Gradual Rewarming Preservation of Liver and Kidney Grafts

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Gradual Rewarming Preservation of Liver and Kidney Grafts

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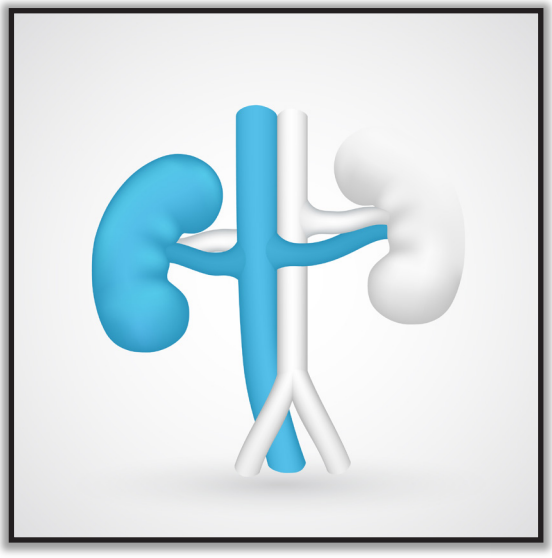
CHAPTER 1

General Introduction and Aims of This Thesis



Transplantation is the only available treatment for end stage liver disease (ESLD) and end stage kidney disease (ESKD). Liver and kidney organs are the two most transplanted organs worldwide. The first successful kidney transplant was performed in 1954 and first liver transplant occurred in 1963 (1,2). In the last few decades, medical advances in the treatment of rejection, improvement in surgical techniques and better post-transplant care have led to enhanced one-year and five-year graft and patient survival rates (3,4).Yet, despite the increase in the number of transplantations and improved clinical outcomes, kidney and liver waiting lists are growing more rapidly than available organs (5). According to the United Network for Organ Sharing (UNOS), more than 95.000 patients currently await kidney transplantation and close to 14.000 patients are on the waiting list for liver transplantation. Many of these patients never receive organs as illustrated by 2017 data wherein only 20.000 kidney transplants and 8.000 liver transplants were performed. This difference in organ supply and demand leaves many patients without access to organ transplant and increases the overall waiting list mortality rate (6,7).

In November of 2000, a new strategy was introduced in an effort to reduce the organ shortage: the use of sub-optimal quality *extended criteria donor (ECD) organs* (8). The characteristics of ECD organs are: organ grafts harboring underlying diseases such as hepatitis B and C, donors derived from donations following circulatory death (DCD) events such as heart attacks or massive strokes. or donors age ≥60 years or over 50 years with at least two of the following conditions – a history of hypertension, serum creatinine level >1.5 mg/dL or cause of death from cerebrovascular accident. An ECD organ has inferior quality in comparison with a standard criteria donor (SCD) organ. As a result of this impaired quality, ECD organs are more susceptible to preservation and ischemia reperfusion injuries in the post-transplant phase which may manifest as primary non-function (PNF), delayed graft function (DGF) and biliary complications in the case of liver transplant (9,10). Subsequently, a considerable number of ECD organs are declined by transplant centers (11). It is possible that a significant number of the declined organs may prove suitable for transplantation if organ quality could be verified or improved before implantation. One possibility would be to confirm and improve organ quality during the preservation phase.

Currently, the most common organ preservation method in clinical practice is cold storage (CS) in which harvested organs remain on ice at the 4°C low metabolism range which keeps the organ in a less-than-10%-of-active-metabolism status. This leads to waste product build-up in the organ during CS which often damages cellular integrity and results in cold ischemic injury(12). In addition, because of the low temperatures, organ quality cannot be confirmed during CS.

Over the past decade, machine perfusion has been introduced as an alternative to CS and offers more advanced perfusion techniques with the opportunity for organ quality improvement and effective assessment (13,14). In this technique, machine perfusion is categorized by temperature, such as hypothermic machine perfusion (HMP) and normothermic machine perfusion (NMP). HMP is a safe and technically-simple protocol which reduces the decrease in organ quality during preservation, by providing for minimal organ nutritional demand associated with low temperature storage and facilitating waste product wash out (13,15). However, there is limited opportunity to determine organ quality or conduct viability assessments during HMP. NMP at body temperature (37°C) is a more advanced technique that offers the possibility to treat the warm ischemia injury in DCD grafts, as well as organ viability assessment (16,17). Nevertheless, it is also known that sudden increase in temperature following cold preservation likely triggers mitochondrial and cellular injury and contributes to organ dysfunction after transplantation (18). Therefore, a more sophisticated perfusion protocol temperature including the advantages of both HMP and NMP perfusion protocols while avoiding disadvantages of sudden changes to NMP appear preferable. The **aim of this thesis** is to assess the feasibility of gradual organ rewarming to improve organ quality during preservation prior to transplantation in preclinical rodent models. A corollary focus is ensuring graft oxygenation during gradual rewarming by testing the value of a hemoglobin-based oxygen carrier (HBOC) in the perfusion protocol.

In **Chapter 2**, we describe two temperature and pressure controlled rodent perfusion systems developed at the University Medical Center of Groningen (UMCG). The aim of **Chapter 3** then is to study the feasibility of *gradual rewarming preservation* in a rat kidney model after extended CS time and compare the results to *immediate rewarming to body temperature* after CS. Energy depletion and waste product accumulation in the organ during CS causes alteration in cellular metabolism and likely cellular injury called cold ischemic injury. The introduction of warm blood during organ implantation after CS results in the release of accumulated waste products and formation of reactive oxygen species (ROS) known as reperfusion injury. Thus, an approach of a gradual increase in temperature and pressure after CS and before reperfusion at body temperature might ameliorate organ reperfusion injury.

Similarly, the aim of **Chapter 4** is to test the perfusion system developed, study the effect of different perfusion protocols including gradual rewarming on DCD rat livers immediately following CS. The particular target is to assess the impact on biliary tract preservation: A very common complication following liver transplant is the development of biliary strictures which results in non-anastomosis strictures (NAS) and is one of the major post-transplant complications in DCD liver graft recipients. Patients with NAS may suffer episodes of

cholangitis as a result of bile duct necrosis or fibrosis which may lead to re-transplantation or death and increase post-transplantation morbidity and mortality rates (11,19,20). Literature data demonstrates that machine perfusion is superior to CS in DCD livers with enhanced outcomes on the quality of graft and bile duct preservation (21,22). However, there is still limited information about the most effective perfusion model on DCD liver grafts and whether gradual rewarming could further improve the quality of DCD livers and add better bile duct preservation, which is studied in this chapter.

One of the main elements in machine perfusion is to provide a sufficient oxygen level that sustains the organ's metabolic needs. During gradual rewarming preservation, temperatures varies between 8°C (HMP) up to 37°C (NMP), wherein active organ metabolism ranges from about 10% to almost 100% (23). This active metabolism highlights the need for sufficient oxygen with a proper oxygen carrier during the preservation process regardless of preservation temperature. Applying a suitable oxygen carrier during gradual rewarming challenges practitioner decision-making due to wide temperature alterations. Therefore, the oxygen carrier must function consistently and without failure in different temperature ranges from HMP to NMP.

Red blood cells (RBCs), the only clinically available oxygen carriers, currently are used in NMP, however, the use of RBCs is associated with complications such as the risk of transmitting blood born infections and RBC hemolysis during perfusion. Furthermore, the biophysical restrictions of RBCs limit their use in temperatures below 37°C (24,25). Artificial oxygen carriers which transport oxygen and unload it to tissue, might be considered as an effective alternative to RBCs during organ preservation. HBOCs are a relatively new generation of oxygen carriers consisting of a hemoglobin which is not inside red blood cells and can function in a wide temperature range from 4°C to 37°C (23,26). In particular, HBOC-201 is a polymerized bovine-hemoglobin-based oxygen carrier that has been successfully used in liver subnormothermic machine perfusion (SNP) and NMP protocols. However, there exists very limited data regarding the use of HBOC in gradual rewarming protocol. Prior liver rewarming studies in literature exclude rewarming all the way to normothermia because of the lack of a proper oxygen (27,28). **Chapter 5** therefore addresses the question of feasibility and efficacy of using an artificial oxygen carrier during gradual rewarming in a rat liver model.

Based on the results of the liver studies, **Chapter 6** then extends the testing of HBOC in a kidney gradual rewarming study. The results are also compared to the results of Chapter 3 of this thesis, in which gradual rewarming was performed up to normothermia but with only diffused oxygen in the perfusion media.

Chapter 7 provides a summary of relevant findings and results of all chapters followed by a general discussion that highlights the opportunity for future research centering on advancing contemporary perspectives associated with the field of organ preservation in transplantation.

REFERENCES

1. Hatzinger M, Stastny M, Grützmacher P, Sohn M. [The history of kidney transplantation]. Urol Ausg A. 2016 Oct;55(10):1353–9.
2. Meirelles Júnior RF, Salvalaggio P, Rezende MB de, Evangelista AS, Guardia BD, Matielo CEL, et al. Liver transplantation: history, outcomes and perspectives. Einstein Sao Paulo Braz. 2015 Mar;13(1):149–52.
3. Hart A, Smith JM, Skeans MA, Gustafson SK, Wilk AR, Robinson A, et al. OPTN/SRTR 2016 Annual Data Report: Kidney. Am J Transplant. 18(S1):18–113.
4. Kim WR, Lake JR, Smith JM, Schladt DP, Skeans MA, Harper AM, et al. OPTN/SRTR 2016 Annual Data Report: Liver. Am J Transplant. 18(S1):172–253.
5. Clayton LM, Rizzolo D, Nair V. Kidney transplant wait list: Review and current trends. JAAPA Off J Am Acad Physician Assist. 2018 Oct;31(10):1–5.
6. Asrani SK, Larson JJ, Yawn B, Therneau TM, Kim WR. Underestimation of liver-related mortality in the United States. Gastroenterology. 2013 Aug;145(2):375-382.e1-2.
7. Augustine J. Kidney transplant: New opportunities and challenges. Cleve Clin J Med. 2018 Feb;85(2):138–44.
8. Rege A, Irish B, Castleberry A, Vikraman D, Sanoff S, Ravindra K, et al. Trends in Usage and Outcomes for Expanded Criteria Donor Kidney Transplantation in the United States Characterized by Kidney Donor Profile Index. Cureus. 2016 Nov 22;8(11):e887.
9. Eren EA, Latchana N, Beal E, Hayes D, Whitson B, Black SM. Donations After Circulatory Death in Liver Transplant. Exp Clin Transplant Off J Middle East Soc Organ Transplant. 2016 Oct;14(5):463–70.
10. Zhao H, Alam A, Soo AP, George AJT, Ma D. Ischemia-Reperfusion Injury Reduces Long Term Renal Graft Survival: Mechanism and Beyond. EBioMedicine. 2018 Feb 2;28:31–42.
11. Op den Dries S, Sutton ME, Lisman T, Porte RJ. Protection of bile ducts in liver transplantation: looking beyond ischemia. Transplantation. 2011 Aug 27;92(4):373–9.
12. Guibert EE, Petrenko AY, Balaban CL, Somov AY, Rodriguez JV, Fuller BJ. Organ Preservation: Current Concepts and New Strategies for the Next Decade. Transfus Med Hemotherapy. 2011 Apr;38(2):125–42.
13. Dutkowski P, Schlegel A, de Oliveira M, Müllhaupt B, Neff F, Clavien P-A. HOPE for human liver grafts obtained from donors after cardiac death. J Hepatol. 2014 Apr;60(4):765–72.
14. Bruinsma BG, Avruch JH, Weeder PD, Sridharan GV, Uygun BE, Karimian NG, et al. Functional human liver preservation and recovery by means of subnormothermic machine perfusion. J Vis Exp JoVE. 2015 Apr 27;(98).
15. De Deken J, Kocabayoglu P, Moers C. Hypothermic machine perfusion in kidney transplantation. Curr Opin Organ Transplant. 2016;21(3):294–300.
16. Ceresa CDL, Nasralla D, Jassem W. Normothermic Machine Preservation of the Liver: State of the Art. Curr Transplant Rep. 2018;5(1):104–10.
17. Weissenbacher A, Hunter J. Normothermic machine perfusion of the kidney. Curr Opin Organ Transplant. 2017 Dec;22(6):571–6.
18. Leducq N, Delmas-Beauvieux MC, Bourdel-Marchasson I, Dufour S, Gallis JL, Canioni P, et al. Mitochondrial permeability transition during hypothermic to normothermic reperfusion in rat liver demonstrated by the protective effect of cyclosporin A. Biochem J. 1998 Dec 1;336 (Pt 2):501–6.
19. Karimian N, Westerkamp AC, Porte RJ. Biliary complications after orthotopic liver transplantation. Curr Opin Organ Transplant. 2014 Jun;19(3):209–16.

20. Seehofer D, Eurich D, Veltzke-Schlieker W, Neuhaus P. Biliary complications after liver transplantation: old problems and new challenges. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2013 Feb;13(2):253–65.

21. Op den Dries S, Sutton ME, Karimian N, de Boer MT, Wiersema-Buist J, Gouw ASH, et al. Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after circulatory death. PloS One. 2014;9(2):e88521.

22. Op den Dries S, Karimian N, Westerkamp AC, Sutton ME, Kuipers M, Wiersema-Buist J, et al. Normothermic machine perfusion reduces bile duct injury and improves biliary epithelial function in rat donor livers. Liver Transplant Off Publ Am Assoc Study Liver Dis Int Liver Transplant Soc. 2016;22(7):994–1005.

23. Hosgood SA, Nicholson HFL, Nicholson ML. Oxygenated kidney preservation techniques. Transplantation. 2012 Mar 15;93(5):455–9.

24. Kameneva MV, Undar A, Antaki JF, Watach MJ, Calhoon JH, Borovetz HS. Decrease in red blood cell deformability caused by hypothermia, hemodilution, and mechanical stress: factors related to cardiopulmonary bypass. ASAIO J Am Soc Artif Intern Organs 1992. 1999 Aug;45(4):307–10.

25. Matton APM, Burlage LC, van Rijn R, de Vries Y, Karangwa SA, Nijsten MW, et al. Normothermic Machine Perfusion of Donor Livers Without the Need for Human Blood Products. Liver Transplant Off Publ Am Assoc Study Liver Dis Int Liver Transplant Soc. 2017 Dec 27;

26. Tolich DJ, McCoy K. Alternative to Blood Replacement in the Critically Ill. Crit Care Nurs Clin North Am. 2017 Sep;29(3):291–304.

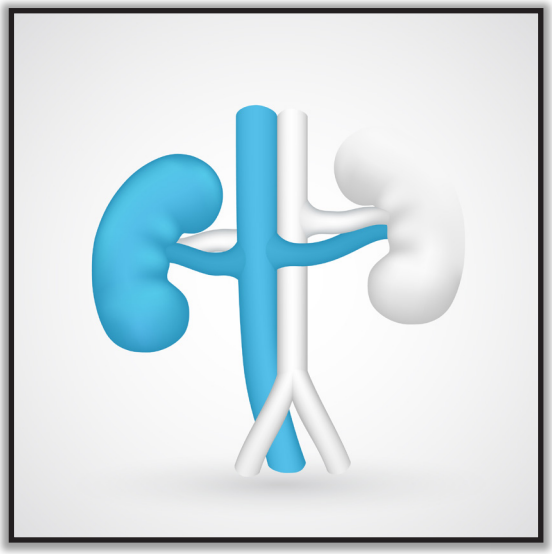
27. Minor T, Efferz P, Fox M, Wohlschlaeger J, Lüer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2013 Jun;13(6):1450–60.

28. Hoyer DP, Mathé Z, Gallinat A, Canbay AC, Treckmann JW, Rauen U, et al. Controlled Oxygenated Rewarming of Cold Stored Livers Prior to Transplantation: First Clinical Application of a New Concept. Transplantation. 2016 Jan;100(1):147–52.

CHAPTER 2

Rodent Organ Perfusion Systems

Paria Mahboub, Sanna Op den Dries



INTRODUCTION

Ex-vivo machine perfusion is gaining popularity as a promising alternative to cold storage (1-6). The potential advantages of machine perfusion range from increased protection from ischemic injury to providing organ assessment before transplantation and improving organ viability during preservation.

Machine perfusion can be performed in many different ways, and it is yet to be determined which type of perfusion system, perfusion fluid, duration and temperature is most beneficial (7,8). The decision will ultimately depend on the goal of perfusion (protection, prolonged preservation, assessment or improvement of the organ, or a combination), the type of organ involved and the financial cost. In order to study these variables of machine perfusion in a controlled setting, a small-size model such as a rodent organ perfusion system can be very helpful.

An important advantage of animal studies, when compared to clinical studies in humans, is the genetic homogeneity of laboratory animals, which significantly reduces the number of cofounding factors in a study. Moreover, rodents express many genetic and biological similarities with humans, making them an appropriate model for medical research (9). The potential of altering the genetic makeup of laboratory animals, particularly in mice, allows researchers to create animal models that are not just 'workable' approximations, but are, in fact, close replicas of human disease under study (10). Essential for translational research, rodent organ perfusion systems can be easily adjusted to have the same characteristics as larger sized perfusion systems for human organs.

In this chapter we discuss our experience with two rodent organ perfusion systems, one for livers and one for kidneys.

BACKGROUND

Several rodent liver and kidney perfusion systems have been described in medical literature. The first *ex vivo* organ perfusion systems for rodent organs were developed a long time ago, in the late 1800's (11). However, these systems were not built to study aspects of machine perfusion. Instead, they were used as a tool for exploring the physiology and pathophysiology of the organ and these systems were often referred to as IPL (isolated perfused liver) and IPK (isolated perfused kidney) systems (12-16).

It was not until the last decade that machine perfusion became an increasingly studied alternative to static cold storage, and subsequently new rodent organ perfusion systems have been developed to study the effects of machine perfusion on donor organs in a smaller-sized model.

In the same way that a variety of larger-sized human organ machine perfusion devices is available, a range of smaller perfusion systems for rodent organs have been developed by different research groups (16-20). With regard to the technical aspects of a rodent organ perfusion system, the relevance of a smaller-sized model is increased when the system more accurately reflects the larger human organ perfusion system used by the research group. A few key changes/adjustments in the development of rodent organ perfusion systems will be discussed for the kidney and liver separately. This chapter will not review the range of available rodent organ perfusion systems, but rather explain and discuss two systems that have been used for rodent organ perfusion by the authors.

General System Characteristics

The rodent kidney and liver perfusion systems as described in this chapter were designed in the University Medical Center Groningen (UMCG), The Netherlands. They have thus far only been used for the perfusion of rat organs, but could be adjusted to allow perfusion of other rodents' organs, therefore the systems will be referred to as "rodent organ perfusion systems".

The rodent kidney perfusion system contains a single pump for arterial flow via the renal artery. The rodent liver perfusion system, on the other hand, contains two pumps for dual perfusion via the hepatic artery and portal vein. Both systems provide pulsatile flow to the artery (the renal artery and hepatic artery, respectively), and in case of the liver perfusion system, continuous flow to the liver's portal vein. The systems are temperature and pressure controlled, the latter providing a physiological flow through the organ, regulated by the organ's vascular resistance. Constant pressure at variable flow rates minimizes the risk of vascular injury and shear stress in the organ (21). Ohm's formula was used to correct for additional resistance caused by the (cannulas of the) perfusion system: $P_{total} = P_{organ}$ $(1 + (R_{cannula}/(R_{total} - R_{cannula})))$. In both systems, inline sensors detect flow, pressure and temperature, and data are analyzed by and displayed in real-time on a connected laptop. The systems use tubular membrane oxygenators as a method for providing oxygen and removing of CO_2. Such oxygenators are considered safe as they minimize the risk of foaming and air bubble formation in the perfusion solution during organ perfusion. To facilitate perfusate sampling and to provide a site for adding medication during perfusion, several three-way connectors are situated within both systems.

RODENT KIDNEY PERFUSION SYSTEM

System Setup

The design of early rodent kidney perfusion systems was based on Bekersky and Bowman's IPK system; a gravity and recirculation system which utilized the effect of gravity to create

pressure and subsequent flow through the organ (13,14). Instead of using gravity, the rodent kidney perfusion system described here contains a roller pump, an inline pressure probe and a connected laptop, delivering a pressure-controlled, pulsatile flow to the kidney (**Figure 1 and 2**). The flow is measured via inline flow sensors. In order to maintain the desired temperature of the perfusion fluid, the system incorporates an inline temperature probe, a heat exchanger (connected to an automated water bath) and a fan heater which responds to a thermostat connected to a laptop. In addition, the rodent kidney perfusion system is surrounded by an insulated double walled box facilitating a stable temperature. The organ chamber is covered by a Perspex lid which helps to maintain a humid environment for the perfused rat kidney. A 100 mL solution reservoir is placed below the organ chamber in order to create a slightly negative pressure in the renal vein. Oxygenation of the perfusion fluid is accomplished by a tubular membrane oxygenator and oxygen saturation is measured by inline oxygen sensors in both arterial and venous perfusion fluid.

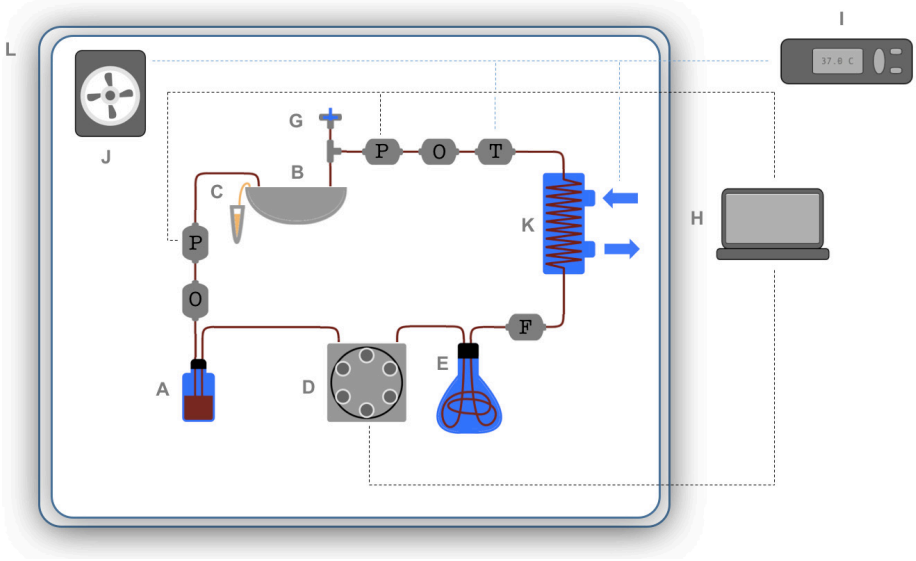


Figure 1. Graphic representation of the rodent kidney perfusion system. The solution reservoir (**A**) is placed bellow the organ chamber (**B**) in order to create a slightly negative pressure in the renal vein. Ultrafiltrate is collected in Eppendorf tubes (**C**). A roller pump provides a pulsatile flow (**D**) to the renal artery. Oxygenation of the perfusion fluid is accomplished by an oxygenator (**E**) and oxygen saturation is measured by inline oxygen sensors in both arterial and venous perfusion fluid (**O**). Several bubble traps (3-way connectors) are used to eliminate air bubbles in the perfusion solution (**G**). Flow (**F**), pressure (**P**), and temperature (**T**) are detected by inline sensors, and data are analyzed by and displayed in real-time on a connected laptop (**H**). The thermostat (**I**), a fan heater (**J**), heat exchangers (**K**) and a Plexiglas box (**L**) encapsulating the perfusion system ensure a stable temperature.

