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ORIGINAL ARTICLE

Preserving the Morphology and Evaluating the Quality of Liver Grafts by Hypothermic Machine Perfusion: A Proof-of-Concept Study Using Discarded Human Livers

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The wider use of livers from expanded criteria donors and donation after circulatory death donors may help to improve access to liver transplantation. A prerequisite for safely using these higher risk livers is the development of objective criteria for assessing their condition before transplantation. Compared to simple cold storage, hypothermic machine perfusion (HMP) provides a unique window for evaluating liver grafts between procurement and transplantation. In this proof-of-concept study, we tested basic parameters during HMP that may reflect the condition of human liver grafts, and we assessed their morphology after prolonged HMP. Seventeen discarded human livers were machine-perfused. Eleven livers were nontransplantable (major absolute contraindications and severe macrovesicular steatosis in the majority of the cases). Six livers were found in retrospect to be transplantable but could not be allocated and served as controls. Metabolic parameters (pH, lactate, partial pressure of oxygen, and partial pressure of carbon dioxide), enzyme release in the perfusate [aspartate aminotransferase (AST) and lactate dehydrogenase (LDH)], and arterial/portal resistances were monitored during HMP. Nontransplantable livers released more AST and LDH than transplantable livers. In contrast, arterial/portal vascular resistances and metabolic profiles did not differ between the 2 groups. Morphologically, transplantable

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATP, adenosine triphosphate; AUC, area under the curve; BSEP, bile salt export pump; CA-DCD, cardiac arrest during controlled donation after circulatory death; CCT, craniocerebral trauma; CV, central vein; DCD, donation after circulatory death; ECD, expanded criteria donor; END1, endothelin 1; HMP, hypothermic machine perfusion; ICAM1, intercellular adhesion molecule 1; ICB, intracranial bleeding; IL, interleukin; LDH, lactate dehydrogenase; LT, liver transplantation; MAPK, mitogen-activated protein kinase; MDR1, multidrug resistance 1; mRNA, messenger RNA; PBD, postanoxemic brain death; PCO_2, partial pressure of carbon dioxide; PCR, polymerase chain reaction; P_{HA}, hepatic artery pressure; PO_2, partial pressure of oxygen; P_{PV}, portal vein pressure; PT, portal tract; Q_{HA}, hepatic artery flow; Q_{PV}, portal vein flow; SCS, static cold storage; SEC, sinusoidal endothelial cell; TNF-α, tumor necrosis factor α; ZO-1, zona occludens 1.

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livers remained well preserved after 24 hours of HMP. In conclusion, HMP preserves the morphology of human livers for prolonged periods. A biochemical analysis of the perfusate provides information reflecting the extent of the injury endured. *Liver Transpl 18:1495-1507, 2012.* © **2012 AASLD.**

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Expanded criteria donors (ECDs) and donation after circulatory death (DCD) donors represent an increasing proportion of the donor pool.[1,2] However, livers from these donors remain underused because of the higher risk of poorer outcomes and the difficulty in assessing this risk.

To date, no objective tools are available for assisting clinicians in the evaluation of human liver grafts. The decision to accept or decline a particular liver offer is still based on the judgment of the surgeon, who takes into account the medical history of the donor, the liver biochemistry, the macroscopic appearance of the liver, and, if necessary, the microscopic appearance of the liver.

Compared to static cold storage (SCS), hypothermic machine perfusion (HMP) provides a window between procurement and transplantation during which the graft quality can be directly assessed ex vivo. Certain biomarkers in the perfusate and the vascular resistance during kidney HMP independently correlate with outcomes, and this additional information can help clinicians in decision making.[3,4] In contrast, data on liver HMP are extremely scarce. Today, only 1 pilot trial has proven the feasibility of preserving standard livers with HMP and successfully transplanting them.[5]

The aim of this proof-of-concept study was to explore whether certain aspects of the quality of human livers could be assessed during their perfusion with standard biochemical, metabolic, and hydrodynamic parameters. For this purpose, we developed an HMP system for human livers and compared nontransplantable livers to control livers that could not be allocated, even though their quality was found to be good in retrospect. We also sought to determine whether the ultrastructural morphology of these livers thought to be transplantable could be preserved after prolonged periods of HMP.

PATIENTS AND METHODS

Livers Discarded for Clinical Transplantation

From September 2003 to June 2009, 17 human livers initially accepted for liver transplantation (LT), which had been procured and allocated by Eurotransplant to particular recipients but eventually discarded, were machine-perfused. The reasons for discarding included unexpected macroscopic/microscopic findings during and/or after procurement, problems during transportation, and premature termination of the recipient operation (Fig. 1 and Table 1). According to Eurotransplant guidelines, all these declined grafts were re-allocated to the next-in-line recipients at various centers but without success. The donor characteristics [age, sex, aspartate aminotransferase (AST), alanine aminotransferase (ALT), sodium, and total bilirubin] are shown in Table 1. By design, these 17 discarded livers reached our laboratory after a prolonged period of SCS (mean = 13 hours 48 minutes, range = 3 hours 2 minutes to 24 hours 55 minutes).

Classification As Nontransplantable or Transplantable Livers

Discarded livers were classified as nontransplantable or (in retrospect) transplantable according to generally accepted clinical criteria, medical data, surgical data, and macroscopic and microscopic appearances after their arrival at our laboratory and before the data analysis (Table 1).

The majority of the livers classified as nontransplantable (n = 8) were massively steatotic (>60% macrovesicular steatosis). In 2 other nontransplantable livers, other contraindications were present in addition to moderate (<30%) macrovesicular steatosis: high sodium levels and cholestasis/bilirubinostasis in one and diffuse Mallory bodies in another. Finally, 1 liver had severe histological signs of recent alcohol intake and acute hepatitis.

The livers that were potentially transplantable but could not be allocated included 2 livers that could not be re-allocated after the recipient operation was aborted because of the recipient's death or the intraoperative discovery of extrahepatic metastases. In 1 case, the ice box containing the liver fell off the trolley when it was leaving the elevator, and the box was trapped and crushed between the closing doors. This liver was macroscopically and microscopically intact. In 3 cases, livers initially had been found to be nontransplantable by the procuring teams, and on the basis of this first evaluation, they were successively declined by other centers and could not be allocated. Additional macroscopic and/or microscopic analyses showed only mild changes, and these livers were transplantable in retrospect. The reasons reported for discarding these livers included poor flush-out, mild steatosis, and subcapsular hematoma in one case and a suspicious cholestatic appearance of the liver along with a minimal elevation of the donor's total bilirubin level in another case. The third liver was initially not offered for transplantation because of elevated transaminase levels after a short period of hemodynamic instability; however, at the time of kidney procurement, the liver appeared intact, and it was realized that although the transaminase levels were initially elevated, they were rapidly decreasing. This

