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von Horn, Charlotte; Baba, Hideo A.; Hannaert, Patrik; Hauet, Thierry; Leuvenink, Henri G.D.; Paul, Andreas; Minor, Thomas; COPE Consortium Partners

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Controlled oxygenated rewarming up to normothermia for pretransplant reconditioning of liver grafts

Charlotte von Horn[1] | Hideo A. Baba[2] | Patrik Hannaert[3] | Thierry Hauet[3] | Henri Leuvenink[4] | Andreas Paul[1] | Thomas Minor[1] | on behalf of COPE consortium partners

[1]Department for Surgical Research, General Visceral and Transplantation Surgery, University Hospital Essen, Essen, Germany

[2]Institute of Pathology, University Hospital Essen, Essen, Germany

[3]RTOMIT, INSERM, Université de Médecine et de Pharmacie de Poitiers, Poitiers, France

[4]Department of Surgery, University Medical Center Groningen, The Netherlands

Correspondence
Thomas Minor, Department for Surgical Research, General Visceral and Transplantation Surgery, University Hospital Essen, Essen, Germany.
Email: chirfor@uk-essen.de

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Abstract

Controlled oxygenated rewarming (COR) up to 20°C during ex vivo machine perfusion limits reperfusion-induced tissue injury upon graft implantation. Rewarming up to normothermia might add further benefits and provide better prediction of post-transplantation organ function. The effect of 90 minutes of oxygenated machine perfusion with Aqix RS-I after cold storage combined with gentle rewarming up to 20°C (COR20) or 35°C (COR35) was studied in rat livers and compared with cold storage alone (CS, n = 6, resp). Postpreservation recovery was evaluated upon warm reperfusion using an established in vitro system. COR generally resulted in significantly improved energetic recovery, increased bile flow, less activities alanine aminotransferase (ALT) release, and improved histopathology upon reperfusion as compared to only cold-stored livers, without significant differences between COR20 and COR35. Parameters obtained during COR, especially during COR35, also allowed for prediction of hepatic recovery upon reperfusion. For instance, ulterior bile production upon reperfusion was found closely correlated to bile flow observed already during COR35 (R^2 = 0.91). COR significantly improved liver quality after static cold storage. Elevation of machine perfusion temperature up to 35°C may prove promising to refine ex vivo evaluation of the graft prior to transplantation.

KEYWORDS
controlled oxygenated rewarming, liver preservation, machine perfusion, organ reconditioning

1 | INTRODUCTION

One of the major restrictions in transplantation medicine is the limited number of available donor organs. Due to this imbalance between donor organs and waiting recipients, the criteria of organ selection were extended and grafts of lesser quality became increasingly accepted to be transplanted.[1] The use of these "less-than-optimal" grafts provides new challenges in transplantation research with regard to organ preservation and conditioning.[2] New ex vivo strategies for maintenance and improvement of organ function could prevent storage and reperfusion-induced tissue injury and could therefore result in decreased post-transplantation graft dys- or nonfunction.[3]

Organ preservation traditionally includes flush-out with appropriate preservation solutions followed by cold storage under hypoxic conditions.[4] Problems associated with this technique are hypoxia-induced apoptotic events as well as activation of pro-inflammatory mediators, which influence post-transplantation organ function and integrity.[5] Previous research evidenced that main destructive effects rather establish during exposure of organs to blood circulation after implantation than during the ischemic cold storage period itself.[6]

The quality loss of organs during ex vivo organ preservation resulted in the invention of new preservation techniques with the aim to recondition grafts prior to transplantation. It was found that brief hypothermic oxygenated machine perfusion (HMP) prior to organ transplantation significantly improves organ integrity and leads to decreased numbers of cases of post-transplantation graft dys- and nonfunction.[7,8] This optimization is related to a reduced release of reactive oxygen species (ROS) during HMP and less oxidative stress upon restoration of blood flow at normothermic conditions.[9]