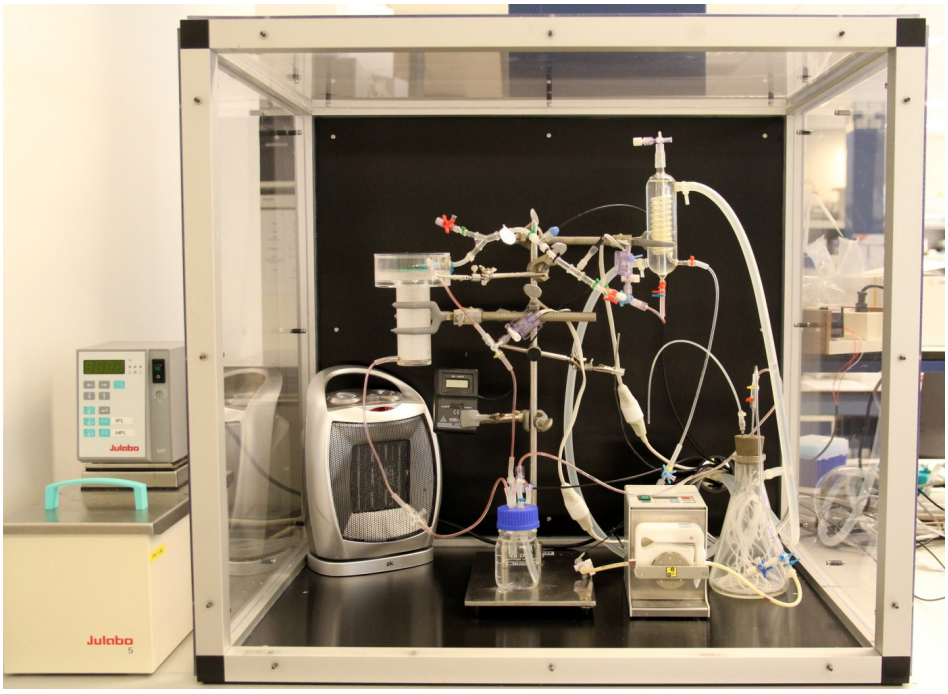


Figure 2. Overview of rodent kidney perfusion system. The system is located in a custom-made climate box. The front cover of the climate box has been removed for this photo. The flow meter, a laptop, and a water bath are connected into the enclosed system via an entry port.

System Componentry

- Custom-made Perspex organ chamber.
- Perspex organ chamber lid.
- Glassware 100 mL flacon used as perfusion fluid reservoir.
- Roller pump with 6 rollers (Ismatec MS-2/6-160; IDEX Health and Science).
- Tubing for roller pump (Ismatec Pharmed BPT NSF-51; IDEX health and Science).
- Glassware coil type heat exchanger (Radnoti Heating coil; 10 mL).
- Two inline pressure sensors (Truwave Tranducer PX600FPR; Edwards Lifesciences Corporation).
- Flow meter (Transonic System Inc. Model T402; 2 channels).
- One inline flow sensor (Transonic System Inc. Type 1PXN).
- Two optical oxygen meters (Fibox 4&Fibox 4 trace; PreSens).
- Two oxygen sensors (type PSt3; PreSens).
- Inline temperature sensor (MEDOS NTC).
- Digital thermostat (Lucky Reptile. Thermo Control Pro 2).

- Fan-driven heater (Ok company, Netherlands).
- Automated water bath (JulaboLabortechnik GMBH MP-5; 2.1 Kilowatt).
- Porous silicon tubing for the oxygenator (Rubber BV).
- Glass Buchner flask with rubber bung for the oxygenator (Schott Duran; 500 mL).
- Three-way connectors (Cole-Parmer Y-form Fitting; 35mm by 21 mm).
- Custom-made plastic lead for the organ chamber.
- Laptop with (pressure and temperature regulation) software (provided by Organ Assist, Groningen, Netherlands).
- PreSensoxygen software (Provided by PreSens company, Germany).
- Custom-made Perspex climate box.
- Arterial, venous and ureter cannulas (Arterial 20 Gauge Cathether, Venous 18 .

Gauge Cathether, Ureter 0.28mm ID and 0.61 OD Polythene tubing, Protex, Smiths medical).

Rat Nephrectomy

In order to perform a nephrectomy, the rat is anesthetized using 2-3% isoflurane. The abdomen is opened by a thoracic transverse decision and the ureter is dissected and cannulated. After heparinizing the rat with 1ml 0.9% NaCl and 500 IU of heparin via the dorsal penile vein, the abdominal aorta is ligated above the left renal artery. Subsequently, the renal artery and renal vein are approached and cannulated. After cannulation, the kidney is flushed via the renal artery with 5 ml of cold (4°C) saline (Baxter, The Netherlands) followed by 5 ml of cold (4°C) preservation solution, such as University of Wisconsin (UW) (Viaspan, Belzer™). Depending on the experimental protocol, the kidney might be subjected to a period of static cold storage at (4°C) in preservation solution or immediately connected to the rodent kidney perfusion system.

To initiate machine perfusion of the kidney, the organ is placed on the organ chamber and connected to the perfusion system via the cannulated renal artery and vein. Ultrafiltrate is collected from the cannulated ureter.

RODENT LIVER PERFUSION SYSTEM

System Setup

Many rodent liver perfusion systems provide single perfusion, which means that the liver is connected to the system only via the portal vein (17,18). The system described here, however, allows for dual perfusion, delivering flow through both the hepatic artery and the portal vein (**Figure 3 and 4**). This adjustment is relevant, as the biliary system's blood supply relies to a large extent on the arterial blood flow. Dual liver perfusion via portal vein and hepatic artery aims to provide better perfusion, more adequate nutrition and oxygen

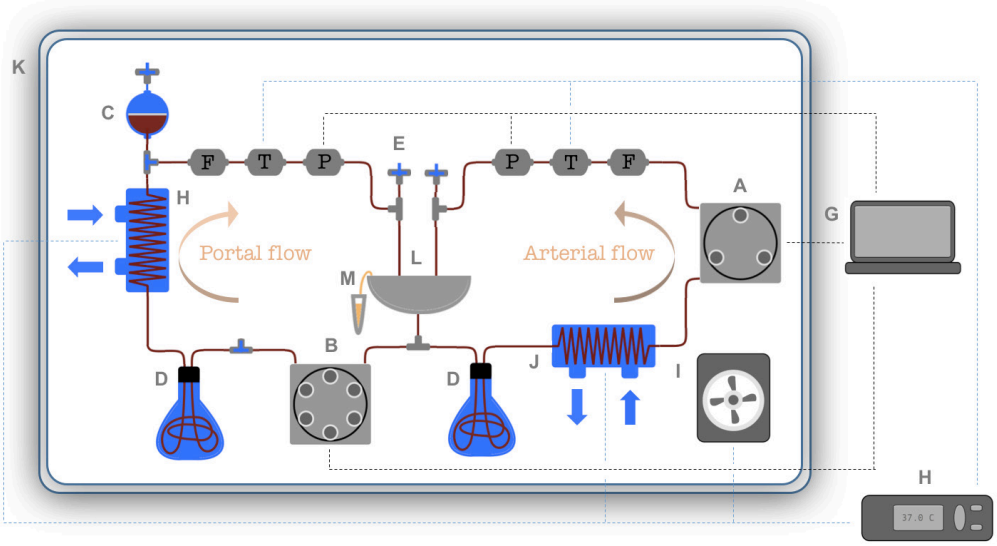


Figure 3. Graphic representation of the rodent liver perfusion system with dual (arterial and portal) perfusion of the liver. Two roller pumps provide a pulsatile flow (**A**) to the hepatic artery and a continuous flow (**B**) to the portal vein, after eliminating pulses with elastic tubing and an air chamber (**C**). Two tubular membrane oxygenators provide oxygenation of the perfusion solution, as well as removal of CO_2 (**D**). Several bubble traps (3-way connectors) are used to eliminate air bubbles in the perfusion solution (**E**). Flow (**F**), pressure (**P**), and temperature (**T**) are detected by inline sensors, and data are analyzed by and displayed in real-time on a connected laptop (**G**). The thermostat (**H**), a fan heater (**I**), heat exchangers (**J**) and a Plexiglas box (**K**) encapsulating the perfusion system ensure a stable temperature. The liver is placed into an organ chamber (**L**) and protected with a transparent cover to maintain a moist and warm environment. Bile is collected in an Eppendorf tube (**M**).

supply to the organ and prevent biliary injury (17,18). Where human liver perfusion devices employ dual perfusion, this should be reflected in the smaller-sized perfusion systems used by these research groups.

The system is pressure-controlled by a computer algorithm allowing auto regulation of blood flow through the liver, with constant pressure at variable flow rates. Flow and pressure are monitored by in-line sensors and data is analyzed by and displayed in real time on a connected laptop. Flow to the hepatic artery and the portal vein is provided by two roller pumps. One roller pump with 3 rollers is located near the entry of the hepatic artery, resulting in a pulsatile flow entering the artery. Using a roller pump with 3 rollers results in a (for a rat) physiological arterial pulse of 250/min at a physiological arterial flow of 6 mL/min. The second roller pump contains 6 rollers (this results in reduced pulsation) and provides flow to the portal vein. This second roller pump is placed far from the entry of the portal vein in order to reduce pulsations to a minimum; a special pulse-reducing air chamber, elastic tubing and one of the two tubular membrane oxygenators are placed between the roller

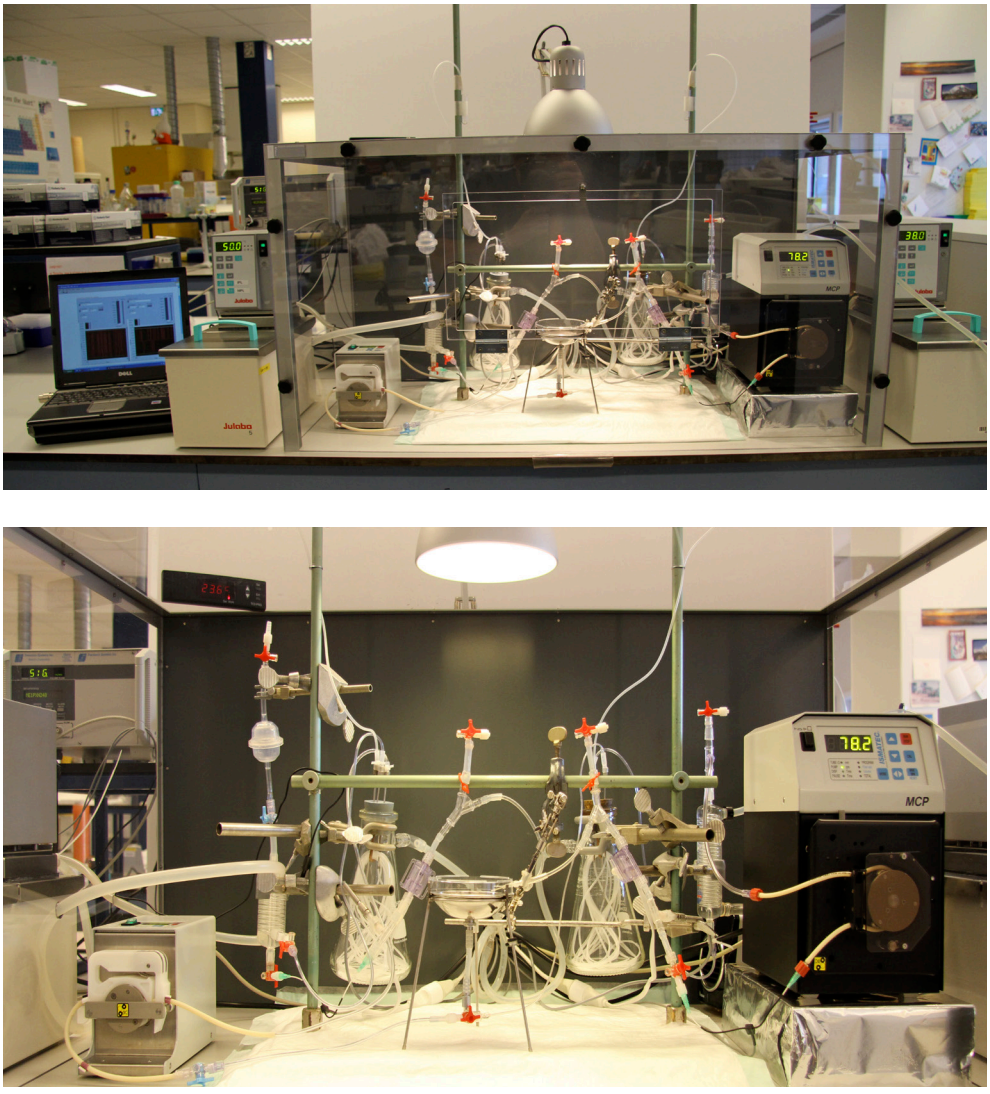


Figure 4. General overview of the rodent liver perfusion system. The system consists of a portal and arterial side and the whole system is situated inside in a climate box. The laptop, flow meter and two water baths are located outside the box, and are connected into the enclosed system via an entry port.

pump and the portal vein, resulting in a continuous flow entering the portal vein. The hepatic vein is not cannulated, allowing for a low-resistance outflow. The rodent liver perfusion system is temperature controlled via a thermostat and the temperature is monitored by two inline temperature probes. The combination of a climate box, heat exchangers, a fan heater

and an automated water bath enable the system to be stable at a range of temperatures (subnormothermic, normothermic). Both rodent organ perfusion systems can be upgraded to allow for a wider range of temperatures (including hypothermic) and controlled gradual rewarming or cooling (19,20) (see below in the *System Componentry* section for details of the upgraded components).

The organ chamber is covered by a Perspex lid which helps to maintain a humid environment for the perfused liver. A metal grid at the base of the organ chamber is covered with parafilm to prevent damage to the organ. Oxygenation of the perfusion fluid is accomplished by two tubular membrane oxygenators. Air bubble traps are located at the highest points in the system (above the organ); one after each oxygenator and one right before the perfusion fluid enters the liver on both the arterial side and the portal side. To facilitate perfusate sampling and to provide a site for adding medications during perfusion, several three-way connectors are located within the system.

System Componentry

- Custom-made stainless organ chamber on a tripod + metal grid.
- Perspex organ chamber lid.
- One roller pump with 6 rollers for the portal vein (Ismatec MS-2/6-160; IDEX Health and Science).
- One roller pump + pump head with 3 rollers for the hepatic artery (Ismatec ISM404 + ISM719; IDEX Health and Science).
- Tubing for roller pumps (Ismatec Pharmed BPT NSF-51; IDEX health and Science).
- Air chamber with membrane.
- Two inline pressure sensors (Truwave Tranducer PX600FPR; Edwards Lifesciences Corporation).
- Flow meter (Transonic Systems Inc. Model T402; 2 channels).
- Two inline flow sensors (Transonic System Inc. Type 1PXN).
- Two inline temperature sensors (MEDOS NTC).
- Digital thermostat (Lucky Reptile. Thermo Control Pro 2).
- Fan-driven heater (Euromacbv. Personal Heater 200; 200 Watt).
- Two water baths (Julabo Labortechnik GMBH MP-5; 2.1 Kilowatt).
- Two glassware coil type heat exchangers (Radnoti Heating coil; 5.5 mL).
- Porous silicon tubing for the oxygenator (Rubber BV).
- Glass Buchner flask with rubber bung for the oxygenator (Schott Duran; 500 mL).
- Three-way connectors (Cole-Parmer Y-form Fitting; 35mm by 21 mm).
- Laptop with (pressure and temperature regulation) software (provided by Organ Assist, Groningen, Netherlands).
- Custom-made Perspex climate box (Research Instrument Manufacturing Department UMCG).

- Portal, arterial and common bile duct cannulas (Insyte, Becton Dickinson BV. Portal 18 Gauge IV Cathether; Arterial 20 Gauge).

Optional upgrade for improved temperature control

- Ministat 230 thermostat and the fan (Huber, USA).
- Inline temperature sensor (Huber, USA) (Electronic Thermometer; ama-digit ad 15th).

Rat Hepatectomy

Inhalation anesthesia with isoflurane and oxygen is used before and during the procurement (2-3% isoflurane). The extrahepatic bile duct is cannulated and 1 ml 0.9% NaCl with 500 IU heparin is administered via the dorsal penile vein. The hepatectomy is performed by ligation of the splenic vein, mesenteric artery and mesenteric vein and cannulation of the celiac trunk. After clamping of the infra-hepatic inferior vena cava and the portal vein, the latter is cannulated and flushed *in situ* with 10 ml 0.9% NaCl (37ºC). Subsequently, the supra-hepatic inferior vena cava is transected, followed by a cold flush out with 5 mL preservation fluid (4ºC) via the portal vein cannula. The liver is removed and flushed with an additional 20mL of cold (4ºC) preservation fluid via the portal vein cannula and 5mL of cold (4ºC) preservation fluid via the hepatic artery (celiac trunk cannula). Depending on the experimental protocol, the liver might be subjected to a period of static cold storage or immediately connected to the rodent liver perfusion system (20,22).

To initiate machine perfusion of the liver, the organ is placed in the organ chamber and connected to the perfusion system via the cannulated hepatic artery and portal vein. Bile is collected from the cannulated bile duct.

Preparing the Perfusion Systems

Before the start of each experiment, the systems should be prepared for use.

- Switch on the roller pumps and the laptop. Open the pressure control software.
- Flush the system with 70% alcohol, unhook the tubing from the roller pumps and dry the system using pressurized air.
- Flush the system with demineralized water and subsequently circulate NaCl 0.9% for 15 minutes.
- Turn on the water bath(s) and set to your desired temperature.
- Switch on the flow meter, the oxygen meter and the fan heater and set the thermostat to the preferred temperature.
- Fill the system with the prepared perfusion fluid (about 100mL). Check the system for air bubbles and use the bubble traps to remove any air if needed. The system should be entirely air bubble free before the organ is connected.
- Start a low carbogen flow (95% O_2, 5% CO_2) through the oxygenator(s) 15 minutes prior connecting the organ.

- Calibrate the pressure sensors.
- Calibrate the oxygen sensor (in the rodent kidney perfusion system).
- Close the climate box and make sure that the temperature is stable.

Systems' Perfusion Procedures

Rodent Kidney Perfusion System

- Before connecting the kidney, double check whether the system (especially the arterial cannula) is air bubble free and make sure that the pressure is calibrated to zero in the system.
- Place the kidney in the organ chamber and connect the arterial cannula to the system, subsequently connect the venous cannula, wait for few seconds until the pressure is stable in the kidney and then change the pressure to the desired pressure as described in the study protocol.
- Direct the ureter cannula in an empty weighed Eppendorf tube.

Rodent liver perfusion system

- Before connecting the liver, double check the system, the hepatic artery and portal vein cannulas for air bubbles and make sure that the pressure is calibrated to zero in the system.
- Place the liver in the organ chamber, first connect the portal vein cannula to the system and subsequently connect the hepatic artery cannula, wait for few seconds until the pressure is stable and then change the pressure to the desired level as described in the study protocol.
- Direct the bile duct cannula in an empty weighed Eppendorf tube.

Systems' Cleaning Procedure

Disconnect the organ from the system and remove it.

- Remove the perfusion fluid from the system, rinse with warm demineralized water (single-pass) until the water exiting the system is clear. Continue the cleaning process with a filtered Biotex soap solution followed by a flush with at least 1L of warm demineralized water, and finalize by a flush with 70% alcohol.
- At the end dry the system with pressure air and be sure that the pump tubing is unhooked from the roller pumps. It is important to keep the system dry until the next experiment. In order to prevent stretching and tearing of the pump tubing, the tubing should remain unhooked after cleaning until the next experiment.