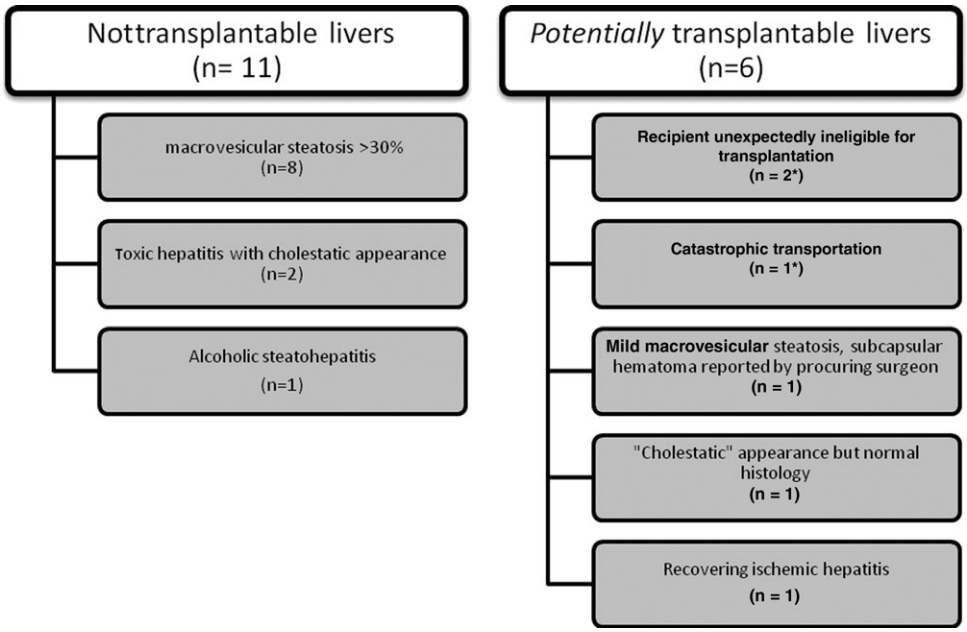

Figure 1. Classification of livers as nontransplantable or transplantable according to the reasons reported to Eurotransplant for the livers being discarded. An asterisk indicates that the liver originated from a DCD donor.

liver was thus offered for transplantation but was declined by all centers, probably in part because of the short notice. Notably, these 6 discarded livers that were found in retrospect to be transplantable reached our laboratory after they were exposed to prolonged periods of SCS, which precluded their transplantation at our own center.

HMP Setup

A liver HMP device was derived from a LifePort kidney transporter (Organ Recovery System, Des Plaines, IL; Fig. 2). The livers were perfused by pressure-controlled continuous perfusion (20-30 mm Hg for the hepatic artery and 7 mm Hg or less for the portal vein). To prevent excessive shear stress on the endothelium, the portal flow was limited to 0.5 mL/g of liver/minute, and along with the arterial flow, this resulted in flows similar to those reported by Guarrera et al.[5] Experimental data on the oxygenation of the perfusate are conflicting. Both beneficial and detrimental effects have been reported.[6-10] In line with the only clinical experience available so far (reported by Guarrera et al.[5]), no active oxygenation of the perfusate was used. All livers were machine-perfused for 24 hours with 2 L of University of Wisconsin machine perfusion solution. The temperature of the circulating perfusate was kept constant at approximately 4°C to 6°C.

Evolution of the Metabolic Profile During HMP

The pH, lactate level, partial pressure of oxygen (PO_2), and partial pressure of carbon dioxide (PCO_2) were measured with an ABL 625 analyzer (Radiometer, Copenhagen, Denmark).

Evolution of Biochemical Parameters During HMP

Perfusate samples were collected at the start of HMP and then after 30 minutes and 1, 6, and 24 hours. As a surrogate of hepatocellular injury, we measured AST and lactate dehydrogenase (LDH) in the perfusate with standard absorption techniques.

Evolution of Vascular Resistances During HMP

Arterial and portal vascular resistances were calculated on the basis of perfusion pressures and flows. The perfusion flows were calculated by the multiplication of the roller pump rotations per minute by the volume delivered per revolution of the pump and were corrected for the size of the tubing used for the hepatic artery and portal vein. Vascular resistances were determined at the start of HMP and after 30 minutes and 1, 3, 6, 12, and 24 hours.

Assessment of Steatosis and Correlation With AST in the Perfusate

The degree of steatosis according to baseline biopsy samples was quantified with (1) the stereological point counting method described by Franzén et al.[11] and (2) semiquantitative scoring.

For the stereological point counting, 2 independent observers analyzed light microscopy images with morphometric imaging software (AnalySIS D, Olympus, Germany). Every observer scored 5 images of nonoverlapping and randomly selected lobules (magnification ×400). Within each image, 1 periportal zone and 1 centrilobular zone were selected and captured distinctively. A point grid (108 crossings 35 μm apart) was superimposed onto each image. All vacuoles

TABLE 1. Donor Characteristics for Livers Preserved by HMP and Reasons for Discarding

Case Number	HMP Date	Sex	Age (Years)	AST (IU/L)	ALT (IU/L)	Sodium (mmol/L)	Bilirubin (mg/dL)	Cause of Death	SCS Time (Minutes)	Transplantable	Reasons for Discarding*
1	September 2003	Male	49	42	34	141	0.3	CCT	611	No	Severe steatosis (>60%)†
2	November 2003	Male	82	58	98	144	0.68	ICB	1207	Yes	Recipient not eligible for transplantation because of extrahepatic hepatocellular carcinoma
3	November 2003	Male	19	46	36	156.8	7.78	CCT	663	No	Hepatomegaly, bilirubinostasis,† and microscopic steatosis†
4	November 2003	Female	42	35	34	144	0.44	ICB	846	No	Severe steatosis (>60%)†
5	January 2004	Female	58	46	22	151.8	0.35	CCT	182	No	Panlobular mediovesicular steatosis† with Mallory bodies†
6	July 2004	Male	43	23	28	149	0.54	ICB	705	No	Severe steatosis (>60%)†
7	July 2004	Male	75	32	15	150	0.4	CCT	1065	No	Severe steatosis (>60%)†
8	October 2004	Female	55	40	31	139	0.24	ICB	700	Yes	Poor flush-out, subcapsular bleeding, and mild steatosis (<30%)
9	October 2004	Female	71	42	108	144	0.37	ICB	851	No	Severe steatosis (>60%)†
10	May 2005	Male	51	94	53	136	0.7	ICB	464	No	Severe steatosis (>60%)†
11	May 2005	Female	64	36	65	142	0.3	PBD	315	No	Severe steatosis (>60%)†
12	June 2005	Male	58	50	23	128	0.7	ICB	1394	No	Steatosis (>60%)†
13	January 2006	Male	46	19	54	141	2.14	CCT	1495	No	Alcoholic intoxication (alcoholic steatohepatitis†)
14	January 2006	Male	59	31	157	142.6	1.91	ICB	864	Yes	Cholestatic appearance of donor liver†
15	March 2006	Female	17	251	508	135		Cerebral ischemia	842	Yes	Poor hepatic circulation secondary to previous ischemic trauma
16	March 2009	Male	39	13	12	147	0.32	CA-DCD	882	Yes	DCD and accidental transportation of icebox containing graft
17	June 2009	Female	47	62	17	143	0.3	CA-DCD	990	Yes	DCD and death of recipient during anhepatic phase

NOTE: The livers are listed in chronological order.
*As reported by the procuring or transplanting surgeons to Eurotransplant.
†Additional histological evidence for discarding livers for transplantation.

