfusion fluid was achieved with a carbogen mixture of 95% oxygen and 5% carbon dioxide (2 L/min), also resulting in an oxygen pressure of 50–80 kPa (375–600 mmHg). The perfusion

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fluid used for NMP was a mixture of red blood cells, heparinized human plasma with the addition of nutrients, trace elements, and antibiotics as described previously.[26]

Gene expression of endothelial proteins

Liver parenchyma biopsies were obtained either at the end of SCS (in the SCS alone group), or after SCS plus two hours of HMP (in the SCS + HMP group), as well as after six hours of NMP. Biopsies were snap-frozen in liquid nitrogen. Total RNA was extracted from snap-frozen liver biopsies using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. The RNA concentration was determined with a NanoDrop ND−1000 UV−Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Subsequently, equal amounts of RNA were converted to cDNA using M-MLV Reverse Transcriptase (Invitrogen, Basel, Switzerland). For qualitative real-time detection, sense and antisense primers were designed using Primer Express® software (version 2.0, Applied Biosystems). Amplification and detection were performed with the ABI Prism 6900HT Sequence Detection System (Applied Biosystems). Relative expression of the mRNA of interest was normalized to the housekeeping gene GAPDH (sense ACCCACTCCTCCACCTTTGA and antisense CATACCAGGAAATGAGCTTGACAA). Primer sequences, sense and antisense respectively, were as follows: CD31 (GACCTCGCCCTCCACAAA and CGTGTCTTCAGGTTGGTATTTCAC), KLF2 (GCAAGACCTACACCAAGAGTTCG and TCCCA GTTGCAGTGGTAGGG), TM (TGATTCCCTCCCGAACAGTT and ACTCTACCGGGCTGTCTGTACTCT), ENOS (TGTATGGATGAGTATGACGTGGTGT and TGCAAAGCTCTCTCCATTCTCC), ET-1 (AACCATCTTCACTGGCTTCCAT and TTTCTGCTGAGAGGTCCATTGTC), VEGF-A (CCTGGGACTCGCCCTCA and CAGAACTAGTGGTTTCAATGGTGTG), HIF-2α (AGCTATGTGACTCGGATGGTCTTT and TGCATGAATTCCCGTCTAAACC) and HO-1 (GCTCAGCCTCAAATGCAGTATTTT and ACCCACGCATGGCTCAA).

Assessment of endothelial function

Nitric oxide is rapidly oxidized to its stable oxidation products nitrate and nitrite. To determine nitrate concentration in the perfusate samples, endogenous nitrate was subtracted from the total nitrate value. A total nitric oxide and nitrate/nitrite parameter assay kit (R&D systems, Minneapolis, MN) was used to quantify nitrate and nitrite in the samples. Values were corrected for nitrate/nitrate levels present in the perfusate at baseline. Assays were performed according to manufacturer's instructions.

Assessment of endothelial integrity

Thrombomodulin (TM) is a transmembrane glycoprotein, and its extracellular part can be shed, for example during endothelial injury. Increased soluble TM (sTM) concentrations in the fluid

Table 1 Comparison of donor characteristics of livers preserved only with static cold storage (SCS) or with SCS plus hypothermic machine perfusion (SCS + HMP)

Variables	SCS alone n = 12	SCS + HMP n = 6	*p*-Value
Age (years)	61 (52–64)	64 (57–70)	0.21
Gender (male)	8 (67%)	3 (50%)	0.49
Type of donor liver			0.18
- DCD	9 (75%)	6 (100%)	
- DBD	3 (25%)	0	
Reasons of rejection for transplantation			0.29
- DCD and age >60 years	5 (42%)	5 (84%)	
- DCD and high BMI	5 (42%)	1 (17%)	
- DCD and high transaminases	2 (16%)	0	
Type of preservation solution			0.18
- UW solution	9 (75%)	6 (100%)	
- HTK solution	3 (25%)	0	
Liver weight (kg)	2.11 (1.81–2.30)	1.83 (1.15–2.23)	0.29
Eurotransplant donor risk index	2.79 (2.24–3.21)	3.11 (2.77–3.39)	0.38
Cold ischemia time (hr:min)[a]	9:04 (7:01–11:14)	7:18 (6:10–8:32)	0.15
Total preservation time (hr:min)[b]	9:04 (7:01–11:14)	11:37 (10:01–12:25)	0.10

