

The influence of storage temperature during machine perfusion on preservation quality of marginal donor livers[☆]

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ABSTRACT

Background: Although non-heart-beating donors have the potential to increase the number of available organs, the livers are used very seldom because of the risk of primary non-function. There is evidence that machine perfusion is able to improve the preservation of marginal organs, and therefore we evaluated in our study the influence of the perfusate temperature during oxygenated machine perfusion on the graft quality.

Methods: Livers from male Wistar rats were harvested after 60-min warm ischemia induced by cardiac arrest. The portal vein was cannulated and the liver flushed with Lifor[®] (Lifeblood Medical, Inc.) organ preservation solution for oxygenated machine perfusion (MP) at 4, 12 or 21 °C. Other livers were flushed with HTK and stored at 4 °C by conventional cold storage (4 °C-CS). Furthermore two groups with either warm ischemic damage only or without any ischemic damage serve as control groups. After 6 h of either machine perfusion or cold storage all livers were normothermic reperfused with Krebs–Henseleit buffer, and functional as well as structural data were analyzed.

Results: Contrary to livers stored by static cold storage, machine perfused livers showed independently of the perfusate temperature a significantly decreased enzyme release of hepatic transaminases (ALT) during isolated reperfusion. Increasing the machine perfusion temperature to 21 °C resulted in a marked reduction of portal venous resistance and an increased bile production.

Conclusions: Oxygenated machine perfusion improves viability of livers after prolonged warm ischemic damage. Elevated perfusion temperature of 21 °C reconstitutes the hepatic functional capacity better than perfusion at 4 or 12 °C.

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Introduction

The widening gap between potential organ recipients and donated organs results in an increased waiting list mortality worldwide. According to the Eurotransplant registry in 2006, altogether 1436 cadaveric liver transplantations were performed compared to 2319 patients on the waiting lists. Almost 500 of these patients died before a suitable organ was available (Eurotransplant Annual report 2006). Since the number of available organs from brain dead donors has remained stable, renewed interest in organs from donors after cardiac death (DCD) has originated [22]. However, not only the increasing demand of organs, but also the poten-

tial wish for organ donation of a deceased person after cardiac death provoked discussions on this topic [20].

Organs from donors after cardiac death are exposed to a prolonged time of warm ischemia, which leads to several metabolic disorders. Lack of oxygen results in conversion of aerobic metabolism into anaerobic metabolism with well-known aftermaths like accumulation of lactate and finally intracellular acidosis. The rapid decline of adenosine triphosphate under anoxic conditions provokes a malfunction of the sodium/potassium pump, thereby causing loss of electrolyte gradients and membrane integrity which leads to cellular edema [6]. Additionally, the degradation product of adenosine, namely hypoxanthine, is converted under post-ischemic conditions by xanthine oxidase into xanthine and urate. Hereby a release of oxygen free radicals causes lipid peroxidation of the cell membranes and structural changes of the parenchymal and endothelial cells [9].

After warm ischemia organs are flushed during retrieval with cold perfusion solutions such as University of Wisconsin solution

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(UW) or Histidine–Tryptophan–Ketoglutarate solution (HTK) to slow down the metabolism to 4 °C. Cold ischemia augments the cellular injury which occurred during the warm ischemic period. Especially the sinusoidal endothelial lining cells are susceptible towards structural changes during cold ischemia [11].

The aim of his study was to evaluate the influence of oxygenated machine perfusion at different temperatures on the organ viability after a prolonged time of warm ischemia. There are some reports indicating a potential benefit of preserving livers at higher temperatures than 4 °C to diminish the above mentioned negative consequences of cold ischemic storage [29]. For the perfusion of livers at temperatures higher than 4 °C a recently developed new organ preservation solution (Lifor, Lifeblood Medical, Inc., Freehold, NJ) was used. The effectiveness of Lifor for preserving hearts at mild hypothermic temperatures was shown in an isolated guinea pig heart model [24].

Materials and methods

All animal experiments were performed according to the regulations of the federal law. The principles of laboratory animal care (NIH Publication No. 85–23, revised 1985) were followed.