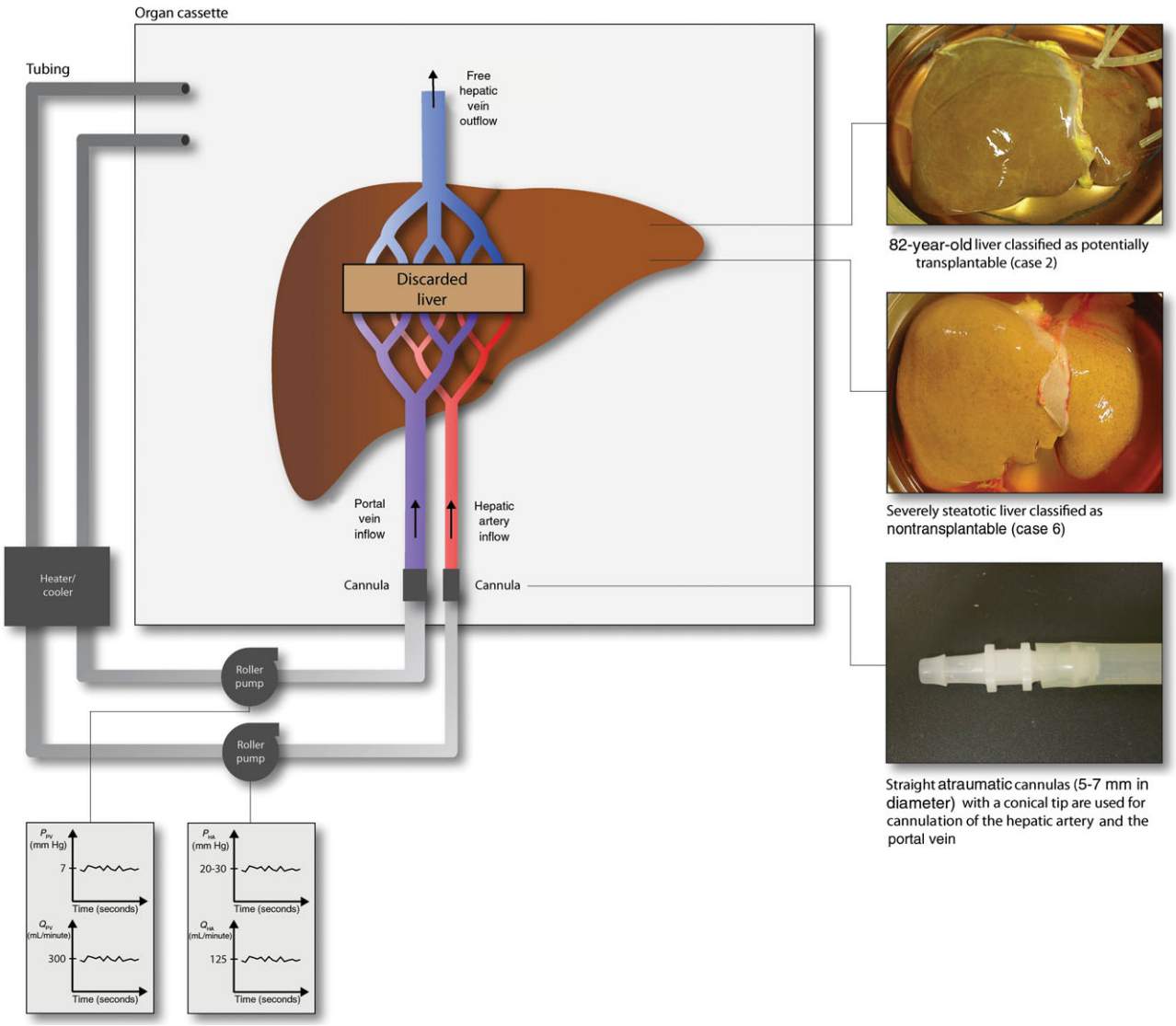

Figure 2. Schematic diagram of the HMP setup with 2 separate roller pumps integrated into 1 device. Livers were perfused with a pressure-controlled continuous perfusion of 20–30 mm Hg through the hepatic artery and 7 mm Hg or less through the portal vein. On the portal inflow side, the perfusion flow was limited to 0.5 mL/g of liver/minute, and this resulted in a total flow of 0.6 to 0.7 mL/g of liver/minute. Two representative livers discarded for transplantation are shown. Case 2 involved a liver from an 82-year-old donor with a normal macroscopic appearance (classified as transplantable), and case 6 involved a liver with major (>60%) macrovesicular steatosis (classified as nontransplantable). The perfusate was chilled with a heater/cooler included in the circuit. Straight atraumatic cannulas (5-7 mm in diameter) with an appropriate conical tip were used for cannulation of the hepatic artery and the portal vein.

bonding to the grid crossings were counted and expressed as a percentage of the total number of crossings. Crossings outside the hepatocellular parenchyma [eg, the portal tracts (PTs), central vein (CV), sinusoids, and artifacts] were excluded.

The semiquantitative scoring was performed by experienced liver pathologists. Macrovesicular steatosis was defined as the presence of a single vacuole that was larger than the nucleus and displaced it in the vicinity of the cell membrane. Microvesicular steatosis was diagnosed when single vacuoles were smaller than half of the cytoplasm. Analogously to Spitzer et al.,[12] macrovesicular steatosis and microvesicular steatosis were expressed in 5% intervals with respect to the total liver parenchyma.

Analogously to a previous study that we conducted with a porcine model,[13] we examined whether a correlation between steatosis and AST release existed.

Correlation Between the Cold Storage Duration and AST Release in the Perfusate

It was inherent to the experimental design that most of the studied livers reached our laboratory after a relatively prolonged period of SCS, during which all attempts were made by Eurotransplant to allocate

these livers. To investigate a potential bias due to the duration of SCS, we investigated whether there was a correlation between the duration of SCS and AST release [as assessed by the area under the curve (AUC)] in the perfusate.

Adenosine Triphosphate (ATP) Tissue Content

Snap-frozen liver samples taken before and after HMP were stored at −80°C. Subsequently, the samples were homogenized with 4.2% perchloric acid and 1 mM diethylenetriamine pentaacetic acid and centrifuged at 14,000*g*, and the supernates were brought to pH 6 with 69% K_2CO_3. Then, ATP was measured with an ATPlite 1-step kit (PerkinElmer, Zaventem, Belgium) according to the manufacturer's instructions with a Fluoroskan Ascent FL (Thermo LabSystems, Beverly, MA). ATP levels were expressed as nanomoles per gram of liver tissue.

