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Equal performance of HTK-based and UW-based perfusion solutions in sub-normothermic liver machine perfusion

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Machine perfusion (MP) is gaining importance in liver transplantation, the only cure for many end-stage liver diseases. Varieties of different MP protocols are available. Currently, various MP protocols are available, differing not only in perfusion temperature but also in the specific perfusion solution required. We aimed to investigate the performance of an HTK-based perfusate during sub-normothermic MP (SNMP) of discarded human liver grafts compared to that of a UW-based solution. Twenty discarded livers (rejected for transplantation by all centers) were subjected to ex-vivo SNMP at 21°C with either HTK- or UW-based solution for 12 h. Perfusate and tissue samples collected before the start, after 6 h, and at the end of SNMP were analyzed for liver enzymes, along with mRNA expression of perfusate and tissue markers associated with organ damage. Hepatocellular viability was assessed by measuring bile production, monitoring pH stability, and analyzing histological changes in HE stained tissue sections. After propensity score matching 16 livers were analyzed. Overall, no differences between HTK- and UW-based solution were detected, except for an increased MLKL mRNA expression and impaired pH stability during SNMP with HTK-based perfusate. No other investigated parameters of cell injury, inflammation or hepatocellular viability supported this finding. Bile production was higher in the 6 HTK-perfused livers compared to the three UW-perfused livers that produced bile. Overall, these findings suggest that HTK performs comparably to a UW-based solution during 12 h of liver SNMP.

Keywords HTK solution, UW solution, Machine perfusion, Liver transplantation, Sub-normothermic machine perfusion

Liver transplantation, the only cure for many end-stage liver diseases, faces numerous challenges in healthcare. Beyond the limitations posed by organ shortage and logistic constraints, a key challenge in transplantation is the time-dependent ischemic tissue damage that occurs during organ transport from the procurement center to the recipient. In the history of solid organ transplantation, many techniques have been investigated to overcome the limitations of conventional static-cold storage (SCS), and thus improving transplantation outcomes significantly. Within this context, machine perfusion (MP) has gained more and more importance, not only because of its ability to safely delay implantation, and thus overcome logistical challenges, but also as platform to mitigate ischemia-reperfusion injury (IRI), particularly in organs of sub-optimal quality[1–3].

In the literature, numerous MP protocols, using different machines, temperature settings and perfusion solutions are described[1,4,5], and comprehensively reviewed elsewhere[6]. As perfusion solutions need to fulfill different requirements depending on the temperature settings during MP[7], the choice of solution is dependent on the target temperature[8]. Each temperature and perfusion solution combination has specific advantages and disadvantages[9], making it challenging to determine the optimal protocol, particularly during sub-normothermic MP (SNMP). Hypothermic MP (HMP) has facilitated the safe expansion of perfusion durations of up to 20 h[10]. However, organ assessment remains limited due the the reduced metabolic activity. Normothermic MP (NMP) offers the key advantage of mimicking physiological conditions, making it more suitable for effective

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viability assessment. With the emergence of combined perfusion protocols, SNMP fits within the intermediate temperature range, and therefore making it a promising approach for further exploration[9]. It offers potentially new insights into viability assessment markers.

Liver SNMP does not require oxygen carriers, as the temperature is low enough to ensure adequate tissue oxygenation, while still high enough to partially maintain metabolic activity, and thus supports extended viability assessment compared to HMP[11–13]. Although several solutions have been investigated for the cold-storage of several organs[14], evidence in context of SNMP is scarce[15]. Therefore, the aim of our study was to compare two commercially available solutions, HTK-based Custodiol® (intended for use in SCS) and UW-based Belzer MPS® (intended for use during MP at 4–8 °C), concerning their performance during 12 h liver SNMP at 21 °C.

Results

Included organs and propensity score matching

In total, 20 consecutive non-transplantable livers were included in the study. Propensity score matching (PSM) was performed to adjust for baseline differences in organ characteristics. The propensity scores were estimated using logistic regression based on the duration of SCS (cut-off 12 h) prior to the beginning of SNMP and the reason for being discarded (steatosis or other, non-liver related, reasons). Two-to-one nearest neighbor matching with replacement was used. All livers perfused with Belzer MPS® (n = 7) were matched to two livers perfused with Custodiol®. Good balance was achieved between the Belzer MPS® and Custodiol® groups, with all standardized mean differences (SMD) below 0.1 after matching. The characteristics of donors and livers before and after PSM are listed in Table 1.

Parameters of liver quality are similar in SNMP with both types of perfusate

Perfusate levels of liver enzymes (AST, ALT, γ-GT, AP, LDH and lactate) were measured to estimate liver quality during SNMP. As shown in Fig. 1, there are no statistically significant differences between perfusion with Custodiol® or Belzer MPS®. Additional perfusate parameters were investigated (Figure S1), supporting the comparability of the two tested perfusion solutions during SNMP, particularly in terms of oxidative stress and inflammation. Donor and organ characteristics, as well as bile production and histological assessment in terms of steatosis, fibrosis, inflammation and necrosis of HE stained liver sections before SNMP and after 12 h of SNMP for PSM livers are presented in Table 2 . Representative pictures of HE stained liver sections are shown in supplementary Figure S2. Histological assessment revealed no deterioration over time in both groups.

