Strategies to improve outcome after partial liver resection

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Liver protection by hypothermic perfusion at different temperatures during total vascular exclusion

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Abstract

Introduction. In situ hypothermic perfusion (HP) can be applied to attenuate ischemia and reperfusion (I/R) injury during liver resection under total vascular exclusion (TVE). This study examines the protective effect of cooling by HP at 20°C and 28°C as compared to no HP during TVE in a porcine liver I/R model.

Methods. Twenty-one pigs underwent 60min TVE of the liver followed by 24h reperfusion. HP was performed via the portal vein using ringer-lactate solution of 4°C. Pigs were assigned to three groups: TVE without HP (no-HP, n=9), TVE with HP at 28°C (HP-28, n=6) and TVE with HP at 20°C (HP-20, n=6).

Results. Perfusion volumes during TVE were 5.1±0.5 L and 17.3±1.7 L in HP-28 and HP-20, respectively (p<0.05). Aspartate aminotransferase (AST) after 24h reperfusion was 1172±440 U/L in no-HP as compared to 223±69 U/L and 180±22 U/L in HP-28 and HP-20, respectively (p<0.05). No differences in liver function or histopathology were found between the HP-28 and HP-20 groups.

Conclusion. HP at 20°C is equally effective in preserving liver function and preventing hepatocellular injury under TVE as compared to HP at 28°C. HP at 28°C is advised, because of the lesser perfusion volume necessary for cooling of the liver.
Introduction

Operative blood loss is a significant factor causing morbidity after partial liver resections. One way to prevent blood loss during partial liver resection is to clamp the portal vein and hepatic artery (Pringle's maneuver). When backflow from the caval vein still causes important blood loss or when part of the caval vein is involved in the tumour requiring caval reconstruction, total vascular exclusion (TVE) can be applied. TVE consists of clamping of the portal vein, the hepatic artery and the supra-and infrahepatic caval vein. A drawback of these clamping techniques is that ischemia and reperfusion (I/R) injury of the liver is induced. Especially in patients with underlying liver disease, hepatic ischemia for periods of 60 minutes or longer leads to increased risk of postoperative liver failure and death.

In situ hypothermic perfusion (HP) of the liver during TVE is a technique that can be applied to reduce liver I/R injury. The protective effect of HP is based upon the concept that hypothermia leads to diminished metabolism and therefore decreased energy and oxygen demand. However, from organ preservation studies it is known that parenchymal liver cells and sinusoidal endothelial cells (SECs) respond differently to hypothermic conditions. Parenchymal cells and SECs are both damaged after prolonged periods of warm ischemia, whereas cold ischemia (4°C) mostly affects SECs and is associated with cell vacuolization and necrosis. This implies that the temperature at which the liver is optimally protected during ischemia lies somewhere between 37°C and 4°C.

In previous studies from our laboratory, using a partial hepatectomy model in the pig, it was found that HP under TVE at subnormothermic temperature (28°C), attenuated hepatocyte damage compared to perfusion at 37°C or no perfusion at all, and that SEC function was spared. The question then arises whether HP below 28°C increases liver protection, bearing in mind that cold storage of liver grafts prior to transplantation is undertaken at 4°C. It is known that in mice, 90 minutes of partial liver ischemia at 4°C does not impair the microcirculation. However, these results were never confirmed in a large animal model. Hence, the aim of this study was to assess the effect of cooling of the liver by in situ HP at 20°C in a pig liver model of 60 minutes TVE, focusing on parenchymal and SEC damage and function.

Materials and methods

Animals

Twenty-one male pigs (Vendrig, Amsterdam, The Netherlands) were used, weighing 40-50 kg. All pigs were allowed to acclimatize to the laboratory environment for 7 days with free access to standard laboratory food (Blok, Woerden, The Netherlands) and water. Pigs were fasted overnight with free access to water before use in experiments. The experiments were approved by the Animal Ethics Committee of the Academic Medical Center, University of Amsterdam, The Netherlands.
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Anesthesia
Methods concerning premedication and anaesthesia were described previously. Phenylephrine was not used in this study.