Messenger RNA (mRNA) Level Quantification

We analyzed the mRNA levels of various cytokines [tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6)], adhesion molecules [intercellular adhesion molecule 1 (ICAM1)], transcription factors involved in inflammation and apoptosis [jun proto-oncogene (JUN) and mitogen-activated protein kinase 1 (MAPK1)], proteins involved in signal transduction at tight junctions [zona occludens 1 (ZO-1)], endothelin 1 (END1), and bile salt transporters [multidrug resistance 1 (MDR1) and bile salt export pump (BSEP)]

Total RNA was isolated from snap-frozen liver tissue taken before and after HMP with the TRIzol reagent method (Invitrogen, Merelbeke, Belgium). The concentration and purity of the RNA were determined spectrophotometrically at 260 and 280 nm. With 1 μg of total RNA, complementary DNA was synthesized with random primers (Invitrogen; 25 mM), a nucleotide mix (Promega, Leiden, the Netherlands; 0.5 mM), a recombinant RNase inhibitor (Invitrogen; 150 U), and Moloney murine leukemia virus reverse transcriptase (Invitrogen; 10,000 U). Primers and probes for beta-actin, BSEP, ICAM1, MDR1, TNF-α, and ZO-1 were designed with Primer3 version 0.4.0. For IL-6, JUN, MAPK1, and END1, we used gene expression assays (Applied Biosystems, Lennik, Belgium). Real-time polymerase chain reaction (PCR) amplification was performed with the ABI 7500 fast real-time PCR system (Applied Biosystems). The reaction was performed in duplicate in a total volume of 10 μL with TaqMan fast universal PCR master mix (Applied Biosystems). The thermocycling conditions consisted of 20 seconds at 95°C followed by 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C. The fold change was calculated with the $\Delta\Delta C_t$ method.

Effect of Prolonged HMP on the Liver Morphology and Ultrastructure

Standard and electron microscopy examinations were performed on biopsy samples taken after 24 hours of HMP, and the results were compared to those for baseline biopsy samples. For standard microscopy, paraffin sections were stained with hematoxylin and eosin.

For electron microscopy, small liver biopsy fragments were trimmed into slices (thickness ≤ 1 mm) and immediately fixed in 2.5% glutaraldehyde and a 0.1 mol/L phosphate buffer (pH 7.2) at 4°C. After 1 hour of postfixation in 1% osmium tetroxide and a 0.1 mol/L phosphate buffer (pH 7.2) at 4°C, the samples were dehydrated in a graded alcohol series and were embedded in an epoxy resin. Ultrathin sections (50-60 nm), which were cut and stained with uranyl acetate and lead citrate, were examined at 50 kV with a Zeiss EM 900 electron microscope. Images were recorded digitally with a Jenoptik Progress C14 camera system and Image-Pro Express software.

Ethical Approval

This study was approved by the ethics committee of University Hospitals Leuven (Catholic University of Leuven, Leuven, Belgium) and by the Belgian Liver and Intestine Committee, which represented all Belgian LT centers that provided livers for this study. The decisions to discard these livers were made by the procurement surgeons, transplant surgeons, physicians, and allocation organization (Eurotransplant) on the basis of the available information. These decisions were made completely independently of the possibility of the inclusion of these livers in a research protocol. After the termination of the experiment, the livers were sent to the pathology department.

Statistical Analysis

Data are expressed as means and standard deviations. The evolution of biochemical, metabolic, and hydrodynamics parameters was determined by the calculation of AUCs with Slide Write Plus (Advanced Graphics Software, Inc., Encinitas, CA). A Mann-Whitney test was used for comparisons at separate time points and for AUCs between groups. A sign test was used for comparisons between time points within groups. At every time point, there were sufficient data from both groups for adequate statistical analysis. The Pearson correlation coefficient was used to study the relationship between the degree of steatosis and AST release in the perfusate. Statistica 8.0 (StatSoft, Inc., Tulsa, OK) was used to perform the statistical analysis. A P value < 0.05 was considered significant.

RESULTS

Donor Data

There were no differences between the livers of the 2 groups with respect to donor age, AST, ALT, sodium, total bilirubin, liver weight, or duration of SCS before HMP (Table 2).

TABLE 2. Representative Donor Characteristics and SCS Times for Nontransplantable and Transplantable Livers

	Nontransplantable Livers (n = 11)	Transplantable Livers (n = 6)	*P* Value
Age (years)	52.4 ± 15.6 (19-75)	49.8 ± 21.7 (17-82)	0.81
AST (IU/L)	42.3 ± 19.7 (19-94)	75.8 ± 87.7 (13-251)	0.66
ALT (IU/L)	42.9 ± 26.4 (15-108)	137.2 ±190.2 (12-508)	0.73
Sodium (mmol/L)	144.0 ± 7.9 (128-157)	141.8 ± 4.2 (135-147)	0.52
Bilirubin (mg/dL)	1.3 ± 2.2 (0.3-7.8)	0.7 ± 0.7 (0.2-1.9)	0.38
Liver weight before HMP (g)	2043 ± 512 (1680-3030)	1738.83 ± 404.95 (1290-2294)	0.48
SCS time (minutes)	785.1 ± 415.7 (182-1495)	914.2 ± 170.9 (700-1207)	0.30

NOTE: The data are presented as means and standard deviations with ranges in parentheses.

Evolution of the Metabolic Profile During HMP

During HMP, the perfusate pH for all livers (nontransplantable and transplantable) decreased from 7.28 ± 0.13 at the baseline to 6.73 ± 0.11 at 6 hours (P = 0.003 for the baseline versus 6 hours) and to 6.40 ± 0.19 at 24 hours (P = 0.003 for 6 hours versus 24 hours). The lactate level increased from 0.9 ± 0.22 mmol/L at the baseline to 7.94 ± 3.69 mmol/L at 6 hours (P = 0.004 for the baseline versus 6 hours) and to 14.47 ± 4.92 mmol/L at 24 hours (P = 0.004 for 6 hours versus 24 hours). PO_2 decreased from 183.5 ± 68.9 mm Hg at the baseline to 36.6 ± 12.5 mm Hg at 6 hours (P = 0.004 for the baseline versus 6 hours) and to 36.5 ± 14.9 mm Hg at 24 hours (P = 0.75 for 6 hours versus 24 hours). Finally, PCO_2 increased from 8.0 ± 7.1 mm Hg at the baseline to 23.4 ± 8.0 mm Hg at 6 hours (P = 0.008 for the baseline versus 6 hours) and to 20.1 ± 6.3 mm Hg at 24 hours (P = 0.75 for 6 hours versus 24 hours). There were no differences in the evolution of these metabolic parameters between the nontransplantable livers and the transplantable livers according to comparisons of their AUCs or their values at single time points ($P > 0.05$).

Evolution of Biochemical Parameters During HMP

The AST release from the nontransplantable livers was remarkably higher than the release from the transplantable livers according to comparisons of the AUCs (9.39 ± 6.31 × 10^6 versus 1.24 ± 0.84 × 10^6, P = 0.006) and values at single time points: 30 minutes (4561 ± 1853 versus 539 ± 485 IU/L, P = 0.009), 1 hour (4620 ± 2062 versus 631 ± 516 IU/L, P = 0.003), 6 hours (7086 ± 2928 versus 928 ± 657 IU/L, P = 0.006), and 24 hours (8671 ± 7294 versus 946 ± 573 IU/L, P = 0.002; Fig. 3A). Similarly, the LDH release from the nontransplantable livers was higher than the release from the transplantable livers according to comparisons of the AUCs (28.97 ± 19.51 × 10^6 versus 3.76 ± 1.72 × 10^6, P = 0.005) and values at single time points: 30 minutes (15,107 ± 8164 versus 1564 ± 1105 IU/L, P = 0.008), 1 hour (14,442 ± 8996 versus 1868 ± 1167 IU/L, P = 0.004), 6 hours (20,213 ± 10,838 versus 2526 ± 1348 IU/L, P = 0.009), and 24 hours (26,188 ± 20,100 versus 3322 ± 1311 IU/L, P = 0.008; Fig. 3B).

