The Groningen hypothermic liver perfusion system for improved preservation in organ transplantation
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Chapter 9

The Groningen Hypothermic Liver Perfusion Pump: Functional Evaluation of a New Machine Perfusion System


9.1 Introduction

The success of the UW concept and the introduction of a number of modifications has significantly contributed to a better understanding of ischemia/reperfusion injury. Nevertheless, the limits of static CS preservation in organ transplantation appear however to be reached. Until to date, the majority of donor livers used for transplantation originate from brain-dead donors only. Livers from marginal or non-heart-beating donors have a lesser quality and are only infrequently used for transplantation due to an expected decreased organ viability after transplantation. In kidney preservation, hypothermic machine perfusion is now used in a number of centers for preservation of non heart-beating and so-called marginal donor kidneys\textsuperscript{1,8,15,26}. In the laboratory, with this technique even 5-7 days success-
ful preservation and transplantation of canine kidneys can be achieved\textsuperscript{23,24}. Due to these successes and the potential to increase the donor pool and prolong storage times, continuous preservation of the liver has gained renewed interest. Continuous machine perfusion of the liver could contribute to better preservation of brain dead donor livers, facilitate the use of marginal donor livers and might result in the use of more non heart-beating donors as well\textsuperscript{16,21}. Furthermore, HMP could allow the use of ‘on-line’ viability markers by analysis of pressure and flow characteristics and perfusate. HMP could, thus, allow a refined clinical decision to include or discard marginal and non-heart-beating donor organs in the transplantation procedure.

In the late 1960’s Belzer\textsuperscript{2}, Slapak\textsuperscript{32} and Brettschneider\textsuperscript{6} experimented with continuous hypothermic machine perfusion of the liver in an experimental setting with results comparable or even better than livers preserved with static CS. In 1986 D’Alessandro et al\textsuperscript{8} and later Pienaar et al\textsuperscript{28} managed to transplant good quality canine livers after 72 hours preservation in a HMP dog model.

However, despite these successes, until now a clear definition and validation of optimal perfusion settings has not become available. Previously, our group reviewed the HMP literature for liver, and defined three determining factors for effective HMP: the type of preservation solution, the characteristics of perfusion dynamics, and the necessity for oxygenation\textsuperscript{30}. We concluded that, to date, Belzer’s University of Wisconsin machine preservation solution (UW-MP) is the most suitable perfusate. Perfusion through the portal vein alone has been shown to result in good short term liver viability upon transplantation\textsuperscript{5,7,20,38}. Nevertheless, to improve single blood vessel perfusion, simultaneous perfusion of portal vein and hepatic artery, might prevent ischemic-type biliary lesions leading to biliary strictures and retransplantation\textsuperscript{29}. Data, which pressure and flow should be used differ between authors, but there is agreement about the use of a lower than physiological perfusion pressure and flow during HMP to limit any shear stress induced endothelial cell injury. Based on an educated guess and proved by our own experiments comparing different perfusion pressures using fluorescence microscopy\textsuperscript{12}, we found a portal venous perfusion pressure of 4 mmHg and a pulsatile hepatic arterial pressure of 30/20 mmHg at 60 BPM give a complete and uniform perfusion of the liver with minimal endothelial and/or hepatocyte damage. Previously, many authors have pointed at the potential importance of additional oxygenation during liver HMP\textsuperscript{9,10,13,25,31}. Based on the data of Fujiya et al\textsuperscript{10} as well as our own experiments\textsuperscript{13} the required partial oxygen pressure was determined at 55 kPa\textsuperscript{31} to comply with total liver oxygen demand during HMP. In addition to an adequate perfusion, we concluded that the hypothermic liver uses oxygen and thus oxygenation of the perfusate, even at

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low temperatures, remains necessary. Physiological oxygen distribution in the liver is 65% through the hepatic artery and 35% through the portal vein. Mimicking this situation in our HMP system, a $pO_2$ of 35.8 kPa and 19.2 kPa is needed in the hepatic arterial and portal venous line, respectively.