Hepatocellular assessment criteria during SNMP suggest differences between Custodiol® and Belzer MPS®

Bile production was similar between SNMP with Custodiol® and Belzer MPS® solution, trending towards increased bile production during SNMP with Custodiol® (Fig. 2a). The amount of sodium bicarbonate needed to maintain physiologic pH, apart from the initial pH adjustment within the first hour of perfusion, was significantly higher during SNMP with Custodiol® when compared to Belzer MPS® (Fig. 2b). Perfusate pH and bicarbonate concentration showed a similar pattern during SNMP with both perfusates (Fig. 2c). Furthermore, pO_2 and pCO_2 were similar for both solutions during 12 h of SNMP, although the pO_2 levels in the Custodiol® group tended to be lower at the time of the back-table procedure (Fig. 2d).

For the 6 Custodiol®-perfused and the three Belzer MPS®-perfused livers, which produced bile during the period of SNMP, pH, bicarbonate and glucose levels (relative to the respective levels in the perfusate) were similar in both groups supporting the hypothesis of similar performance of the two tested perfusion solutions (Figure S3). Bile production started earlier in Custodiol®-perfused livers (mean: 1.8 h; range: 1–5 h) compared to Belzer MPS®-perfused livers (mean: 3.7 h; range: 1–6 h).

mRNA expression in liver tissue confirms similarity of HTK-based Custodiol® and UW-based Belzer MPS® for use in SNMP

mRNA expression of most cell survival parameters validates the outcomes of the perfusate parameters. The mRNA expression of the necroptosis effector MLKL (mixed lineage kinase domain-like pseudokinase) was the only marker that showed an increase in livers perfused with Custodiol® compared to Belzer MPS®. The difference reached statistical significance after 6 h of SNMP, but returned to a non-significant trend at the end of the 12 h SNMP (Fig. 3). This finding, was not supported by the expression of RIPK1 (receptor-interacting serine/threonine-protein kinase 1) or any other investigated markers, including those for oxidative stress and inflammation (Figure S4). Further analysis of protein expression by western blotting revealed similar MLKL

		Custodiol® (n = 8)	Custodiol® (n = 13)
	Belzer MPS® (n = 7) PS matched	PS matched	Non-matched
Donor age (years)	67 (55; 77)	70 (64; 77)	68 (57; 77)
Gender (m/f)	4 / 3	3 / 5	8 / 5
Organ weight (kg)	1.50 (1.43; 1.92)	1.50 (1.28; 2.28)	1.90 (1.39; 2.40)
SCS (< 12 h/ ≥ 12 h)	2 / 5	4 / 4	6 / 7
Steatosis/Other	3 / 4	4 / 4	8 / 5

Table 1. Baseline characteristics of discarded livers included for SNMP. Data are presented as median (Q1; Q3). *SCS* static cold storage.