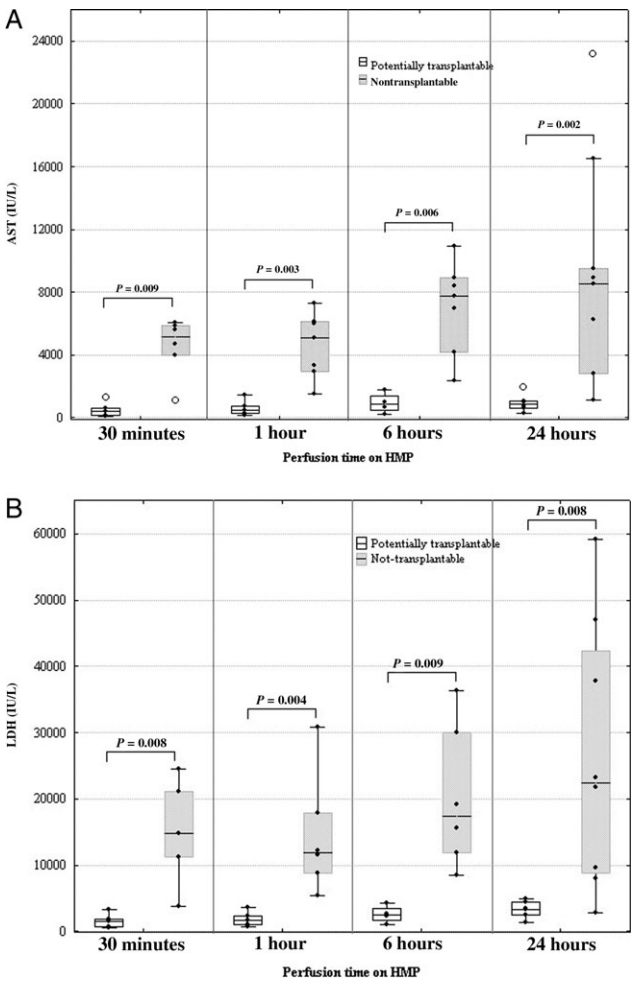

Figure 3. (A) During HMP, AST release in the perfusate was higher for nontransplantable livers versus transplantable livers. (B) Similarly, nontransplantable livers released more LDH than transplantable livers.

Evolution of Vascular Resistances During HMP

During HMP, the AUC for arterial resistance (698.7 ± 702.8) was higher than the AUC for portal resistance

(16.2 ± 20.8, $P < 0.001$). The arterial resistance gradually decreased during HMP from 1.29 ± 0.67 to 0.52 ± 0.47 mm Hg minute/mL. A similar phenomenon was observed, albeit to a lesser extent, for the portal resistance, which decreased from 0.25 ± 0.30 to 0.13 ± 0.13 mm Hg minute/mL. The AUCs for both portal and arterial resistances were similar for the nontransplantable and transplantable livers ($P = 0.94$ and $P = 0.48$, respectively).

Correlation Between Steatosis and AST in the Perfusate

Steatosis on baseline biopsy samples that was assessed by stereological point counting correlated with the AUC of AST release after 24 hours of HMP ($P = 0.006$, correlation coefficient = 0.9). In contrast, macrovesicular steatosis that was quantified semiquantitatively did not correlate with the AUC of AST release after 24 hours of HMP ($P = 0.13$, correlation coefficient = 0.45). Microvesicular steatosis that was semiquantitatively quantified did not correlate with the AUC of AST release in the perfusate after 24 hours of HMP ($P = 0.12$, correlation coefficient = 0.47).

Correlation Between the Cold Storage Duration and AST Release in the Perfusate

No correlation was observed between the duration of SCS before HMP and the AUC of AST release during HMP ($P = 0.44$, correlation coefficient = 0.25).

ATP Tissue Content

Before HMP, the ATP tissue content was 7.2 ± 8.8 nmol/g in transplantable livers and 1.6 ± 1.8 nmol/g in nontransplantable livers ($P > 0.05$). After HMP, the ATP tissue content was 12.7 ± 22.7 nmol/g in transplantable livers and 2.5 ± 2.3 nmol/g in nontransplantable livers ($P = 0.55$).

mRNA Level Quantification

No changes in the mRNA expression of TNF-α, IL-6, ICAM1, JUN, MAPK, ZO-1, END1, MDR1, or BSEP were observed after HMP versus the start of HMP ($P > 0.05$).

Morphology and Ultrastructure After 24 Hours of HMP

According to standard microscopy, the parenchymal architecture of the livers deemed to be transplantable was maintained after 24 hours of HMP (Fig. 4A-D). All livers showed normal PTs without histological evidence of necrosis and/or apoptosis (Fig. 4C). A variable degree of sinusoidal dilatation was seen. Limited hepatocellular vacuolation was observed (Fig. 4D). Some anoxic vacuoles were observed in various zones extending from the CV into the PT but on other occasions remained mostly concentrated around the CV (Fig. 4C,D). The distribution of this vacuolation was heterogeneous throughout the parenchyma, with some lobules containing numerous vacuoles and others containing no vacuoles.

According to electron microscopy, the ultrastructure of the hepatocytes and sinusoidal cells and the endothelial cell lining of the transplantable livers were well preserved after 24 hours of HMP (Fig. 5A-F). Some but not all hepatocytes contained anoxic vacuoles (Fig. 5D). The sinusoidal endothelial cells (SECs) and Kupffer cells showed no features of ischemic damage. The space of Disse and the sinusoidal lumina appeared slightly enlarged (Fig. 5D,E). Occasionally, slightly swollen mitochondria with a less electron-dense matrix (suggestive of some degree of autolytic yet reversible changes) were observed within certain hepatocytes (Fig. 5F). Mitochondria with flocculent densities (suggestive of more irreversible changes) were rarely seen. Other cytoplasm organelles and glycogen particles appeared normal (Fig. 5F).

In contrast to the transplantable livers, massive macrovesicular steatosis (>60%) was prominently present in 8 steatotic, nontransplantable livers according to standard and electron microscopy.

In 1 nontransplantable liver (case 13), there was evidence of alcoholic steatohepatitis at a septal stage. Another nontransplantable liver (case 3) displayed extensive coagulation necrosis (up to 40%) of the parenchyma together with hepatocellular ballooning and bilirubinostasis. After HMP, macrovesicular steatotic lesions remained unchanged, and no obvious changes in the limited remaining hepatocellular parenchyma (including anoxic vacuolation) were observed. Similarly but to a lesser extent in comparison with the transplantable livers, the space of Disse and sinusoidal lumina were enlarged. In these nontransplantable livers, more mitochondria tended to be irreversibly damaged in comparison with transplantable livers.