Experimental protocol

Male Wistar rats weighing between 250 and 280 g were housed in temperature and light controlled cages with access to tap water and food ad libitum. Anesthesia was performed by isoflurane inhalation. The abdomen was opened by a midline incision and the liver mobilized from all ligamentous attachments. The common bile duct was cannulated with 27G polyethylene tubing, allowing for collection of total bile outflow during reperfusion. Cardiac arrest was induced by phrenotomy. After 60-min warm ischemia the portal vein was cannulated and the liver flushed with 20 ml of Lifor (Lifeblood Medical, Inc.) organ preservation solution (at 4, 12 and 21 °C) or 60 ml of Histidine–Tryptophan–Ketoglutarate (HTK) solution at 4 °C. Livers flushed with Lifor solution were connected to a machine perfusion circuit and perfused for 6 h also with Lifor solution. The recirculating perfusion setup comprised a non-pulsatile roller pump to maintain a flow rate of 1 ml/min/g liver weight and an oxygenator (SafeMini[®], Polystan, Denmark) for creating an oxygen partial pressure of $pO_2 > 400$ mmHG. In general the perfusion strategy, constant flow vs. constant pressure, is an intensively debated topic. We decided for a constant flow model with a flow rate of 1 ml/min/g liver weight as an average of different previously used study protocols for hypothermic machine perfusion as reviewed by Dutkowski et al. [8]. The perfusate temperature was adjusted according to our study protocol at 4, 12 or 21 °C by means of a Ministat[®] (Huber, Offenburg, Germany) water bath. Conventional stored livers were submerged in HTK for 6 h at 4 °C. Control livers without any ischemic damage were connected immediately after harvesting to the oxygenated reperfusion circuit. Other control livers were subjected to 1-h warm ischemia and afterward connected to the reperfusion circuit without any cold ischemic period.

Oxygenated reperfusion

In order to account for the period of slow rewarming of the organ during surgical implantation in vivo, all livers were exposed to 21 °C for 30 min before reperfusion started. Immediately before reperfusion, all livers were flushed with 10 ml saline solution via the portal vein.

Sixty-minute portal venous normothermic (37 °C) reperfusion was performed in vitro in a recirculating system at a constant flow

of 3 ml/min/g liver weight with oxygenated (95%O₂/5%CO₂) Krebs–Henseleit solution. Perfusion with Krebs–Henseleit solution is a well established model for evaluation of ischemia–reperfusion damage for solid organs [10]. In contrast to organ transplantation models isolated reperfusion allows a high rate of reproducibility and a close control of the experimental setting. As we reperfused the livers for a limited time of 60 min, no additives like albumin were included, which for longer reperfusion times or detecting minor differences in between experimental groups is recommended [16]. Nevertheless for better classification of the presented results two further control groups with livers without any ischemic damage and livers with 1-h warm ischemia only are shown. Here a constant flow model was chosen for the purpose of using the resulting portal venous resistance as a marker for the endothelial integrity. The liver was placed floating in the Krebs–Henseleit solution in order to assure homogenous perfusate distribution within the liver. The infrahepatic vena cava was ligated and the suprahepatic vena cava cannulated, allowing for sample collection and recirculation of the effluent perfusate.

Analysis

Vascular resistance upon reperfusion

Portal venous pressure (PVP) was continuously measured during isolated perfusion by means of a water column connected to the portal inflow line and precalibrated to the calculated flow of 1 ml/min/g liver during machine perfusion and 3 ml/min/g liver during isolated reperfusion using PE-catheters of length and size identical to the one used for the perfusion of the livers. Portal venous resistance was calculated according to flow rate and liver weight, and given in dyn s cm^5 .

Bile flow

Functional integrity and homogenous flow distribution within the livers was estimated by the collection of the bile produced and the overall bile production determined in $\mu\text{l/g liver weight/60 min}$.

Liver enzymes

Liver enzyme release of alanine-aminotransferase (ALT) in the effluent perfusate was assessed photometrically using commercialized standard kits. The liver enzyme release was determined in mU/g liver weight .