Surgical procedure
Experiments were performed under sterile conditions. Cannulae were placed for administration of anaesthetics and fluids, for continuous measurement of mean arterial pressure (MAP), heart rate and blood temperature, and for blood sampling. A physiological body temperature (in pigs 37-38°C) was maintained by means of a heating lamp and a heating mat. A midline laparotomy was performed followed by dissection (Force™ 20, Valleylab, Boulder, USA) and cannulation of the common bile duct for continuous measurement of bile production. Another cannula was placed into the distal common bile duct and the duodenum to return bile to the animal. The portal vein, the hepatic artery and the caval vein were dissected free and total vascular exclusion (TVE) was applied by clamping the infra- and suprahepatic caval vein, the common hepatic artery and the portal vein. To prevent splanchnic congestion and venous congestion in the lower limbs, a situation that is lethal to pigs, a polyethylene prosthesis with one side port was used to bypass blood from the infrahepatic caval vein and portal vein to the suprahepatic caval vein. After a transverse incision into the infrahepatic caval vein, the prosthesis was inserted and guided towards the cranial side. The prosthesis was fixed around the caval vein with two slings, which were placed suprahepatic as well as infrahepatic. Then the portal vein was divided and the proximal end was positioned over the side port of the prosthesis after which the clamps were removed from the caval vein and portal vein. There was no need for systemic heparinization using the prosthesis. The distal end of the portal vein was used to perfuse the liver with cold (4°C) ringer-lactate solution. The perfusate was allowed to flow through the transverse incision of the caval vein into the abdominal cavity and taken out by suction (fig 1a).

Five minutes before the end of TVE, perfusion was stopped and the distal portal vein was reconnected with the proximal side by interposition of a polyethylene tube. A ligature was placed cranial from the side port around the infrahepatic caval vein and the sling around the suprahepatic caval vein was removed. Then reperfusion of the liver was applied by releasing the clamps from the portal vein and the common hepatic artery and the abdomen was closed (fig 1b).

Pilot experiments
Pilot experiments were performed to validate the model. It was found that perfusion via the portal vein leads to a more rapid and deeper cooling than perfusion via the hepatic artery. For this reason the portal vein was used for HP during further experiments. Also, it was found that when applying a maximal flow of 500 mL/hour during 1 hour TVE without extra isolating measures, the lowest possible mean core liver temperature is 20°C. To examine the effect of perfusion without cooling, perfusion at 37°C with ringer-lactate was applied during 60 min. TVE in 3 pigs. All animals died directly after TVE due to massive congestion of the liver leading to non-treatable shock and death.
Figure 1. Situation created during total vascular exclusion (TVE) (a) and during reperfusion (b)

1a: During TVE, a clamp is placed on the hepatic artery (HA). A polyethylene prosthesis (PP) is placed inside the caval vein (CV). It is fixed into the caval vein using two slings, which were placed in suprahepatic as well as in infrahepatic position. The portal vein (PV) is divided and the proximal end is connected to the side port of the prosthesis. The distal end of the portal vein is connected to a perfusion line (PL) for hypothermic perfusion. The perfusate is allowed to drain through the transverse incision of the caval vein into the abdominal cavity. 1b: During reperfusion, a ligature is placed cranial from the side port around the infrahepatic caval vein and the sling around the suprahepatic caval vein is removed. Both sides of the portal vein are reconnected. The clamp from the hepatic artery is removed.
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Experimental design

All 21 pigs underwent 60 minutes of TVE followed by 24 hours of reperfusion. The animals were randomly divided into three groups. The first group did not undergo HP during TVE (no-HP; n=9), the second group was perfused with 4°C ringer-lactate solution maintaining a mean core liver temperature of 28°C (HP-28; n=6) and the third group received HP with 4°C ringer-lactate solution maintaining a mean core liver temperature of 20°C (HP-20; n=6). The core liver temperature was measured by two temperature probes (Metatemp, Industrial Temperature Measurement, Steenbergen, The Netherlands) and continuously monitored (Thermistor 4070, Mallinckrodt, St. Louis, USA). The probes were placed within the right lateral and the left medial lobes. The tip was placed 4-5 cm within the liver tissue. The average temperature was used for adjusting flow of the perfusion solution. Perfusion was powered by a roller pump (Gambro Instrumenta AB, Lund, Sweden) and the flow was adjusted from 50 to 500 ml/hour depending on the core liver temperature. Flow was started at 200-400 ml/hour and when the desired core liver temperature was achieved, flow was adjusted to 50 ml/hour, which was suitable to maintain core liver temperature at the desired level. In the group without perfusion, the liver temperature was maintained at 37-38°C. After TVE, the pigs remained under anesthesia for 24 hours when they were sacrificed.