DISCUSSION

Because of the shortage of standard criteria donors, livers from ECDs (eg, donors with steatosis, older donors, and donors with hypernatremia) and DCD donors are increasingly being considered for transplantation. In the absence of objective criteria for transplantability, the final decision to accept or decline these higher risk livers remains subjective and is based solely on surgeons' judgment. Although transplant surgeons are urged to consider higher risk grafts that may benefit the recipient population as a whole, they may be reluctant to expose individual patients to uncertain risks of poor outcomes. Consequently, a substantial number of grafts that may actually be transplantable are not used. The development of objective tools capable of assessing liver graft quality is a prerequisite for a real and safe expansion of the donor liver pool. In this proof-of-concept study, we have shown that a biochemical analysis of the perfusate during HMP of human livers provides

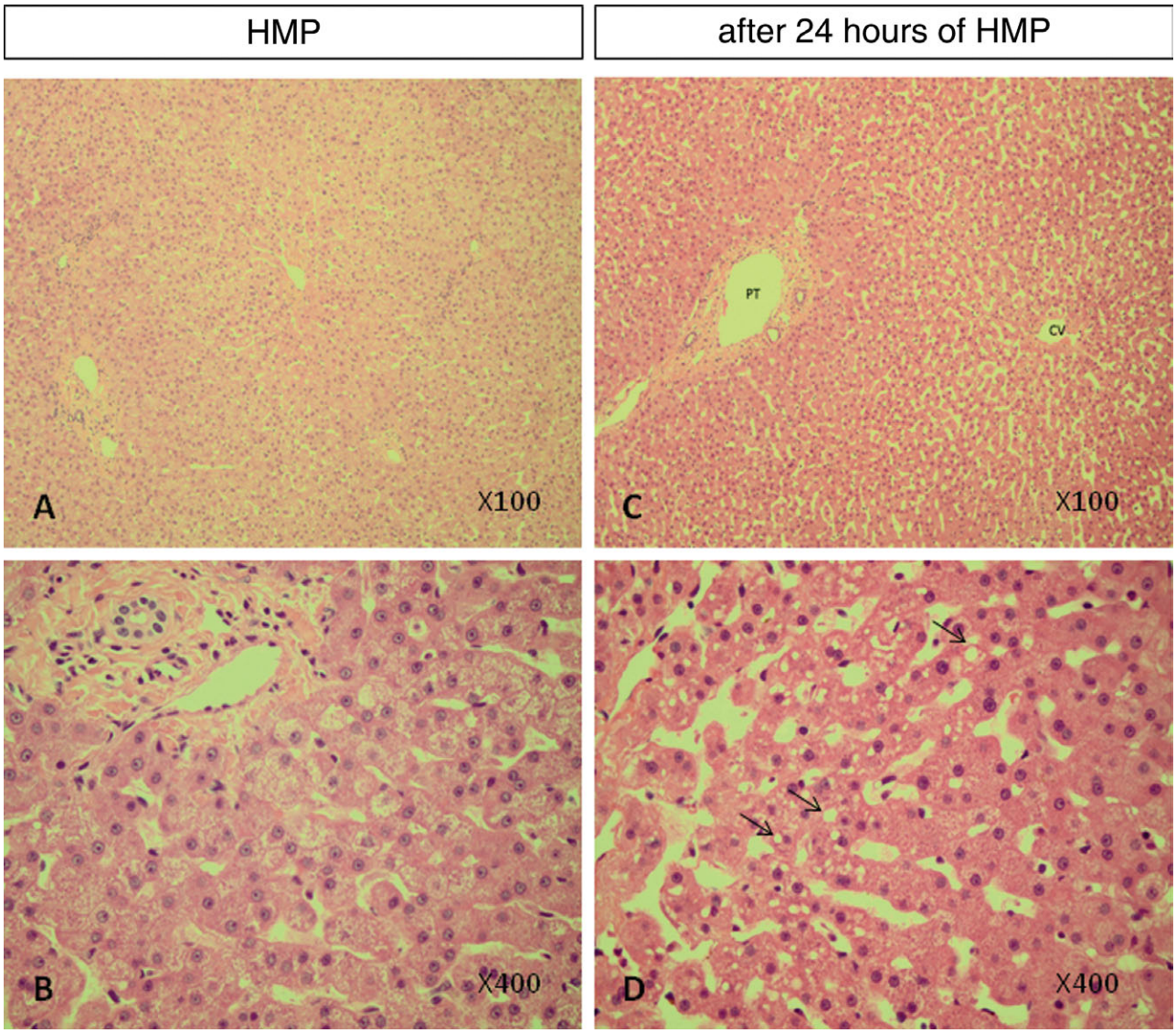

Figure 4. Representative histopathological findings (A,B) before and (C,D) 24 hours after HMP of a transplantable liver from a DCD donor that was rejected after the recipient was found to be unsuitable for transplantation (case 17). Panel A shows that the parenchyma was generally well preserved (magnification ×100). Panel B shows that at a higher magnification (×400), the parenchyma next to the PT appeared normal. Panel C shows a variable degree of sinusoidal dilatation around the CV together with a variable degree of hepatocellular vacuolation in contrast to a rim of normal parenchyma around the PT after 24 hours of HMP (magnification ×100). Panel D shows sinusoidal dilatation (arrows) and limited hepatocellular vacuolation after 24 hours of HMP, as occasionally observed, at a higher magnification (×400).

information that may assist clinicians in decision making. We have also shown that the ultrastructural morphology of good-quality livers can be maintained for prolonged periods by HMP.

In comparison with SCS, HMP preservation has 2 potential advantages. First, continuously perfusing an organ with a cold preservation solution may provide better preservation than simple cold storage of the organ. A meta-analysis and randomized control trials have demonstrated that kidneys preserved by HMP function better and longer than those preserved by SCS.[14,15] Second, HMP allows us to directly evaluate preserved organs and may provide predictive information on the posttransplant outcome. The concentration of certain biomarkers reflecting cell necrosis (glutathione *S*-transferase, alanine aminopeptidase, *N*-acetyl-β-D-glucosaminidase, and heart-type fatty acid binding protein) in the perfusate of machine-perfused kidneys[3] and the vascular resistance during perfusion correlate with posttransplant function.[4] The need to better preserve and assess ECD and DCD human livers is also critical, and the revival of kidney HMP has also contributed to increased interest in liver HMP. A few animal studies have suggested a benefit for liver HMP versus SCS,[6,16] and a pilot clinical trial recently conducted by Guarrera et al.[5] showed for the first time the feasibility (and potential superiority) of liver HMP. However, in contrast to kidney transplantation,