Data presented as median ± IQR for continuous variables or numbers (percentages) for categorical variables.
Abbreviations: SCS, static cold storage; HMP, hypothermic machine perfusion; DCD, donation after circulatory death; DBD, donation after brain death; BMI, body mass index; UW, University of Wisconsin; HTK, histidine–tryptophan–ketoglutarate.
[a] Time between *in situ* cold flush out in the donor and start of machine perfusion (either HMP or NMP) of the liver.
[b] Time between *in situ* cold flush in the donor and start of NMP.

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phase are, therefore, a marker of decreased vascular integrity. Perfusate concentrations of TM were determined using a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) kit, human thrombomodulin/BDCA-3 (DY3947) (R&D systems, Minneapolis, MN). Samples were diluted 1:40 and the ELISA was performed according to manufacturer's instructions.

Immunohistochemistry

Liver parenchyma biopsies were obtained either at the end of SCS (in the SCS alone group), or after SCS plus two hours of HMP (in the SCS + HMP group), as well as after six hours of NMP. Samples were fixated in 10% formalin and paraffin embedded. Slides were prepared for hematoxylin and eosin (H&E) staining. Additional slides were prepared for immunohistochemical detection of thrombomodulin and CD31. The antibody against thrombomodulin (QBEND/40, Novus Biologicals, Littleton, CO, USA) was applied in a dilution of 1:50. All steps were performed according to manufacturer's guidelines. The antibody against CD31 (Clone JC70A, DAKO, Glostrup, Denmark) was applied in a dilution of 1:40 in an automated immunoperoxidase staining system (Roche Ventana Medical Systems, Basel, Switzerland). All chemicals applied during this staining process were purchased from Roche Ventana Medical Systems. Pretreatment for CD31 immunostaining was 36 min of treatment with Ultra CC1 which is integrated in the automated staining procedure.

Histological analysis of endothelial injury of the microvasculature

Intrahepatic degree of endothelial injury of the microvasculature was blindly assessed using a semi-quantitative histological scoring system for H&E stained slides using light microscopy. In the absence of a established histological scoring system for the assessment of endothelial cell and vascular injury, our experienced liver pathologist (ASHG) developed a semi-quantitative histological scoring system based on generally accepted microscopic characteristics of endothelial cell injury (Table 2). Per slide, 10 portal venous and 10 arterial branches within a portal triad, as well as 10 central vein branches, of the same size were scored separately. The size of the portal triads was related to the diameter of the bile duct. In case multiple vessels of the same type were observed in one portal triad, the vessel with the worst injury score was chosen. Injury was scored with a minimum of five and a maximum of twelve points.

Statistical analysis

Continuous variables were presented as median and interquartile range (IQR). Comparison of unpaired continuous data was performed using the Mann−Whitney U test and paired continuous variables were compared using the Wilcoxon test. Categorical data were compared using the Pearson chi-square or Fisher's exact test. The level of significance was set at a p value of 0.05. All statistical analyses were performed using IBM SPSS Statistics software version 22 for Windows (SPSS, Inc., Chicago, IL, USA).