Hepatocellular injury

To assess hepatocellular injury, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations were measured in arterial blood samples taken before TVE and after 1, 6, 12 and 24 hours of reperfusion using routine laboratory techniques.

Hepatocellular function

Bile production was measured before as well as during and after TVE as a parameter of excretory function of the hepatocytes and hepatic blood flow. To assess clearance function of the liver, indocyanine green (ICG) clearance was measured before TVE and 6 and 24 hours after TVE. After a bolus intravenous injection of 0.5 mg/kg ICG, dissolved in 10 ml 5% glucose solution (Infracyanine, Laboratoires Pharmaceutiques, Paris, France), blood samples were taken every 5 minutes until 20 minutes. ICG concentration was measured by spectrophotometric analysis (λ=805nm). ICG clearance was expressed as % of ICG cleared from the circulation 15 minutes after bolus injection.

Sinusoidal endothelial cell (SEC) function

Clearance of hyaluronic acid (HA) was used as a parameter for SEC function. HA clearance was measured before and 24 hours after TVE. A bolus intravenous injection of 5 mg HA (Healon' GV, Pharmacia & Upjohn AB, Uppsala, Sweden) was followed by blood sampling after one minute and every 10 minutes until 60 minutes. HA clearance was expressed as % HA cleared within 60 minutes after bolus injection. During TVE, HA concentrations were measured at 0, 15, 30, 45 and 60 minutes without bolus injection. HA concentration was measured in heparinized plasma samples using a commercially available ELISA kit (Corgenix, Inc., Westminster, USA).
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Inflammatory response
To evaluate the inflammatory response, interleukin-6 (IL-6) was measured in heparinized plasma samples using a commercially available ELISA kit (Quantikine®, R&D Systems, Minneapolis, USA). Also, leukocyte count was measured in arterial blood samples using routine laboratory techniques.

Histopathology
Liver biopsies were taken from the rim of the right medial liver lobe. They were taken before, 10 minutes after and 1, 6 and 24 hours after TVE. After fixation in 4% buffered formaldehyde, biopsies were embedded in paraffin and 4 μm sections were cut. Sections were stained with haematoxylin and eosin for semi-quantitative microscopic analysis. Histopathology was examined blindly by 2 independent observers scoring necrosis, cellular vacuolization and leukocyte accumulation. Necrosis and cellular vacuolization were scored as percentage of cells per 10 microscopic fields (20 times magnification). A score of 0 to 5 was applied: 0=0%, 1=1-10%, 2=11-20%, 3=21-30%, 4=31-50% and 5=51-100%. Leukocyte accumulation was scored from 0 to 4 according to extensiveness per 10 microscopic fields (20 times magnification). The maximum score was 14.

Statistical analysis
Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 12.0.1 for Windows (SPSS Inc., Chicago, Ill, USA). One-way ANOVA, followed by Bonferroni post-test was used for differences between groups. Repeated measures ANOVA was used for measurements at multiple time-points followed by Bonferroni post-test for differences between groups or paired T-test for differences within groups at different time points. The Kaplan-Meier method was used to construct survival curves and the log-rank significance test was used for comparison of survival between groups. A p-value <0.05 was considered significant.