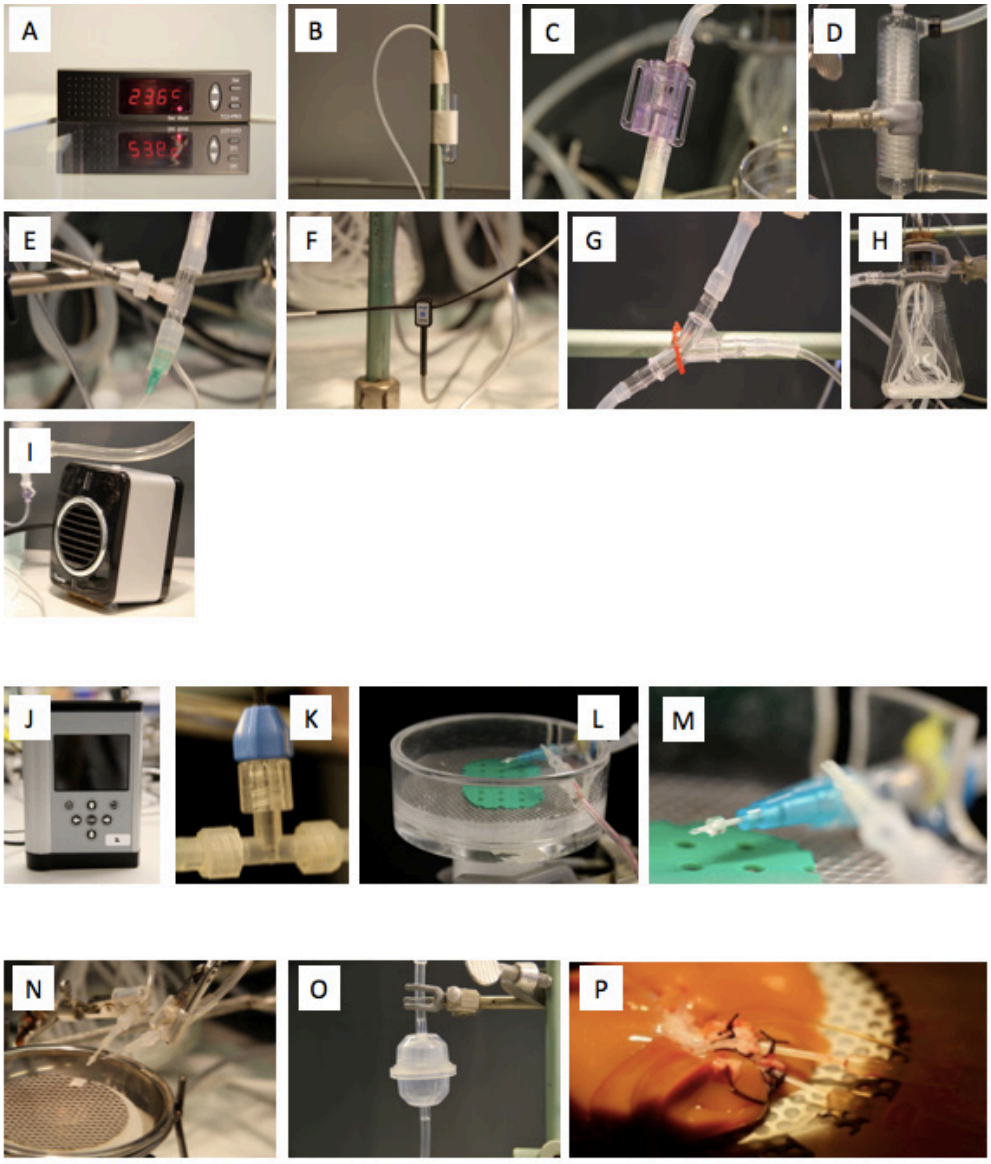


Figure 5. Photographic details of the rodent perfusion systems. Both systems contain: A thermostat (A), an outlet (B) for the 95% oxygen used in the tubular membrane oxygenators (H) inside the cabinet. An inline pressure sensor (C), temperature sensor (E) and flow sensor (F). A three-way connecter with a small tube inside, used as a bubble-trap (G), a heat exchanger (D) and a fan heater (I). The rodent kidney perfusion system includes: An oxygen meter (J) and inline oxygen sensor (K), and a kidney organ chamber (L) with cannulas for the renal artery and vein (M). The rodent liver perfusion system contains: An air chamber to minimize pulses in the portal flow, also functioning as an additional bubble-trap (O), a liver organ chamber (N) and cannulas for the hepatic artery and portal vein (P).

CONCLUSION

The rodent organ perfusion devices described here have been used and continue to be used for a range of studies at the University Medical Center in Groningen (19,20,22). The systems have delivered stable, reproducible outcomes at a wide range of temperatures, using organs donated after brain death (DBD) and after circulatory death (DCD). Over time, various improvements have been made, ranging from improved temperature control to placing oxygen sensors in the renal artery and vein tubing for real-time oxygen consumption measurements during perfusion (19,20). As new ideas for improvement arise, and as human organ perfusion systems develop, rodent organ perfusion systems can be easily adjusted and improved to study relevant machine perfusion related research questions. One such adjustment that shows much potential is to enable rodent perfusion systems to perfuse mice organs, which would open up new possibilities for research, given the broad range of genetically modified mice available.

The improvement of rodent perfusion systems is an ongoing process and there remains much scope for further modifications based on future experimental design.

REFERENCES

(1) Chadha R, Ayaz M, Bagul A. Optimising organs for transplantation: is normothermic machine perfusion the answer? Expert Rev Med Devices 2016 Jan 25.

(2) Dutkowski P, Polak WG, Muiesan P, Schlegel A, Verhoeven CJ, Scalera I, et al. First Comparison of Hypothermic Oxygenated PErfusion Versus Static Cold Storage of Human Donation After Cardiac Death Liver Transplants: An International-matched Case Analysis. Ann Surg 2015 Nov;262(5):764-771.

(3) Bruinsma BG, Yeh H, Ozer S, Martins PN, Farmer A, Wu W, et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. Am J Transplant 2014 Jun;14(6):1400-1409.

(4) Op den Dries S, Karimian N, Sutton ME, Westerkamp AC, Nijsten MW, Gouw AS, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. Am J Transplant 2013 May;13(5):1327-1335.

(5) Minor T, Olschewski P, Tolba RH, Akbar S, Kocalkova M, Dombrowski F. Liver preservation with HTK: salutary effect of hypothermic aerobiosis by either gaseous oxygen or machine perfusion. Clin Transplant 2002 Jun;16(3):206-211.

(6) Hoyer DP, Mathe Z, Gallinat A, Canbay AC, Treckmann JW, Rauen U, et al. Controlled Oxygenated Rewarming of Cold Stored Livers Prior to Transplantation: First Clinical Application of a New Concept. Transplantation 2016 Jan;100(1):147-152.

(7) Martinez J, Bachler JP, Moisan F, Torres J, Duarte I, Perez RM, et al. Outcomes using two preservation solutions (UW/HTK) in liver transplantation from brain death donors. Rev Med Chil 2014 Oct;142(10):1229-1237.

(8) Selzner M, Goldaracena N, Echeverri J, Kaths JM, Linares I, Selzner N, et al. Normothermic Ex Vivo Liver Perfusion Using Steen Solution as Perfusate for Human Liver Transplantation-First North American Results. Liver Transpl 2016 Jun 24.

(9) Doorschodt BM, Teubner A, Kobayashi E, Tolba RH. Promising future for the transgenic rat in transplantation research. Transplant Rev (Orlando) 2014 Oct;28(4):155-162.

(10) Hogenes M, Huibers M, Kroone C, de Weger R. Humanized mouse models in transplantation research. Transplant Rev (Orlando) 2014 Jul;28(3):103-110.

(11) Gores GJ, Kost LJ, LaRusso NF. The isolated perfused rat liver: conceptual and practical considerations. Hepatology 1986 May-Jun;6(3):511-517.

(12) Taft DR. The isolated perfused rat kidney model: a useful tool for drug discovery and development. Curr Drug Discov Technol 2004 Jan;1(1):97-111.

(13) Bekersky I, Popick AC. Disposition of bumetanide in the isolated perfused rat kidney: effects of probenecid and dose response. Am J Cardiol 1986 Jan 24;57(2):33A-37A.

(14) Bowman RH. Renal secretion of [35-S]furosemide and depression by albumin binding. Am J Physiol 1975 Jul;229(1):93-98.

(15) ‘t Hart NA, van der Plaats A, Moers C, Leuvenink HG, Wiersema-Buist J, Verkerke GJ, et al. Development of the isolated dual perfused rat liver model as an improved reperfusion model for transplantation research. Int J Artif Organs 2006 Feb;29(2):219-227.

(16) Compagnon P, Clement B, Campion JP, Boudjema K. Effects of hypothermic machine perfusion on rat liver function depending on the route of perfusion. Transplantation 2001 Aug 27;72(4):606-614.

(17) Berendsen TA, Bruinsma BG, Lee J, D’Andrea V, Liu Q, Izamis ML, et al. A simplified subnormothermic machine perfusion system restores ischemically damaged liver grafts in a rat model of orthotopic liver transplantation. Transplant Res 2012 May 9;1(1):6-1440-1-6.

(18) Dutkowski P, Furrer K, Tian Y, Graf R, Clavien PA. Novel short-term hypothermic oxygenated perfusion (HOPE) system prevents injury in rat liver graft from non-heart beating donor. Ann Surg 2006 Dec;244(6):968-76; discussion 976-7.

(19) Mahboub P, Ottens P, Seelen M, T Hart N, Van Goor H, Ploeg R, et al. Gradual Rewarming with Gradual Increase in Pressure during Machine Perfusion after Cold Static Preservation Reduces Kidney Ischemia Reperfusion Injury. PLoS One 2015 Dec 2;10(12):e0143859.

(20) Westerkamp AC, Mahboub P, Meyer SL, Hottenrott M, Ottens PJ, Wiersema-Buist J, et al. End-ischemic machine perfusion reduces bile duct injury in donation after circulatory death rat donor livers independent of the machine perfusion temperature. Liver Transpl 2015 Oct;21(10):1300-1311.

(21) Gallinat A, Fox M, Luer B, Efferz P, Paul A, Minor T. Role of pulsatility in hypothermic reconditioning of porcine kidney grafts by machine perfusion after cold storage. Transplantation 2013 Sep;96(6):538-542.

(22) Op den Dries S, Karimian N, Westerkamp AC, Sutton ME, Kuipers M, Wiersema-Buist J, et al. Normothermic machine perfusion reduces bile duct injury and improves biliary epithelial function in rat donor livers. Liver Transpl 2016 Jul;22(7):994-1005.

CHAPTER 3

Gradual Rewarming with Gradual Increase in Pressure During Machine Perfusion After Cold Static Preservation Reduces Kidney Ischemia Reperfusion Injury

PLOSONE 2015; Dec 2

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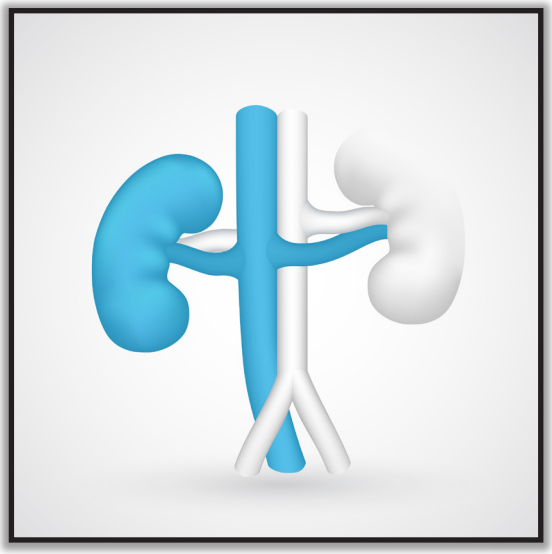

ABSTRACT

In this study we evaluated whether gradual rewarming after the period of cold ischemia would improve organ quality in an Isolated Perfused Kidney Model. Left rat kidneys were statically cold stored in University of Wisconsin solution for 24 hours at 4°C. After cold storage kidneys were rewarmed in one of three ways: perfusion at body temperature (38°C), or rewarmed gradually from 10°C to 38°C with stabilization at 10°C for 30 min and rewarmed gradually from 10°C to 38°C with stabilization at 25°C for 30 min. In the gradual rewarming groups the pressure was increased stepwise to 40 mmHg at 10°C and 70 mmHg at 25°C to counteract for vasodilatation leading to low perfusate flows. Renal function parameters and injury biomarkers were measured in perfusate and urine samples. Increases in injury biomarkers such as aspartate transaminase and lactate dehydrogenase in the perfusate were lower in the gradual rewarming groups versus the control group. Sodium re-absorption was improved in the gradual rewarming groups and reached significance in the 25°C group after ninety minutes of perfusion. HSP-70, ICAM-1, VCAM-1 mRNA expressions were decreased in the 10°C and 25°C groups. Based on the data kidneys that underwent gradual rewarming suffered less renal parenchymal, tubular injury and showed better endothelial preservation. Renal function improved in the gradual rewarming groups versus the control group.

INTRODUCTION

Current preservation in organ transplantation is based on hypothermic preservation. The standard practice is to preserve organs by static cold storage (SCS) at 4°C until the time of implantation. Although metabolism is reduced during hypothermia, it is not completely arrested. Even at 4°C, cells continue to consume oxygen and utilize adenosine triphosphate (ATP) at a metabolic rate of approximately 5% of baseline.(1,2) This leads to a gradual depletion of ATP and adenosine diphosphate (ADP), which stops almost all energy-dependent processes and also initiates early damage. All these factors contribute to cold ischemia injury in the organ during static cold preservation. At the time of reperfusion, graft rewarming and re-oxygenation induces even more damage than the initial tissue damage caused by ischemia due to formation of reactive oxygen species.
Alternative preservation approaches to improve graft quality during organ preservation (mainly liver) are currently being studied by many groups. Major developments are machine perfusion methods such as hypothermic, sub-normothermic and even normothermic perfusion. It is shown that a period of hypothermic oxygenated machine perfusion (3,4) or subnormothermic machine perfusion (5) prior to the reperfusion has been beneficial in increasing the ATP content of the graft which later helps to protect the organ against ischemia reperfusion injury (6,7). Alongside hypothermic and sub-normothermic machine perfusion, normothermic machine perfusion (NMP) has been applied prior to reperfusion. Adding a period of NMP after SCS and before implantation of the organ offers potential to assess graft viability prior to transplantation.(8,9) NMP includes a pulsatile flow of oxygenated perfusion solution in the organ which supports cellular metabolism at body temperature restores the energy content of the organ, and washes out waste products prior to reperfusion in the recipient body. Nicholson and colleagues have shown the benefits of kidney NMP in several studies and the method has been applied in human organs with success.(10,11) Although machine perfusion is associated with better graft function after transplantation and may protect against ischemia reperfusion injury, there has been little attention on strategies to protect the organ from sudden graft rewarming and reoxygenation during machine perfusion.(5)

In this study we investigated whether a strategy of a gradual increase in temperature and pressure after cold storage, prior to reperfusion at body temperature improves kidney graft quality.

METHODS

Animals Used

Male Lewis rats (Harlan, The Netherlands) weighing 290-350 g were used in this study. Animals received care according to the Dutch Law on animal experiments. The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Groningen (IACUC-RuG).

Rats were anesthetized using 5% isoflurane and 1ml 0.9% NaCl with 500 IU of heparin was administrated via the dorsal penile vein. The rats were sacrificed after left nephrectomy. The renal artery and ureter were cannulated. The kidneys were then flushed via the renal artery with 5 ml of cold (4°C) saline (Baxter, The Netherlands) followed by 5 ml of cold (4°C) University of Wisconsin (UW) preservation solution (Viaspan, Belzer ™). The kidneys were cold stored at 4°C for a period of 24 hours in UW in a 25 mL flask. After CS, kidneys were placed in an isolated kidney perfusion (IPK) device.

The Isolated Perfused Kidney (IPK) Device and Perfusion Settings.

The IPK device consists of a roller pump (Ismatec MS-2/6-160; IDEX Health and Science), heat exchanger (Radnoti Heating coil, 5.5 mL), one tubular membrane oxygenator, 100 mL solution reservoir, an inline temperature probe and pressure probe (Edwards Lifescience Corporation). The device was pressure and temperature controlled. Pressure was monitored continuously by a probe connected to a lap top during the IPK experiment. The heat exchanger was connected to two (one cold and one warm) water baths (Julabo Labortechnik). The organ chamber was covered by a Perspex lid which helped to provide a moist environment for the perfused rat kidney.

The kidneys were placed in the organ chamber and connected to the IPK device and perfused through the renal artery with oxygenated William's medium E (WME). The ureter was cannulated and the ultra-filtrate (urine) was collected.

Experimental Groups

Following 24 hours of SCS (4°C) kidneys were connected to the IPK device and perfused during 90 minutes according to one of the following protocols.

Control Group (immediate rewarming) (n=8)

Kidneys were immediately perfused at 38°C at a mean arterial pressure of 100 mm Hg during 90 minutes perfusion "Table 1".

Table 1 | This table illustrates the details of study design including duration of cold storage, perfusion temperature, perfusion pressure, rewarming and reperfusion phase.

Groups n=8	Cold Storage	Rewarming	Reperfusion
Control	24 h	38°C/100mmHg/30 min	38°C/100mmHg/60 min
10°C	24 h	10°C/40mmHg/25min 25°C/70 mmHg/5min	38°C/100mmHg/60 min
25°C	24 h	10°C/40mmHg/5min 25°C/70 mmHg/25min	38°C/100mmHg/60 min

Gradual Rewarming from 10°C to 38°C (n=8)

Kidneys were first perfused at a temperature of 10°C for 25 minutes. Afterwards, the temperature was gradually increased to 38°C in two steps. First it was increased to 25°C for a few minutes, and next it was raised to 38°C and perfused at 38°C for additional 60 minutes. Parallel to increasing the temperature, the pressure was gradually elevated from 40 mm Hg to 70 mm Hg at 25°C and to 100 mm Hg at 38°C “Table 1”.

Gradual Rewarming from 25°C to 38°C (n=8)

Kidneys were placed in the IPK set-up and the temperature was set on 10°C in the beginning and then gradually raised from 10°C to 25°C and was stabilized at 25°C for 25 minutes. Alongside to this, pressure was increased from 40 mm Hg to 70 mm Hg. After first 30 minutes the temperature was adjusted at 38°C with pressure set to 100 mm Hg for 60 minutes “Table 1”.

Cold Preservation Group (n=6)

Followed nephrectomy kidneys were subjected to 24 hours SCS in UW solution at 4°C without rewarming. After SCS tissue samples were taken and stored at -80°C for further analysis.

Perfusion Solution

The perfusion solution consists of William’s Medium E (Life technologies, USA) 100 mL, Creatinine (Sigma-Aldrich, The Netherlands) 0.08 g/dL, bovine serum albumin (PAA Laboratories GmbH, Austria) 5g/dL, HEPES (Sigma-Aldrich, The Netherlands) 0.7149 g/dL. This solution was used for the 90 minutes perfusion period. Prior to the experiments, the perfusion solution was oxygenated during 15 minutes with carbogen (95%O_2 and 5%CO_2) to achieve an oxygen pressure of at least 60 kPa and it was kept actively oxygenated. After this equilibration the pH was adjusted to 7.4. During the IPK perfusion no further adjustments were made to the pH.

Temperature Hemodynamic Monitoring

Temperature and renal flow were recorded every 10 minutes during the IPK perfusion.

Perfusate and Ultrafiltrate Sampling and Analysis

Perfusate samples were collected after 15, 30, 60 and 90 minutes of perfusion and stored at -80°C for further analysis. Ultrafiltrate production was measured at the same time points and the samples were stored at -80°C. Fractional re-absorption of sodium ((perfusate sodium-ultrafiltrate sodium) / (perfusate sodium) ×100) and creatinine clearance (ultrafiltrate creatinine × ultrafiltrate volume/perfusate creatinine) were calculated. Lactate level and arterial pH were measured by an ABL800 FLEX analyzer (Radiometer, Brønshøj, Denmark).

Renal Injury Biomarkers

Indicators of renal cellular injury were analyzed in the perfusate and ultrafiltrate.(12,13) Aspartate transaminase (AST) and lactate dehydrogenase (LDH) were measured in the perfusate. N-acetyl-ß-D-glucosamine (NAG) was measured in the ultrafiltrate samples as it is an indicator of ischemic tubular damage in kidney.(14) The methodology for these biochemical analyses has been described in detail previously.(15)

Lipid Peroxidation

Oxygen free radical (OFR) induced injury was measured by the level of lipid peroxidation in the perfusate samples. The methodology has been described previously.(16)

mRNA Expression Assay

Details of real-time reverse transcription polymerase chain reaction (qRT-PCR) have been reported previously.(17) Gene expression of kidney injury molecule-1 (KIM-1), heat shock protein-70 (HSP-70), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), P-selectin and β-actin (as housekeeping gene) were measured. Based on the mean of β-actin mRNA content, gene expression was normalized and calculated. Results were represented as 2-ΔCT (CT threshold cycle). Primers are listed in “Table 2”.

Tissue Energy State

Tissue concentration of ATP was used as an indicator of the energy status of the grafts. Kidney samples were taken after cold storage in the reference group and after perfusion in the control, and in the experimental groups. Samples were snap frozen in liquid nitrogen. Frozen tissue was cut into 20 µm slices and a total amount of ± 50 mg was homogenized in 1 mL of SONOP (0.372g EDTA in 130 mL H_2O and NaOH (pH 10.9) + 370 mL 96% ethanol) and sonificated. The precipitate was removed by centrifugation (13,000 rcf for 10 min). In order

Table 2 | qRT PCR primers of the housekeeping gene (β-actin), KIM-1, HSP-70, ICAM-1, VCAM-1 and P-selectin primers and their sequences.

Primers	Forward	Reverse	Amplicon (bp)
β-actin	5'-GGAAATCGTGCGTGACATTAAA-3'	5'-GCGGCAGTGGCCATCTC-3'	109
KIM-1	5'-AGAGAGAGCAGGACACAGGCTTT-3'	5'-ACCCGTGGTAGTCCCAAACA-3'	89
HSP-70	5'-GGTTGCATGTTCTTTGCGTTTA-3'	5'-GGTGGCAGTGCTGAGGTGTT-3'	97
ICAM-1	5'-CCAGACCCTGGAGATGGAGAA-3'	AAGCGTCGTTTGTGATCCTCC	251
VCAM-1	5'-TCTCTGGGTCTTCGTGTTTCTTATCT-3'	5'-GTGTCCCCCTAGTACCATCTGAA-3'	80
P-selectin	5'-TGTGGAAGTGTGCCCGAAA-3'	5'-ACGAGCCATTAACAGACTTTAGCA-3'	84

to achieve a protein concentration of 200-300 mg/mL (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL) supernatant was diluted with SONOP and mixed with 450 mL of 100 mM phosphate buffer (Merck; pH 7.6-8.0). Fifty microliters of phosphate buffered supernatant was used for ATP measurement using ATP Bioluminescence assay kit CLS II (Boehringer, Mannheim, Germany) and a luminometer (Victor^{3TM} 1420 multilabel counter, PerkinElmer). ATP concentrations were calculated from a calibration curve constructed on the same plate, corrected for the amount of protein, and values were expressed as µmol/g protein.

Histology

Renal tissues were collected at the end of the perfusion and were fixed in 10 percent formalin. The tissue blocks were embedded in paraffin and were cut at 4 µm and stained with the Periodic acid-Schiff (PAS) methods for evaluation using light microscopy. Slides were scored at 4 fields in order to assess changes in morphological parameters by two independent investigators.

Statistical Analysis

The data is represented as mean ± standard deviation. P value is analyzed using Mann-Whitney U test. Analyses is performed using SPSS software version 16.0 (Inc., Chicago, IL, USA). A p-value of equal or less than 0.05 was considered significant.

RESULTS

Temperature and Hemodynamic Monitoring:

Temperature profiles are shown in "Figure 1A". The graph represents the gradual temperature increase in the gradual rewarming groups and the temperature status of the control group during the perfusion time.