Table 2 Histological scoring system for microvascular injury

Vascular component	Injury score		
	Score 1	Score 2	Score 3
Presence of endothelial cells	Present	Absent	–
Pyknotic endothelial cells	None	Present	N/A
Lifted endothelial cells	None	Present	N/A
Swollen vessel wall[a]	None	Present	–
Necrotic vessel wall	None	Present	–
Inflammation[b]	None	Mild	Moderate

A histological injury score, developed by the authors, to assess microvascular injury of endothelial cells lining the vasculature and the vessel walls of hepatic arteries, portal veins and central veins in hematoxylin and eosin stained liver biopsies. Endothelial cells were scored were scored on presence (1 point) or absence (2 points), and on indications of cell death (pyknosis or endothelial lifting) (2 points). Vessel wall was scored on presence of swelling and/or necrosis (2 points). Inflammation was scored categorically: none (1 point), mild (2 points) or moderate (3 points).
Abbreviations: N/A, not applicable.
[a] Only applicable for hepatic artery branches.
[b] Not applicable for hepatic artery branches.

Results

Donor characteristics

The comparison of donor characteristics between livers only preserved by SCS and livers that underwent two hours of additional HMP is shown in Table 1. Between groups there were no significant differences in donor variables with regard to age, type of donor graft, warm ischemia time (in case of DCD), type of preservation solution, and the Eurotransplant donor risk index.[16]

Flow patterns

Arterial and portal flow increased during the first half hour of NMP and remained stable thereafter in both SCS alone and SCS + HMP livers. At the end of six hours viability testing, both portal and arterial flow were significantly higher in SCS + HMP livers compared to livers preserved by SCS alone, with a median portal flow of 883.5 (804.6−979.4) vs. 411.4 (302.7−679.0) mL/min/kg ($p = 0.001$) and a median arterial flow of 335.2 (182.8−462.5) vs. 121.9 (95.2−152.9) mL/min/kg ($p = 0.032$), respectively (Fig. 1c and d).

Presence of endothelial cells

In all liver biopsies, presence of endothelial lining of the microvasculature was confirmed by positive staining of CD31. Moreover, as both staining and mRNA content of the pan endothelial cell marker CD31 did not differ between groups, equal endothelial content in all biopsies was assumed (Fig. 1 and Table 4, respectively).

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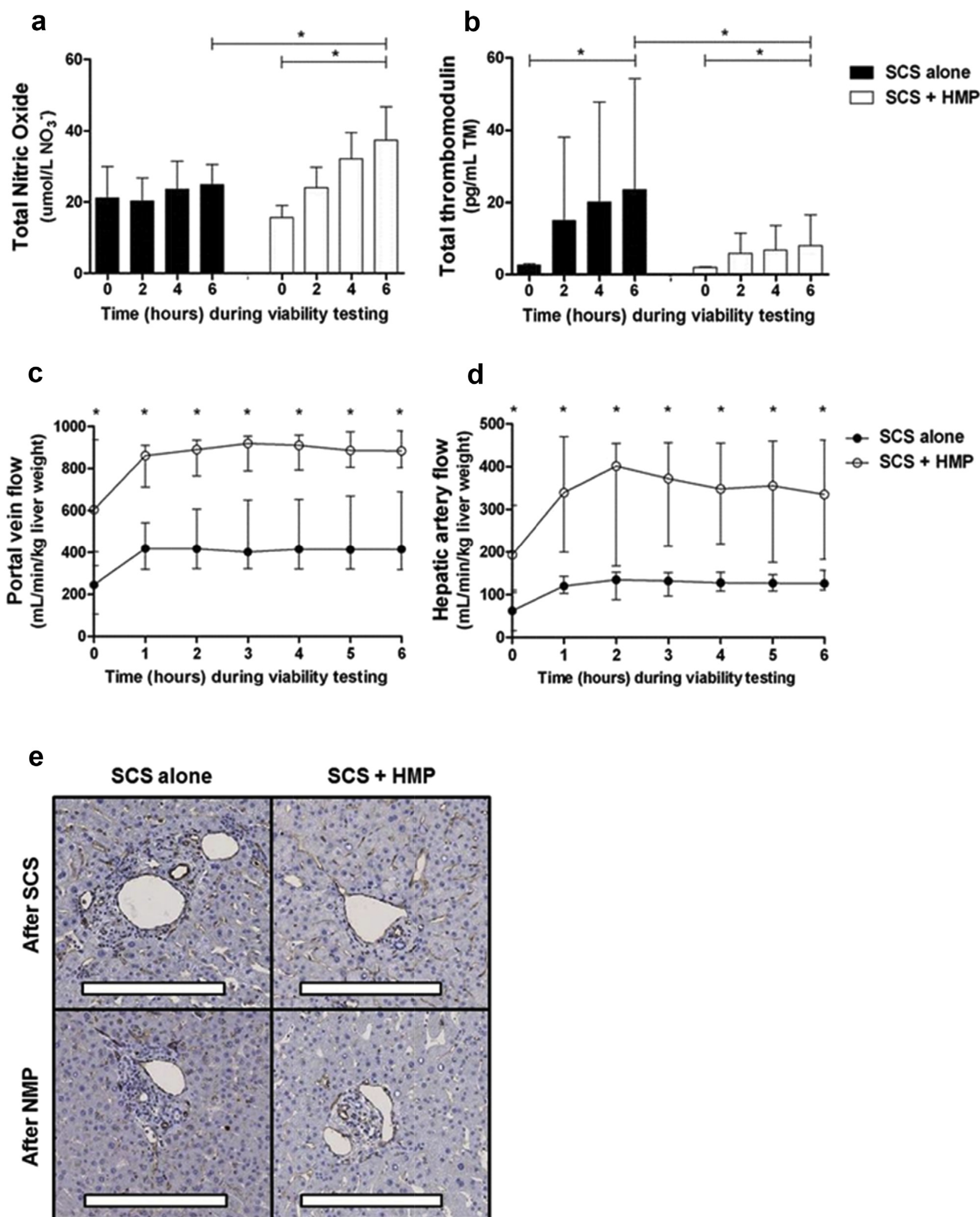