Histology

For evaluation of histopathological changes, tissue biopsies were taken at the end of isolated reperfusion. Liver tissue was cut into small blocks (3 mm thickness) and fixed by immersion in 4% buffered formalin overnight at 4 °C. The blocks were embedded in paraffin and cut into 2 μm sections using a microtome. After hematoxy-eosin staining, histological changes focusing on hepatocyte necrosis and endothelial swelling were evaluated by means of a semi quantitative score from 0 to 3. Endothelial cell damage was graded by the extension of swelling and detachment from the sinusoidal lining. Hepatocyte damage was graded by the extension of vacuolation of hepatocellular cytoplasm, condensation of chromatin and loss of cell contacts.

Statistics

The results are expressed as means \pm SE. For all data, one-way ANOVA with adequate post hoc analysis (Student–Newman–Keuls test) was performed. Differences were considered significant at $p < 0.05$.

Results

Portal venous resistance

Vascular flow characteristics were approximated by the portal pressure, which was continuously measured at constant flow rates of 1 ml/min/g liver weight during machine perfusion, and 3 ml/min/g liver weight during normothermic reperfusion. During the 6 h machine perfusion a significant reduced portal venous resistance (PVR) was observed for livers perfused at 21 °C (21 °C-MP) compared to perfusion at 4 or 12 °C (Fig. 1A). With the normothermic reperfusion at 37 °C livers stored at 4 °C by conventional static storage in HTK (4 °C-CS) and machine perfusion at 4 and 12 °C revealed elevated portal venous resistances. In contrast, for livers stored at 21 °C (21 °C-MP) a marked reduction of PVR was shown. In fact the PVR for this group was as low as for non-ischemic control livers (Fig. 1B).

Bile flow

Cumulative bile flow during 6 h of machine perfusion within the 21 °C-MP group was significantly higher ($2 \pm 0.2 \mu\text{l/g}$ liver weight/6 h) compared to preservation at 4 or 12 °C (4 °C-MP 0.7 ± 0.4 and 12 °C-MP $0.8 \pm 0.2 \mu\text{l/g}$ liver weight/6 h) (Fig. 2A). Accordingly, with the normothermic reperfusion livers preserved

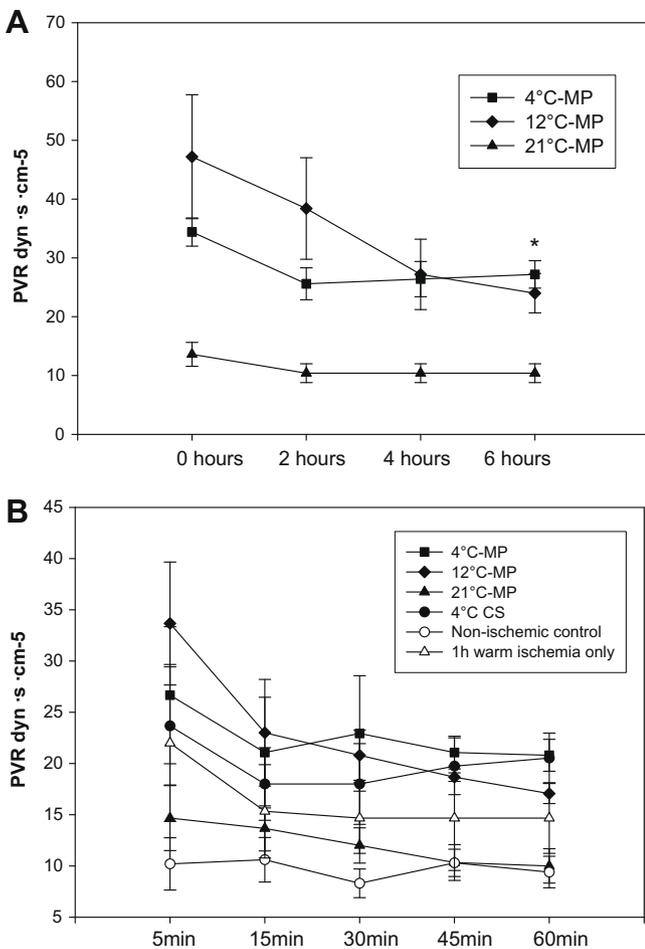


Fig. 1. Portal venous resistance during 6 h machine perfusion (A) and subsequent 60 min normothermic reperfusion (B). Machine perfusion was performed at 4, 12 and 21 °C. Control livers were stored by conventional static cold storage at 4 °C, further results for non-ischemic controls and livers subjected to 1-h warm ischemia only are shown; values are given as means \pm SE; $p < 0.05$ vs. 21 °C-MP; $n = 5$.