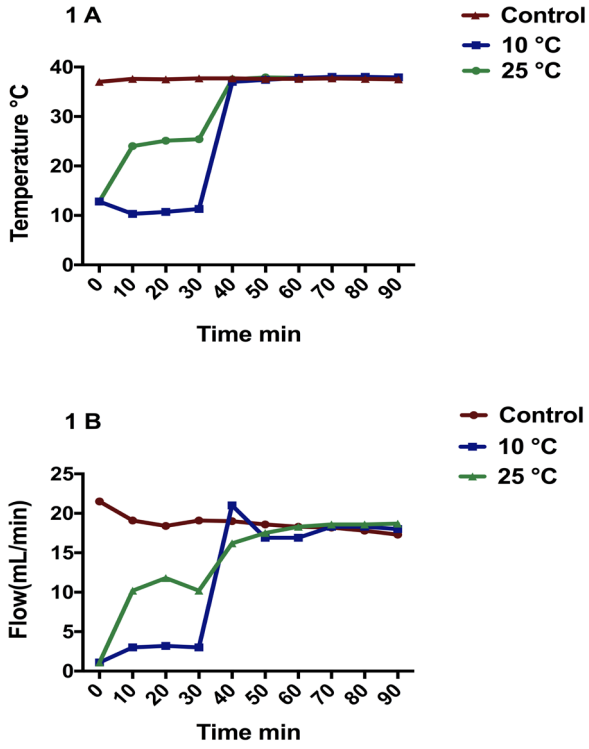

Figure 1 | A) Thermal variation in the control and gradual rewarming groups during the perfusion period. Values are mean ± standard deviation. B) Flow variation in the control group and the rewarming groups during 90 minutes of perfusion. Values are mean ± standard deviation.

Renal flow was gradually increased in the gradual rewarming groups in the first 30 minutes of rewarming. During perfusion and until the end of the perfusion at 38°C there was no difference in flow between the control group and the gradual rewarming groups “Figure 1B”.

Functional Parameters

Ultrafiltrate production was higher in the control group compared to the gradual rewarming groups “$p<0.05$; Figure 2A”. Fractional re-absorption of sodium however was improved in all the gradual rewarming groups compared to the control group and this, reached statistical significance in the 25°C group at the end of reperfusion (t=90) “Table 3”. There were no differences in glomrerular filtration rate (GFR) between the control group versus the gradual rewarming groups “Table 3”. After 90 minutes of perfusion there was a significantly lower lactate level in the gradual rewarming groups compared to the control group “Table 3”. In all three groups pH was decreased at the end of the perfusion compared to the beginning of the perfusion “Table 4”.

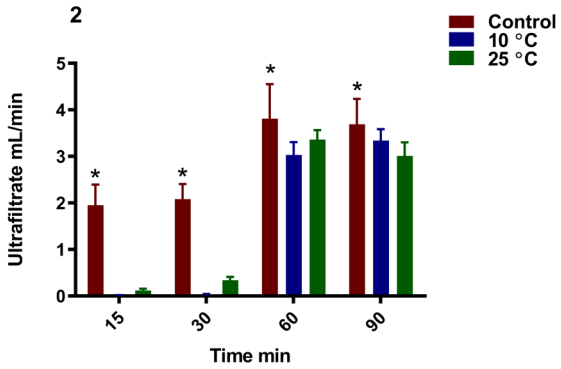

Figure 2 | Ultrafiltrate Production at 15, 30, 60 and 90 minutes of the perfusion in the control and gradual rewarming groups. * P<0.05 vs control group. Values are mean ± standard deviation.

Table 3 | Fractional re-absorption of sodium and lactate and LPO level in the perfusate and GFR after 90 minutes of perfusion in the control group and in the gradual rewarming groups. * P<0.05 vs control group. Values are mean ± standard deviation.

In the end of perfusion	Control	10°C	25°C	p-value
Fractional re-absorption of sodium	29.98±9	42.59±16	46.5±11*	0.005
GFR	0.181±0.06	0.202±0.08	0.194±0.08	0.015
Lactate	0.8±0.13	0.4±0.05*	0.5±0.04*	P<0.0001
LPO	1.05±0.0.8	1.03±0.04	0.9±0.07	0.37

Table 4 | Acid-base balance in the perfusate at the end of the perfusion period in the control group and the gradual rewarming groups. * P<0.05 vs control group. Values are mean ± standard deviation.

pH	Control	10°C	25°C
Pre-perfusion	7.41±0.03	7.43±0.03	7.42±0.03
Post-perfusion	7.33±0.05	7.17±0.08	7.20±0.05
p-value	0.015	P<0.001	P<0.001

Renal Injury Biomarkers

Concentrations of AST in the perfusate gradually increased in all four experimental groups during the course of 90 minutes perfusion with the steepest rise observed in the control group versus all gradual rewarming groups “P<0.05; Figure 3A”. The level of LDH in the perfusate was higher in the control group compared to all gradual rewarming groups during the 60 minutes reperfusion in 38°C “Figure 3B”. NAG in the ultrafiltrate was lower in the

gradual rewarming groups (10°C and slow 38°C) compared to the level of NAG in the control group “P<0.05; Figure 3C”.

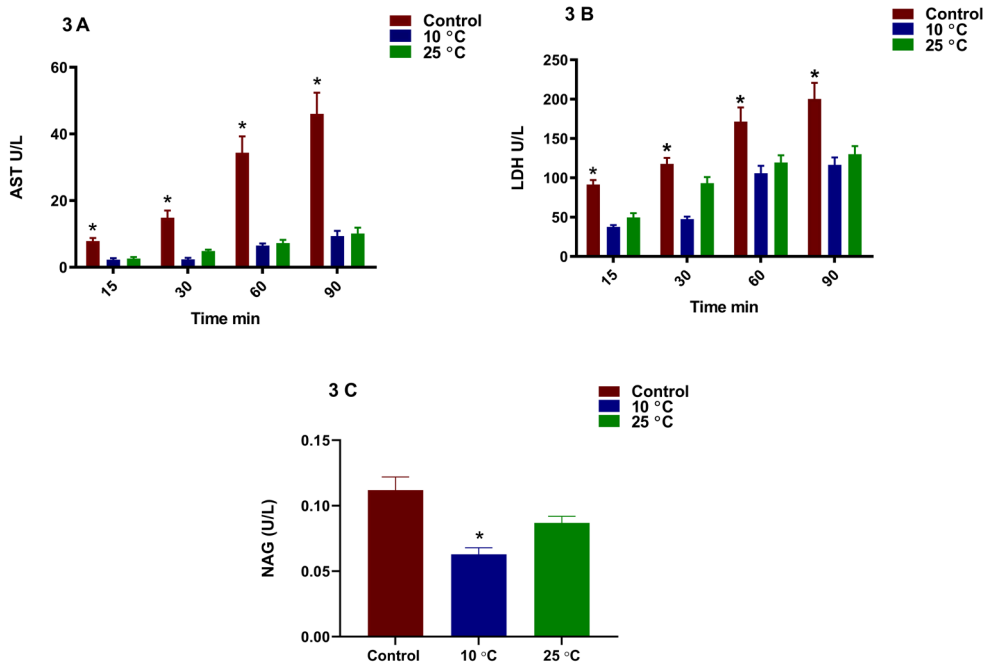

Figure 3 | A) Perfusate level of AST during 90 minutes of perfusion in the control and gradual rewarming groups. B) Perfusate level of LDH during 90 minutes of perfusion. C) The level of NAG in the ultrafiltrate during perfusion in the control and gradual rewarming groups. * P<0.05 vs control group. Values are mean ± standard deviation.

Lipid Peroxidation

The results from lipid hydroperoxide (LPO) measurements in the perfusate samples collected at the end of the perfusion (T=90 min) showed no statistical difference between the control group and gradual rewarming groups “Table 3”.

mRNA Expression

By the end of perfusion, the level of KIM-1, ICAM-1, VCAM-1 and HSP-70 expression was reduced in the gradual rewarming groups compared to the control group. Also, the expression of P-selectin was numerically reduced in all gradual rewarming groups compared to the control group “Table 5".

Table 5 | mRNA expression level of KIM-1 and HSP-70 in the kidney biopsies specified by real-time PCR in the frozen sections from the control group and the gradual rewarming groups. * P<0.05 vs control group. Values are mean ± standard deviation.

mRNA expression	Control	10°C	25°C	p-value
KIM-1	0.005±0.001	0.002±0.0007*	0.003±0.002*	P≤0.05
HSP-70	64.0±12.5	43.6±8.1*	42.2±8.3*	P≤0.05
ICAM-1	1.56±0.60	0.74±0.15*	0.97±0.10*	P≤0.05
VCAM-1	0.63±0.17	0.29±0.07*	0.34±0.08*	P≤0.05
P-selectin	0.18±0.07	0.05±0.02*	0.05±0.02*	P≤0.05

Tissue Energy State

ATP content was significantly elevated after 90 minutes of perfusion in the control group and gradual rewarming groups in comparison to the cold static preservation group. There was no difference between control group and the gradual rewarming groups “Table 6”.

Table 6 | Renal ATP content after SCS in the reference, control and the gradual rewarming groups after 90 minutes perfusion in the frozen tissue samples. * P<0.05 vs Reference group. Values are mean ± standard deviation.

	Reference	Control	10°C	25°C	p-value
ATP level	7±2.4	71±29*	73±9*	70±32*	P<0.0001

Histology

Light microscopy performed on tissue samples obtained at the end of the experiments did not reveal significant differences among the rewarming groups versus the control group. Overall only slight alterations of normal structural appearance were observed in any group including limited tubular dilation and epithelial shredding “Figure 4”.

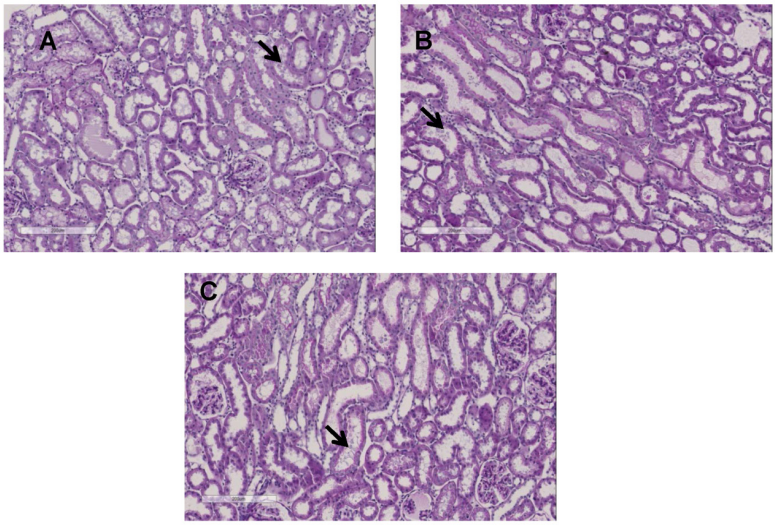

Figure 4 | Examples of H&E staining of kidney tissue subjected to: **"Panel A"**, immediate rewarming (control group) or gradual rewarming at 10°C **"Panel B"**, 25°C **"Panel C"**. Epithelial shredding is pointed in all the images.

DISCUSSION

Alternations in cellular metabolism and likely cellular injury occur due to energy depletion and accumulation of waste products in an organ during SCS. During graft implantation the re-introduction of warm (37°C) oxygenated blood to the cold (4°C) ischemic organ causes a major release of reactive oxygen species (ROS) and accumulated waste products known as reperfusion injury. Reperfusion injury could result in a delayed graft function and loss of graft viability after transplantation. (18) In liver perfusion, Minor and his colleagues have demonstrated that controlled oxygenated re-warming in an ex-vivo liver perfusion model is correlated with better preservation of liver grafts and improved liver function.(5) Our results are in line with this study as better results were obtained in the gradual rewarming groups. After reperfusion, lower AST and LDH level in the gradual rewarming groups suggest that the gradual increase in temperature induces less thermal stress that is associated with less parenchymal injury. The results obtained from HSP-70 also support less cellular stress in the gradual rewarming groups. HSP-70 is a heat shock protein which is expressed in the presence of different stress stimuli in cell lines.(19). Some studies indicate that higher expression of HSP-70 protein is associated with the activation of protective mechanisms. (9) However less expression could also be sign of decreased organ injury.

Acute renal tubular injury is one of the consequences of reperfusion and it might lead to acute kidney failure.(20) It was shown in a study by Han and his colleagues that KIM-1 gene expression as a proximal tubular injury biomarker is undetectable in healthy kidneys. However, the gene is up-regulated after ischemic injury and it is noticeably high after 24-48 hours.(21) Higher gene expression is associated with cellular epithelium differentiation which is the early cellular response to injury. Based on the tubular injury biomarker outcomes such as the lower KIM-1 expression and NAG release, the gradual rewarming strategy used here to reduce tubular injury is promising. The kidneys in the gradual rewarming groups were metabolically more stable as indicated by the lower lactate level which means that these kidneys exert adequate aerobic metabolism.(22) In all three groups a slight acidosis was observed at the end of the perfusion period, this could be resulting from the closed perfusion system used by us, in which the solution was recirculated during the ninety minutes of perfusion.

During kidney perfusion an adequate perfusion pressure is needed in order to support kidney metabolism and to deliver oxygen to the tissue.(23) On the other hand there are some studies showing a correlation between perfusion pressure and endothelial damage due to vascular shear stress.(24,25) Therefore focusing on potential endothelial damage caused by perfusion pressure was another goal of this study. The kidney podocyte cells are very sensitive to shear stress and damage to these cells can lead to organ dysfunction after renal transplant.(26) Shear stress caused by perfusion flow could induce endothelial cellular detachment and subsequently lead to vascular endothelial damage. Damaged endothelial cells play an important role in inflammation and reactive ROS formation after reperfusion by providing an adhesion cite for inflammatory mediators like monocyte-derived macrophages.(27) In order to maintain vascular integrity and to prevent endothelial damage induced by pressure in the rewarming groups we gradually increased the pressure alongside increasing the temperature. Although there was no difference in flow between the groups during the reperfusion period, kidneys in the control group demonstrated higher endothelial damage indicated by higher expression of I-CAM, V-CAM and P-Selectin.

Kidney ex vivo perfusion is a well-established model to perform different machine perfusion methods in animal study (pig and rat) and even human organs.(9,10,23,28) Oxygenated WME solution was used as perfusion solution in this study. The composition of this solution makes it an applicable candidate as an acellular solution for organ perfusion at normothermic or near normothermic temperature. It has already been shown that warm perfusion with an acellular, nutrient rich solution is helpful to recover from ischemia injury.(29) WME solution used by us in this study has been previously tested as a preservation and perfusion solution and has demonstrated satisfactory results.(6,30) The rationale behind using an oxygenated solution even at lower temperature is based on experimental and even clinical evidence that mitochondrial function and the energy status of the organ during perfusion can be improved

by short term re-oxygenation reducing oxidative stress reaction and further tissue injury both in kidneys and livers.(3,18,31)

Our findings demonstrate that adding an oxygenated perfusion period after SCS is beneficial in improving renal ATP, without the formation of ROS as indicated by the absence of changes in LPO levels measured as an oxidative stress marker. Leducq and colleagues showed that transition in temperature from hypothermia to normothermia is associated with rapid fall of ATP content in the organ and increased mitochondrial permeability.(32)

The main limitation of our study is the short reperfusion time (90 min), which may not be sufficient to see improvement in functional parameters and histological changes between the groups. Although the IPK Model enables the obtainment of multiple samples for assessment of injury prior to transplantation while avoiding the use of recipient animals, the short reperfusion time (90 min) is not sufficient to see sustainable improvements in functional parameters and histological changes between the groups. In future studies a transplantation model will be needed to fully investigate the potential of our findings.

In conclusion, our data demonstrate that post SCS gradual rewarming and gradual increase in pressure during perfusion is beneficial in decreasing injury compared to sudden reperfusion at body temperature. Temperature and pressure controlled oxygenated perfusion of kidneys prior to reperfusion could provide a better recovering strategy especially for kidneys at risk for delayed graft function. As the best results were obtained from gradual rewarming from 10 to 38°C future studies demonstrating the potential of this strategy in a relevant transplant model are needed before implementation in the clinical situation.

REFERENCES

(1) Bickford RG, Winton FR. The influence of temperature on the isolated kidney of the dog. J Physiol 1937 Mar 5;89(2):198-219.

(2) BURG MB, ORLOFF J. Active Cation Transport by Kidney Tubules at O C. Am J Physiol 1964 Nov;207:983-988.

(3) Dutkowski P, Graf R, Clavien PA. Rescue of the cold preserved rat liver by hypothermic oxygenated machine perfusion. Am J Transplant 2006 May;6(5 Pt 1):903-912.

(4) Moers C, Pirenne J, Paul A, Ploeg RJ, Machine Preservation Trial Study Group. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med 2012 Feb 23;366(8):770-771.

(5) Minor T, Efferz P, Fox M, Wohlschlaeger J, Luer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. Am J Transplant 2013 Jun;13(6):1450-1460.

(6) Berendsen TA, Bruinsma BG, Lee J, D'Andrea V, Liu Q, Izamis ML, et al. A simplified subnormothermic machine perfusion system restores ischemically damaged liver grafts in a rat model of orthotopic liver transplantation. Transplant Res 2012 May 9;1(1):6-1440-1-6.

(7) Op den Dries S, Sutton ME, Karimian N, de Boer MT, Wiersema-Buist J, Gouw AS, et al. Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after circulatory death. PLoS One 2014 Feb 14;9(2):e88521.

(8) op den Dries S, Karimian N, Sutton ME, Westerkamp AC, Nijsten MW, Gouw AS, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. Am J Transplant 2013 May;13(5):1327-1335.

(9) Hosgood SA, Patel M, Nicholson ML. The conditioning effect of ex vivo normothermic perfusion in an experimental kidney model. J Surg Res 2013 Jun 1;182(1):153-160.

(10) Hosgood SA, Nicholson ML. First in man renal transplantation after ex vivo normothermic perfusion. Transplantation 2011 Oct 15;92(7):735-738.

(11) Nicholson ML, Hosgood SA. Renal transplantation after ex vivo normothermic perfusion: the first clinical study. Am J Transplant 2013 May;13(5):1246-1252.

(12) Jochmans I, Lerut E, van Pelt J, Monbaliu D, Pirenne J. Circulating AST, H-FABP, and NGAL are early and accurate biomarkers of graft injury and dysfunction in a preclinical model of kidney transplantation. Ann Surg 2011 Nov;254(5):784-91; discussion 791-2.

(13) Danpure CJ. Lactate dehydrogenase and cell injury. Cell Biochem Funct 1984 Jul;2(3):144-148.

(14) Kotanko P, Margreiter R, Pfaller W. Urinary N-acetyl-beta-D-glucosaminidase and neopterin aid in the diagnosis of rejection and acute tubular necrosis in initially nonfunctioning kidney grafts. Nephron 2000 Mar;84(3):228-235.

(15) Moers C, Varnav OC, van Heurn E, Jochmans I, Kirste GR, Rahmel A, et al. The value of machine perfusion perfusate biomarkers for predicting kidney transplant outcome. Transplantation 2010 Nov 15;90(9):966-973.

(16) Minor T, Kotting M. Gaseous oxygen for hypothermic preservation of predamaged liver grafts: fuel to cellular homeostasis or radical tissue alteration? Cryobiology 2000 Mar;40(2):182-186.

(17) van Rijt WG, Nieuwenhuijs-Moeke GJ, van Goor H, Jespersen B, Ottens PJ, Ploeg RJ, et al. ARA290, a non-erythropoietic EPO derivative, attenuates renal ischemia/reperfusion injury. J Transl Med 2013 Jan 9;11:9-5876-11-9.

(18) Manekeller S, Minor T. Possibility of conditioning predamaged grafts after cold storage: influences of oxygen and nutritive stimulation. Transpl Int 2006 Aug;19(8):667-674.

(19) Wang Z, Gall JM, Bonegio RG, Havasi A, Hunt CR, Sherman MY, et al. Induction of heat shock protein 70 inhibits ischemic renal injury. Kidney Int 2011 Apr;79(8):861-870.

(20) Ma Z, Wei Q, Dong G, Huo Y, Dong Z. DNA damage response in renal ischemia-reperfusion and ATP-depletion injury of renal tubular cells. Biochim Biophys Acta 2014 Jul;1842(7):1088-1096.

(21) Han WK, Bailly V, Abichandani R, Thadhani R, Bonventre JV. Kidney Injury Molecule-1 (KIM-1): a novel biomarker for human renal proximal tubule injury. Kidney Int 2002 Jul;62(1):237-244.

(22) Bellomo R. Bench-to-bedside review: lactate and the kidney. Crit Care 2002 Aug;6(4):322-326.

(23) Hosgood S, Harper S, Kay M, Bagul A, Waller H, Nicholson ML. Effects of arterial pressure in an experimental isolated haemoperfused porcine kidney preservation system. Br J Surg 2006 Jul;93(7):879-884.

(24) Cerra FB, Raza S, Andres GA, Siegel JH. The endothelial damage of pulsatile renal preservation and its relationship to perfusion pressure and colloid osmotic pressure. Surgery 1977 May;81(5):534-541.

(25) Grundmann R, Raab M, Meusel E, Kirchhoff R, Pichlmaier H. Analysis of the optimal perfusion pressure and flow rate of the renal vascular resistance and oxygen consumption in the hypothermic perfused kidney. Surgery 1975 Mar;77(3):451-461.

(26) Srivastava T, Celsi GE, Sharma M, Dai H, McCarthy ET, Ruiz M, et al. Fluid flow shear stress over podocytes is increased in the solitary kidney. Nephrol Dial Transplant 2014 Jan;29(1):65-72.

(27) Miravete M, Dissard R, Klein J, Gonzalez J, Caubet C, Pecher C, et al. Renal tubular fluid shear stress facilitates monocyte activation toward inflammatory macrophages. Am J Physiol Renal Physiol 2012 Jun 1;302(11):F1409-17.

(28) van den Eijnden MM, Leuvenink HG, Ottens PJ, 't Hart NA, van Oeveren W, Morariu AM, et al. Effect of brain death and non-heart-beating kidney donation on renal function and injury: an assessment in the isolated perfused rat kidney. Exp Clin Transplant 2003 Dec;1(2):85-95.

(29) Brasile L, Stubenitsky BM, Booster MH, Lindell S, Araneda D, Buck C, et al. Overcoming severe renal ischemia: the role of ex vivo warm perfusion. Transplantation 2002 Mar 27;73(6):897-901.