Figure 1 Vascular function, integrity and flow patterns during 6 h of viability testing using normothermic machine perfusion (NMP). Panel a: During NMP, total nitric oxide production significantly increased significantly in the SCS + HMP group (p = 0.028) but not in the SCS alone

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Table 3 Gene transcription of endothelial specific genes in liver parenchyma after 6 h of viability testing (NMP) as a ratio relative to baseline

	SCS alone	*p*-Value[a]	SCS + HMP	*p*-Value[b]	SCS alone vs. SCS + NMP *p*-Value
CD31	0.83 (0.34–1.33)	0.79	1.10 (0.75–1.33)	0.60	0.69
KLF2	2.43 (1.01–2.97)	**0.026**	2.50 (1.08–7.23)	**0.043**	0.76
TM	2.18 (0.75–5.58)	0.06	12.75 (8.05–31.03)	**0.028**	**0.016**
ENOS	3.33 (0.32–7.17)	0.11	28.05 (12.13–112.88)	**0.028**	**0.009**
ET-1	1.42 (0.51–2.81)	0.18	1.04 (0.57–2.40)	0.46	0.58
HIF-2α	0.82 (0.66–0.95)	0.09	3.10 (1.45–3.75)	**0.028**	**0.003**
VEGF-A	0.67 (0.57–0.97)	0.13	3.73 (1.82–5.68)	**0.027**	**0.001**
HO-1	1.65 (0.96–2.97)	**0.016**	2.67 (1.47–19.29)	**0.028**	0.32

During six hours of viability testing, gene expression of KLF2 and HO-1 significantly increased in the SCS alone group compared to baseline. In the SCS + HMP group, gene expression of KLF2, TM, eNOS, HIF-2α, VEGF-A and HO-1 increased significantly during six hours of viability testing compared to baseline. At the end of six hours of viability testing, gene expression of TM, eNOS, HIF-2α and VEGF-A were significantly higher in the SCS + HMP group compared to the SCS alone group. Data are presented as median ± IQR and as a ratio relative to baseline. Bold font indicates significance of $*p < 0.05$.
[a] SCS alone versus baseline.
[b] SCS + HMP versus baseline.