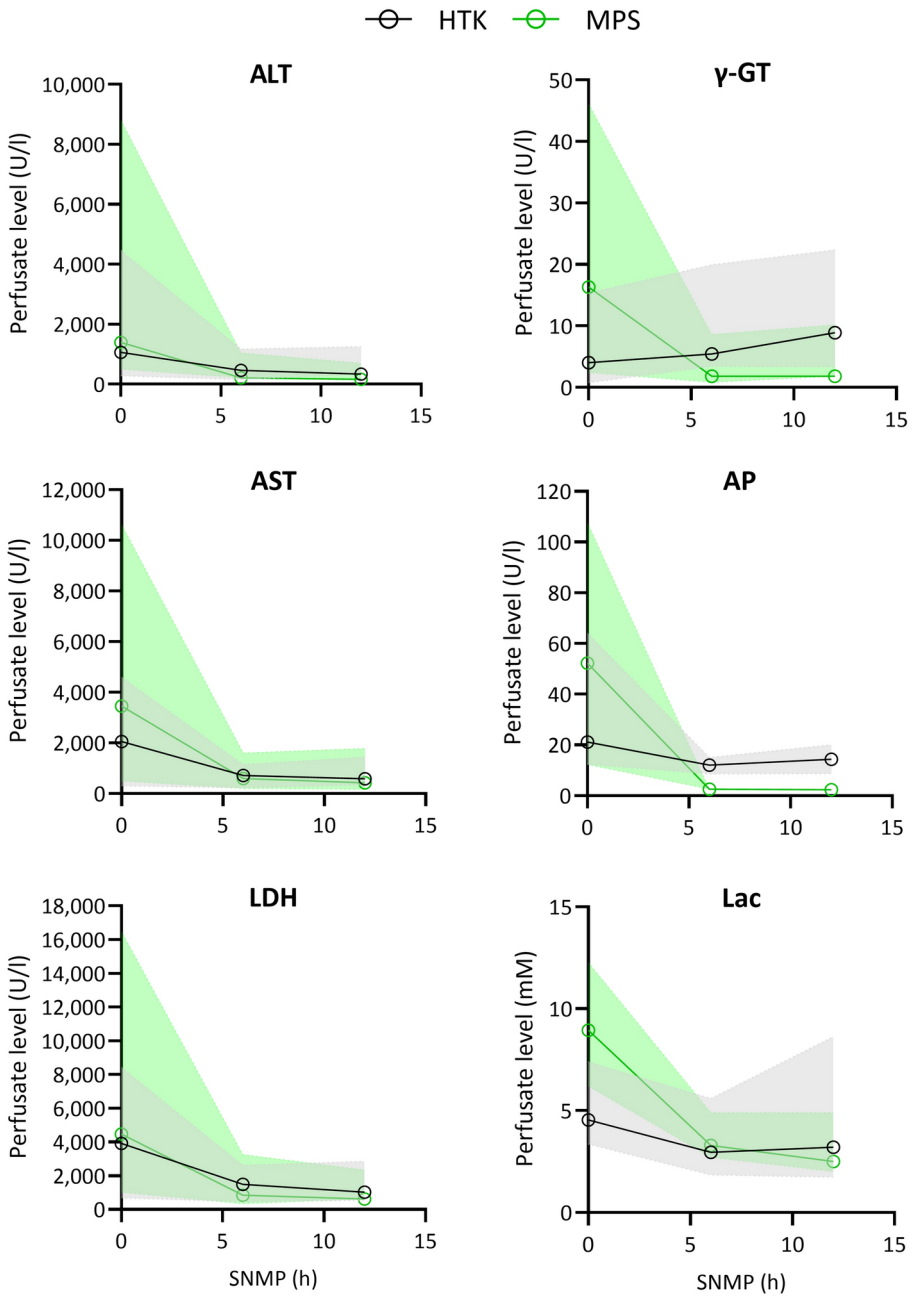

Fig. 1. Liver Parameters of PS matched livers during SNMP. ALT (Alanine transaminase), AST (Aspartate aminotransferase) LDH (Lactate dehydrogenase), γ-GT (gamma-glutamyltransferase) AP (alkaline phosphatase), Lac (lactate). HTK: Custodiol®; MPS: Belzer MPS®. SNMP: sub-normothermic machine perfusion. Data are presented as median and 95% CI of Custodiol® (HTK, n = 8; black line, grey area) compared to Belzer MPS® (MPS, n = 7; green line, green area) groups.

protein expression in both groups (Figure S5) and phosphorylated-MLKL (pMLKL) was only detectable in one liver of the Belzer MPS® group.

Discussion

MP, with its diverse protocols, has become a key approach in state-of-the-art organ preservation and reconditioning. This techniques have shown superiority compared to conventional SCS. In this studywe investigated the suitability of Custodiol®, a perfusion solution mainly used for perfusion of donor organs during organ retrieval or SCS, as a perfusion solution during SNMP and compared it to Belzer MPS®, which is intended for use during HMP at temperatures between 4 and 8 °C. Our data provides evidence, that both solutions support liver SNMP. All investigates parameters showed a similar trend, except for sodium bicarbonate requirement for pH stability, and MLKL mRNA expression.

	Donor		Organ characteristics before SNMP onset							Histological tissue quality assessment			
Liver ID	age (y)	sex	Rejection	SCS	Liver wt (kg)	Perfusate	ET-DRI	SNMP (h)	Bile	Steatosis	Fibrosis	Inflammation	Necrosis
1	72	m	Non-liver related	< 12 h	2.60	Custodiol®	2.018	0	–	5–30%	None	None	None
								12	y	5–30%	None	None	Isolated
2	68	f	Steatosis	< 12 h	1.20	Custodiol®	1.631	0	–	5–30%	Low grade	None	None
								12	y	5–30%	Low grade	None	Isolated
3	77	m	Steatosis	≥ 12 h	2.40	Custodiol®	2.150	0	–	30–60%	None	None	Isolated
								12	y	> 60%	None	None	None
4	44	f	Steatosis	≥ 12 h	1.60	Custodiol®	1.965	0	–	5–30%	None	None	None
								12	n	5–30%	None	None	None
5	59	f	Non-liver related	< 12 h	1.33	Custodiol®	1.473	0	–	< 5%	None	None	None
								12	y	< 5%	None	None	None
6	80	f	Non-liver related	≥ 12 h	1.26	Custodiol®	2.534	0	–	< 5%	None	None	None
								12	y	< 5%	None	None	Isolated
7	54	f	Steatosis	≥ 12 h	1.84	Belzer MPS®	1.762	0	–	> 60%	None	None	None
								12	n	> 60%	None	None	Isolated
8	76	f	Steatosis	≥ 12 h	1.50	Belzer MPS®	1.990	0	–	5–30%	None	None	None
								12	y	5–30%	None	None	Isolated
9	56	f	Non-liver related	≥ 12 h	1.36	Belzer MPS®	1.850	0	–	5–30%	None	None	Isolated
								12	n	< 5%	None	None	Isolated
10	81	m	Non-liver related	≥ 12 h	1.45	Belzer MPS®	2.216	0	–	< 5%	None	None	None
								12	y	< 5%	None	None	None
11	49	m	Non-liver related	≥ 12 h	1.92	Belzer MPS®	1.657	0	–	< 5%	None	None	None
								12	y	< 5%	None	None	None
12	78	m	Non-liver related	< 12 h	1.43	Belzer MPS®	1.843	0	–	< 5%	None	None	None
								12	n	< 5%	None	None	None
13	67	m	Steatosis	< 12 h	2.01	Belzer MPS®	1.644	0	–	5–30%	None	None	Isolated
								12	n	5–30%	None	None	None
17	78	m	Steatosis	< 12 h	1.90	Custodiol®	1.615	0	–	> 60%	None	Present	None
								12	n	> 60%	None	Present	Isolated
20	66	f	Non-liver related	≥ 12 h	1.39	Custodiol®	2.037	0	–	< 5%	None	None	None
								12	y	< 5%	None	None	None