Further experiments have indicated that rewarming of the organ during oxygenated machine perfusion could further contribute to improved organ integrity and in vivo functionality through an adapted increase in metabolic activity of mitochondria and tissue cells.[10] Controlled oxygenated rewarming (COR) to 20°C was already shown to have protective capacities and improves organ recovery upon reperfusion.[11]

So far, extracorporeal temperature rise during COR had been limited to 20°C, as perfusion solutions had been used that were specifically designed for organ protection at hypothermia and might become inappropriate perfusates at increasing temperatures. However, COR up to normothermia might be advantageous over COR 20°C and could potentially provide the detection of functional markers for a more reliable prediction of post-transplantation organ quality.

Aqix RS-I is a novel extracellular-type solution, which has the capability to maintain pH stability over a wide temperature range and has proven an appropriate preservation solution up to normothermic conditions.[12]

This study thus was designed to compare the suitability of liver reconditioning by COR up to varying temperatures (20°C and 35°C) by machine perfusion. The potential and optimal regeneration conditions for damaged livers by short-term preimplantation ex vivo liver perfusion were evaluated.

2 | MATERIALS AND METHODS

The experiments were performed according to the federal law regulating the protection of animals and follow the principles of laboratory animal care (NIH publication vol 25, No 28, revised 1996). Male Wistar rats with a weight between 250 and 300 g were anesthetized using isoflurane (induction with 4%). Ketamine (75 mg/kg bw) was intraperitoneally injected as additional analgesia. The abdomen was exposed by midline incision and exsanguination of the animal induced by incision of the infrarenal aorta. Thirty minutes after cardiac arrest, the portal vein was cannulated and the liver rinsed via the portal vein with 60 mL of histidine-tryptophan-ketoglutarate (HTK) solution (Köhler Chemie, Germany). After hepatectomy, the liver was finally cold stored overnight in HTK for 18 hours at 4°C.

2.1 | Controlled oxygenated rewarming (COR)

After 18 hours of static preservation at 4°C, some grafts were put on a home-made machine perfusion circuit, and 200 mL of Aqix RS-I solution was recirculated through the portal vein in a constant pressure mode imitating the way described earlier in larger organs.[11] The solution was oxygenated through a thin-wall silicone tubing curled in a box, which was ventilated with a 95% O_2-5% CO_2 gas mixture to allow for sufficient tissue aerobiosis during the rewarming process.[13] Thus, oxygen partial pressures, measured with a temperature compensated fiber optic oxygen meter (Fibox3 LCD, PreSens precision sensing, Regensburg, Germany) at the venous effluent, did always exceed 200 mm Hg. After 30 minutes of hypothermic perfusion, controlled rewarming of the perfusate was performed up to 20°C (COR20; n = 6) or to 35°C (COR35; n = 6) by means of a programmable, external circulating cryo-thermostat. Overall perfusion time was 90 minutes; the maximal temperature was reached within the first 60 minutes. Portal pressure was adjusted to 5 cm H_2O, for temperatures above 20°C, portal perfusion pressure was elevated to 8 cm H_2O. Untreated livers that were only cold stored without end-ischemic rewarming served as controls (CS; n = 6).

2.2 | Isolated liver perfusion

Postpreservation recovery of all livers was evaluated after preservation upon warm reperfusion in vitro in a recirculating system for 120 minutes at 37°C. Prior to reperfusion, all livers were exposed to room temperature on a Petri dish for 20 minutes prior to reperfusion to simulate the ischemic period during surgical implantation in vivo. Oxygenated (95% O_2 – 5% CO_2; pO_2 > 500 mm Hg) Williams E solution (Sigma Aldrich, Germany), supplemented with 3 mg/100 mL of bovine serum albumin, was pumped through the portal vein at a constant flow of 3 mL/g min while the liver was placed floating in a bath of perfusion solution at 37°C. This setup has been previously validated and was shown to allow for adequate approximation of tissue integrity and detection of structural changes in rat livers after hypothermic preservation.[14] Perfusate samples were taken after 15, 45, 90, and 120 minutes of warm reperfusion.