So far, there is no clinical liver perfusion machine commercially available to improve organ viability and seriously challenge the limits of liver preservation by optimizing perfusion and transportation during cold storage, comparable to machine preservation of kidneys in dedicated transplant centers. We, therefore, have developed a hypothermic liver perfusion machine using oxygenated UW-MP solution and dual vascular perfusion technique that is able to perfuse the liver for 24 hours and attempts to maintain the quality of donor livers. The ultimate clinical goal with this machine is to obtain an increase of the donor pool, maintain viability and prolong storage times. The aim of the study presented here was to test the technical performance of a prototype of the Groningen Liver Perfusion Pump. This includes adequate perfusion of the liver (i.e. uniform perfusion with no or minor cellular injury), maintenance of hypothermia, pressure and sterility, and supply of the liver tissue with sufficient oxygenation during 24 hours of continuous preservation.

9.2 Materials and Methods

9.2.1 HMP design

The Groningen hypothermic liver perfusion pump consists of a reservoir in which the liver is placed, two miniature centrifugal pumps (Deltastream DPII, MEDOS Medizintechnik AG, Stolberg, Germany) delivering continuous and pulsatile flow, respectively, a miniature hollow fibre membrane oxygenator (HILITE 800LT, MEDOS Medizintechnik AG, Stolberg, Germany), an oxygen cylinder, a battery pack and a measurement and control unit which is connected to an interface (Figure 9.1). The pulsatile pump directs the preservation solution (UW-MP) from the reservoir through the oxygenator and into the hepatic artery; the continuous pump perfuses the portal vein without oxygenation. Both pumps are pressure-controlled, and recirculate the preservation solution with a constant perfusion pressure. The sterile disposable unit of the Groningen hypothermic liver perfusion pump, including the reservoir, pump heads, oxygenator, cannulas and tubing, is situated inside a polystyrene cooling box (Wolters kunststoffen, Enter, the Netherlands) which is filled with melting ice to secure a hypothermic temperature of 0-4°C for more than 24 hours.
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Figure 9.1: Prototype of the Groningen hypothermic liver perfusion pump (top: artist impression, bottom: schematic set-up).
9.2. Materials and Methods

9.2.2 Experimental design

Six female pigs weighing 40-50 kg were used. A porcine model was chosen since pigs are phylogenetically more similar to humans than rodents or other domestics. All animal procedures were approved by the animal research ethics committee of the Faculty of Medical Sciences of the University of Groningen. The care and handling of the animals were in accordance with National Institutes of Health guidelines. The experiment (Figure 9.2) started with the procurement and blood wash-out of the donor liver. After procurement, the liver was submerged in the reservoir, containing UW-MP and the portal vein, celiac trunc and common bile duct were cannulated. The sterilized disposable unit, including the reservoir, pump heads, oxygenator and tubing was subsequently attached to the HMP system. The perfusion pressure was set at 4 mmHg and 30/20 mmHg for portal vein and hepatic artery, respectively. After 24 hours perfusion was stopped and the liver was taken out of the reservoir. Just prior to and after preservation, biopsies of the liver were taken for histology. During preservation, at time points 0, 2, 4, 8, 12 and 24 hours, samples of the perfusate were taken to assess the partial oxygen pressure and lactate dehydrogenase (LDH) levels.

The performance of the Groningen HMP liver perfusion system was assessed by judging:

1. perfusion dynamics, with a controlled constant pressure and resulting flow, maintained temperature at 0-4°C, and uniform perfusion without loss of tissue integrity,

2. oxygenation, showing $pO_2 > 35.8$ kPa and 19.2 kPa for hepatic artery and portal vein, respectively,

3. microbiology, to demonstrate sterility inside the disposable unit throughout the entire preservation period.