<table>
<thead>
<tr>
<th>Table 1. Temperature and hemodynamic characteristics</th>
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<tr>
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<tr>
<td>Mean core liver temperature during TVE (°C)</td>
</tr>
<tr>
<td>no HP (n=6)#</td>
</tr>
<tr>
<td>HP 28 (n=6)</td>
</tr>
<tr>
<td>HP 20 (n=6)</td>
</tr>
<tr>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>Body temperature (°C)</td>
</tr>
<tr>
<td>Pre-TVE</td>
</tr>
<tr>
<td>37.5 ± 0.2ABC</td>
</tr>
<tr>
<td>38.1 ± 0.1</td>
</tr>
<tr>
<td>35.9 ± 0.2DEF</td>
</tr>
<tr>
<td>35.1 ± 0.3</td>
</tr>
<tr>
<td>83.0 ± 1.3H</td>
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<tr>
<td>83.5 ± 6.5HI</td>
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<tr>
<td>73.9 ± 2.7</td>
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<tr>
<td>83 ± 3M</td>
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<tr>
<td>122 ± 8MN</td>
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<td>89 ± 5</td>
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Temperature and hemodynamic characteristics. Mean core liver temperature was significantly different between all groups. Hemodynamic instability directly after TVE was most pronounced in the no HP group.
# Only pigs that survived the whole experiment are analysed. Values with equal fonts are significantly different.
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Results

Temperature and hemodynamic characteristics

During TVE, mean core liver temperature was maintained within the physiological range in the no-HP group (Table 1). In both HP groups, mean core liver temperature was kept within 0.5°C of the desired temperature. Body temperature directly after TVE was significantly decreased in the HP-28 group and the HP-20 group compared to pre-TVE as well as compared to the no-HP group. No significant differences in mean body temperatures were found directly after TVE between HP-28 and HP-20. MAP was significantly decreased at 10 min reperfusion compared to pre-TVE values in all groups. MAP was significantly lower in no-HP compared to the other groups at 10 min reperfusion. Heart rate was significantly increased after 10 min reperfusion in no-HP compared to pre-TVE as well as compared to both other groups. Body temperature, MAP and heart rate were adjusted and were within physiological ranges after 4 hours reperfusion.

Table 2. Perfusion characteristics

<table>
<thead>
<tr>
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<th>HP 28 (n=6)</th>
<th>HP 20 (n=6)</th>
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<tbody>
<tr>
<td>Mean flow during HP (mL/min)</td>
<td>85.0 ± 13.9</td>
<td>287.5 ± 29.8*</td>
</tr>
<tr>
<td>Range (mL/min)</td>
<td>50 – 300</td>
<td>50 – 500</td>
</tr>
<tr>
<td>Total volume of HP solution (L)</td>
<td>5.1 ± 0.5</td>
<td>17.3 ± 1.7*</td>
</tr>
<tr>
<td>Range (L)</td>
<td>3.7 - 5.6</td>
<td>12.3 - 20.0</td>
</tr>
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</table>

Perfusion characteristics. Significantly more perfusion solution was needed to cool the liver to 20°C as compared to 28°C. * Significantly different from HP 28 group (p<0.05).

Perfusion characteristics

Cooling of the liver to an average temperature of 28°C required a mean flow of 85 ± 14 ml per minute, while cooling to 20°C required a mean flow of 288 ± 30 ml per minute (Table 2). During the 60min perfusion period, this resulted in a mean total perfusion volume of 5.1 and 17.3 liter in the HP-28 and HP-20 groups, respectively (p<0.05).

Figure 2. Survival analysis

All animals of both the HP-28 (n=6) and HP-20 (n=6) groups survived (dotted line). In the no-HP group, 3 of 9 pigs (33%) died within the first 40 minutes of reperfusion (continuous line). The difference in survival time between no-HP and HP groups is significant.
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Survival
In the no-HP group, 3 of the 9 pigs (33%) died within the first 40 minutes after TVE (fig 2). The cause of death in these animals was massive congestion of the liver leading to non-controllable hypotensive shock, highly elevated heart rate and eventually, cardiac arrhythmia and death. This massive congestion of the liver did not occur in the in HP-28 and HP-20 groups in which all animals survived during the experiment.

Hepatocellular damage
AST values were significantly decreased in the HP-28 and HP-20 groups compared to no-HP (fig 3a). No significant differences were found at any time point between animals of the HP-28 group and the HP-20 group. ALT values showed the same pattern (data not shown).

Hepatocellular function
During TVE, bile production was minimal (0-8 mL). During reperfusion, bile production gradually increased in all groups (fig 3b). In contrast to the no-HP group, bile production at 24 hours reperfusion was not significantly different from pre-TVE bile production in the HP-28 and HP-20 groups. Bile production was significantly higher in the HP-28 group and the HP-20 group as compared to the no-HP group.