2.3 | Detection of injury biomarkers

Enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the perfusate were assessed in a routine fashion at the laboratory center of the University Hospital.

Perfusate pH, levels of glucose, and lactate were assessed using an acid base laboratory blood gas analyzer (ABL 800, Radiometer, Copenhagen, Denmark).

2.4 | Liver function

The common bile duct of the livers was cannulated with a 27-gauge polyethylene tubing. Bile was collected during the whole reperfusion period, and hepatic bile production was calculated as μL/g/h.

2.5 | Energetic status

Tissue specimens for assessment of high-energy phosphates were taken with precooled steel tongs, immersed in liquid nitrogen, and

stored at −80°C for later analysis. Wet weight of the frozen tissue samples was measured before they were lyophilized in a vacuum freezer (−60°C; <0.025 mbar) for at least 7 days to evaporate tissue water. Freeze dried specimens were weighed again, and proteins were extracted with perchloric acid as described previously.[15] Aliquots of the neutralized supernatant were used for determination of adenosine triphosphate (ATP) by means of a commercial test kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. The results were corrected for the respective dry weight to wet weight ratio of the tissue samples and expressed as μmol/g dry weight.

2.6 | Histology

Liver tissue was collected at the conclusion of the experiments, cut into small blocks (3 mm thickness), and fixed by immersion in 4% buffered formalin. The blocks were embedded in paraffin, and 2-4 mm tissue slides were prepared using a microtome (SM 2000R, Leica Instruments, Nußloch, Germany). Hematoxylin and eosin (H & E) staining was conducted adherent to in-house standards and used to assess morphological integrity of the parenchyma. Sections were examined at 200-fold magnification (Nikon Eclipse E800, Nikon, Tokyo, Japan) and the extent of necrotic injury was semiquantitatively graded in a 4-stage system ranging from 0 (no necrosis) to 3 (severe necrosis with disintegration of hepatic cords) as described elsewhere[16] by two independent examiners.

2.7 | Statistics

All values are expressed as means ± SEM of n = 6 animals per group. Differences among the groups were tested by analysis of variances followed by the Student-Newman-Keuls test. Statistical significance was set at *P* less than .05.

3 | RESULTS

3.1 | Controlled oxygenated rewarming (COR)

Figure 1 shows the temperature kinetics during controlled rewarming up to 20°C or 35°C, respectively, as well as the corresponding flow volumes via the portal vein in the two groups. Venous effluent pO_2 always stayed above 200 mm Hg, and perfusate concentrations of lactic acid remained below 1 mmol/L throughout the procedure in any group. Of note, perfusate concentrations of potassium remained stable during the first 60 minutes of perfusion but then showed a slight declining tendency suggesting net absorption by hepatic metabolism. This pattern was similarly observed in both groups but did not reach statistical significance. Perfusate pH during COR remained in the physiological range (7.35-7.40) in both groups, and no additional equilibration was necessary.

Supplementary parameters related to liver integrity at the end of the rewarming machine perfusion are depicted in Table 1.

3.2 | Liver recovery upon reperfusion

Perfusate levels of hepatocellular enzymes, released upon reperfusion, were determined to characterize the effect of the different treatment arms on parenchymal liver damage (Figure 2). Notable concentrations of AST were found already early upon reperfusion of cold-stored livers and steadily increased thereafter. Controlled rewarming prior to reperfusion notably reduced the enzyme loss during the whole observation period. This reduction seemed to be more prominent after COR20 than after COR35, but no significant differences were disclosed between the two groups. Similar results were obtained with regard to ALT, the leakage of which culminated to 338 ± 58, 123 ± 31*, and 185 ± 24* (CS vs COR20 vs COR35; *P < .05 vs CS). Resumption of metabolic activity of the livers is summarized in Table 1. Controlled rewarming up to either 20°C or 35°C significantly improved hepatic bile production upon reperfusion and also led to a better recovery of energetic status during reperfusion (cf. Table 2). While no difference could be substantiated between COR20 and COR35 for energetic recovery, bile yield was somewhat lower after COR35 than after COR20.