(30) Roskott AM, Nieuwenhuijs VB, Leuvenink HG, Dijkstra G, Ottens P, de Jager MH, et al. Reduced ischemia-reoxygenation injury in rat intestine after luminal preservation with a tailored solution. Transplantation 2010 Sep 27;90(6):622-629.

(31) 't Hart NA, van der Plaats A, Faber A, Leuvenink HG, Olinga P, Wiersema-Buist J, et al. Oxygenation during hypothermic rat liver preservation: an in vitro slice study to demonstrate beneficial or toxic oxygenation effects. Liver Transpl 2005 Nov;11(11):1403-1411.

(32) Leducq N, Delmas-Beauvieux MC, Bourdel-Marchasson I, Dufour S, Gallis JL, Canioni P, et al. Mitochondrial permeability transition during hypothermic to normothermic reperfusion in rat liver demonstrated by the protective effect of cyclosporin A. Biochem J 1998 Dec 1;336 (Pt 2)(Pt 2):501-506.

CHAPTER 4

End-Ischemic Machine Perfusion Reduces Bile Duct Injury in Donation After Circulatory Death Rat Donor Livers

Liver Transplantation 2015; Jun 19

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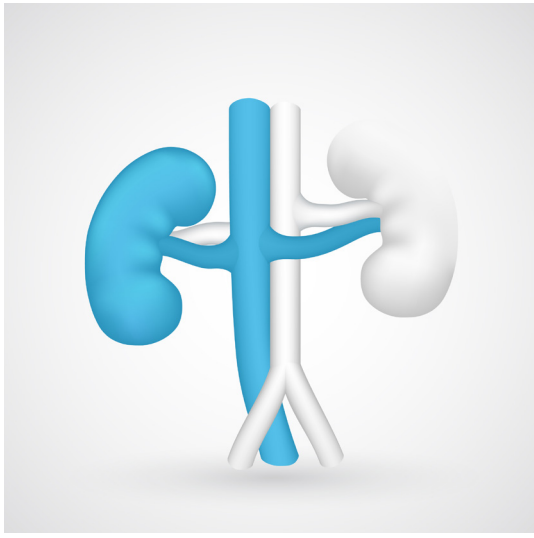

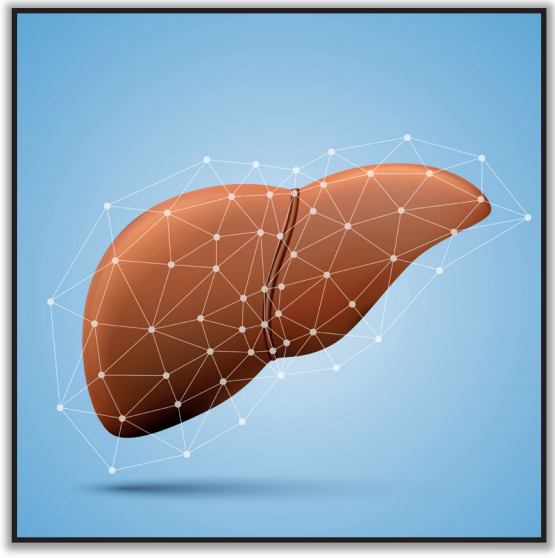

ABSTRACT

Background

A short period of oxygenated machine perfusion (MP) after static cold storage (SCS) may reduce biliary injury in donation after circulatory death (DCD) donor livers. However, the ideal perfusion temperature for protection of the bile ducts is unknown. In this study, the optimal perfusion temperature for protection of the bile ducts was assessed.

Methods

DCD rat livers were preserved by SCS for 6 hours. Thereafter, 1 hour of oxygenated MP was performed using either hypothermic (HMP), subnormothermic (SNP) or with controlled oxygenated rewarming (COR) conditions. Subsequently, graft and bile duct viability were assessed during 2 hours of normothermic *ex situ* reperfusion.

Results

In the MP study groups, lower levels of transaminases, LDH and TBARS were measured compared to SCS. In parallel, mitochondrial oxygen consumption and ATP production were significantly higher in the MP groups. Biomarkers of biliary function, including bile production, biliary bicarbonate concentration and pH, were significantly higher in the MP groups, whereas biomarkers of biliary epithelial injury (biliary gamma-GT and LDH) were significantly lower in MP preserved livers. Histological analysis revealed less injury of large bile duct epithelium in the MP groups, compared to SCS.

Conclusion

Compared to SCS, end-ischemic oxygenated MP of DCD livers provides better preservation of biliary epithelial function and morphology, independent of the temperature at which MP is performed. End-ischemic oxygenated MP could reduce biliary injury after DCD liver transplantation.

INTRODUCTION

Ischemic cholangiopathy, also known as non-anastomotic biliary strictures (NAS), is one of the most prevalent and troublesome complication after liver transplantation. During NAS formation, in particular, the large (extrahepatic) bile ducts become fibrotic and/or necrotic. Patients with NAS may suffer from recurrent jaundice and episodes of cholangitis and retransplantation may be the only curative treatment (1). The combination of ischemia and ischemia/reperfusion (I/R) injury has been shown to be a major risk factor for the development of NAS after transplantation (2). The combination of ischemia and I/R injury can lead to impaired regeneration of the biliary epithelium with subsequently NAS formation as clinical consequence (3,4).

A short period of end-ischemic machine perfusion (MP) after the regular period of static cold storage SCS has been shown to reduce I/R injury, compared to SCS alone (5). Although the exact mechanisms underlying the protective effects of end-ischemic MP are not fully known, an important feature of end-ischemic MP is the resuscitation of mitochondrial respiration and resynthesize of cellular adenosine triphosphate (ATP) (5,6). Restoration of cellular ATP improves metabolic function after reperfusion, making the hepatocytes and cholangiocyte more resistant to the effects of I/R injury (5,6). Although recent data from animal models and (discarded) human donor livers have provided promising results suggesting that end-ischemic MP has relevant protective effects on the bile ducts of DCD liver grafts, the most optimal perfusion temperature during end-ischemic MP has not been investigated (7-17). Aim of this study was, therefore, to assess the optimal perfusion temperature during end-ischemic oxygenated MP for protection of the large bile ducts against I/R injury in a DCD rat liver model.

MATERIALS AND METHODS

Animals

Male Lewis rats (LEW/Han®Hsd) (290-320 g) were obtained from Harlan Laboratories (Boxmeer, the Netherlands). Animals received care according to the guidelines set by the US National Institutes of Health (1985). The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Groningen, the Netherlands (IACUC-RUG).

Experimental Design

Thirty rat livers were divided into four experimental groups and a reference group (n = 6 per group). The four experimental groups were used to study the effects of end-ischemic MP,

while the reference group was used for *in vivo* collection of bile during 2 hours of anesthesia (Figure 1). In the four experimental groups, livers were procured from DCD donors and subsequently preserved by SCS in histidine-tryptophan-ketoglutarate (HTK) preservation solution (Custodiol, Essential Pharmaceuticals, Ewing, NJ, USA) for 6 hours. To study the effects of end-ischemic MP, the 6 hours of SCS were followed by 1-hour end-ischemic oxygenated MP at 3 different perfusion temperatures. The fourth group consisted of livers that were preserved by 6 hours of SCS alone (Figure 1). MP was performed at hypothermic conditions (8°C [HMP]), subnormothermic conditions (20°C [SNP]) or with controlled oxygenated rewarming [COR], using Belzer machine perfusion solution (MaPerSol, Bridge-to-Life, Ltd. Northbrook, IL, USA). During COR, the temperature of the perfusate was kept at 8°C for the first 20 minutes and then gradually increased from 8 to 20°C in the next 20 minutes. For the last 20 minutes of the COR phase, the perfusion temperature was kept at 20°C. Prior to reperfusion, all livers were flushed with 10 mL of cold saline and subsequently stored on a petri dish, covered with a wet gauze at room temperature for 60 minutes, to mimic surgical implantation of the liver. Subsequently, liver grafts were reperfused *ex situ* for 2 hours with a perfusion fluid consisting of 25 mL human red blood cell concentrate (final hematocrit 25%) (Sanquin, Amsterdam, the Netherlands), 53.9 mL William's Medium E solution (Life Technologies Europe, Bleiswijk, the Netherlands), 20 mL human albumin (200 g/L Albuman, Sanquin, Amsterdam, the Netherlands), 1 mL insulin (100 IE/mL Actrapid, Novo Nordisk, Alphen aan den Rijn, the Netherlands) and 0.1 mL unfractionated heparin (5000 IE/mL, LEO Pharma A/S, Ballerup, Denmark), adding up to a total volume of 100 mL.

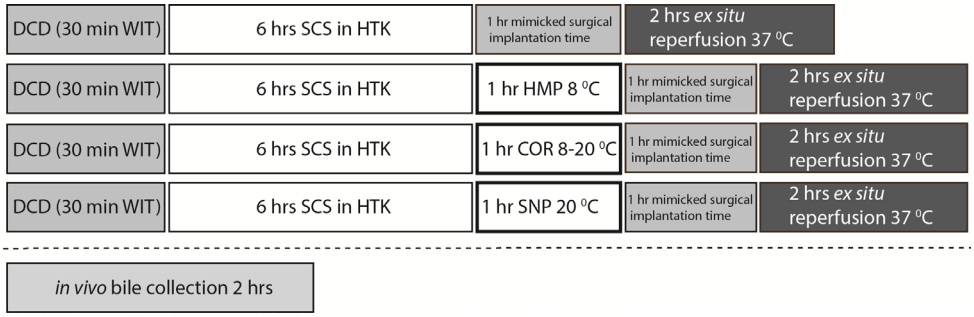

Figure 1 | Schematic representation of the experimental groups to examine the effects of end-ischemic machine perfusion (MP) at three different perfusion temperatures: hypothermic 8°C (HMP), controlled oxygenated rewarming 8-20°C (COR), and subnormothermic 20°C (COR). A group of DCD livers underwent only static cold storage without end-ischemic MP (SCS alone). For normal values of bile composition, bile was collected 2 hours *in vivo* in the reference group. All groups contained 6 rat livers.

In vivo Bile Collection and Procurement of DCD Donor Livers

Inhalation anesthesia with isoflurane and oxygen was used before and during the procurement (2-3% isoflurane). First, the large bile duct was cannulated. For the reference groups, the rats were supported with mechanical ventilation and bile was collected for 2 hours in Eppendorf tubes. The procedures of DCD and *in situ* warm ischemia time (WIT) (30 min) have been described previously (18). In brief, 1 mL 0.9% NaCl with 500 IU of heparin was administered via the dorsal penile vein. After heparinization, cardiac arrest was induced by external compression of the heart (tamponade) until contractions ceased. Subsequently, the aorta was closed using a vascular clamp rostral from the heart for 30 minutes. The thoracotomy and laparotomy wounds were covered with gauze, moistened with 0.9% NaCl. Using a heating lamp prevented cooling of the rat. After 30 minutes *in situ* WIT, the hepatectomy was performed by ligation of the splenic vein, mesenteric artery, and mesenteric vein. Thereafter, the celiac trunk was cannulated. After clamping of the infra-hepatic vena cava and the portal vein, the portal vein was cannulated and via the portal vein cannula, the liver was flushed *in situ* with 10 mL 0.9% NaCl (37°C). Subsequently, the supra-hepatic vena cava was transected, followed by a cold flush out with 5 mL HTK preservation solution (4°C) via the portal vein cannula. The liver was removed and flushed with an additional 20 mL of cold (4C) HTK via the portal vein cannula and 5 mL of cold (4°C) HTK via the hepatic artery (celiac trunk cannula) before preservation by SCS.

Static Cold Storage, End-Ischemic Machine Perfusion and Ex situ Reperfusion

For SCS, livers were stored in bags with ice-cold HTK (4°C) on melting ice for 6 hours. End-ischemic MP and *ex situ* reperfusion of rat donor livers were performed with a liver machine perfusion system that enabled dual perfusion via both the hepatic artery and the portal vein using a closed circuit (Figure 2). Two roller pumps (Ismatec ISM404 + ISM719 and MS-2/6-160; **IDEX Health & Science,** Wertheim-Mondfeld, Germany) provided pulsatile flow through the hepatic artery and continuous flow through the portal vein. Continuous flow to the portal vein was achieved by the combination of elastic tubing and a pulse damper to remove pulses from the roller pump. Two tubular membrane oxygenators provided oxygenation of the perfusion solution and removal of CO_2. Perfusion box and perfusate temperature was maintained stable at the indicated temperatures using a thermostat pump (Huber, Offenburg, Germany) and radiator/ventilator combination (Freezing Hardware, Losser, the Netherlands). The system was pressure-controlled by a computer algorithm allowing auto regulation of blood flow through the liver, with constant pressure at variable flow rates. In-line sensors monitored flow, pressure, and temperature. Data were displayed in real-time on a connected laptop.

During HMP (8°C), the portal pressure was 3 mmHg and mean arterial pressure was 25 mmHg. During SNP (20°C), the portal pressure was set at 4 mmHg and mean arterial

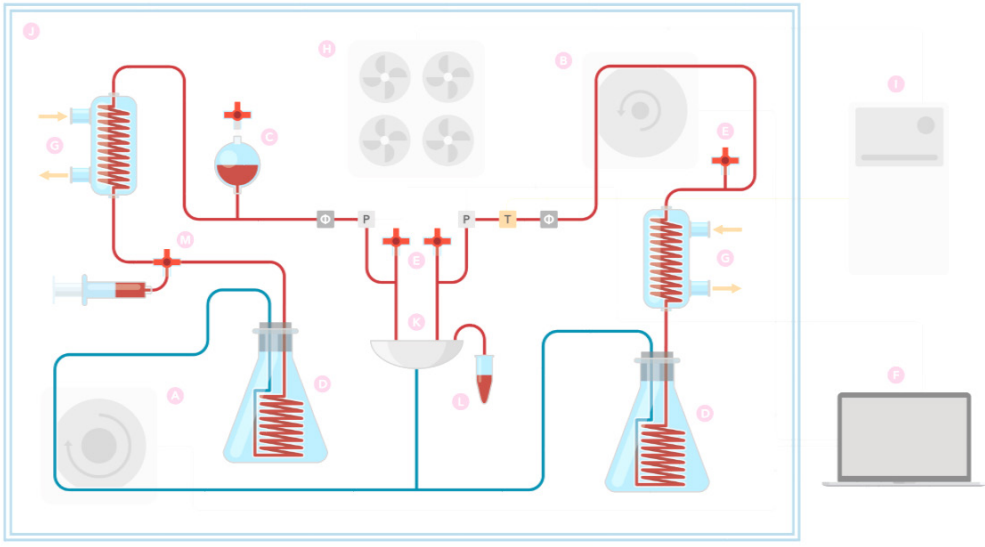

Figure 2 | Schematic presentation of the rat liver machine perfusion system providing a combination of arterial and portal perfusion of the liver. Two roller pumps provide a continuous flow to the portal vein (A) and a pulsatile flow to the hepatic artery (B). Pulses in the portal flow were eliminated with elastic tubing and a pulse damper (C). Two tubular membrane oxygenators provide oxygenation of the perfusion solution, as well as removal of CO_2 (D). Several bubble traps (three-way connectors) were used to eliminate air bubbles in the perfusion solution (E). Flow (Φ) and pressure (P) were detected by in-line sensors and data were displayed and analyzed in real-time on a connected laptop (F). The perfusion temperature was maintained constant by two heat exchangers (G) and a radiator/ventilator combination (H), all connected to the thermostat pump (I). For real time control of the perfusion temperature, one in-line temperature sensor (T) was connected to the thermostat pump. The isolated box encapsulated the perfusion system (J) preventing loss of warm or cold air. The rat liver was placed into an organ chamber (K). Bile was collected in Eppendorf tubes (L). By the three-way connector at the portal side, samples of the perfusion solution were taken every 30 min for analysis of the perfusate (M).

pressure at 40 mmHg. During the first 20 minutes of COR, the same portal and arterial pressure were used as during HMP. Thereafter, the perfusion solution was gradually warmed up to 20°C in 20 minutes and for the last 20 minutes the pressures were set at levels used during SNP (4 and 40 mmHg, respectively). *Ex situ* reperfusion (37°C) was performed with a mean arterial pressure of 110 mmHg and 11 mmHg at portal side. During end-ischemic MP and *ex situ* reperfusion, the perfusion fluid was oxygenated with 100% 02 and the pO2 was 60-80 kPa (450-600 mmHg), as described previously (8,11,15,19).

Biochemical Markers of Function and Injury

During *ex situ* reperfusion, flow and temperature were registered every 10 minutes. Before reperfusion and after every 30 minutes of reperfusion, samples were taken from the

perfusion fluid. Samples were centrifuged (2700 g for 5 min at 4°C) and the supernatant was collected, frozen and stored at -80°C for determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH), using standard biochemical methods.

Bile production was measured at 60-minute intervals by weighing Eppendorf tubes in which bile was collected from the biliary drain. Biliary epithelial cell function was assessed by measuring pH and bicarbonate concentration in bile (20). For this purpose, bile samples were collected under mineral oil and analyzed immediately using an ABL800 FLEX analyzer (Radiometer, Brønshøj, Denmark). Biliary concentration of gamma-glutamyl transferase (gamma-GT) and LDH were measured as biomarkers of biliary epithelial cell injury (21), and biliary bilirubin concentration was measured as biomarker of hepatocellular secretory function (21), using standard biochemical methods.

Thiobarbituric acid reactive substances (TBARS) were measured in perfusate samples after 2 hours of reperfusion, as a marker for oxidative stress. The method for TBARS measurements has been described previously (12).

Isolation of Mitochondria and Mitochondrial Oxygen Consumption Analysis

Mitochondria isolation and mitochondrial oxygen consumption analysis were performed after 2 hours *ex situ* reperfusion. The protocol for isolation and oxygen consumption measurement has been described before (22). In brief, oxygen consumption was measured with a Clark type electrode (Strathkelvin Instruments LTD, North Lanarkshire, UK). This electrode was placed in a double walled respiration chamber with continuous stirred suspension of the isolated mitochondria (2 mg/mL) and oxygen consumption buffer at 37°C. Basal oxygen consumption of the mitochondria (state 2 respiration) was measured after addition of glutamate (10 mM) and malate (2 mM) as substrates to stimulate complex I, III, and IV. Maximal respiration rate (state 3 respiration) was measured after addition of adenosine diphosphate (ADP) (5 mM), which causes a sudden burst of oxygen uptake as the ADP is converted into ATP by ATPase synthase (complex V). Oligomycin (2.5 µM) was used to stop ATPase synthase by blocking complex V (defined as state 4 respiration). Rates of oxygen consumption were expressed as µmol O2/min/mg liver. The mitochondria respiratory control ratio (RCR) was defined as the ratio of state 3 and state 4. The function of the RCR is to reflect the viability of the mitochondria (23).

Adenosine triphosphate (ATP) Extraction and Measurement

Hepatic concentration of ATP was used as an indicator of the energy status of grafts after 2 hours of *ex situ* reperfusion. Method for extraction and measurement has been described previously (12).

Histological Evaluation of the Large Bile ducts

After 2 hours of *ex situ* reperfusion, a segment of the large bile duct proximal from the tip of the biliary catheter (and therefore not mechanically injured) was dissected and stored in 10% formaldehyde for inclusion in paraffin. Paraffin-embedded slides were cut with 0.5 mm interspaces, resulting in 3 slides per bile duct. In addition, staining was performed with hematoxylin and eosin (H&E) staining. Large bile duct injury was semi-quantified using a systematic scoring system described by Hansen *et al*. (24) with adjustments according to op den Dries *et al*. (4). All bile duct sections were examined in a blinded fashion by two investigators (ACW and SLM) under supervision of an experienced liver pathologist (ASHG) using light microscopy.

Statistical Analyses

Continuous data were presented as median and interquartile range (IQR). Mann-Whitney *U* test was used to compare groups and the Kruskal-Wallis Test was used for statistical comparison of >2 groups. Categorical data were expressed as numbers and percentage and groups were compared using Pearson chi-square test or Fischer's exact test as appropriate. The level of significance was set at $p < 0.05$. Analyses were performed using SPSS software version 22.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

End-ischemic MP Provides Better Protection of Hepatocellular Function

Portal vein and hepatic artery resistance during 2 hours of *ex situ* reperfusion were influenced by end-ischemic MP treatment. At the portal side (Figure 3A), the vascular resistance was significant lower after end-ischemic COR and SNP preservation in comparison to livers only preserved with SCS. Although the portal vascular resistance after end-ischemic HMP was similar to the SCS alone group during the first hour of reperfusion, resistance during the second hour of reperfusion was significantly lower in the end-ischemic HMP group, compared to only SCS preservation. At the side of the hepatic artery, end-ischemic HMP, COR, and SNP resulted in a significant reduction of the vascular resistance during the second hour of reperfusion (Figure 3B). Taken the vascular resistance characteristics of the portal vein and hepatic artery during the second hour of reperfusion together, the results suggest better vascular hemodynamics after end-ischemic MP (HMP, COR, and SNP) compared to only SCS preservation.