9.2.3 Operative procedure

After anesthesia using 10 mg/kg ketamine i.m., the animal was intubated and mechanically ventilated with a mixture of oxygen and isoflurane. The procurement of the liver followed the standard techniques for human multiorgan retrieval. Briefly, the donor operation was performed through a midline laparotomy. The abdominal aorta was isolated and encircled with a ligature distally of the renal artery for insertion of an infusion cannula (18 Fr, Tyco Healthcare, Zaltbommel, The Netherlands). The aorta was dissected from the surrounding tissue cranially of
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the celiac trunc to allow crossclamping during infusion of lactated-Ringer’s solution. The supra- and infrahepatic vena cava were dissected and prepared for ligation. The splenic and left gastric arteries were dissected and ligated. The celiac trunc was dissected free from its surrounding tissue towards the aorta. The common bile duct was mobilized and transected. The portal vein was then freed and prepared for cannulation. At this point the pig was fully heparinized (20,000 units), the aorta was crossclamped cranially of the celiac trunc and the aorta was cannulated caudally of the renal artery followed by infusion of lactated-Ringer’s solution. After three liters of aortic infusion a cannula (18 Fr, Tyco Healthcare, Zaltbommel, The Netherlands) was placed into the portal vein and infused with one liter of lactated-Ringer’s solution. The intrapericardial inferior vena cava was transected\textsuperscript{33,34}. The celiac trunc was dissected in continuity with a circumferential piece of aorta.

9.2.4 Back table procedure

Cannulation with an 18 Fr cannula of both the portal vein and aortic stump was performed with the liver submerged in four liters 0-4°C UW-MP solution. The cystic duct was ligated and a catheter (6 Fr) was placed in the common bile duct for decompression and collection of bile formed during preservation. At this point samples and biopsies were taken for microbiological examination, histology, electron microscopy (EM) and fluorescence microscopy (FM). Biopsies were taken in the liver hilus and periphery in four liver lobes, i.e. right medial lobe, right lateral lobe, left medial lobe, left lateral lobe. A peripheral biopsy in the left lateral lobe was used for EM. Acridine Orange (AO: 5 mg/l) and Propidium Iodine (PI: 10 mg/l) were subsequently added to the UW-MP solution.
9.2.5 Data collection

Perfusion pressure (Truwave, Edwards Lifesciences, Irvine, USA), flow rates (H7C, Transonic Systems, Ithaca, USA) and temperature (10k3D(X), BetaTherm, Galway, Ireland) were continuously recorded for 24 hours. Samples of the perfusion solution were taken at 0, 2, 4, 8, 12 and 24 hours of machine perfusion. LDH as a marker for liver damage, and partial oxygen pressure ($pO_2$) were measured in these samples. Liver biopsies were taken after 24 hours of machine perfusion close to the previous location of biopsies to determine tissue integrity using light microscopy, electron microscopy and fluorescence microscopy.

9.2.6 Microscopic techniques

Cryosections (4 µm) were examined to identify the location and amount of dead cells, stained with the exclusion-dye PI and to disclose minute staining with AO. A fluorescence microscope (Leica DC300F, Rijswijk, The Netherlands) with 495/519 nm (FITC) and 547/572 nm (TRITC) filter was used, with a magnification of 20 times. Light microscopy (magnification 20 times) of hematoxyline and eosine stained sections was used to demonstrate changes in morphology. Tissue was collected, fixated in 4% formaline, subsequently paraffin embedded and cut into 4 µm thick sections.

9.2.7 Microbiology

Samples of perfusion solution, liver, bile and ice, taken before and after 24 hours machine preservation, were smeared on blood agar plates and cultured at 37°C in aerobic and anaerobic environments for seven days. The agar plates were subsequently macroscopically examined for colony forming bacteria and hence bacterial contamination.