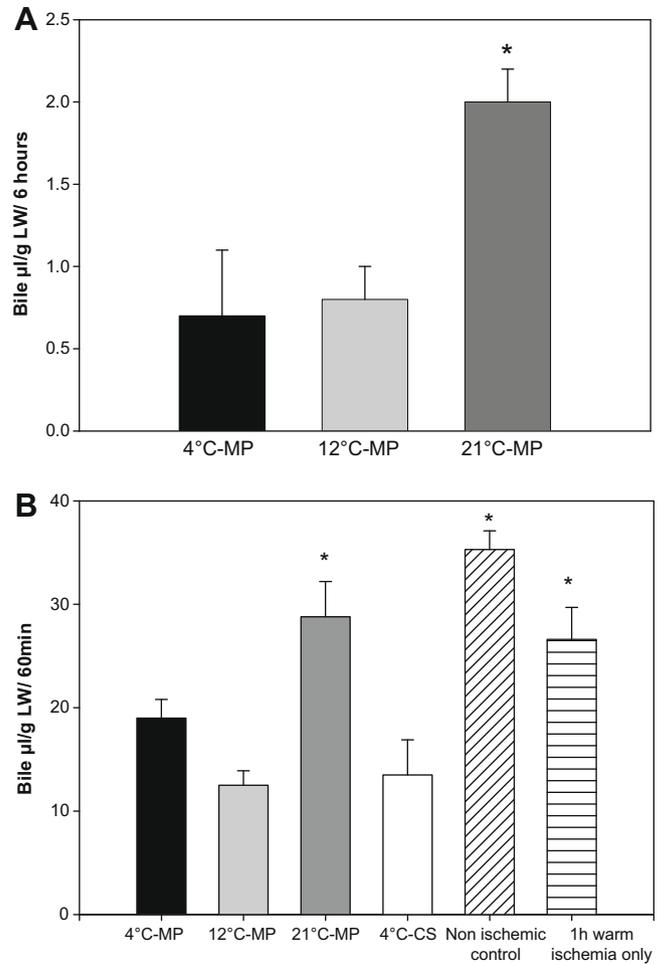


Fig. 2. Bile production during 6 h machine perfusion (A) and subsequent 60 min normothermic reperfusion (B). Machine perfusion was performed at 4, 12 and 21 °C. Control livers were stored by conventional static cold storage at 4 °C, further results for non-ischemic controls and livers subjected to 1-h warm ischemia only are shown; values are given as means \pm SE; $p < 0.05$ vs. 4 °C-MP; 12 °C-MP, 4 °C-CS; $n = 5$.

at 21 °C showed a marked increase in post-ischemic recovery of biliary flow, whereas no significant difference was observed between static storage at 4 °C and machine perfusion at 4 or 12 °C. The difference between group 21 °C-MP and non-ischemic control livers or livers exposed to 1-h warm ischemia only was not significant (Fig. 2B).

Lactate production and oxygen consumption

To differentiate between aerobic and anaerobic metabolism during machine perfusion at 4, 12 and 21 °C lactate concentration and oxygen consumption were recorded. There was a constant increase of lactate concentration in all groups and perfusion at 21 °C after 6 h revealed a significant higher lactate production compared to perfusion at 4 or 12 °C (Fig. 3). The calculated oxygen consumption was almost equal in all groups taking into account the reduced physical solubility of gaseous oxygen at higher temperatures (4 °C-MP $18.25 \pm 2.04 \mu\text{l/g/min}$ LW; 12 °C-MP $19.36 \pm 1.05 \mu\text{l/g/min}$ LW; 21 °C-MP $20.57 \pm 0.95 \mu\text{l/g/min}$ LW, data not shown).