After 2 hours of reperfusion, no statistically significant differences were found in the mitochondrial respiration and ATP production between the three end-ischemic MP groups. By adding ADP to stimulate ATP synthesis in the isolated mitochondria from livers that

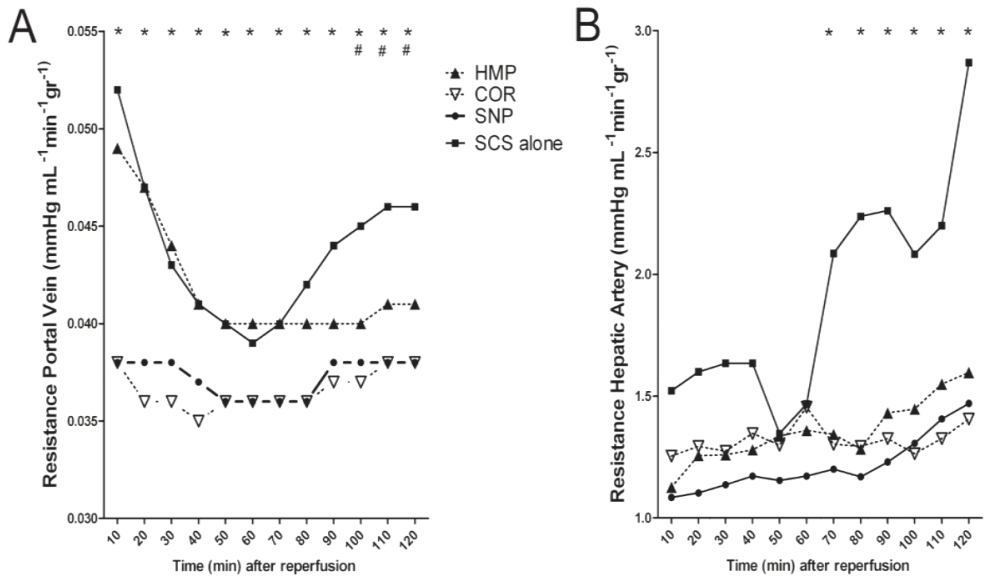

Figure 3 | Vascular flow resistance in the portal vein and the hepatic artery during 2 hours of *ex situ* reperfusion. Panel A) During reperfusion, livers treated with end-ischemic MP controlled oxygenated rewarming (COR) and subnormothermic machine perfusion (SNP) had significantly lower portal vein resistance in comparison to liver preserved by SCS alone (*p<0.05). Livers treated with hypothermic machine perfusion (HMP) displayed a significantly lower portal vein resistance during the last 30 minutes of reperfusion, compared to SCS alone (#p<0.05). Panel B) During the last hour of reperfusion, livers that were preserved by SCS alone had a significant higher hepatic artery resistance compared to liver that underwent end-ischemic HMP, COR, or SNP (*p< 0.05). Data are represented as medians.

underwent one of the three temperature protocols of end-ischemic MP (HMP, COR, and SNP), the levels of oxygen consumption in mitochondria (state 3 respiration) were significantly higher compared to SCS alone (Figure 4A). In parallel, the RCR, a marker for mitochondria viability, was significantly higher in the three end-ischemic MP preserved groups (HMP, COR, and SNP), compared to preservation by only SCS (Figure 4B). In accordance with these isolated mitochondrial function analyses, cellular ATP levels were significantly higher in the three groups of end-ischemic MP compared to SCS alone (Figure 4C).

In line with the enhanced mitochondrial function after end-ischemic MP, also hepatic bile production after reperfusion was significantly higher in the three end-ischemic MP groups (HMP, COR, and SNP), compared to the group with SCS alone (Figure 5A). No significantly differences in bile production were found between the 3 end-ischemic MP groups. Nevertheless, in all the end-ischemic MP groups' bile production during *ex situ*

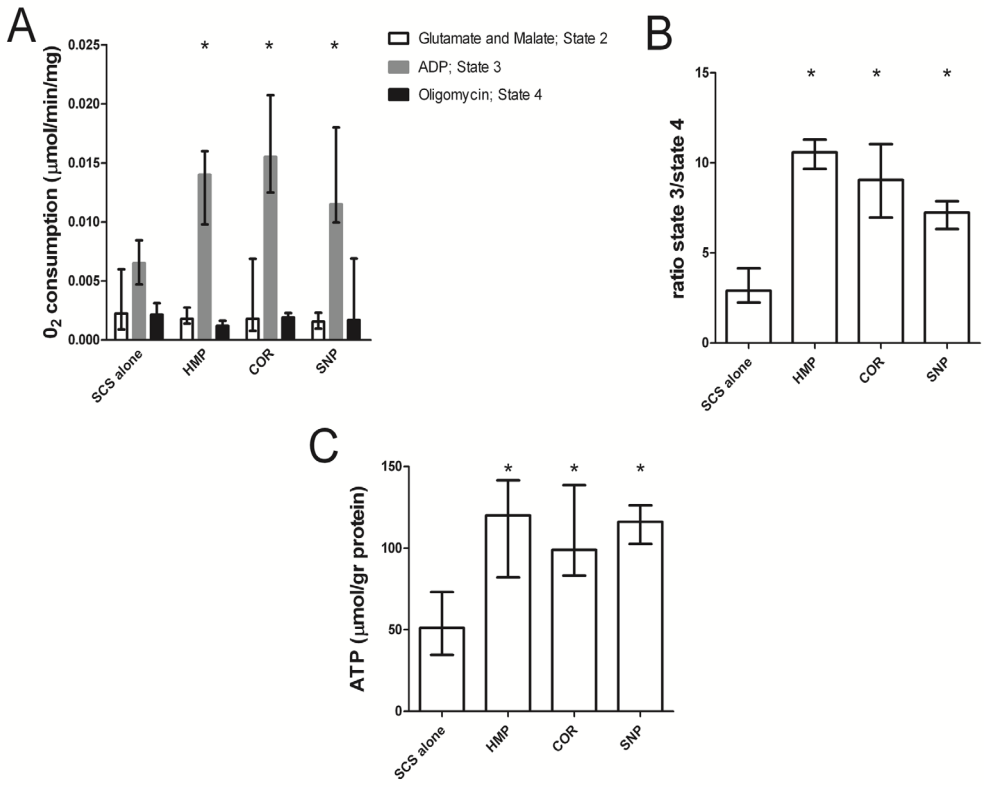

Figure 4 | Mitochondrial respiration function and hepatocellular ATP concentrations after 2 hours *ex situ* reperfusion. Panel A: Oxygen consumption rates after the addition of adenosine diphosphate ADP (state 3 respiration) was significantly higher in livers that underwent 1-hour end-ischemic hypothermic MP (HMP), end-ischemic controlled oxygenated rewarming (COR) MP, and subnormothermic machine perfusion (SNP) compared to livers that underwent SCS alone (*p<0.05). There were no significant differences between the three end-ischemic MP groups. Panel B) The respiratory control ratio (RCR) was significantly higher in the three end-ischemic MP groups, compared to SCS alone (*p<0.05). The RCR value was not significantly different between the three end-ischemic MP groups. Panel C: Cellular ATP concentration (µmol/gr protein) after 2 hours of reperfusion was significantly higher in livers that first underwent 1 hour of end-ischemic MP in comparison to SCS alone (*p<0.05). Again, there were no significant differences in ATP production between the three groups with different temperatures protocols for end-ischemic MP (HMP, COR, and SNP). Data are represented as medians with IQR (error bars).

reperfusion remained lower than the *in vivo* bile production in the reference group. Biliary bilirubin concentrations were comparable among the three experimental groups (HMP, COR, and SNP), and *in vivo* reference group. However, the SCS preserved livers demonstrated significantly lower levels of biliary bilirubin (Figure 5B).

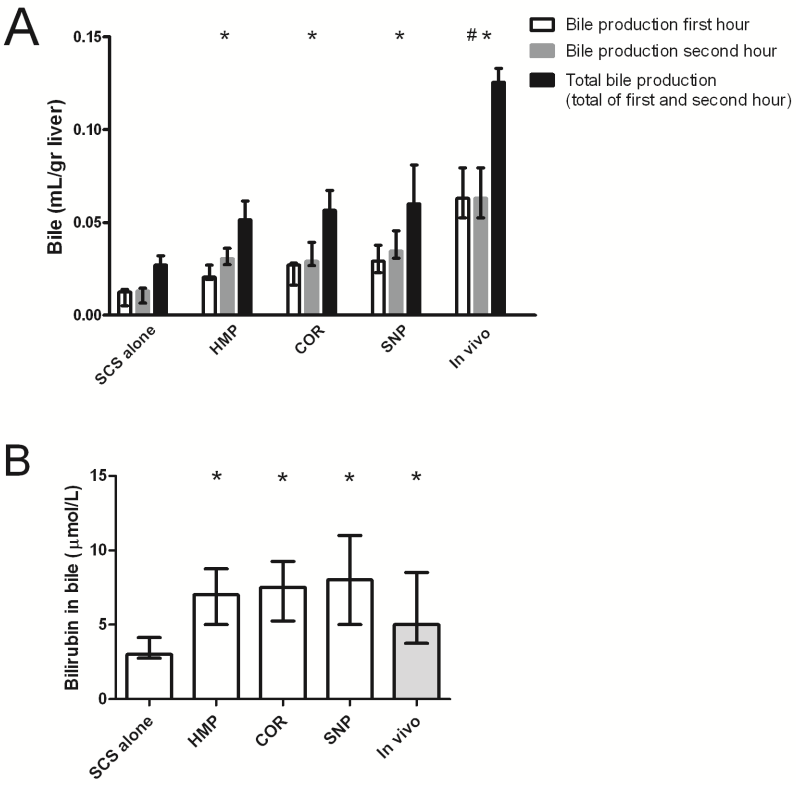

Figure 5 | Bile production and bilirubin concentration in bile after 2 hours of *ex situ* reperfusion. Panel A) End-ischemic hypothermic (HMP), controlled oxygenated rewarming (COR) and subnormothermic machine perfusion (SNP) resulted in significantly higher bile volumes during the first and second hour of reperfusion compared to SCS alone (**p*<0.05). There were no significant differences in bile production between the three end-ischemic MP groups. In general, however, bile production was significantly lower in livers that were preserved by HMP, COR, SNP or SCS alone, compared to values obtained *in vivo* (#*p*< 0.05). Panel B) The concentration of biliary bilirubin was significantly lower in the group with only SCS preservation compared to the three MP groups and the reference group with *in vivo* bile measurements (**p*<0.05). Between the three MP study groups and reference group no significant differences were measured in the biliary bilirubin concentration. Data are represented as medians with IQR (error bars).

End-ischemic MP Reduces Hepatocellular Injury

Hepatocellular injury after *ex situ* reperfusion was significantly less in the three end-ischemic MP groups, compared to livers preserved by SCS alone. In the first 30 minutes of reperfusion, markers for hepatocellular injury, including levels of AST, ALT, and LDH in the perfusion fluid, were significantly lower in the end-ischemic MP groups (HMP, COR, and SNP), compared to livers preserved by SCS alone (Figure 6A). Similarly, after 2 hours of reperfusion, levels of the oxidative stress biomarker TBARS were significantly lower in the end-ischemic MP treated groups (HMP, COR, and SNP) (Figure 6B).

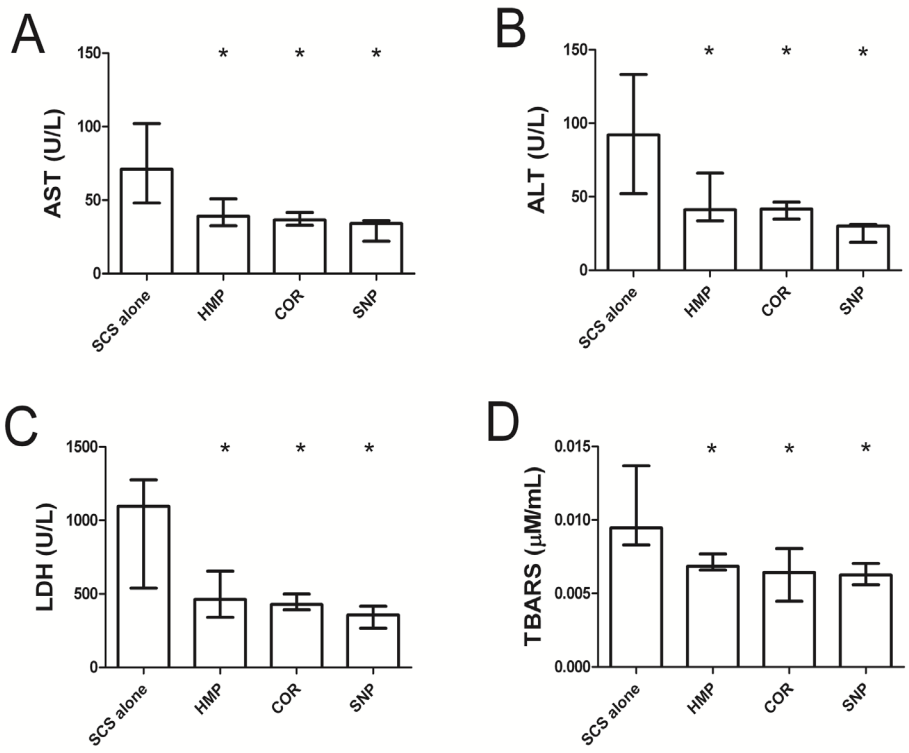

Figure 6 | Biochemical markers of hepatocellular injury and a marker of oxidative stress (TBARS) measured in the perfusion fluid during 2 hours *ex situ* reperfusion. Panel A-C) Concentrations of AST, ALT, and LDH into the perfusion solution during the first 30 minutes of reperfusion. Concentrations of AST, ALT, and LDH during the first 30 minutes of reperfusion were significantly lower in livers that underwent end-ischemic hypothermic MP (HMP), controlled oxygenated rewarming (COR) MP, or subnormothermic MP (SNP), compared to livers that underwent only SCS (*p<0.05). There were no significant differences in levels of AST, ALT, and LDH between end-ischemic HMP, COR, and SNP preserved livers. Panel D) After 2 hours of reperfusion, levels of TBARS were significantly higher in the SCS alone preserved livers versus the end-ischemic MP treated groups (HMP, COR, and SNP) (*p<0.05). No differences in TBARS levels were noted between the end-ischemic MP groups. Data are represented as medians with IQR (error bars).

End-ischemic MP Improves Cholangiocyte Function and Reduces Cholangiocyte Injury

Biliary bicarbonate concentration after 2 hours of reperfusion was significantly higher in the three groups of end-ischemic MP (HMP, COR, and SNP), compared to the group with SCS alone (Figure 7A). No significant differences were observed between the three end-ischemic MP groups. However, in all four experimental groups (HMP, COR, SNP, and SCS alone) biliary bicarbonate concentrations after reperfusion were significantly lower compared to values obtained in bile samples from *in vivo* measurements (reference group).

Biliary pH after 2 hours reperfusion was significantly higher in the three end-ischemic MP groups (HMP, COR, and SNP) versus the SCS alone group (Figure 7B). In fact, there was no significant difference in biliary pH between the three experimental MP groups and the *in vivo* reference group.

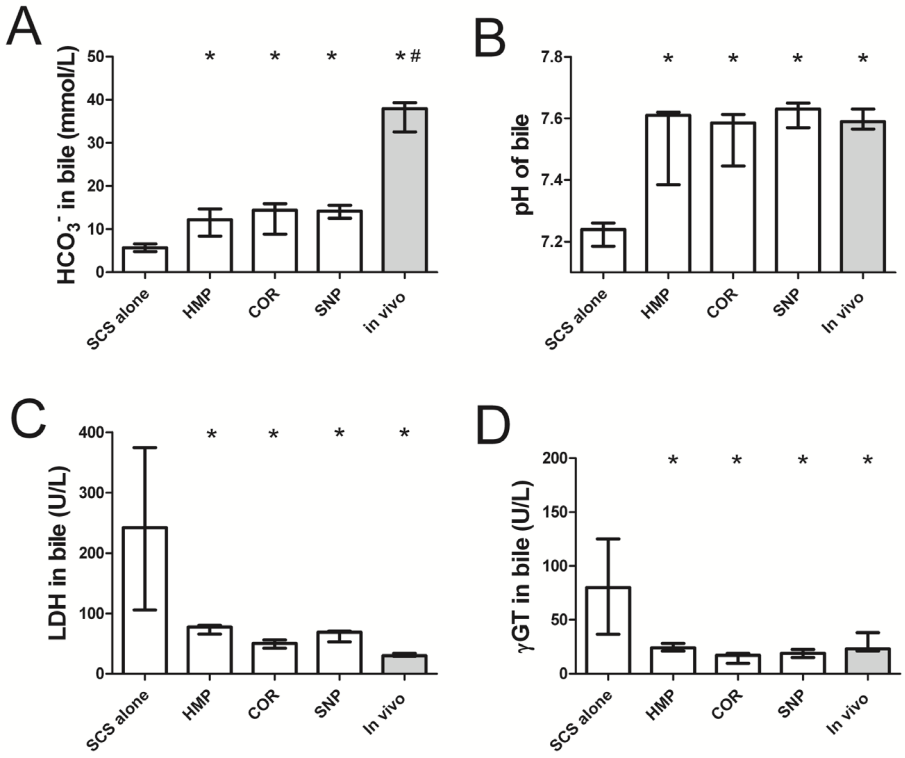

Figure 7 | Biochemical markers of cholangiocyte function and injury measured in bile after 2 hours of *ex situ* reperfusion. Panel A and B) Markers of cholangiocyte function: bicarbonate (HCO_3^-) concentration and pH detected in bile. Biliary bicarbonate concentrations were significantly higher in livers that had been preserved by end-ischemic hypothermic MP (HMP), controlled oxygenated rewarming (COR) MP or subnormothermic MP (SNP), compared to livers that underwent only SCS (*p<0.05). There were no significant differences in biliary bicarbonate concentrations among the three machine perfusion groups (HMP, COR, and SNP). The concentration of biliary bicarbonate collected *in vivo* was significantly higher compared to the three end-ischemic MP groups (HMP, COR, and SNP) and the SCS alone group ($^{\#}p$<0.05). Panel C) The biliary pH was in the three end-ischemic MP groups comparable with the pH measured in the bile samples collected from the reference group with *in vivo* bile collection. Moreover, the livers with only SCS as preservation demonstrated significantly lower biliary pH than the three MP study groups and the group with *in vivo* bile collection (*p<0.05). There were no significant differences in biliary pH between the three end-ischemic MP groups. Panel C and D) The biomarkers for cholangiocyte injury, LDH and gamma-GT, were similar in the three end-ischemic MP groups (HMP, COR, and SNP) and the reference group with *in vivo* bile collection. In bile samples of livers preserved only with SCS, significantly higher values of LDH and Gamma-GT were measured, compared to the end-ischemic MP groups and the group with *in vivo* collected bile (*p<0.05). Data are represented as medians with IQR (error bars).

End-ischemic MP Provides Better Preservation of the Biliary Epithelial Lining of Large Bile Ducts

Injury of the epithelial lining of the lumen of large bile ducts was assessed by an established semi-quantitative histological grading using H&E staining. Livers that were preserved by SCS alone displayed significantly more grade 1 (<50% of the circumferential lining) epithelial cell loss and significantly higher degrees of mural stroma necrosis (grade 1: < 25% and grade 2: 25-50%), compared with livers that underwent end-ischemic MP (HMP, COR, or SNP) (Figure 8 and 9). In fact, large bile duct biopsies of livers that underwent end-ischemic MP revealed only minimal injury of the epithelial cell layer and no signs of mural stroma necrosis. No differences were observed in the degree of vascular injury, incidence of vascular thrombosis, incidence of intramural bleeding, or degree of peribiliary gland injury among all study groups.

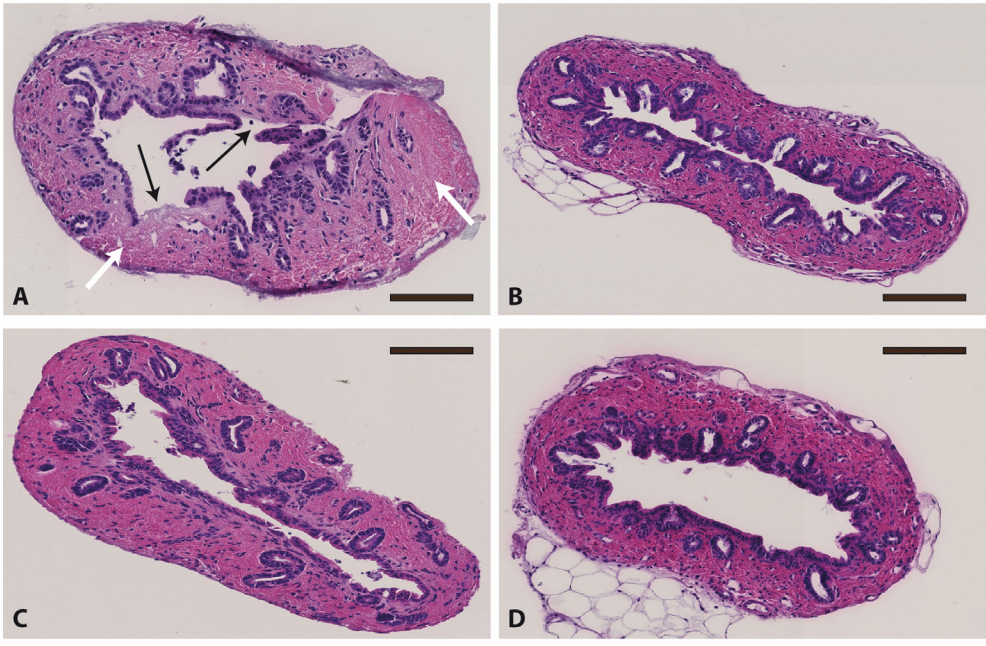

Figure 8 | Hematoxylin and eosin (H&E) staining of the large bile duct biopsies after 2 hours *ex situ* reperfusion. Panel A) SCS preservation only. Black arrows indicate biliary epithelial cell loss (epithelium injury grade 1). White arrows indicate mural stroma necrosis (stroma necrosis grade 2). Panel B) End-ischemic hypothermic MP. Panel C) End-ischemic controlled oxygenated rewarming MP. Panel D: End-ischemic subnormothermic MP. Scale bars indicate 100um.

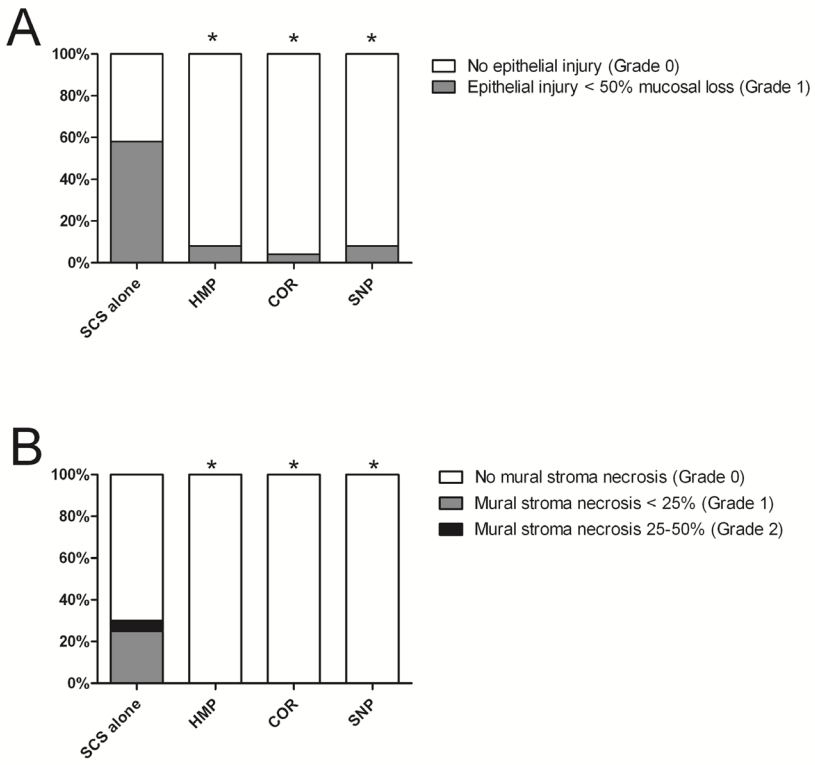

Figure 9 | Overview of the distribution of epithelium injury and mural stroma necrosis of the large bile ducts. Panel A) Livers that were preserved by SCS displayed a significantly higher frequency of epithelium injury grade 1 (< 50% mucosal loss) (**p*<0.05). Panel B) Livers that were preserved by SCS displayed significantly more mural stroma necrosis grade 1 (< 25% stroma necrosis) and mural stroma necrosis grade 2 (stroma necrosis 25-50%), compared with the three end-ischemic MP preserved groups (hypothermic MP, controlled oxygenated rewarming MP, and subnormothermic MP) (**p*<0.05). There were no significant differences in epithelium injury or mural stroma necrosis between the end-ischemic MP groups.