Figure 3. AST values (a) and bile production (b) measured before TVE (0), during TVE (T) and during reperfusion

![Graph A](image)

3a: AST values were significantly lower in HP-28 and HP-20 groups as compared to no-HP. No differences were found between the HP-28 and HP-20 groups. * Significantly different compared to HP-28 and HP-20 (repeated measures ANOVA, p<0.05). 3b: Bile production during 24 hours of reperfusion was significantly higher in the HP-28 and HP-20 groups as compared to no-HP. * Bile production is significantly more increased compared to no-HP (repeated measures ANOVA, p<0.05).
Figure 4. Indocyanine green clearance (a) and hyaluronic acid clearance (b) rates, measured before TVE and during reperfusion.

4a: ICG clearance rates are presented as percentage of ICG cleared from the blood 15 minutes after bolus injection. After 6 hours of reperfusion, ICG clearance in the HP-28 (grey bars) and in HP-20 (white bars) groups was significantly greater than in no-HP (black bars) (* p<0.05). 4b: Hyaluronic acid (HA) clearance rates are presented as percentage HA cleared from the blood 60 minutes after bolus injection. A significantly higher percentage of HA was cleared in the HP-28 (grey bars) and HP-20 (white bars) groups as compared to no-HP (black bars) (* p<0.05).

Mean pre-TVE ICG clearance rates did not differ significantly between the groups (fig 4a). After 6 hours reperfusion, ICG clearance rates were significantly higher in the HP-28 and HP-20 groups, as compared to no-HP. After 24 hours reperfusion, ICG clearance was partially restored in surviving animals of the no-HP group. No statistical differences were found between the HP-28 group and the HP-20 group.

SEC function
After 24 hours reperfusion, HA clearance rates were significantly higher in the HP-28 and HP-20 groups as compared to the no-HP group (fig 4b). No significant differences were found in HA clearance rate between HP-28 and HP-20 groups after 24 hours reperfusion.

Inflammatory response
IL-6 concentration in plasma samples peaked after 1 hour reperfusion in the no-HP and HP-28 groups (fig 5a). After 12 and 24 hours reperfusion, IL-6 decreased to normal levels in all groups. Overall, significantly lower concentrations IL-6 concentrations were found in the HP-20 group as compared to the no-HP and HP-28 groups.

Histopathology
Parenchymal necrosis, cellular vacuolization and leukocyte influx score were combined to create a total histopathologic score of parenchymal damage and inflammation (fig 5b). This total score was significantly higher in the no-HP group as compared to the HP-28 and HP-20 groups. The difference between the groups can largely be attributed to cellular
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Figure 5. Plasma IL-6 concentrations (A) and histopathology score (B) before TVE and after 5 min (R), 1, 6, 12 and 24 hours of reperfusion

5a: IL-6 values were significantly lower in the HP-20 group as compared to the no-HP and HP-28 groups (* p<0.05, repeated measures ANOVA). No differences were found between no-HP and HP-28. 5b: Histopathology scores were significantly higher in the no-HP group as compared to the HP-28 and HP-20 groups (* p<0.02, repeated measures ANOVA).

vacuolization. Cellular vacuolization was significantly increased 10 min. after TVE in the no-HP group compared to both HP groups. After 10 min. reperfusion, cellular vacuolization decreased in all groups. 24 Hours after TVE, cellular vacuolization had increased again and was significantly elevated in the no-HP group as compared to the HP 20 group. Hardly any necrosis was detected in the sections and no significant differences were found between the groups. Leukocyte accumulation gradually increased in all groups during reperfusion, but no significant differences were found.