Table 2. Donor and organ characteristics and histological assessment of liver tissue prior to (0) and at the end of sub-normothermic machine perfusion (SNMP; 12) of propensity score matched (PSM) livers perfused with Custodiol® or Belzer MPS®. For detailed information regarding rejection of the respective livers for transplantation refer to Table S2. *ET-DRI* Eurotransplant donor risk index calculated according to[16].

The liver enzymes investigated within this study showed a similar trend in livers perfused with Custodiol® and Belzer MPS® during 12 h of SNMP. This was supported by the histological assessment, as well as the extended liver assessment panel. MPO, a widely used marker for oxidative stress[17], as well as HMGB-1, representing liver cell damage[18], and FMN as a measure of mitochondrial damage[19] did not reveal any differences between the two tested solutions. Calprotectin and Calgranulin-C, reflecting inflammatory damage further supported the findings[20]. While Custodiol® required significantly more sodium bicarbonate to stabilize pH in the perfusion circuit, bile production was not only slightly higher, but also set on earlier and occurred more frequently in livers perfused with Custodiol® compared to those perfused with Belzer MPS®. The increased need of sodium bicarbonate to maintain pH stability has been linked to cell viability during NMP[21] During SNMP, bile production has been reported as a discriminative factor for viability assessment[22].

Cell survival was reflected by tissue mRNA expression of RIPK1, CASP 8 and MLKL as representatives of necroptotic cell death[23,24], as well as BCL2 and BAX representing apoptosis[25]. These two pathways are well described examples for regulated pathways of cell death in liver disease[26]. Although the mRNA expression of the necroptosis effector MLKL was significantly upregulated in livers at 6 h SNMP perfused with Custodiol®, no other cell death parameter along with MLKL protein expression did reflect this result. Two other markers of cell viability, namely NOTCH1 and MAPK7, showed similar mRNA expression patterns in both groups during SNMP. NOTCH1, involved in hepatocyte injury and metabolism in non-alcoholic fatty liver disease[27], has been shown to suppress apoptosis induced by oxidative stress[28]. In murine orthotopic liver transplantation NOTCH1 prevented hepatocellular apoptosis[29]. MAPK7 expression, which is activated by laminar shear stress in endothelial cells, leads to the expression of protective genes with anti-apoptotic and anti-inflammatory functions[30]. Additionally, it plays a regulating role in hepatocyte proliferation during liver regeneration[31] and modulates stellate-cell activities[32]. Taken together, most markers suggest no differences in cell survival rates during SNMP with Custodiol® when compared to Belzer MPS®.