DISCUSSION

Currently, there are three different perfusion temperature conditions that are most frequently applied in the context of end-ischemic MP: hypothermic (<10°C), subnormothermic (20°C), and controlled oxygenated rewarming (8-20°C). However, these three methods have not been examined head to head, and it remains unknown which temperature protocol provides the best protection against biliary injury. In the current study, we, therefore, examined which perfusion temperature provides the best protection against bile duct injury in DCD donor livers.

Our study demonstrates that end-ischemic oxygenated MP significantly improves biliary function and morphology and reduces biliary cellular injury, compared to conventional SCS alone. Interestingly, this beneficial effect was independent from the temperature protocol used during end-ischemic MP. Also, hepatocellular mitochondria oxygen consumption was enhanced after a short period of end-ischemic MP, resulting in higher cellular ATP concentrations. Moreover, end-ischemic MP mitigated hepatocellular injury during reperfusion and lowered oxidative stress, again independent from the temperature protocol used.

It is well known that biliary epithelial cells (cholangiocytes) and the bile duct stroma cells are very sensitive to ischemia and relatively short periods of ischemia result in a rapid depletion of intracellular concentrations of ATP (25,26). Due to ATP depletion, cholangiocytes lose their attachment to the basement membrane, resulting in sloughing of the epithelium layer and denudation of the bile duct luminal surface. In our study end-ischemic MP significantly minimized the degree of biliary epithelial cell loss and reduced the degree of mucosal stroma necrosis in the large bile ducts to a minimum, compared to only SCS preservation. Recently, three independent clinical studies have demonstrated that major mucosal cell loss (> 50% biliary epithelial injury) and mural stroma necrosis (< 50% necrotic cells) of the large bile duct is present in more than 80% of human donor livers (both DBD and DCD) at the end of SCS and subsequent reperfusion (4,24,27). Although biliary preservation injury is apparently almost universally present, only a minority of liver recipients develop NAS after transplantation. This observation has led to the hypothesis that insufficient proliferation and regeneration of cholangiocytes from the peribiliary glands are important factors in the pathogenesis of NAS (3,4). In current study, we did not observe differences in injury of the peribiliary glands between the groups with end-ischemic MP and only SCS preservation. These findings are in contrast with our histological study in human donor livers demonstrating substantial injury of the more peripheral layers of the bile duct wall, including the peribiliary biliary glands, after SCS preservation (4). This study indicated that the severity of bile duct injury decreases from the most central, periluminal layers toward the periphery of the bile ducts (4). This is compatible with the fact that blood supply to the bile ducts enters from the periphery, resulting in the lowest oxygen tension in central structures situated near the lumen. Peribiliary glands are situated in the peripheral layers of the bile duct wall. Apparently, rat liver bile ducts are more resistant to ischemic injury than human bile ducts. Therefore, the rat DCD liver model used in the current study did not cause detectable injury of peribiliary glands. Although we did not observe differences in injury of the peribiliary glands between the groups, end-ischemic MP significantly minimized the degree of biliary epithelial cell loss and reduced the degree of mucosal stroma necrosis to a minimum, compared to only SCS preservation. In parallel with these differences in histopathological assessment, we observed significantly lower biliary concentrations of biochemical markers

of biliary epithelial injury, such as gamma-GT and LDH in bile. Moreover, in contrast to livers that were preserved by SCS alone, all three groups of end-ischemic MP revealed a normal alkalotic biliary pH and improved bicarbonate secretion after reperfusion. Biliary secretion of bicarbonate by biliary epithelial cells is considered an important protective mechanism against the cytotoxic effects of hydrophobic bile salts, which is known as the “bicarbonate umbrella” (20,28). Early recovery of the biliary function after SCS is clinically relevant since hydrophobic bile salts have been shown to play a role in worsening biliary injury and subsequent formation of NAS after transplantation (29,30). Taken together, these findings indicate that a short period of oxygenated MP after conventional SCS not only reduces the amount of biliary epithelial injury after reperfusion, but also contributes to an early recovery of biliary bicarbonate secretion, which helps cholangiocytes to protect themselves against the cytotoxic effects of hydrophobic bile salts.

So far, temperature conditions for end-ischemic MP have only been examined in animal studies, without special focus on biliary viability (15-17). Minor and coworkers (15) used a pig model to study liver graft viability during 4 hours of *ex vivo* reperfusion after living donation. During reperfusion, end-ischemic MP with COR was identified as the most protective temperature strategy. Interestingly, bile production, an important marker for liver function, was only significantly improved by end-ischemic COR and not by HMP or SNP. Moreover, bile production after end-ischemic MP at hypothermic or subnormothermic conditions was almost similar to the group with only SCS preservation. In this respect, our findings are different from those made by Minor *et al.* as we did not observe significant differences in bile production between livers that underwent end-ischemic HMP, SNP, or COR prior to reperfusion. A possible explanation for this could be a difference in organ quality, due to variations in the donation models. Minor and colleagues used livers obtained from living donation procedures, whereas we have used livers from DCD donors who had suffered a period of 30 min warm ischemia prior to donor hepatectomy. The main difference between living donor and DCD donor is the inevitable period of warm ischemia after cardiac arrest in the DCD donor. During this period of warm ischemia, the liver has to switch from aerobic to anaerobic conditions for ATP production. However, anaerobic production of ATP is by far not enough to fulfill the metabolic demand of the liver. This results in a severe ATP depletion prior to cold preservation. In contrast, during living donation, the liver is directly flushed with cold preservation solution, reducing metabolism and ATP consumption (31,32). Therefore, we hypothesize that DCD liver grafts used in our experiment have developed a more severe oxygen debt and sustained more hepatocellular injury at baseline, compared to liver grafts from a living donor. Because of this higher degree of cellular injury in DCD grafts, the effects of resuscitation by end-ischemic MP may be different and even more pronounced, compared to end-ischemic MP in living donor grafts. Our data indicate that a short period of oxygenated machine perfusion after conventional SCS of liver grafts obtained

from DCD donors results in effective restoration of mitochondrial function and cellular ATP concentrations, which is independent of the temperature at which the liver is perfused.
Some limitations of this study should be mentioned: we did not examine the effects of end-ischemic MP at body temperature (normothermic, 37°C). Other studies have demonstrated that short periods of normothermic MP after SCS are not better than SCS preservation alone. In fact, a short period of normothermic MP after conventional SCS may not be any different from immediate implantation and reperfusion of a liver graft in the recipient. In both situations the whole cascade of I/R injury will be activated without a conditioning or protective effect of MP (5,33,34). Another limitation of our study is the *ex situ* reperfusion model. Although this model, which has also been used by other groups (9,12,14,15), allowed us to assess viability and injury of hepatocytes and cholangiocytes, we did not confirm our findings in a transplantation model. In particular, a longer follow-up period is required to assess biliary complications, which can only be achieved in a transplantation study.
In conclusion, this is the first study demonstrating that end-ischemic oxygenated MP of DCD livers provides better preservation of biliary epithelial function and morphology, independent of the temperature at which MP is performed. By significantly reducing biliary injury, especially in the large bile ducts, as well as restoring ATP levels in DCD liver grafts prior to transplantation, end-ischemic oxygenated MP could improve outcome after DCD liver transplantation.

REFERENCES

(1) Verdonk RC, Buis CI, van der Jagt EJ, Gouw AS, Limburg AJ, Slooff MJ, et al. Nonanastomotic biliary strictures after liver transplantation, part 2: Management, outcome, and risk factors for disease progression. Liver Transpl 2007;13:725-732.

(2) Dubbeld J, Hoekstra H, Farid W, Ringers J, Porte RJ, Metselaar HJ, et al. Similar liver transplantation survival with selected cardiac death donors and brain death donors. Br J Surg 2010;97:744-753.

(3) Karimian N, Op den Dries S, Porte RJ. The origin of biliary strictures after liver transplantation: is it the amount of epithelial injury or insufficient regeneration that counts? J Hepatol 2013;58:1065-1067.

(4) op den Dries S, Westerkamp AC, Karimian N, Gouw AS, Bruinsma BG, Markmann JF, et al. Injury to peribiliary glands and vascular plexus before liver transplantation predicts formation of non-anastomotic biliary strictures. J Hepatol 2014;60:1172-1179.

(5) Schlegel A, Dutkowski P. Role of hypothermic machine perfusion in liver transplantation. Transpl Int 2015;28:677-689.

(6) Dutkowski P, Odermatt B, Heinrich T, Schonfeld S, Watzka M, Winkelbach V, et al. Hypothermic oscillating liver perfusion stimulates ATP synthesis prior to transplantation. J Surg Res 1998;80:365-372.

(7) Guarrera JV, Henry SD, Samstein B, Odeh-Ramadan R, Kinkhabwala M, Goldstein MJ, et al. Hypothermic machine preservation in human liver transplantation: the first clinical series. Am J Transplant 2010;10:372-381.

(8) Dutkowski P, Schlegel A, de Oliveira M, Mullhaupt B, Neff F, Clavien PA. HOPE for human liver grafts obtained from donors after cardiac death. J Hepatol 2014;60:765-772.

(9) Ferrigno A, Rizzo V, Boncompagni E, Bianchi A, Gringeri E, Neri D, et al. Machine perfusion at 20 degrees C reduces preservation damage to livers from non-heart beating donors. Cryobiology 2011;62:152-158.

(10) Tolboom H, Izamis ML, Sharma N, Milwid JM, Uygun B, Berthiaume F, et al. Subnormothermic machine perfusion at both 20 degrees C and 30 degrees C recovers ischemic rat livers for successful transplantation. J Surg Res 2012;175:149-156.

(11) Schlegel A, Graf R, Clavien PA, Dutkowski P. Hypothermic oxygenated perfusion (HOPE) protects from biliary injury in a rodent model of DCD liver transplantation. J Hepatol 2013;59:984-991.

(12) Op den Dries S, Sutton ME, Karimian N, de Boer MT, Wiersema-Buist J, Gouw AS, et al. Hypothermic oxygenated machine perfusion prevents arteriolonecrosis of the peribiliary plexus in pig livers donated after circulatory death. PLoS One 2014;9:e88521.

(13) Knaak JM, Spetzler VN, Goldaracena N, Boehnert MU, Bazerbachi F, Louis KS, et al. Subnormothermic ex vivo liver perfusion reduces endothelial cell and bile duct injury after donation after cardiac death pig liver transplantation. Liver Transpl 2014;20:1296-1305.

(14) Bruinsma BG, Yeh H, Ozer S, Martins PN, Farmer A, Wu W, et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. Am J Transplant 2014;14:1400-1409.

(15) Minor T, Efferz P, Fox M, Wohlschlaeger J, Luer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. Am J Transplant 2013;13:1450-1460.

(16) Shigeta T, Matsuno N, Obara H, Kanazawa H, Tanaka H, Fukuda A, et al. Impact of rewarming preservation by continuous machine perfusion: improved post-transplant recovery in pigs. Transplant Proc 2013;45:1684-1689.

(17) Matsuno N, Obara H, Watanabe R, Iwata S, Kono S, Fujiyama M, et al. Rewarming preservation by organ perfusion system for donation after cardiac death liver grafts in pigs. Transplant Proc 2014;46:1095-1098.

(18) van den Eijnden MM, Leuvenink HG, Ottens PJ, 't Hart NA, van Oeveren W, Morariu AM, et al. Effect of brain death and non-heart-beating kidney donation on renal function and injury: an assessment in the isolated perfused rat kidney. Exp Clin Transplant 2003;1:85-95.

(19) Luer B, Koetting M, Efferz P, Minor T. Role of oxygen during hypothermic machine perfusion preservation of the liver. Transpl Int 2010;23:944-950.

(20) Hohenester S, Wenniger LM, Paulusma CC, van Vliet SJ, Jefferson DM, Elferink RP, et al. A biliary HCO3- umbrella constitutes a protective mechanism against bile acid-induced injury in human cholangiocytes. Hepatology 2012;55:173-183.

(21) Vajdova K, Smrekova R, Kukan M, Lutterova M, Wsolova L. Bile analysis as a tool for assessing integrity of biliary epithelial cells after cold ischemia--reperfusion of rat livers. Cryobiology 2000;41:145-152.

(22) Silva AM, Oliveira PJ. Evaluation of respiration with clark type electrode in isolated mitochondria and permeabilized animal cells. Methods Mol Biol 2012;810:7-24.

(23) Brand MD, Nicholls DG. Assessing mitochondrial dysfunction in cells. Biochem J 2011;435:297-312.

(24) Hansen T, Hollemann D, Pitton MB, Heise M, Hoppe-Lotichius M, Schuchmann M, et al. Histological examination and evaluation of donor bile ducts received during orthotopic liver transplantation--a morphological clue to ischemic-type biliary lesion? Virchows Arch 2012;461:41-48.

(25) Noack K, Bronk SF, Kato A, Gores GJ. The greater vulnerability of bile duct cells to reoxygenation injury than to anoxia. Implications for the pathogenesis of biliary strictures after liver transplantation. Transplantation 1993;56:495-500.

(26) Doctor RB, Dahl RH, Salter KD, Fouassier L, Chen J, Fitz JG. ATP depletion in rat cholangiocytes leads to marked internalization of membrane proteins. Hepatology 2000;31:1045-1054.

(27) Brunner SM, Junger H, Ruemmele P, Schnitzbauer AA, Doenecke A, Kirchner GI, et al. Bile duct damage after cold storage of deceased donor livers predicts biliary complications after liver transplantation. J Hepatol 2013;58:1133-1139.

(28) Beuers U, Hohenester S, de Buy Wenniger LJ, Kremer AE, Jansen PL, Elferink RP. The biliary HCO(3)(-) umbrella: a unifying hypothesis on pathogenetic and therapeutic aspects of fibrosing cholangiopathies. Hepatology 2010;52:1489-1496.

(29) Buis CI, Verdonk RC, Van der Jagt EJ, van der Hilst CS, Slooff MJ, Haagsma EB, et al. Nonanastomotic biliary strictures after liver transplantation, part 1: Radiological features and risk factors for early vs. late presentation. Liver Transpl 2007;13:708-718.

(30) Yska MJ, Buis CI, Monbaliu D, Schuurs TA, Gouw AS, Kahmann ON, et al. The role of bile salt toxicity in the pathogenesis of bile duct injury after non-heart-beating porcine liver transplantation. Transplantation 2008;85:1625-1631.

(31) Monbaliu D, Pirenne J, Talbot D. Liver transplantation using Donation after Cardiac Death donors. J Hepatol 2012;56:474-485.

(32) Perera MT, Richards DA, Silva MA, Ahmed N, Neil DA, Murphy N, et al. Comparison of energy metabolism in liver grafts from donors after circulatory death and donors after brain death during cold storage and reperfusion. Br J Surg 2014;101:775-783.

(33) Reddy SP, Bhattacharjya S, Maniakin N, Greenwood J, Guerreiro D, Hughes D, et al. Preservation of porcine non-heart-beating donor livers by sequential cold storage and warm perfusion. Transplantation 2004;77:1328-1332.

(34) Reddy S, Greenwood J, Maniakin N, Bhattacharjya S, Zilvetti M, Brockmann J, et al. Non-heart-beating donor porcine livers: the adverse effect of cooling. Liver Transpl 2005;11:35-38.

CHAPTER 5

Gradual Rewarming with Hemoglobin-Based Oxygen Carrier (HBOC) in a Rat DCD Liver Model

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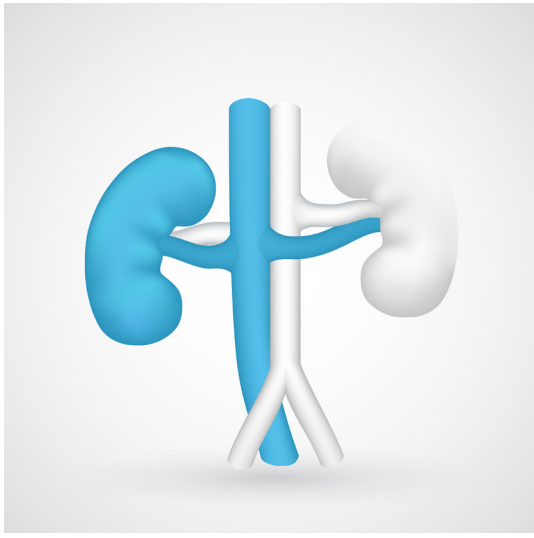

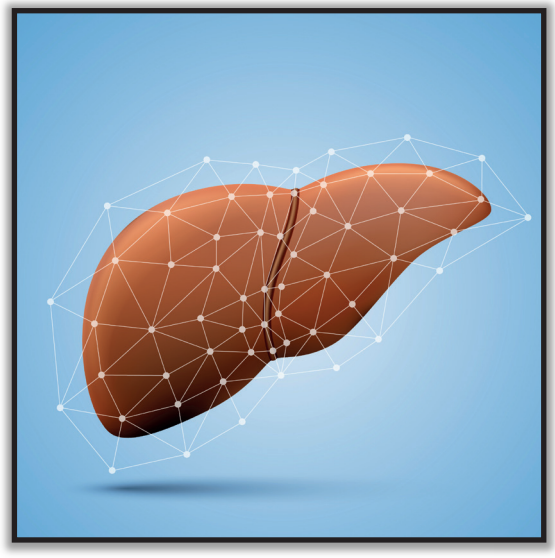

ABSTRACT

Donors after circulatory death (DCD) grafts are of great interest in increasing the number of available donor organs for transplant. Machine perfusion strategies aim to treat the warm ischemic injury and increase the transplant success of these grafts. Gradual rewarming machine perfusion has shown to reduce reperfusion injury and improve graft quality upon implantation. The main limitation of gradual rewarming has been the lack of an oxygen carrier that functions and maintains the integrity over the temperature change. Therefore, in this study we tested the efficacy and safety of using Hemoglobin Based Oxygen Carrier (HBOC) during gradual rewarming of DCD rat livers. Liver grafts were procured after 30 minutes of warm ischemia. The effect of 90 minutes of oxygenated rewarming perfusion from ice cold temperature to 37°C with HBOC after CS was evaluated and the results were compared with CS alone. Reperfusion at 37°C was performed to assess the post preservation recovery. This study suggests the safety of using HBOC during rewarming perfusion of rat livers, as no harmful effect was detected. Overall, gradual rewarming with HBOC led to better recovery, reduced vascular resistance, decreased lactate and increased bile flow during reperfusion as compared to cold stored liver grafts.

INTRODUCTION

DCD grafts are contributing significantly to expanding the organ pool (1). However, DCD grafts are associated with post-transplant complications such as primary non-function, ischemia reperfusion injury and biliary strictures (2,3). Therefore, the improvement of DCD graft quality before implantation is an essential step towards improving the outcome after transplant.

DCD grafts undergo warm ischemia in addition to a period of CS which itself is injurious. The combination of warm and cold ischemia injury is suggested to increase ischemia reperfusion injury which explains the inferior quality of DCD grafts after transplantation (3). Machine perfusion is a new preservation method currently in clinical trials across the world, and offers the likelihood of treating these DCD livers (4,5). Different machine perfusion protocols from hypothermic to sub-normothermic and normothermic have been studied before and the results have shown that machine perfusion is beneficial in reducing reperfusion injury (6–8). Among different perfusion protocols normothermic machine perfusion with red blood cells (RBCs) is the most common method in clinical trials as it offers viability assessment during graft preservation period (5,9,10).

Gradual rewarming during organ machine perfusion from hypothermia to normothermia has been described as a successful preservation method for eliminating reperfusion injury (11,12). A direct comparison of gradual rewarming with normothermic perfusion has also reported superior results of gradual rewarming in terms of better graft preservation and improved function (13). However, in the earlier rewarming studies, no oxygen carrier was added to the perfusion solution as the temperature change during rewarming limits the use of RBCs. In this study, the gradual rewarming from hypothermia to normothermia, with the supplementation of HBOC as the oxygen carrier was designed to assess the feasibility and safety of using an artificial oxygen carrier in the temperature range from 4°C to 37°C in DCD liver grafts.

MATERIAL AND METHODS

Experimental Animals and Liver Procurement

Male Lewis rats weighing 290-350 g were used in this study. Animals received care according to the national research Council guidelines on animal experiments. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the Massachusetts General Hospital. Anesthesia and surgical details of recovery are provided elsewhere (14). Briefly, the animals were anesthetized using 5% isoflurane and 1ml 0.9% NaCl with 500 IU of heparin was administrated via the dorsal penile vein. In order to induce DCD procedure,

cardiac arrest was performed by squeezing of the heart till heart contraction was stopped. Then, the animal was kept at 37°C for 30 minutes. The bile duct was cannulated during 30 minutes of warm ischemia. Portal vein was cannulated using an 18-gauge intravenous catheter and the liver was flushed through the portal vein with 10 mL 0.9% NaCl at room temperature followed by 30 mL University of Wisconsin (UW) preservation solution at 4°C. The liver was removed and stored in cold UW (4°C) during CS preservation.

Experimental Design

A total number of 10 rat livers were divided into 2 experimental groups after DCD procurement (n = 5 per group). In the **rewarming with HBOC group (rewarm&HBOC)**, after 270 minutes CS in UW media, the liver grafts underwent gradual rewarming perfusion from 8°C to 37°C for 90 minutes. Afterwards, the grafts were flushed with 10 mL of cold saline and stored in a petri dish covered with wet gauze at room temperature for 30 minutes, in order to mimic surgical implantation period. Subsequently, the grafts were reperfused at 37°C for 120 minutes, with the protocol detailed below. In the **CS group**, the grafts were kept in ice cold UW for 360 minutes, followed by 30 minutes of storage at room temperature to mimic implantation, and then were subjected to reperfusion at 37°C for 120 minutes, same as in rewarming group.