9.2.8 Statistics

Comparison between the results was done by means of an unpaired two-tailed Student’s t-test. Differences were considered to be significant for $P < 0.05$. Values are represented in mean ± SEM.
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9.3 Results

9.3.1 Perfusion dynamics

Temperature measurements showed a constant temperature inside the cooling box of 0°C at time points 0, 2, 4, 8, 12 and 24 hours for all experiments. Mean hepatic arterial perfusion pressure was constant (25 mmHg) (Figure 9.3) at these time points. Portal venous perfusion pressure was 4 mmHg for time points 0 to 12 hours, but increased gradually between time points 12 and 24 hours, in two cases. In the other four cases portal venous pressure was constant throughout the preservation period.

![Perfusion pressure during 24 hours of perfusion preservation](image)

Figure 9.3: Perfusion pressure during 24 hours of perfusion preservation (mean ± sem).

Hepatic arterial flow measurements showed an initial increase in the first two hours of preservation, from $78 \pm 12$ ml/min to $91 \pm 11$ ml/min (Figure 9.4). After two hours, the mean hepatic arterial flow decreased gradually from a maximum of $91 \pm 11$ ml/min to a minimum of $63 \pm 8$ ml/min after 24 hours perfusion. A similar trend was visible in the portal venous flow, with a small rise between 0 and 2 hours (from $386 \pm 30$ ml/min to $448 \pm 35$ ml/min, respectively) and a subsequent gradual decrease to $160 \pm 65$ ml/min after 24 hours.

Histological evaluation of biopsies taken from hilus and periphery revealed expanded sinusoidal spaces after 24 hours of machine preservation (Figure 9.5). At the start of preservation, widened sinusoids, indicating congestion, were already visible.

Fluorescence microscopy resulted in a uniform staining pattern in both hilus and
9.3. Results

Figure 9.4: Resulting flow during 24 hours perfusion preservation (mean ± sem).

Figure 9.5: Widened sinuoids before (left) and after (right) 24 hours of machine preservation shown with light microscopy using hematoxyline-eosine staining.
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periphery (Figure 9.6). Staining with propidium iodide, showing the number of dead cells, revealed more dead cells in the liver hilus than in the periphery, where only a few dead cells were found. The dead cells found were determined to be non-parenchymal cells, i.e. remaining blood cells, Kupffer cells and endothelial cells.

Finally, electron microscopy was conducted to evaluate the condition of the sinusoidal endothelial cells (Figure 9.7). At 0 hour, the endothelial cells showed their characteristic orientation, with stretched cytoplasm along the sinusoids. After 24 hours of HMP, endothelial cytoplasm retracted, creating fenestrations between the endothelial cells. The cellular nuclei were still intact.

LDH released in the perfusate is a marker for cellular injury and showed an increasing trend from 102 ± 12 U/l to 148 ± 20 U/l over the total preservation period (Figure 9.8). This increase, however, was not significant.

Figure 9.6: Rate of perfusion (left) and number of dead cells (right) after 24 hours machine perfusion, stained with acridine orange and propidium iodide, respectively, for the liver hilus (top) and periphery (bottom).
9.3. Results

![Figure 9.7: Lacto-dehydrogenase (LDH) levels during 24 hours perfusion preservation (mean ± sem).](image)

9.3.2 Oxygenation

The $pO_2$ was constant, 95 ± 3 kPa, in the arterial line, the tube in which the oxygenator is situated, (Figure 9.9). The partial oxygen pressure in the portal venous line was initially low, starting at 17 ± 2 kPa, but increased gradually to a mean $pO_2$ of 65 ± 6 kPa after 8 hours and remained constant.

![Figure 9.8: Partial oxygen pressure in the hepatic arterial line and the portal venous line during 24 hours of perfusion preservation (mean ± sem).](image)
9. Evaluation of the Groningen HMP system

9.3.3 Microbiology

Samples obtained at 0 hours and prior to preservation from liver, bile and perfusion solution showed no bacterial colonies after seven days of culturing. In comparison, as expected, non-sterile ice samples, however, were found to be largely contaminated with bacteria. Also, after 24 hours of preservation, the perfusion solution, liver and bile remained sterile.