Liver enzymes

Release of alanine-aminotransferase (ALT) was measured in the effluent perfusate and taken as a general parameter of parenchymal injury of the liver during machine perfusion and isolated

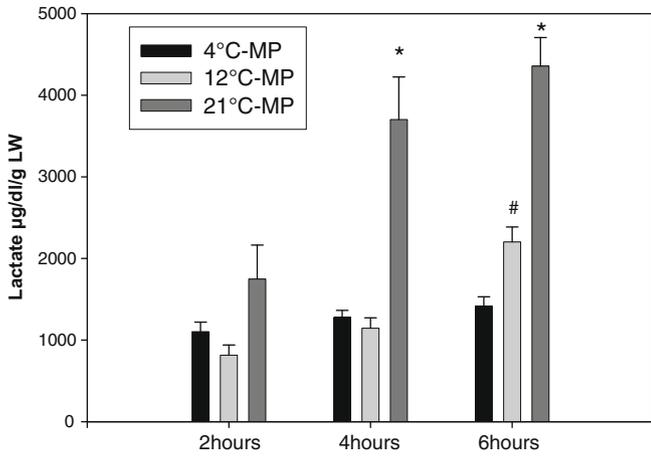


Fig. 3. Lactate concentration during 6 h of machine perfusion. Machine perfusion was performed at 4, 12 and 21 °C. Values are given as means ± SE; $p < 0.001$ vs. 4 °C-MP; 12 °C-MP; # $p < 0.05$ vs. 4 °C-MP; $n = 5$.

reperfusion. As shown in Fig. 4, a constant release of ALT during machine perfusion was observed for all groups. Perfusion at 12 and 21 °C resulted after 6 h machine perfusion in a significant higher enzyme release compared to machine perfusion at 4 °C. For comparison results of non-ischemic controls and livers subjected to 1-h warm ischemia only are shown.

After 60 min of normothermic reperfusion, livers stored static at 4 °C (4 °C-CS) revealed a significant elevated enzyme release compared to livers stored by machine perfusion. But again for livers preserved at 21 °C (21 °C-MP) a more pronounced release of ALT was observed compared to 4 °C-MP and 12 °C-MP (Fig. 5).

Histology

Based on our semi quantitative score from 0 to 3, endothelial cell damage was only found in a low degree in all groups.

Although we observed in the livers in groups 4 °C-CS and 21 °C-MP a tendency of reduced endothelial damage, no statistical significant difference compared to groups 4 °C-MP and 12 °C-MP was detected (Fig. 6A). The degree of histological evident hepatocyte damage also did not differ statistically between the groups (Fig. 6B).

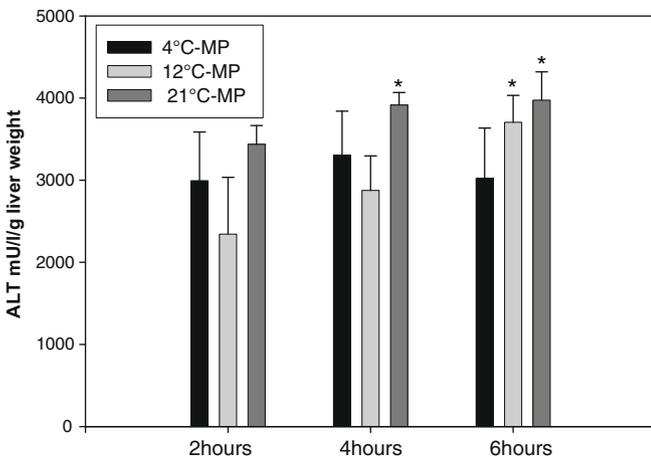


Fig. 4. ALT release during 6 h machine perfusion at 4, 12 and 21 °C; values are given as means ± SE; $p < 0.05$ vs. 4 °C-MP; $n = 5$.