Discussion

HP of the liver can be applied to diminish I/R injury during TVE.9-11. It is based on data that cellular metabolism is reduced 2-fold when temperature is lowered by 10°C.21. This leads to a decrease in ATP and oxygen demand and thereby to a decrease in oxidative stress and prolongation of cellular viability during ischemia.22-24. In pilot experiments however, it was found that in situ HP of the liver without extra isolating measures using a 4°C perfusion solution leads to a minimum mean core liver temperature of 20°C in a period of 1 hour. Isolating material around the liver was not applied, because in the clinical situation this
would interfere with operating procedures. Alternatively, the core liver temperature could have been lowered below 20°C average by increasing flow of the 4°C perfusion solution via the portal vein. However, it was chosen not to exceed a maximum flow rate of 500 mL/min. during HP, because of the high risk of perfusion pressure related injury to the microcirculation, further damaging SECs when perfusing at low temperatures. Ringer-lactate was used as perfusion solution during TVE. It is an isotonic solution containing Na⁺, K⁺, Ca²⁺, Cl⁻ and Lactate ions with a pH of approximately 6.0. It also has been used by other groups as HP solution. University of Wisconsin (UW) solution, which has been used for HP by the group of Hannoun, in our view has important disadvantages such as high viscosity and high potassium content and therefore was not chosen. Furthermore, the UW solution has been designed for use at 4°C and perfusion at higher temperatures is not advised because of the high potassium content. Body temperature decreased after HP which could have contributed to the protective effect of HP in the HP-28 as well as in the HP-20 group (Table 1). However, with use of a heating lamp and heating mat, body temperature returned to normal within 4 hours after TVE in all animals.

In literature, SEC vacuolization and microcirculatory disturbances are found mainly in cold storage experiments when the liver temperature is decreased to 4°C for a period of 24 hours. In this study, no loss of HA clearance function occurred 24 hours after TVE in both HP groups suggesting that when HP is applied for 1 hour at 20°C, SEC function is not affected. Cellular vacuolization was also significantly less pronounced in the HP-20 group as compared to the no-HP group after 24 hours reperfusion. Directly after TVE, all animals in the no-HP group developed massive congestion of the liver within 10-40 mins following reperfusion. This perfusion failure or 'no reflow' phenomenon resulted in hemodynamic instability and shock. It is caused by a combination of factors like cellular vacuolization and leukocyte stasis within sinusoids. During warm ischemia, hypoxia and ATP depletion can lead to specific changes in mitochondrial function and membrane transport. Along with the formation and release of reactive oxygen species (ROS) during reoxygenation, Na⁺-K⁺-ATPase (sodium pump) activity is inhibited resulting in cellular vacuolization. When examining histology after 10 min reperfusion, significantly more vacuolization was found in the no-HP group as compared to both HP groups (Figure 8). Apparently, liver I/R injury leading to perfusion failure and 'no reflow' resulted in shock and subsequent death in three animals in the no-HP group (33%). In both HP groups, shock or hemodynamic instability was not encountered.

The protective effect of HP cannot only be attributed to the wash-out of remnant blood and waste products. To examine hepatocellular damage during HP, the release of AST and ALT were measured in the perfusate. At the end of HP, AST and ALT concentrations in the perfusate never exceeded 20 U/L (data not shown). From liver preservation experiments using machine-perfusion it is also known that AST/ALT release mainly occurs during oxygenated warm-ischemic reperfusion and not during cold perfusion. Furthermore, during preliminary experiments, perfusion at 37°C for 1 hour resulted in massive liver congestion, shock and death of all animals (n=3). The protective effect of HP on hepatocyte cell mass as well as SECs was clearly demonstrated in this study. Animal survival increased to 100%, while AST, histopathology score, liver function parameters and IL-6 levels were significantly attenuated by HP. However, the
beneficial effect of cooling to 20°C as compared to 28°C was found to be only marginal. IL-6 production was significantly decreased in the HP-20 group as compared to HP-28 group. Furthermore, a trend was found towards lower AST levels and increased bile production in the HP-20 group. Also, a trend was found towards more vacuolization after 24 hours reperfusion in the HP-28 group as compared to the HP-20 group. This however, did not result in loss of hepatocyte function as measured by ICG clearance in the HP-28 group.

In conclusion, HP at 20°C equally preserves SECs as well as hepatocyte function and equally prevents the occurrence of microvascular perfusion failure compared to HP at 28°C. Also, to cool the liver for 1 hour to an average of 20°C without isolation, requires an impractically large volume of perfusion fluid (17.3 ± 1.7 L.). Although IL-6 release is decreased in HP at 20°C, a mean core liver temperature of 28°C is both sufficient and practical when applying HP.

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Reference list


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