9.4 Discussion

Hypothermic machine perfusion preservation of kidneys has been shown to improve preservation quality and result in longer preservation times\(^\text{28}\) and a reduced incidence of delayed graft function\(^{18,27}\). As a consequence, HMP allows inclusion of non heart-beating donors in the kidney donor pool. To extend these advantages to the liver donor pool, many authors have been studying HMP of the liver as well\(^{7,16,20,28,32}\). 72 hours successful preservation of good quality dog livers has been reported\(^{28}\), and the first promising experiences with HMP of non-heart-beating donor livers in pigs and rats have been documented\(^{16,21,36}\). However, despite these successes, liver HMP has not become the clinical standard preservation procedure yet. One of the reasons for this is that current machines are non-transportable and/or not easy to use without a stand-alone possibility requiring continuous supervision.

The Groningen hypothermic liver perfusion pump constitutes an attempt towards introduction of machine preservation for the liver into clinical practice. New developments and techniques now make it possible to expand the conventional static cold storage procedure to a portable continuous perfusion system using miniaturized blood pumps, small oxygenators and micro-electronics. Design criteria for the pump system included pressure-controlled oxygenated perfusion with arterial and portal venous pressures that are high enough to enable a uniform perfusion throughout the liver without compromising endothelial cell integrity. Additional design criteria included a continuous cold temperature and sterility for the duration of 24 hours.

The prototype of the Groningen HMP system is able to maintain a temperature of 0-4°C. The cooling box in which the reservoir, oxygenator and pump heads are located, was filled with melting non-sterile ice and can passively maintain its temperature without refilling. The disposable unit remains sterile since neither aerobic nor anaerobic bacterial colonies were found in the perfusate after 24 hours.
9.4. Discussion

preservation. The control unit manages to maintain hepatic arterial pressure at 30/20 mmHg for 24 hours, while portal venous pressure will increase to a certain extent after 12 hours of perfusion. During the total preservation period portal venous flow varied significantly, which was a result of altered resistances in the liver. At 24 hours, portal venous resistance was at such a level that the controller could not maintain a perfusion pressure of 4 mmHg, since the minimal rotational speed of the centrifugal pump was reached.

During the first 12 hours of the preservation period, the controller maintained a perfusion pressure of 4 mmHg with an initial flow of 448 ml/min to a final flow of 160 ml/min at 24 hours. Since perfusion pressure is maintained, flow variation resembled vascular resistance, defined as perfusion pressure divided by resulting flow. This variation of mainly portal venous flow was unexpected and, according to our knowledge, was never reported in literature before. Yamamoto\textsuperscript{38} continuously perfused porcine livers through the portal vein, but found no increase in resistance during 72 hours of machine preservation. The increase in resistance could have been caused by interstitial edema formation. However, light microscopy did not reveal any major edema formation in the microvascular system of the liver. Some spacing of the sinusoids was seen, but appeared already to be present, although to a lesser extent, after the initial flush and before preservation. This phenomenon has been seen before in hypothermic preservation for both cold storage and machine perfusion\textsuperscript{18,37}. In this first try-out experimental series we also found edema formation in the connective tissue of the extrahepatic portal vasculature. The extent of perivascular edema could explain the increased flow resistance, and the subsequent decrease in flow. We postulate that this finding might be the result of a still too high perfusion pressure. We could not confirm this idea, however, by measuring an increase in endothelial cell damage or hepatocyte injury using PI staining. Using static cold storage, pig livers have been preserved for 6-8 hours and transplanted with good survival rates\textsuperscript{4}, while 50-80% of the animals die when transplanted after 10-12 hours preservation. With 24 hours of HMP, the maximum viable preservation time of the pig liver could have been reached, reflected by the increased resistances. Whether the cause of the decreasing flow during our 24 hours HMP is related to a high perfusion pressure or to other species-dependent effects remains to be investigated. Also, we do not know its consequence for liver viability, since no further major damage to the liver was seen. Future experiments in a large animal liver transplantation model will, therefore, be performed.