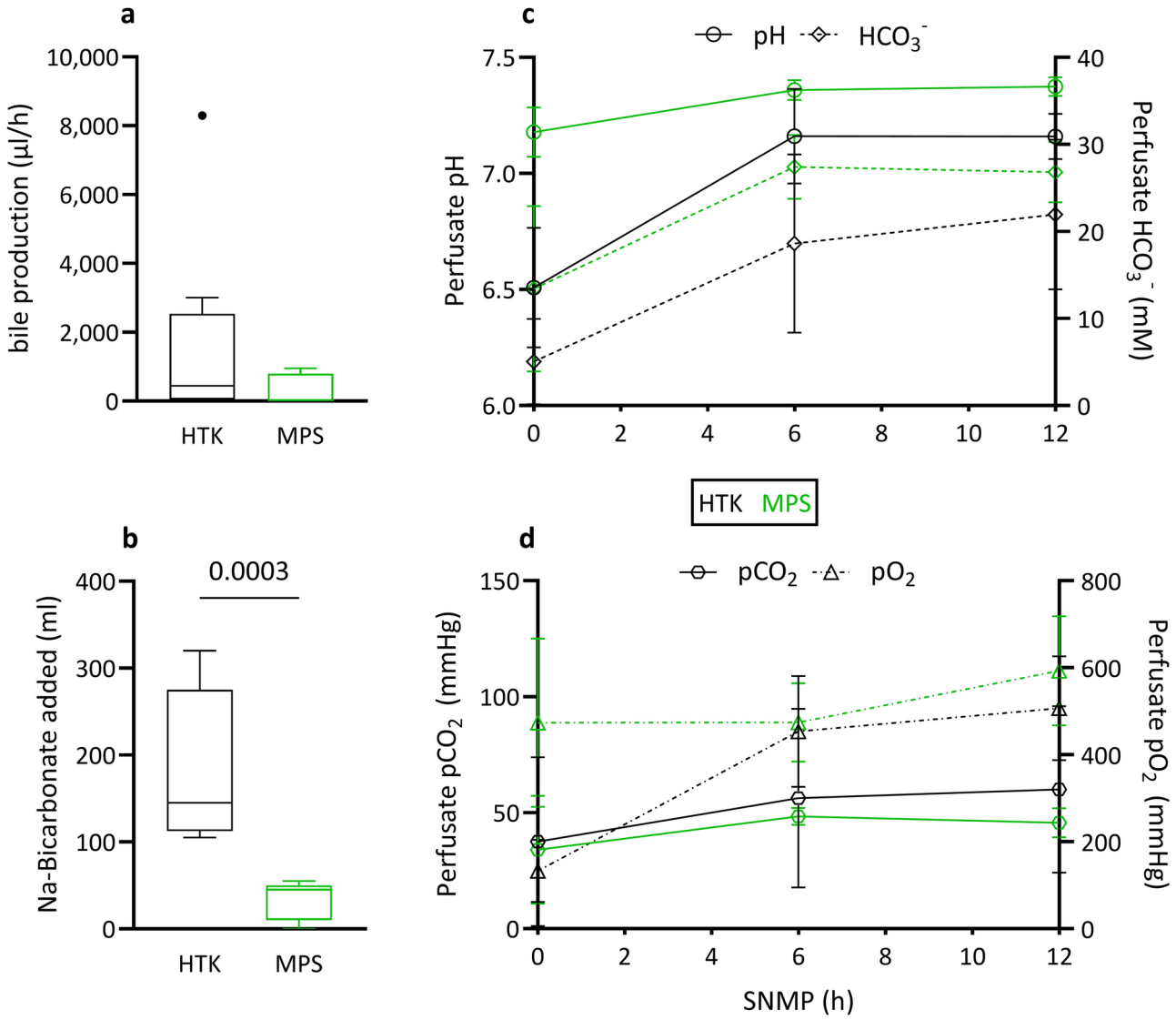

Fig. 2. Bile production, pH stability and blood gases of PS matched liver SNMP. (**a**) Bile production during 12 h of SNMP. (**b**) Cumulative volume of sodium bicarbonate added to stabilize pH after initial pH adjustment within the first hour of SNMP. **c**: Perfusate pH and bicarbonate (HCO_3^-) concentration during SNMP. (**d**) Oxygen (pO_2) and carbon dioxide (pCO_2) partial pressure during SNMP. HTK: Custodiol®; MPS: Belzer MPS®; SNMP: sub-normothermic machine perfusion. Bile production and sodium bicarbonate data are presented as Tukey's Boxplot. pH-stability and blood gas data are presented as median and 95% CI of Custodiol® (HTK, n = 8) compared to Belzer MPS® (MPS, n = 7) group.

As revealed by the expression of cell survival genes, several markers of oxidative stress revealed no differences between Custodiol® and Belzer MPS® in SNMP. SOD2 serves as protector against oxidative stress in neuronal cells[33] and its downregulation results in chronic oxidative stress during aging[34,35]. Another potent antioxidant, HSP60 reduces ROS conveyed oxidative damage either directly or via an SOD2 dependent pathway[36,37]. HSP70 is involved in oxidative stress pathways[38], redox homeostasis[39], and regulates MLKL polymerization, a pre-requisite for its activation as the last step towards necroptosis[24]. This suggest that the increased mRNA expression of MLKL might not have resulted in an increased cell death, as evidenced by the liver enzymes, as well as histological assessment. A study by Shi and colleagues[40] reported increased levels of pMLKL after a short period of SCS followed by 1 h of reperfusion. In our study the expression of eNOS mRNA, which is known to improve disturbed redox homeostasis and mitigates the assicated endothelial dysfunction[41,42]. This further supports the hypothesis of Custodiol® and Belzer MPS® performing similarly during SNMP.

At the inflammatory level, levels of IL10, an anti-inflammatory cytokine[43] that has been shown to enhance the inflammatory response during NMP[44] and plays a role in mitigating IRI-related Fas-driven apoptosis[45], align with the findings from cell survival and oxidative stress markers. The levels of the pro-inflammatory chemoattractant CXCL2, produced by activated Kupffer cells, stimulates neutrophil-driven ROS (reactive oxygen species) production[46,47], along with NRROS, which limits ROS production by phagocytes during inflammatory