Table 4 Microvascular injury according to histological vascular injury score, both end-ischemic and the end of viability testing in both study groups

Type of vessel	SCS alone	SCS + HMP	*p*-Value
End-ischemic injury score			
Hepatic artery	6.6 (6.5–6.7)	6.3 (5.6–6.5)	0.07
Portal vein	7.2 (6.6–7.5)	6.6 (6.0–7.0)	0.09
Central vein	7.0 (6.2–7.4)	6.4 (6.1–7.3)	0.70
Bile duct (μm)	30.3 (27.8–40.1)	37.2 (31.1–40.0)	0.78
Injury score at the end of viability testing			
Hepatic artery	6.6 (6.3–7.0)	5.7 (5.5–6.2)	**0.005**
Portal vein	6.7 (6.5–7.4)	6.5 (6.2–6.8)	0.32
Central vein	6.5 (5.8–7.4)	6.3 (6.1–6.9)	1.00
Bile duct (μm)	35.4 (31.0–36.7)	28.8 (27.0–33.8)	0.18

Total microvascular injury score based on Table 2. The end-ischemic injury score of all vessel branches did not differ between groups at baseline. After 6 h of viability testing, microvascular injury score was significantly lower in hepatic artery branches of HMP + SCS livers ($p = 0.005$). Diameter of the bile ducts in the portal triads was comparable between groups. Data are presented as median ± IQR. Bold font indicates significance of $*p < 0.05$.

Changes in flow-regulated genes

Changes in gene expression of selected genes related to vascular function during NMP are depicted in Table 3. During 6 h of NMP, gene expression of KLF2 expression increased 2-fold compared to baseline in both SCS alone and SCS + HMP livers, 2.43 (1.01–2.97) p = 0.026 and 2.50 (1.08–7.23) p = 0.043, respectively. The increase in KLF2 mRNA expression was not significantly higher in the SCS + HMP compared to the SCS alone group (p = 0.763). Interestingly, after 6 h of NMP, gene expression of TM was 6-fold higher in HMP preserved livers compared to livers only preserved via SCS, 12.75 (8.05–31.03) vs. 2.18 (0.75–5.58), respectively (p = 0.016). Concomitantly, eNOS gene expression was 9-fold higher in HMP preserved livers compared to SCS alone livers, 28.05 (12.13–112.88) vs. 3.33 (0.32–7.17), respectively (p = 0.009). Gene expression of vasoconstrictor endothelin-1 (ET-1) was slightly higher in SCS alone livers 1.42 (0.51–2.81) compared to SCS + HMP livers 1.04 (0.57–2.40), but this difference was not statistically significant (p = 0.580).

Changes in oxygen-regulated genes

Molecular changes of selected genes related to vascular oxygen levels are depicted in Table 3. During six hours of NMP, gene

group. At the end of NMP, total nitric oxide production was significantly higher in the SCS + HMP group (p = 0.027) compared to the SCS group. Panel b: During NMP, thrombomodulin concentration increased in both SCS alone and SCS + HMP group, p = 0.002 and p = 0.028, respectively. Total thrombomodulin increase was significantly higher in the SCS alone group (p = 0.032). Panel c and d: During NMP, both portal and hepatic flows were significantly higher in livers first treated with HMP compared to control livers only preserved by SCS, p = 0.001 and p = 0.032, respectively. Data are presented as median and IQR. $*p < 0.05$. Panel e: Pan endothelial cell marker CD31 confirmed presence of endothelial lining of the microvasculature of all liver biopsies. Positive staining of CD31 antibody in liver biopsies of SCS alone (panels on the left) and SCS + HMP livers (panels on the right), both directly after SCS (upper panels) and at the end of NMP (lower panels). Dark brown indicates CD31 positive staining. Scale bar indicates 300 μm.