Light microscopic evaluation of liver samples at the end of reperfusion was in line with the functional data. Severe alterations like necrotic foci and hepatic chord disintegration were more abundant in the cold-stored livers than after controlled oxygenated rewarming leading

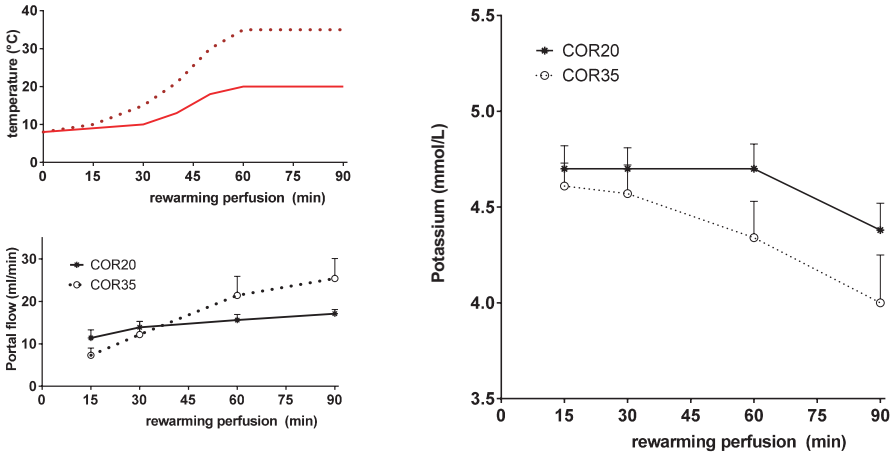

FIGURE 1 Temperature kinetics, hepatic flow development, and perfusate potassium concentrations during controlled oxygenated rewarming up to 20°C (COR20) or 35°C (COR35)

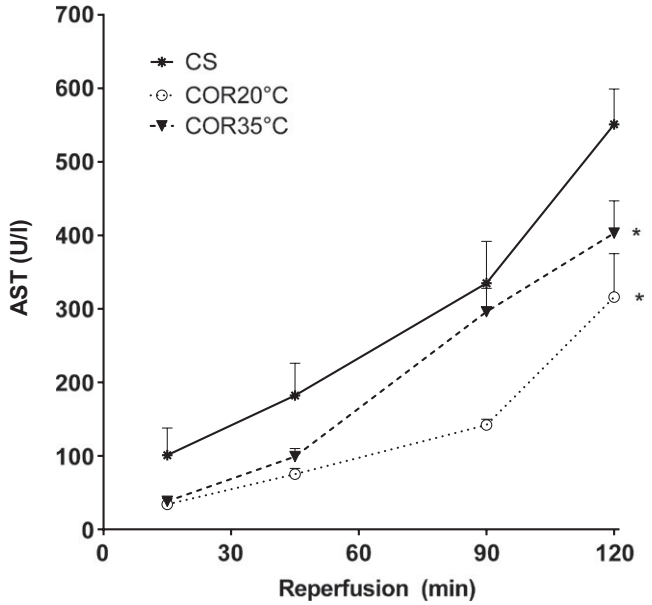

FIGURE 2 Hepatic release of aspartate aminotransferase (AST) upon reperfusion directly after cold storage (CS) and after interposed controlled oxygenated rewarming up to 20°C (COR20) or 35°C (COR35) *P < .05 vs CS

to a significantly higher histological injury score in comparison with both of the treatment groups (cf. Figure 3).