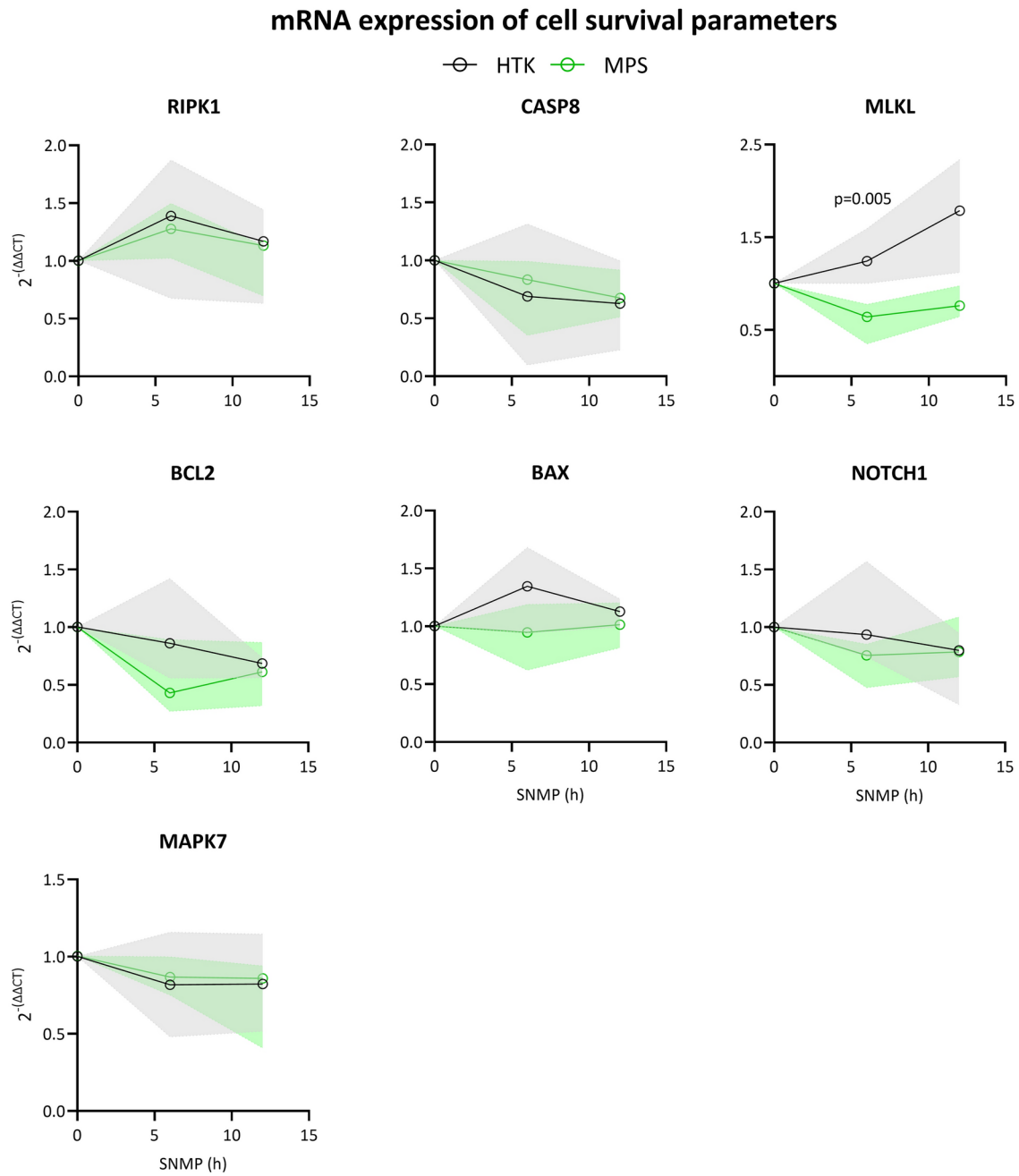

Fig. 3. mRNA expression of cell survival markers of PS matched liver SNMP. *Necroptosis:* RIPK1 (receptor-interacting serine/threonine-protein kinase 1), CASP8 (Caspase-8), MLKL (mixed lineage kinase domain-like Pseudokinase). *Apoptosis:* BCL2 (B-cell lymphoma 2), BAX (Bcl-2-associated X protein). *Cell injury/stress:* NOTCH1 (Neurogenic locus notch homolog protein 1), MAPK7 (mitogen-activated protein kinase 7). Data are presented as median and 95% CI of Custodiol® (HTK, n = 8; black line, grey area) compared to Belzer MPS® (MPS, n = 7; green line, green area) groups. *SNMP* sub-normothermic machine perfusion.

processes[48], further support the similar performance of Custodiol® and Belzer MPS® during SNMP. VCAM1, originally described as a regulator of inflammatory vascular adhesion and subsequent cell transmigration, has more recently been described to play an important role in transplant rejection and immunological disorders[49], and is known to be regulated by oxidative stress[50]. Similarly, ICAM1, an important regulator of inflammatory responses[51] and participant in cytokine production[52], completes the panel of the investigated markers.

Finally, the data of this study aligns with previous reports, in the context of HMP of porcine kidneys. Manekeller and colleagues state that Custodiol® performed similarly to Belzer MPS® in protecting kidneys during hypothermic MP[53]. A Brazilian group conducted a randomized prospective trial and reported similar safety of Custodiol® and Belzer MPS® in kidney transplantation. This study, however, used conventional SCS rather than MP for organ preservation[54]. To date, reports on liver SNMP with Custodiol®[55] or Belzer MPS® remain limited.