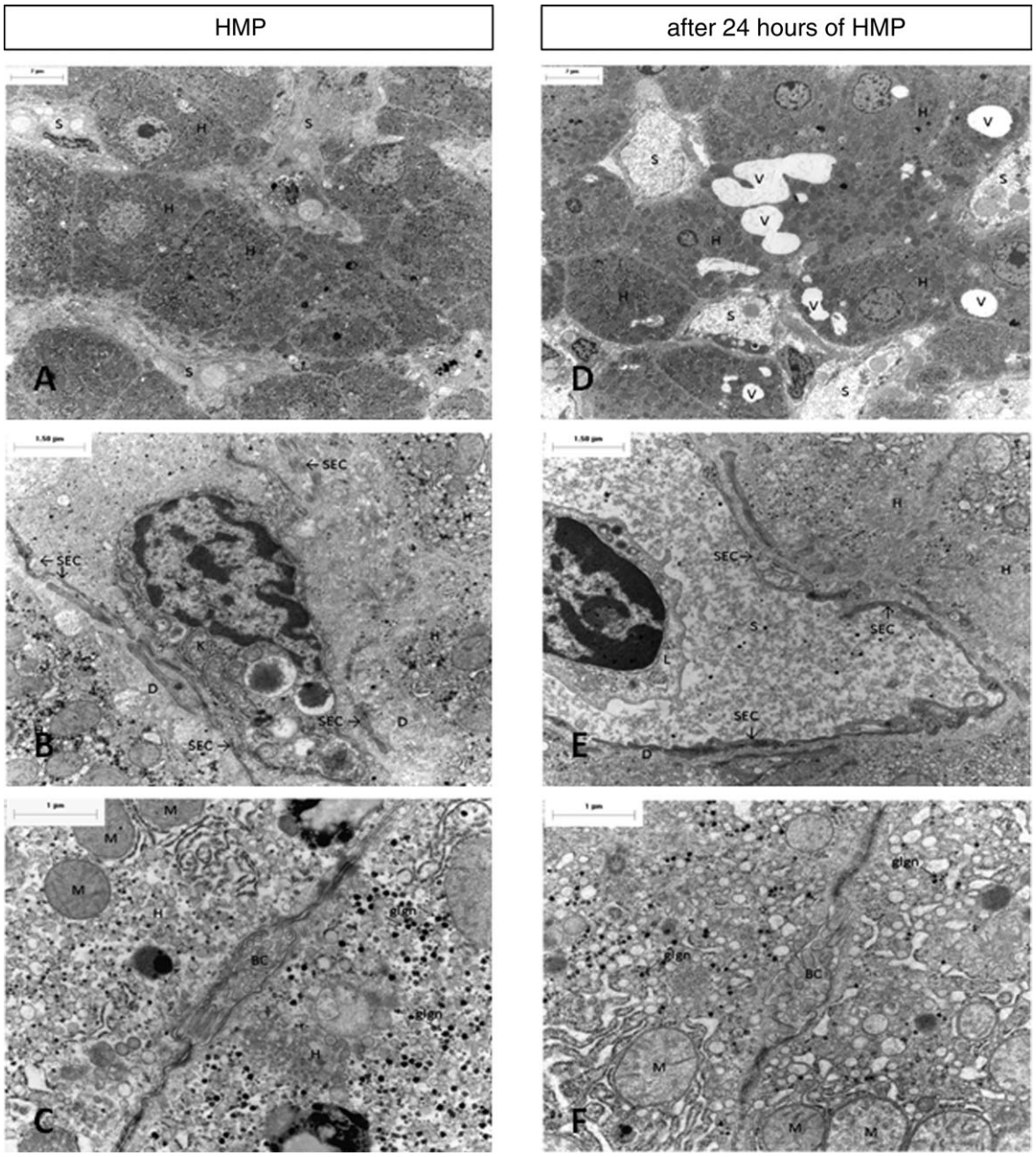

Figure 5. Electron micrographs of liver parenchyma (A-C) before and (D-F) 24 hours after HMP of a transplantable liver (case 17). Panel A presents an overview of ultrastructurally well-preserved hepatocytes and sinusoids before HMP, whereas panel D shows that some hepatocytes with anoxic vacuoles and slightly dilated sinusoids were present after 24 hours of HMP. Panel B presents a detail of the sinusoidal lumen lined by a thin process of SECs before HMP; a Kupffer cell with heterogeneous lysosomes can be seen. Panel C shows a detail of a bile canaliculus formed by 2 adjacent hepatocytes before HMP; the mitochondria and other cytoplasmic organelles as well as glycogen particles (arrows) had a normal ultrastructure. Panel E shows that after 24 hours of HMP, SECs did not present signs of autolytic changes; a lymphocyte was lying in the sinusoidal lumen, and the endothelial cell lining appeared intact. Panel F shows that after 24 hours of HMP, only mitochondria were slightly swollen, with a less electron-dense matrix pointing to reversible changes; the other cytoplasmic organelles as well as the glycogen particles were normal. The following symbols are used in the figure: BC, bile canaliculus; D, space of Disse; glgn, glycogen; H, hepatocyte; K, Kupffer cell; L, lymphocyte; M, mitochondria; S, sinusoid; and V, vacuole. The scale bars represent (A) 7 μm, (B) 1.5 μm, (C) 1 μm, (D) 1.5 μm, (E) 1.5 μm, and (F) 1 μm.

there are currently no data on the possibility of evaluating ECD or DCD liver grafts during HMP, and this was the main aim of this proof-of-concept study.

In analogy to kidneys, we hypothesized that the analysis of markers of cell necrosis in the perfusate of machine-perfused livers and their vascular resistance would provide valuable information on their baseline condition. In a validated pig model of DCD LT, we previously reported that AST in the perfusate correlated well with the length of the warm ischemia to which these porcine livers had been exposed and with the extent of damage and the associated risk of primary nonfunction after transplantation.[17,18] However, in this porcine model (in contrast to what has been

reported for kidneys), we observed no correlation between the severity of the ischemic damage endured and the arterial and portal vascular resistances during HMP.[19]

We have now developed and tested an HMP prototype for human livers. In this proof-of-concept study, we adapted the perfusion protocol developed for renal HMP by performing dual (independent portal and arterial) perfusion and by using independent flows and pressure settings. Like Guarrera et al.,[5] no active oxygenation of the perfusate was applied.

One of the first points of this study is that similarly to what we have observed for porcine livers, HMP is capable of maintaining liver morphology for long periods.[20] Indeed, in good-quality livers not transplanted because of failed allocation, standard and electron microscopy examinations showed well-preserved parenchyma and an intact endothelial cell lining after 24 hours of HMP. The ultrastructure of hepatocytes and SECs was well maintained. Mitochondria displayed mostly mild and reversible changes. This is quite remarkable because these livers had been cold-stored for prolonged periods before they were machine-perfused. Notably, vacuoles appeared in hepatocytes, although this was a limited phenomenon. We previously described this vacuolization phenomenon in hepatocytes of pig livers exposed to warm ischemia.[13] These vacuoles have also been called anoxic vacuoles because they seem to be the result of oxygen deprivation. In support of that, we found in another experiment that adding oxygen to the perfusate reduced the development of these vacuoles.[7] Importantly from the perspective of clinical applications, no technical problems were encountered during prolonged HMP, and in particular, no vascular damage caused by the insertion of the cannulas was observed.

We then explored whether HMP of human livers could provide clinically relevant information on their condition. In accordance with our previous findings in pigs, differences were observed in the release of intracellular enzymes (AST and LDH) between massively steatotic, nontransplantable livers and control transplantable livers. These differences became apparent after only 30 minutes of HMP and persisted thereafter. Threshold values of 1500 and 4000 IU/L for AST and LDH, respectively, within the first hour of HMP differentiated nontransplantable livers from transplantable livers. This short time frame indicates that prolonged machine preservation is not necessarily required to provide clinically useful information. In our pig model, we found that liver-type fatty acid binding protein also correlated with the ischemic damage endured, and this is another potential marker of interest.[18]

Similarly to our earlier report on porcine HMP,[20] PO_2 in the perfusate decreased gradually, whereas PCO_2 increased in both groups. This indicates an initially aerobic metabolism progressively replaced by some degree of anaerobic metabolism (as indicated by rising lactate levels and decreasing pH). However, no differences in metabolic profiles were observed between nontransplantable and transplantable livers. It may be that the long period of cold ischemia that preceded HMP accounted for this absence of differences in the metabolic profiles. Similarly, no significant differences in the tissue levels of ATP before and after HMP were observed when nontransplantable and transplantable livers were compared. In contrast to what has been reported for kidneys, no difference in vascular resistance was observed between nontransplantable and transplantable livers. Similarly to our findings in pigs,[20] the arterial resistance was constantly higher than the portal resistance and decreased during perfusion in both groups.