3.3 | Correlation with functional recovery

Parameters obtained during COR were also scrutinized for their potential to allow for prediction of hepatic recovery upon reperfusion. Inherent to the limitations of the in vitro model used, bile production upon reperfusion was chosen as the main indicator for functional liver recovery. During COR35, all of the livers already consistently exhibited a measurable bile production, which turned out to closely correlate with ulterior bile flow during reperfusion (cf. Figure 4). Bile production during COR20 was less consistent and in two cases not measurable, thus precluding this parameter to be used for prediction of ulterior function. However, perfusate concentrations of potassium at the end of COR20 appeared to serve as a surrogate for functional measurements during COR, showing a correlation with ulterior bile production upon reperfusion of $R^2 = 0.87$.

4 | DISCUSSION

The use of less than optimal organs in consequence to donor organ shortage provides new challenges in transplantation research with respect to graft storage and reconditioning.[17]

During initial organ cold storage at 4°C, deprivation of oxygen[9] as well as hypothermia itself,[18] may adversely influence tissue state and trigger the induction of pro-inflammatory events and ulterior cell death.

Previous research indicated that main influence factors predicting the organ functionality and integrity after engraftment evolve upon reperfusion through reestablishment of the blood circulation.

TABLE 1 Concentrations of lactate, liver enzyme activities (alanine aminotransferase—ALT and aspartate aminotransferase—AST) and differences of glucose concentration at 90 minutes minus concentration at 60 minutes[20] in the perfusate upon controlled oxygenated rewarming up to 20°C (COR20) or 35°C (COR35)

	COR20	COR35	
Lactate (mmol/L)	0.5 ± 0.1	0.8 ± 0.1	ns
ALT (U/L)	7.7 ± 1.0	11.8 ± 1.6	ns
AST (U/L)	18.0 ± 3.6	33.0 ± 2.4	*
Delta Glucose (mg %)	8.7 ± 2.8	5.8 ± 1.1	ns

*P < .05.
ns: not significant—Student's t-test.

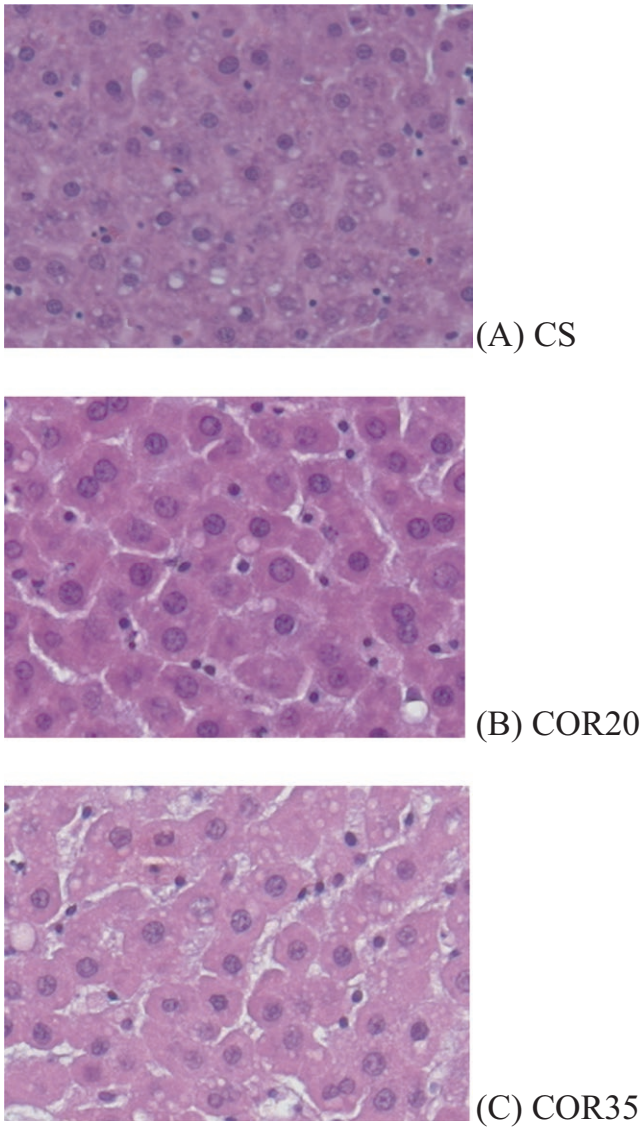

FIGURE 3 Liver tissue histology at the end of reperfusion after (A) cold storage (A, CS) or after controlled oxygenated rewarming up to 20 °C (B, COR20) or 35 °C (C, COR35)