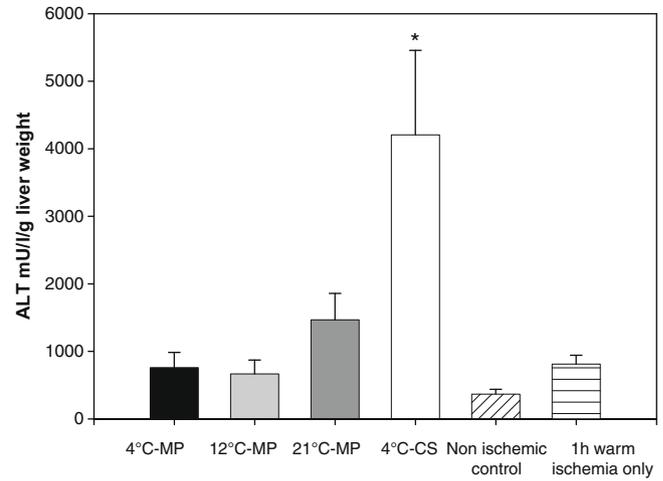


Fig. 5. ALT release during 60 min normothermic reperfusion after machine perfusion at 4, 12 and 21 °C. Control livers were stored by conventional static cold storage at 4 °C, further results for non-ischemic controls and livers subjected to 1-h warm ischemia only are shown; values are given as means ± SE; $p < 0.05$ vs. 4 °C-MP; 12 °C-MP, 4 °C-CS; non-ischemic and 1-h warm ischemia only control groups; $n = 5$.

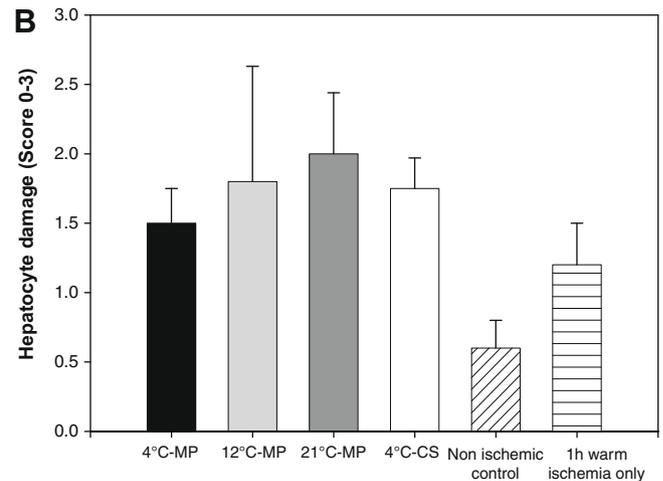
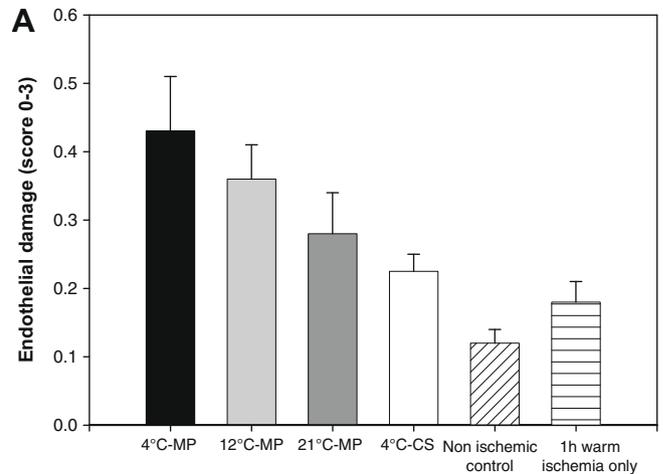


Fig. 6. Endothelial damage (A) and hepatocellular damage (B) as measured by a semiquantitative score from 0 to 3 at the end of normothermic reperfusion after machine perfusion at 4, 12 and 21 °C and static storage at 4 °C, further results for non-ischemic controls and livers subjected to 1-h warm ischemia only are shown; $n = 5$.

Discussion

The aim of this study was to evaluate the influence of oxygenated machine perfusion at different temperatures on the preservation quality of livers from donors after cardiac death (DCD). Due to the shortage of organs and the potential wish for organ donation from donors after cardiac death a renewed interest for these organs prone to prolonged warm ischemic damage has aroused. Multiple studies proved the possibility of successfully grafting organs for kidney transplantations with warm ischemic times longer than 30 min [4,31]. However, a longer ischemic time increases the risk of primary non- or dysfunction [17]. Machine perfusion was shown to be effective in clinical and experimental studies for improvement of the viability of kidneys exposed to prolonged warm ischemic times [14,15]. For liver transplantation with organs donated after cardiac death only limited clinical experience exists. Reported primary graft function rates differ from 50% to 90% [2,5,21] depending on the