The major limitation of the present study is the lack of reperfusion to investigate post-transplant graft function and overall clinical outcomes. Since the organs included in the study had already been rejected for transplantation, the only feasible method to investigate reperfusion would have been NMP. However, this was not part of the study due to financial constraints. Future experimental studies, would benefit from simulating reperfusion using NMP to assess the impact of the different preservation solutions on reperfusion injuries. Another limitation is the quite small sample size, partly due to the heterogeneity of the livers. Given the inherent heterogeneity of discarded organs, we used propensity score matching to balance key confounding factors, thereby minimizing the bias in comparingthe HTK-based and UW-based perfusion solutions.

Conclusions

In conclusion, our findings suggest that the HTK-based Custodiol®, originally designed for SCS, performs similarly to the UW-based Belzer MPS® during SNMP. To validate these findings, further experimental studies and future clinical trials are needed, in particular to evaluate transplantation outcomes, not only after SNMP but also in combination with other preservation strategies, such as SCS or graduation rewarming.

Methods

Organ retrieval and back-table procedure

Human livers were procured from brain-dead organ donors as part of the standard multi-organ donation procedure. Livers that were initially accepted by our center for transplantation but later declined based on pathological biopsy findings were included in the study and used for machine perfusion experiments, in accordance with the institutional ethics committee of the Medical University of Graz (30–493 ex17/18). Obtaining informed consents from the organ donors' relatives was not required according to the Austrian law and the local ethics committee, and additionally not possible as the donors' identities are anonymized by Eurotransplant. Liver procurement was performed in a standard manner without any additional interventions.

For SNMP the hepatica artery (8F, Arterial cannula; Organ Assist, Xvivo, Groningen, Netherlands) and portal vein (25F, LiA portal cannula; Organ Assist, Xvivo, Groningen, Netherlands) were cannulated and fixed with 2-0 Vicryl (Ethicon, Johnson & Johnson Medical N.V., Belgium) ligatures. Furthermore, to collect the produced bile, the common bile duct was cannulated with a polyurethane Nutrifit feeding tube (8F, 125 cm length; Vygon, Paris, France), which was fixed with a 2-0 Vicryl (Ethicon, Johnson & Johnson Medical N.V., Belgium) ligature. Immediately prior to perfusion start the organ was weighted and subsequently flushed with 2L ice cold Custodiol® (Dr. Franz Köhler Chemie GmbH, Bensheim, Germany).

Sub-normothermic machine perfusion and sampling

For liver perfusion the Liver Assist® (Organ Assist, Xvivo, Groningen, Netherlands) was used in combination with the respective disposable set (Organ Assist, Xvivo, Groningen, Netherlands). The 12 h perfusion protocol was adapted from the previously published work by Bruinsma et al.[12]. The system was primed with 2-4L of perfusion solution (HTK-based Custodiol® Dr. Köhler Chemie, Bensheim, Germany or UW-based Belzer MPS®, Bridge to Life, Northbrook, IL, USA), and supplemented with Penicillin/Streptomycin (Sigma-Aldrich; Merck, Darmstadt, Germany) as well as Amphotericin B (Gibco; Thermo Fisher Scientific, Vienna, Austria) to a final concentration of 40,000 U/l, 0.04 mg/l and 1 mg/l, respectively. Machine set-up was done according to the manufacturer's instructions and the temperature set to 21 °C. Oxygenation was performed with 100% O_2 at a constant flow rate of 1L/min. After 6 h of perfusion, 50% of perfusate was replaced by fresh perfusate.

Sampling (perfusate and tissue) was performed after back-table (0 h), 6 h and 12 h of SNMP. Perfusate samples (retrieved via the sampling port of the perfusion machine) were shock frozen in liquid nitrogen and stored at -80 °C until further use. Tissue samples (wedge biopsies) were both fixed in 4% Formaldehyde solution for post-hoc histological evaluation and shock frozen in liquid nitrogen for later RNA isolation.

Perfusate and bile (if produced) pH, bicarbonate, pCO_2, pO_2, Glucose levels were determined by an ePOC handheld blood-gas analysis system (Siemens Healthineers, Erlangen, Germany) on site.

Liver enzymes and viability parameters

Aspartate aminotransferase (AST), Alanine Aminotransferase (ALT), gamma-glutamyltransferase (γ-GT), alanine phosphatase (AP) and lactate dehydrogenase (LDH) were determined from frozen perfusate samples taken at back-table, after 6 h, and 12 h of SNMP and analyzed on a Cobas® 8000 analyzer (Roche Diagnostics GmbH, Mannheim, Germany) with reagents from the same manufacturer. Since the respective methods were not validate for this special sample matrix, we evaluated the analytical performance using a standard addition protocol (for details see supplemental methods).