The sudden temperature change from cold storage at 4°C to normothermic conditions upon transplantation is suggested to negatively impact organ integrity and contribute to reperfusion-induced tissue injury.[19]

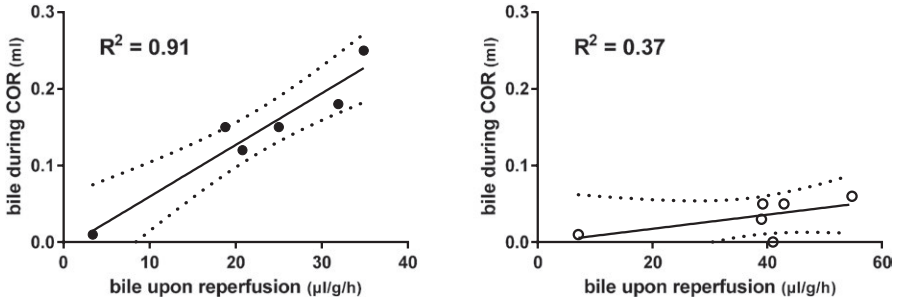

FIGURE 4 Correlation of bile production during controlled oxygenated rewarming up to 35°C (left) or 20°C (right) and subsequent bile flow upon postpreservation reperfusion

TABLE 2 Functional recovery and histological integrity of livers after cold storage (CS) or after interposed controlled oxygenated rewarming up to 20°C (COR20) or 35°C (COR35)

	CS	COR20	COR35
Bile (µL/g/h)	5.1 ± 1.9	37.4 ± 5.9*	22.1 ± 4.3*
ATP (µmol/g dw)	1.79 ± 0.07	2.56 ± 0.32*	2.44 ± 0.27*
Histoscore	2.8 ± 0.2	1.6 ± 0.1*	1.7 ± 0.1*

*$P < .05$ vs CS; ANOVA and SNK-test.

While machine perfusion with cold preservation solutions and controlled oxygenated rewarming up to mid-thermal temperatures has previously been described as a successful method to improve post-transplant outcome and to mitigate "rewarming injury" to the graft,[11,20] this is the first study to investigate controlled warming up from hypothermia to normothermic temperature prior to warm reperfusion.

It was found that liver injury could be significantly reduced by the controlled rewarming technique, but in our model, the therapeutic effect could not be enhanced by extension of the rewarming process up to temperatures higher than 20°C.

However, Banan and coworkers, who only investigated abruptly started postpreservation perfusion with a sanguineous medium at 20°C or 38°C, still found some protective effects in slowly increasing temperature from 20°C to 38°C instead of immediate perfusion at 38°C.[21]

Notwithstanding that cell culture experiments from Rauen et al have demonstrated that the majority of cell damage, induced by rapid warming up of the medium are only observed upon transition from hypo- to midthermia (between 8 and 16°C), suggesting the "rewarming injury" to be operative predominantly at temperatures below approximately 16°C.[22]

Therefore, it might be conjectured that controlling the temperature rise up to 20°C might be a sufficient measure to mitigate rewarming injury in this context. Moreover, adequate restitution of cellular redox-homeostasis would not necessarily require normothermic temperatures.[3,23]

On the other hand, liver reconditioning at normothermia possibly might rely on the presence of oxygen carriers to solicit optimal results. At least for long-term liver perfusions, Alexander et al[24] have suggested oxygen carries to beneficially influence liver injury parameters in a rat liver model at fixed flow conditions. Likewise, Ferrigno et al[25] recently reported that hypoxic signaling (ie, upregulation of HIF-1 alpha) is triggered upon normothermic rat liver perfusion without oxygen carriers at flow rates of 2.6 mL/g/min.