length of warm and cold ischemic time, and even after successful initial function an increased incidence of non-anastomotic biliary strictures is described in the follow-up period [1,12,25]. For improving viability of predamaged livers different experimental strategies like machine perfusion [13,19], venous systemic oxygen persufflation [18] or cardio-pulmonary bypassing [3] were developed and partly transferred into clinical practice [3,27]. Machine perfusion has, despite its promising experimental results, not yet been introduced into the clinical routine due to its complexity. As recently reviewed by Vekemans et al. [30], machine perfusion is either performed at hypothermia of approx. 4 °C or at body temperature at approx. 37 °C. For warm ischemia time of 60 min or longer, machine perfusion at 37 °C with autologous blood [23] or at 4 °C with modified starch omitted UW solution [7] was able to revitalize the liver for successful transplantation. There are some reports on the feasibility of machine perfusion at subnormothermic temperatures of 21 °C with possible advantages like reducing the complexity of the machine perfusion setup

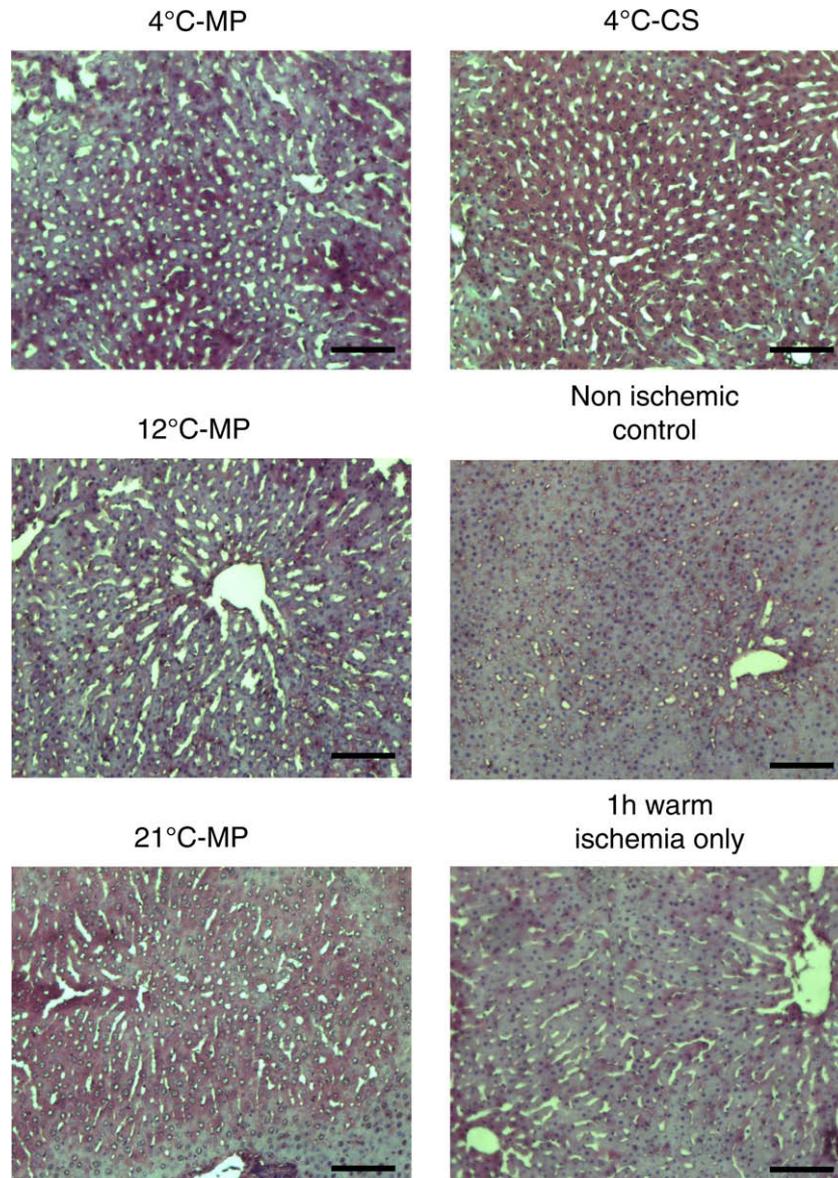


Fig. 7. Representative photomicrographs of livers after either MP at 4, 12 and 21 °C and conventional cold storage at 4 °C are shown. Further totally non-ischemic controls and liver subjected to 1-h warm ischemia only are shown. HE-staining, magnification 100× (scale bars 100 μm). According to Fig. 6, only minor alterations concerning hepatocellular or endothelial damage are observed without obvious differences in between all groups.