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expression of HIF-2α increased 3-fold compared to baseline in livers first treated with two hours of HMP (3.10 [1.45–3.75], p = 0.028), but not in SCS alone livers (0.82 [0.66–0.95], p = 0.091). Concomitantly, VEGF-A gene expression increased 3-fold during NMP in HMP livers (3.73 [1.82–5.68], p = 0.027), but not in SCS alone livers (0.67 [0.57–0.97], p = 0.131). At the end of six hours of NMP, gene expression of both HIF-2α and VEGF-A were significantly higher in HMP treated livers compared to livers only preserved via SCS alone, p = 0.003 and p = 0.001, respectively. During NMP, gene expression of HO-1 increased in both SCS alone and SCS + HMP livers compared to baseline, 1.65 (0.96–2.97) p = 0.016 and 2.67 (1.47–19.29) 0.028, respectively. Although gene expression was slightly higher in SCS + HMP livers compared to SCS alone livers, this was not statistically significant (p = 0.315).

Endothelial cell function

Cumulative NO production during viability testing is presented in Fig. 1a. During six hours of NMP, a 2-fold increase of cumulative perfusate concentration of endothelial vasodilatator nitric oxide (in μmol/L) was observed in SCS + HMP livers compared to baseline (15.7 [12.8–19.0] vs. 37.3 [15.7–18.4]) (p = 0.028). In SCS alone livers, on the other hand, an increase in cumulative nitric oxide production was not observed over the course of NMP (20.8 [15.9–29.1] vs. 22.7 [19.0–30.5]) (p = 0.182). At the end of six hours of NMP, total production of nitric oxide was significant higher in SCS + HMP livers compared to the SCS alone group (37.3 [15.7–18.4]) vs. 22.7 [19.0–30.5] (p = 0.027).

Integrity of the vascular endothelium

Concentration of sTM in the perfusate during viability testing is presented in Fig. 1b. Compared to baseline levels, sTM concentration (in pg/mL) significantly increased during six hours of NMP in both the SCS (2.6 [2.5–3.1] vs. 23.6 [11.9–54.3]) and SCS + HMP group (2.1 [2.0–2.2] vs. 8.1 [5.1–16.1]), p = 0.002 and p = 0.028, respectively. At the end of NMP, cumulative thrombomodulin increase was, however, nearly 3-fold higher in the SCS group compared to the SCS + HMP group (23.6 [11.9–54.3] vs. 8.1 [5.1–16.1]) (p = 0.032).

Histological analysis of vascular injury

The size of the portal triads scored was comparable between groups, as the bile duct diameter (in μm) did not differ between biopsies of SCS alone and SCS + HMP livers taken both directly after SCS (30.3 [27.8–40.1] vs. 37.2 [31.1–40.0], respectively, p = 0.78) and after NMP (35.4 [31.0–36.7] vs. 28.8 [27.0–33.8], respectively, p = 0.18). As expected, directly after SCS, there were no differences in the histological vascular injury score of portal, arterial and central vein branches between the two groups (data not shown). After six hours of NMP, injury of the arterial branches was significantly lower in the SCS + HMP group compared to the SCS alone group, injury score of 5.7 [5.5–6.2] vs. 6.3 [5.6–6.5], respectively (p = 0.005). Differences in injury of both the portal and central vein branches were not observed between groups at the end of NMP.

Discussion

End-ischemic oxygenated HMP is gaining increasing attention as a promising method of organ preservation compared to conventional SCS alone. Although the amount of evidence of pathophysiological mechanisms, explaining the beneficial effect of end-ischemic HMP on liver parenchymal cells, is growing, the effect of HMP on the hepatic vascular endothelium remains largely unknown. In this study we report the effect of two hours of end-ischemic oxygenated HMP on endothelial cell function of ECD livers.