In this proof-of-concept study, only basic parameters similar to those routinely used to evaluate hypothermically perfused kidney grafts were measured. However, HMP technology provides additional possibilities, such as proteomic perfusate studies, more refined evaluations of ATP contents and energy stores, and examinations by magnetic resonance imaging and spectroscopy. Our group and others are studying the value of these more sophisticated methods.[21,22]

A potential intrinsic limitation of HMP, however, is that because of hypothermia, most aspects of cellular activity may remain quiescent. In support of this, we observed during HMP no changes in the expression of various mRNAs (TNF-α, IL-6, ICAM1, JUN, MAPK, END1, MDR1, BSEP, and ZO-1). Tulipan et al. compared mRNA changes in biopsy samples taken before and after preservation by HMP and SCS alone. No changes in monocyte chemoattractant protein 1 or IL-1 receptor antagonist were observed after HMP. In contrast, the monocyte chemoattractant protein 1 level was increased after SCS, and this suggested an active anti-inflammatory effect of HMP instead of metabolic quiescent activity due to hypothermia itself.[23] Furthermore, Guarrera et al.[24] observed that the upregulation of TNF-α, IL-8, and ICAM1, which is normally seen after the transplantation of SCS-preserved livers, was reduced when livers had been preserved with HMP.

It is likely that the real-time assessment of metabolism, inflammation, and function will require more physiological midthermic or normothermic perfusion.[25]

An unexpected but interesting observation is that the concentration of AST in the perfusate had a linear correlation with the degree of steatosis assessed by stereological point counting. The underlying reason that steatotic livers release more AST during HMP is not clear. It may be that steatotic hepatocytes are more vulnerable to ischemic injury[26,27] and that this higher AST release simply reflects the suboptimal quality of these grafts. A correlate of this is that AST release from machine-perfused steatotic grafts may represent an additional and perhaps more reliable surrogate of the overall grade of steatosis in comparison with conventional histology, which is subject to interobserver variation and sampling error as we and others have reported earlier.[13,28]

Our liver HMP setting (no active oxygenation and relatively low flow/pressure perfusion) was similar to the one used by Guarrera et al.,[5] who were the first to

successfully transplant human livers after 4 to 8 hours of machine perfusion. In contrast to our study, Guarrera et al. perfused predominantly standard criteria donor livers (eg, they excluded donors > 65 years of age, DCD donors, and donors with >25% macrovesicular steatosis on biopsy). The primary aim of that trial was not to determine the value of HMP for assessing viability but instead to prove the feasibility of preserving human livers by HMP in lieu of cold storage. Notably, AST in the perfusate of these predominantly standard criteria donor livers after 2 hours of HMP ranged from 307 to 609 IU/L[5]; these values are similar to those measured in the perfusates of the transplantable livers in our study and, therefore, corroborate our findings.

Our experimental design has intrinsic limitations, and our results need to be interpreted with caution. First, the reasons for discarding were multifactorial, and the classification of livers as nontransplantable or transplantable remains subjective. The group of nontransplantable livers was in fact homogeneous because most of them had massive steatosis (>60%). The reasons for discarding in the group of transplantable livers were more heterogeneous. However, in the end, the quality of these livers was comparable and independent of the events leading to discarding. Importantly, the classification of livers as transplantable or nontransplantable was made before the data analysis. The fact that the release of AST and LDH in the perfusate was found a posteriori to be different between the nontransplantable and transplantable livers suggests that the original classification was accurate, and it supports ex post facto the concept that HMP can provide useful and objective data for assessing the degree of injury endured.

Second, HMP was preceded by a prolonged period of SCS that may have masked differences in metabolic profiles. This prolonged SCS may have exacerbated the release of AST and LDH, particularly in livers of poor quality, but no correlation was found between the duration of SCS and AST release. In addition, the prolonged SCS may have played a role in the clear and rapid decrease of PO_2 and the concomitant rise of the lactate level and PCO_2. This decrease was not observed during HMP after shorter periods of SCS by Guarrera et al.,[5] and this may point to the potential importance of active oxygenation during HMP when there is a long preceding period of SCS.

Third, the HMP prototype used in this study was derived from a kidney device, and certain adjustments more specific for the liver require further investigation. Lowering the perfusion pressure (to prevent excessive shear stress) results in better maintenance of ATP, less vacuolation, and eventually better preservation.[7,8] Data on the value of oxygenation for the perfusate are conflicting. We used a setting similar to that of Guarrera et al.,[5] who found that HMP of human livers had a favorable impact, despite the absence of active oxygenation of the perfusate. There are even data indicating that active oxygenation during perfusion may exacerbate the production of radical oxygen species and eventually be detrimental.[9] However, there is also experimental evidence from our group and others showing that oxygenation of the perfusate may be superior to nonoxygenated HMP,[6,7,9] probably because it maintains a certain degree of aerobic metabolism and prevents at least partially a switch to an anaerobic metabolism that we observed in this study. The presence of anoxic vacuoles on electron microscopy images reflects hypoxic trauma and suggests that supplemental oxygenation of the perfusate may have been beneficial and will have to be tested. In support of this, oxygen persufflation of statically cold-stored pig livers has been shown by Minor et al.[10] to be protective.

Fourth, the ultimate test of viability that LT represents could for obvious reasons not be done. To mimic LT ex vivo, we exposed an additional cohort of human livers to isolated and warm oxygenated reperfusion after a period of HMP.[29] In this latter experiment, we observed a linear correlation between AST in the perfusate during HMP and AST release after warm oxygenated reperfusion (data not shown). Notably, the maximum AST concentrations measured by Guarrera et al.[5] in the perfusate of HMP-preserved human livers correlated well with the AST peak that they observed after transplantation.

This proof-of-concept study is only a first step toward the development of new predictors of liver quality, and there is an urgent need for this. During the 6-year study period in Belgium, 60 procured livers were discarded (I. Tieken, MD, Eurotransplant, written communication, January 2010), and high discard rates have also been reported in various European countries and in North America on the basis of subjective grounds alone. For massively steatotic livers, an assessment by HMP is probably superfluous. However, certain transplantable livers in our study had been discarded because of the overinterpretation of minimal changes or insufficient information at the time of procurement. It is likely that knowledge by the transplant surgeon of an objective indicator of minimal baseline damage (eg, low AST/LDH release as described in this study) would have encouraged their acceptance in the first place. Finally, in the future, it is likely that the availability of more objective and more sophisticated predictors of liver graft quality will stimulate LT surgeons to consider more ECD and DCD liver grafts for transplantation.

In conclusion, during HMP, nontransplantable, steatotic human livers release more AST and LDH in the perfusate, but they have similar vascular resistances and metabolic profiles in comparison with transplantable human livers. HMP preserves the morphology of transplantable human livers for prolonged periods. Randomized control trials of the transplantation of machine-perfused livers versus cold-stored livers are now warranted to determine the added value of HMP not only for the preservation but also for the assessment of human liver grafts.

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