by omitting additional dialysis and cooling/heating devices [28]. In this study we could show consistent with previous reports an improved preservation of livers after 1 h of warm ischemia using oxygenated machine perfusion. Livers preserved by simple cold storage at 4 °C revealed significantly higher leakage of ALT as a general parameter of parenchymal damage compared to livers preserved by machine perfusion, irrespectively of the applied temperature. Nevertheless livers perfused at 12 and 21 °C had an accelerated increase of ALT release during machine perfusion compared to livers perfused at 4 °C. This effect also became visible with isolated reperfusion of livers from group 21 °C-MP, which had a markedly, although not significantly, higher enzyme release after 60 min compared to livers at 4 or 12 °C. The hepatocyte oxygen demand during preservation at 21 °C was not completely met by the hemoglobin-free perfusion medium as also reflected by the increased lactate concentration after 6 h machine perfusion.

For evaluating the functional integrity of the liver, bile production was measured during machine perfusion for preservation and during isolated reperfusion. Hereby livers stored at 21 °C-MP revealed a significantly higher bile production already during machine perfusion, but also after isolated reperfusion. The augmented bile production during machine perfusion at 21 °C can be explained by the increased metabolic activity at elevated temperatures. We assume that perfusion at 21 °C enables the liver to build up its energy stores more effectively, resulting in a rapid initial function during reperfusion. Likewise it was shown from Vairretti et al. that after machine perfusion at subnormothermic temperatures the ATP tissue content is higher compared to cold preserved liver grafts [29].

In this study we used a constant flow of 1 ml/min/g liver weight and measured the portal venous resistance as a parameter of sinusoidal endothelial integrity. The endothelium is supposed to be more susceptible to structural damages by cold ischemia [11]. Furthermore machine perfusion may cause endothelial damage by shear stress, especially provoked by elevated perfusion pressures [26]. For livers preserved at 21 °C we observed during machine perfusion and isolated reperfusion a significantly reduced portal venous resistance. Livers preserved by machine perfusion at 4 and 12 °C and also livers stored by static cold storage at 4 °C revealed elevated perfusion pressures. This was partly confirmed by histological evaluation, which demonstrated although not significantly reduced endothelial damage for livers from groups 21 °C-MP and 4 °C-CS. Representative images from each group are shown in Fig. 7. The endothelium of livers preserved by machine perfusion at lower temperatures (4 and 12 °C) appears more often detached from the sinusoidal layer. Altogether histological alterations are only marginal compared to non-ischemic control livers, probably related to the limited time of reperfusion with a cell free medium, e.g. no interactions between inflammatory active and hepatic cells are observable. To further discriminate the different influences of flow rate and perfusate temperature on the sinusoidal integrity further studies using either different flow rates or constant pressure models in conjunction with different temperatures are needed. Nevertheless we conclude with respect to our data that a reduction of the flow rate below 1 ml/min/g liver weight probably will not allow for a sufficient oxygen delivery when using a hemoglobin-free perfusate.

We summarize that oxygenated machine perfusion facilitates the reconstitution of the functional capacity of the liver. In contrast to preservation at 4 or 12 °C machine perfusion at 21 °C has a beneficial effect on the initial organ function and the structural integrity of the sinusoidal endothelium. This advantage has to be balanced against an increase of hepatocellular damage for livers

preserved at 21 °C, probably caused by an oxygen debt when perfusion is performed without specific oxygen carriers. So we finally conclude that further studies are necessary to define the optimal setting with respect to the perfusion medium and flow rate for perfusion of predamaged organs at elevated temperatures.

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