The main finding of this study was the improved endothelial function of livers which were additionally preserved via two hours of end-ischemic HMP. While a short period of only two hours of HMP might seem short to actually increase NO production upon reperfusion, these data demonstrate that NO production significantly increases during *ex situ* NMP of HMP livers, while this increase was not observed in SCS alone preserved livers. This explains, at least partially, why significantly higher flows in both the hepatic artery and portal vein of HMP livers were observed during NMP at all time-points, compared to the flows of the SCS alone preserved livers. The increased phosphorylation of eNOS via KLF2 might explain the increased NO production in HMP livers. Recent studies have demonstrated that lack of biomechanical stimuli occurring during cold preservation for transplantation markedly deteriorates LSEC protective phenotype by down regulating the expression of the transcription factor Kruppel-like Factor 2 (KLF2), which orchestrates the transcription of a variety of protective genes including the endothelial synthase of NO (eNOS) and the antithrombotic molecule thrombomodulin.[2]

We found that KLF2 expression at the end of six hours *ex situ* NMP is significantly increased compared to baseline in livers preserved via two hours of HMP in addition to the static cold preservation as well as in livers only preserved via SCS. Expression of cytoprotective genes downstream in the flow sensitive pathway, eNOS and TM, respectively were, however, only significantly higher in the HMP group compared to the SCS alone livers at the end of *ex situ* NMP. Previously, it has been demonstrated that even 1 h end-ischemic non-oxygenated, pulsatile hypothermic machine perfusion is able to recondition donor kidneys grafts via up regulation of mechano-sensitive transcription factor KLF2.[27] Furthermore, our data suggest that after two hours of end-ischemic oxygenated HMP livers are more capable to withstand I/R injury, via upregulation of HIF-2α and subsequent VEGF-A upon normothermic reoxygenation. In donor kidneys, HIF-2α has a protective role against I/R injury via amelioration of oxidative stress.[28] Moreover, in this study we found that HMP preconditioned livers are able to counterbalance oxidative stress via up regulation of HO-1 gene expression.

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A potential disadvantage of cold perfusion could be inadequate shear stress on, and therefore damage to, the hepatic endothelium.[29] In this study, concentrations of TM in the perfusate, a marker of endothelial damage, were significantly higher at the end of NMP in the SCS alone group compared to the HMP livers. This suggests that vascular integrity was in fact better preserved in the HMP livers. An optimal perfusion pressure is a necessity for adequate perfusion of the graft without causing harm.[30] Since flows were lower in the SCS alone livers at the beginning of NMP while the pressure settings were equal to those in the HMP group, the resistance in the microcirculation was higher and therefore the perfusion pressure might have been relatively too high in the SCS alone group.

The debate about the best method of liver perfusion has been ongoing: single versus dual perfusion as well as continuous versus pulsatile flow.[31] In this study, we perfused livers via a dual perfusion method with continuous flow via the portal vein and pulsatile flow via the hepatic artery. In previous studies, the shear stress regulatory effect of KLF2 has been shown to be flow-pattern specific.[32] In cultured human endothelial cells, pulsatile flow with significant forward direction increased KLF2 expression, while oscillatory flow with little forward direction did not.[33] Interestingly, our study demonstrates better preservation of the arterial endothelial cell morphology of HMP livers compared to SCS alone livers. This advocates a beneficial effect of hepatic artery perfusion during end-ischemic oxygenated HMP.

Due to the *ex vivo* set up of this study, the impact of the improved endothelial function has yet to be investigated *in vivo* including actual transplantation and long-term follow up. Furthermore, all livers were included in this study in a consecutive fashion. Livers between were not matched. However, baseline characteristics do not differ substantially between groups.

In conclusion, two hours of end-ischemic oxygenated HMP results in better endothelial cell function of ECD livers, when compared to SCS preservation alone, via up regulation of mechano-sensitive cytoprotective genes and results in better preservation of arterial endothelial cell morphology.

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Disclosure

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Previous communication

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Conflicts of interest

None declared.

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