HMP as such could induce shear stress to the endothelial cells which are already more sensitive at low temperature and thus prone to injury\textsuperscript{11}. Microscopic
observation of the propidium iodide staining showed a small amount of damaged non-parenchymal cells, mainly near the portal triad of the liver lobules. More damaged cells were found in the hilum of the liver than in the periphery, indicating that the perfusion pressure was chosen too high. The extent of damage to the endothelial cells was studied in more detail using electron microscopy. The endothelial cell structure before HMP and after initial flushing was normal with a cytoplasm stretched along the sinusoidal wall and aligned to hepatocytes. After 24 hours of HMP, the nuclei of the endothelial cells were still intact, but the long-stretched cytoplasm was now retracted. This result has been confirmed by other authors\textsuperscript{18,37} who also measured decreased function of endothelial cells after HMP. Shear stress-induced endothelial cell injury could be prevented by lowering the perfusion pressure. However, staining with acridine orange indicated that the perfusion pressure was well chosen to obtain a complete and uniform perfusion of the liver. Lowering the perfusion pressure could result in a lower incidence of endothelial damage but in an incomplete perfused liver. Since the extent of damage appeared to be moderate (Figure 9.8), perfusion pressure was concluded to suffice.

Another important design criterium for the Groningen hypothermic liver perfusion pump was to obtain sufficient oxygenation. The hollow fibre membrane oxygenator placed in the hepatic arterial line managed to saturate the perfusate immediately after starting HMP. The $pO_2$ in the portal venous line, without oxygenator, increased slowly from room-air saturation to 65 kPa after 8 hours and remained then constant during preservation. The oxygen pressure complied to the design criterium for oxygenation, for both lines. The difference between arterial and portal venous partial oxygen pressures was the amount of oxygen consumed by the liver itself, compensating for the saturation time of the total reservoir. After eight hours reservoir saturation was reached and oxygen uptake by the liver remained constant but still considerable (35 kPa). This finding is in accordance with other authors\textsuperscript{9,10,13}, who showed an improved liver viability after oxygenated vs non-oxygenated HMP indicating that the liver, even under hypothermic conditions, has a considerable oxygen demand.

The injury marker LDH did not significantly increase during the preservation period. Combining this finding with the low amount of damaged hepatocytes, indicated with fluorescent PI staining and electron microscopy, we conclude that the perfusion regime of the Groningen hypothermic perfusion machine did not induce any major injury to the pig liver. However, liver viability can only be judged in a reperfusion model, i.e. either an in vitro isolated reperfused liver.
model or ultimately a transplantation model.
In summary, the Groningen hypothermic liver perfusion pump prototype was able to dually perfuse the liver for 24 hours in a pulsatile (HA) and continuous (PV) manner. The system maintained adequate flow at a controlled pressure. Although an increasing resistance of the liver was observed, no major endothelial cell or hepatocyte damage was induced. Some perivascular extrahepatic edema was, however, found. This phenomenon could either be pressure-induced or species-specific, which will be investigated in another large animal model. The hollow fibre oxygenator oxygenated the arterial line satisfactorily, and even supplied the portal venous line with a sufficient amount of oxygen. Melting non-sterile ice kept the temperature of the disposable section, including reservoir, oxygenator, pump heads and liver, below 4°C during 24 hours, and did not affect the sterility inside the disposable section.

We feel that the Groningen HMP system has proven to comply with the design criteria and requirements that were formulated to optimally preserve a donor liver. Following these functionality tests, the system now must demonstrate the advantages of hypothermic dual perfusion of the liver over cold storage in a transplant model.
If correct, the system would then allow extended preservation times despite lower quality of donors and make liver transplantation a semi-emergency procedure. Preservation outcome would then be improved in such a way that the incidence of initial poor function and primary non-function could decrease and in addition, even non heart-beating donor livers ought to be included in the donor pool on a more regular basis.

9.5 References


9. Evaluation of the Groningen HMP system


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