In our study, hepatic flow rates went somewhat higher and rose up to about 3 mL/g/min during normothermic perfusion and hepatic venous effluent partial pressures of oxygen never fell below 200 mm Hg during the controlled rewarming perfusion, which rather indicates adequate oxygen delivery to the tissue.

Notwithstanding, we cannot completely rule out the possibility that oxygen carriers might have improved the results in the COR35 group and this possibility should be kept in mind in further studies. However, detrimental effects of high oxygen delivery by artificial oxygen carriers have also been reported in postischemic cardiomyocytes.[26]

Another possible criticism of our COR35 protocol might be seen in the increased velocity of warming up in comparison with the COR20 group. In preliminary experiments, we could not observe any improvements by extending the total period of controlled rewarming to 180 minutes instead of only 90 minutes, thus achieving a similar rate of tissue rewarming than in the COR20 group. In fact, the results turned out to be virtually identical to those after only 90 minutes of rewarming (eg, maximal release of AST: 432 ± 35 U/L; bile production upon reperfusion: 23.8 ± 5 µL/g/min). It was hence concluded that slowing down the time kinetic of warming up in the COR35 group is not of essence.

While not adding therapeutic benefits over the COR20 protocol, warming up to normothermia might foster evaluative purposes, as metabolic integrity would probably be best evaluated at circumstances of physiological homeostasis including temperature.

Moreover, the increased metabolism at higher temperatures should globally result in quantitative higher readouts for any parameter under investigation and thus possibly increase diagnostic discrimination.

In our experiments, increased metabolism and enhanced microcirculation at normothermic perfusion resulted in slightly, albeit mostly not significantly more elevated levels of lactate and transaminases in the perfusate. Likewise, hepatic net loss of glucose was reduced during machine perfusion at 35°C as compared to 20°C, putatively relating to incipient enhancement of glucose uptake by viable hepatocytes.

Bile production upon normothermic machine perfusion prior to transplantation has been proposed as easily and readily available predictive parameter,[27] albeit without actual control by consecutive reperfusion experiments.

In our experiments, bile production was consistently observed during warming up to 35°C and did well correlate to ulterior liver function upon reperfusion. Further analysis of bile composition, as

previously proposed by the group of Porte,[28] might have led to an even better quality of viability assessment. However, in our study on rat livers, we did not perform further analyses of the bile, due to the overall very limited quantities of bile produced during COR.

In agreement with the theoretical reduction in hepatic metabolism at lower temperatures, bile collection during COR20 was less consistent. Possibly, discriminative amounts of bile might be easier to obtain in a larger size model or in humans. However, surrogate readouts also easily available on site during machine perfusion and relating to structural liver injury were still useful to help viability assessment even at mid-thermic perfusion. Potassium, for instance, taken up by rewarmed hepatocytes upon energetic activation (eg, by Na^+-K^+-ATPase), results in lower K^+ perfusate concentrations that are linked to resumption of liver metabolism and fairly well correlated with ulterior graft function upon reperfusion.

Due to the technical limitations, immanent to this small animal in vitro model, first conclusions have to be drawn carefully. However, controlled rewarming up to normothermia appears to be a feasible approach to gain functional data on graft viability prior to actual transplantation. Notably, the higher metabolic rate at normothermia also increases the risk of adverse repercussions on graft integrity in case of inadequate perfusion conditions, either by model or by accident. Therefore, further studies are encouraged to validate and optimize technical details prior to final clinical recommendations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Charlotte von Horn: Wrote paper, performed research, and analyzed data; Hideo A. Baba: Performed research and analyzed data; Patrik Hannaert: Analyzed data and wrote paper; Thierry Hauet: Designed study and revised paper; Henri Leuvenink: Designed study and revised paper; Andreas Paul: Designed research and revised paper; Thomas Minor: Designed research and wrote paper.

ORCID

Thomas Minor http://orcid.org/0000-0002-7124-3590

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