Flavin mononucleotide (FMN) was determined as previously described[56]. Briefly, a 7-point standard curve ranging from 10 to 640 ng/ml was prepared by dissolving Firboflavin-5'monophospate (Sigma Aldrich, Vienna, Austria) in the desired blank perfusate. For determination of FMN levels in black 96-well plates Fluorescence was recorded at 485/520 nm (Ex/Em) in duplicate by means of a Fluostar Omega (BMG Labtech, Ortenberg, Germany) plate reader. High-mobility group box 1 (HMGB-1, Biomatik, Delaware, USA; porcine: ByBioSource, SanDiego CA, USA), a marker of liver cell damage, as well as Calprotectin (S100A8/S100A9), Calgranulin-C (S100A12, Immundiagnostik, Bensheim, Germany) markers of inflammation, and Myeloperoxidase (MPO, Immundiagnostik, Bensheim, Germany), a marker of oxidative stress, were determined by commercially available ELISAs according to manufacturer's instructions. ELISA readouts were performed by means of a Spectrostar Omega (BMG Labtech, Ortenberg, Germany) plate reader.

Tissue preparation and evaluation

Formalin fixed paraffin embedded liver samples were prepared in a standard manner. Liver sections (2 µm thick) were de-paraffinized and stained with hematoxylin and eosin (HE) for evaluation by an experienced pathologist. The evaluation was based on the extent of steatosis, fibrosis and changes in inflammation and cellular necrosis throughout SNMP.

RNA isolation, reverse transcription and qPCR

Tissue samples were snap-frozen and stored at − 80 °C until nucleic acid extraction. Tissue (50–100 mg) was homogenized in 1 mL TRIzol reagent in combination with a MagNA Lyser (Roche Diagnostics GmbH, Mannheim, Germany). Isolation of total RNA was done by means of a miRNeasy Kit in combination with the RNase-Free Dnase Set (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer. RNA quantity and quality were determined by Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and 2 µg of RNA used for reverse transcription (High-Capacity cDNA RT Kit; Thermo Fisher Scientific, Waltham, MA, USA) according to the protocol provided by the manufacturer in a final volume of 20 µL.

Real-time PCR amplification and melting analysis were performed using a BioRad CFX384 TouchTM System (Bio-Rad Laboratories Ges.m.b.H., Vienna, Austria). cDNA corresponding to an equivalent of 10 ng RNA was added to a reaction mix containing Promega GoTaq® qPCR Master Mix (Promega, Madison WI, USA) and 1 µM of each primer in a final reaction volume of 10 µL. The PCR reaction mixture was subjected to an initial denaturation at 95 °C for 10 s, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 20 s, and elongation at 72 °C for 30 s followed by a melting curve (60 to 95 °C; increment of 0.5 °C every 5 s). Primers were designed by the primer design tool available on NCBI (using Primer3 and BLAST) and whenever possible designed to span exon/intron boundaries. Primer efficiencies were determined in a range from 1.25 to 20 ng/µl initial RNA on template of 6 different tissue types by means of the same cycling conditions as described above. For detailed information on primers, refer to supplementary Table 1.

Relative gene expression was determined using the Bio-Rad CFX Maestro 3.1 (Bio-Rad Laboratories Ges.m.b.H., Vienna, Austria) using the Cq regression method embedded in the program. All PCR reactions were done in duplicates. Relative gene expression ($2^{-\Delta\Delta Cq}$) was calculated, after correction for primer efficiency and inter-plate variance, using ACTB (beta-actin) and GAPDH (Glyceraldehyd-3-phosphat-Dehydrogenase) as reference genes.

Statistical analysis and graphic representation of data

Statistical analysis was performed with GraphPad Prism9 for Windows (Version 9.1.2). Differences between SNMP groups (Custodiol® vs Belzer MPS®) during perfusion were analyzed by multiple Kolmogorov–Smirnov test with a two-stage set-up correction of Benjamini, Krieger and Yekutieli for multiple comparisons. For simple group comparison Kolmogorov–Smirnov test was used without correction for multiple comparison. A $p < 0.050$ is considered as statistically significant. Categorical variables were presented as count (n) and percentages whereas continuous variables were presented as median (95% CI) unless stated otherwise.

For propensity-score analysis and matching R (version 4.1.3)[57] in combination with R Studio (Version 2022.07.02 + 576) and packages dplyr[58], tidyverse[59], tableone[60], MatchIt[61] and cobalt[62] were used.

Data availability

The dataset is available for download via zenodo.org under the following identifier: 13777843.

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Author contributions
B.L. was responsible for study conceptualization, performed machine perfusion experiments, collected data, performed statistical analysis and wrote the manuscript. SS performed machine perfusion experiments, collected data and critically reviewed the manuscript. K.B., J.W. and L.R. performed laboratory analysis and machine perfusion experiments and critically reviewed the manuscript. A.A. was responsible for pathological evaluation of tissue samples, provided key recommendations and reviewed the manuscript. T.N. was responsible for measurement of liver parameters in perfusate samples and reviewed the manuscript. P.S. was responsible for the conceptualization and critical review of the manuscript. R.S. contributed by critically analyzing the manuscript and provided key recommendations. All the authors approved the final version of the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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The study was approved by the institutional ethics review board of the Medical University of Graz (30-493 ex17/18) and conducted in accordance with the guidelines of the Declaration of Helsinki.

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