Machine Perfusion

The perfusion device is a flow-controlled system for rodent organ perfusion (Fig 1) (14). In the rewarming group, the temperature of the perfusion solution was set at 8°C at the

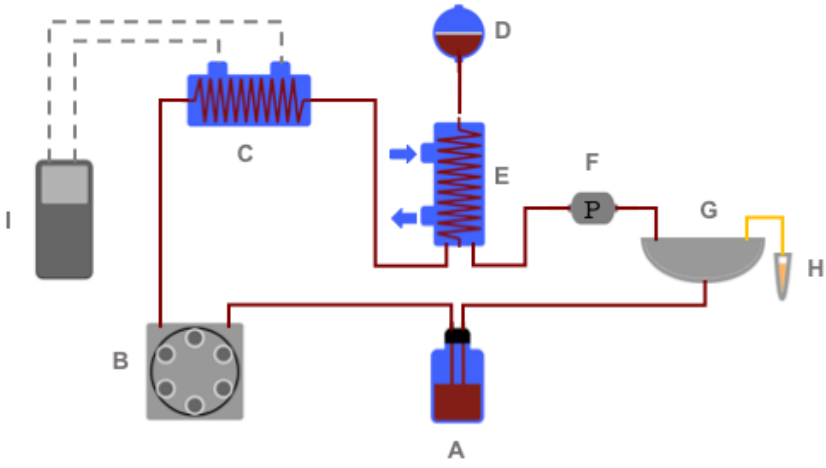

Figure 1 | Graphic representation of the rodent liver perfusion system
(A)Solution reservoir; (B) Roller pump; (C) Oxygenator containing silicon tubing and providing Carbogen and heat exchanger; (D&E) Bubble trap; (F) Pressure probe; (G) Organ chamber; (H) Bile Eppendorf; (I) thermostat which regulates the temperature.

beginning of rewarming, minimum allowed by the equipment, and was gradually increased to 37°C through 60 minutes. The temperature was kept stable at 37°C for the final 30 minutes.

Viability Assessment (Normothermic Reperfusion)

To mimic the transplant process, a 30-minute room temperature period was chosen to reflect the implantation in the recipient as described above, and a following 120 minutes normothermic perfusion duration was considered as a simulated early post-transplantation period. In both the experiment and control groups, the perfusion temperature was set at 37°C and perfusion was continued for a total of 120 minutes. The flow rate during this period was initially set at 8.0 ml/minute and was adjusted according to the portal venous pressure, which was kept constant between 50- and 140-mM H_2O.

Perfusion Solution

The perfusion solution consisted of Williams Medium E (Sigma-Aldrich, St Louis, MO, USA) supplemented with insulin (2 U/L Humulin; Eli Lilly & Co, Indianapolis, IN, USA), L-glutamine (0.292 g/L; Gibco/Invitrogen), heparin (1,000 U/L APP pharmaceuticals, Schaumberg, IL,USA), Albumin 15% (Sigma-Aldrich, St Louis, MO, USA) and 25% HBOC-201 v/v (provided by Hemoglobin Oxygen Therapeutics, Souderton, PA). Different HBOC concentrations were tested and final concentration was determined based on ensuring detectable hemoglobin in the perfusate, as well as a detectable change in dissolved oxygen in the media. During the rewarming and reperfusion experiments, the solution was oxygenated using Carbogen, a mixture of 95% O_2 and 5% CO_2. Note that the same formula was used in both gradual rewarming group and the reperfusion phase. In the rewarm&HBOC group, during the 30min simulated anastomosis time, the perfusion device was flushed clean and the perfusion solution was renewed.

Perfusion Measurements and Injury

Rewarming

During rewarming, temperature, flow and pressure were recorded at 30 minutes intervals and subsequently resistance was calculated. pH, Bicarbonate, Lactate, pO_2 and pCO_2 levels in the perfusate samples were analyzed using I-Stat analyzer (Abbott, USA) every 30 minute, and pH was corrected by adding 8.4% $NaHCO_3^-$. Bile production was observed and recorded at the end of 90 minutes rewarming.

Reperfusion

The same perfusion parameters noted in rewarming procedure in addition to glucose was measured in both groups during 120 minutes of reperfusion.

Liver Injury

Alanine Aminotransferase (ALT) was measured in the perfusate samples using Elisa kit (# MBS041480 MyBioSource, Inc., San Diego, CA) during reperfusion in both rewarming and CS groups.

Oxygen Consumption and Adenosine Tri-Phosphate (ATP) Measurement

Reperfusion

Oxygen consumption during reperfusion was calculated in both groups using the following formula:

([{ApO_2−VpO_2} × K /760] × total flow) + ([{AsO_2−VsO_2} × Hb × c × 0.0001] × flow) / Liver weight × 100. In which pO_2 was in mm Hg, sO_2 in %, Hb in g/dL, Portal vein flow in mL/min and liver weight in g. c the oxygen binding capacity of HBOC (1.26) and K was a constant (0.0225).

The tissue samples for ATP measurement were only taken at the end of reperfusion (t=120 minutes) In order to prevent inducing injury to the liver grafts during rewarming and reperfusion. Method for extraction and measurement has been described previously (15,16).

Bile Production and Cholangiocyte Function

Rewarming

The produced bile was collected in Eppendorf tubes and was measured in milliliter (mL) at the end of 90 minutes rewarming.

Reperfusion

Bile production was recorded and biliary epithelial cell function was assessed by measuring pH and bicarbonate concentration in bile (17). For this purpose, bile samples were collected under mineral oil and were analyzed immediately using I-Stat analyzer.

Histological Evaluation

Reperfusion

Biopsies were obtained from the liver parenchyma at the end of reperfusion phase and were stored in 10% formalin for the histological evaluation. Paraffin-embedded slides of liver biopsies were prepared for hematoxylin and eosin (H&E).

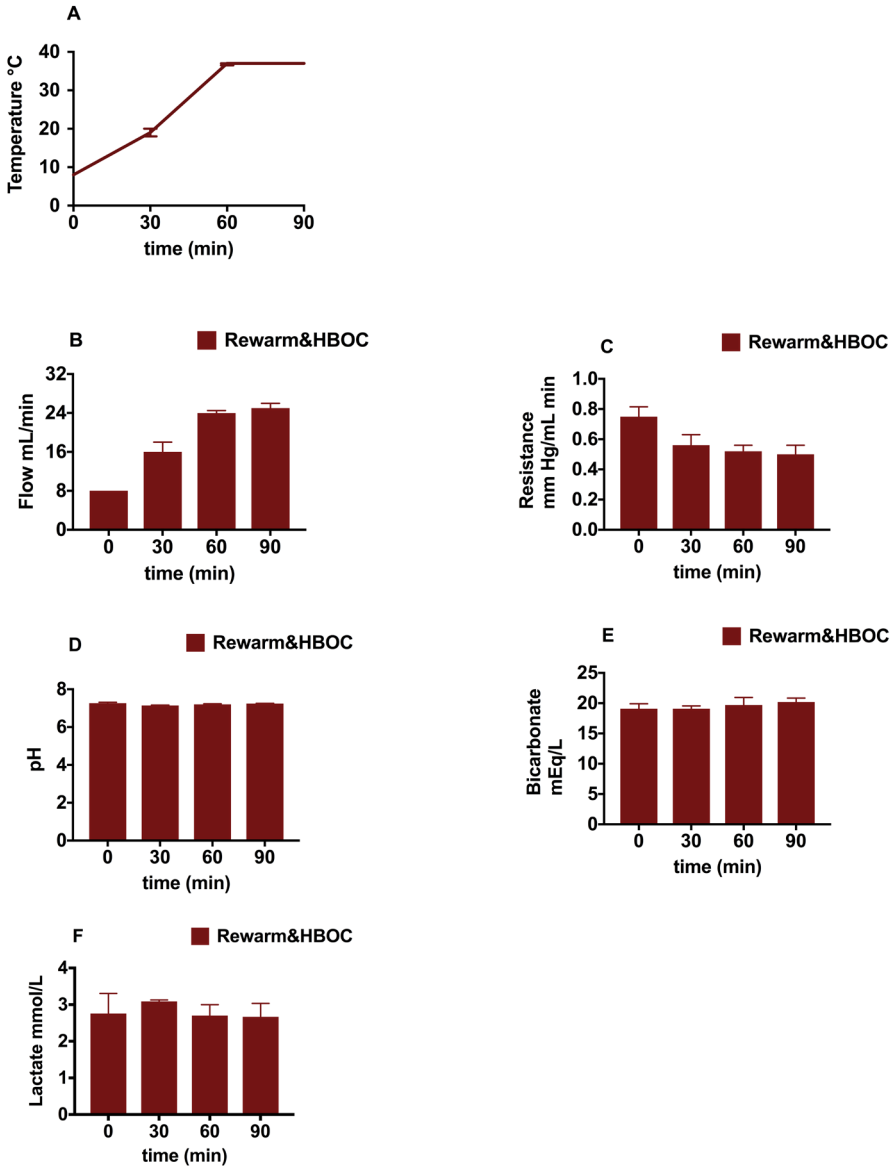

Figure 2 | Liver profile during gradual rewarming in rewarm&HBOC group
A) Temperature; B, C) Flow was increased and portal Resistance slightly reduced during gradual rewarming. D) pH, which normalized by the end of gradual rewarming procedure. E) Bicarbonate levels in perfusate. F) Decreased lactate levels were observed.

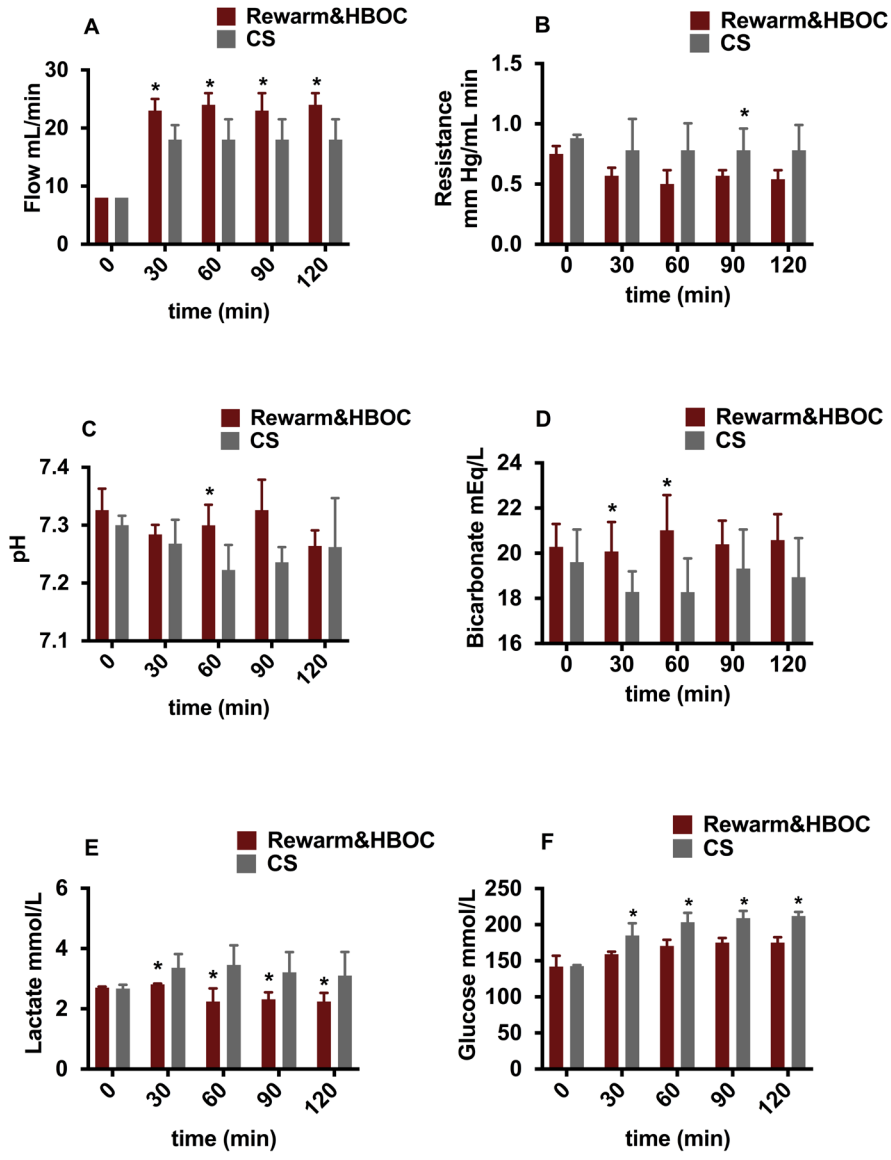

Figure 3 | Livers undergoing Gradual Rewarming show improved function and recovery compared to CS controls during reperfusion

A) Flow was significantly higher in rewarm&HBOC compare to CS group from t-30 to t=120 (p=≤ 0.05). B) Resistance was lower in the rewarm&HBOC group compared to CS group during 120 minutes reperfusion with significant difference at t=90 (p= 0.02). C) pH was better in the rewarm&HBOC group compare to CS group with significant difference t=60 (p=0.016). D) Bicarbonate levels were better in the rewarm&HBOC group with significant difference at t=30 (p=0.05) and t=60 (p= 0.032). E) In contrast to CS group, Lactate level was significantly lower in rewarm&HBOC group between t=30 and t=120 (p=≤ 0.05). F) Glucose concentration remained significantly lower in the rewarm&HBOC group compared to CS group between t=30 and t=120 (p=≤ 0.05). * represents individual time point significances.

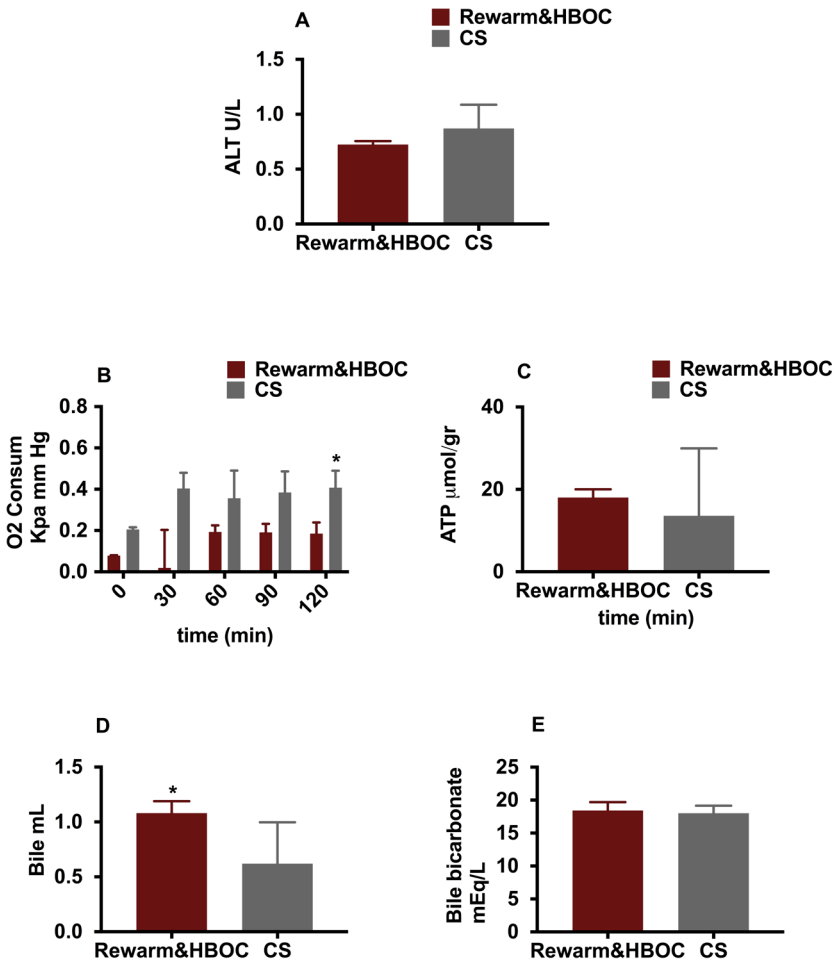

Figure 4 | Graphical presentation of oxygen concentration, ATP level and bile fluid in both rewarming and CS groups

A) There was lower trend of ALT in the rewarm&HBOC compare to CS group during 120 minutes of reperfusion (p=0.056). B) Oxygen Consumption remained higher in CS group in comparison with Rewarming and this difference was significant at t=90 (p=0.024). C) There was no significant difference in ATP levels between the both groups (p=0.55). D) The total volume of bile production measured at the end of 120 minutes reperfusion was meaningfully higher in rewarm&HBOC group compared to CS group. E) There was no difference in the level of bicarbonate in bile samples of rewarm&HBOC and CS groups during 120 minutes reperfusion. * represents individual time point significances.

Statistical Analysis

Continuous data were presented as the median and interquartile range (IQR). Mann-Whitney U test was used to compare groups. A p-value of less than 0.05 was considered significant. Analyses were performed using SPSS software version 22.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Perfusion Profile during Gradual Rewarming

Temperature profile during rewarming is shown in (Fig 2A). Portal vein flow was increased and resistance decreased, stabilized towards the end of rewarming perfusion (Fig 2B&C). Regarding other perfusion parameters, bicarbonate level was slightly improved with the pH within physiological range in all the liver grafts (Fig 2D&E). Lactate level was decreased through the rewarming perfusion in all the liver grafts (Fig 2F). We also observed bile production during rewarming which is an indicator of liver function (0.45±0.125).

Comparison of Gradual Rewarming and Cold Storage during Reperfusion

The flow was increased and resistance in portal vein gradually decreased in both rewarm&HBOC and CS groups with lower trend in the rewarm&HBOC group, t=90 (p=0.02) t=0 and t=120 (p=0.09) (Fig 3A&B). pH was closer to physiological range in the rewarm&HBOC group compared to CS liver grafts during 120 minutes reperfusion with a significant difference at t=60 (p=0.01) (Fig 3C). In the rewarm&HBOC group, bicarbonate level was higher with significant difference at t=30 (p=0.05) and t=60 (p=.03) compared to the CS group (Fig 3D). Lactate concentrations initially increased in both groups but subsequently decreased and the concertation remained significantly lower in the rewarm&HBOC liver grafts compared to CS liver grafts throughout the reperfusion procedure (between t=30 and t=120) ($p \leq 0.05$) (Fig 3E). Glucose concentration was measured in the perfusate samples during 120 minutes of reperfusion and was significantly lower in the rewarm&HBOC from t=30 till the end of reperfusion ($p=\leq 0.05$) (Fig 3F).

Liver Injury

ALT, an indicator of hepatic injury was measured in the perfusate samples during reperfusion. There was lower trend of ALT in the rewarm&HBOC group compared to CS group with a p value 0.056 (Fig 4A).

Oxygen Consumption and ATP

Oxygen consumption was observed during 120 minutes of reperfusion and was slightly higher in CS group in comparison to rewarm&HBOC group with a significant difference at t= 120 (p= 0.008) (Fig 4B). After 120 minutes of reperfusion, no statistical differences were found in the ATP production between the rewarm&HBOC and CS groups (p=0.55) (Fig 4C), although this appeared more a result of the high variability in the CS livers and values trended higher with less variability in the rewarm&HBOC group.

Bile Production and Cholangiocyte Function

Bile production and biliary cholangiocyte function were measured at the end of reperfusion period. The median bile production was higher in the rewarm&HBOC in comparison with CS group (p=.016) (Fig 4D). There was no difference in the level of bicarbonate in the bile samples between the rewarm&HBOC and CS groups (p=0.6) (Fig 4E).

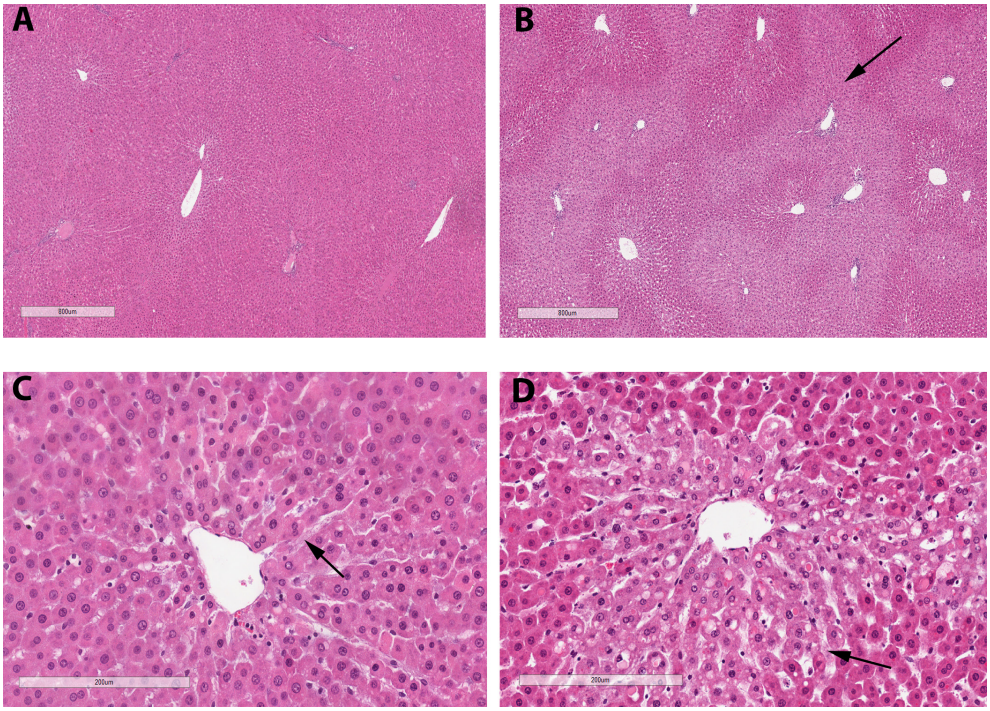

Figure 5. H & E staining of liver tissue from rewarming and CS groups at the end of reperfusion
CS liver tissues (**B**&**D**) demonstrated higher venous congestion (stasis of fluid in parenchyma shown by arrows) compared to rewarm&HBOC liver grafts (**A** &**C**).

Histological Evaluation

In parallel with the observed differences in biochemical markers of hepatic function, livers in the CS group showed more sign of venous congestion, compared to the rewarm&HBOC group (Fig 5).

DISCUSSION

This study shows the feasibility of using HBOC in the gradual rewarming and some end points may suggest improved graft function in the rewarm&HBOC compared to the clinical standard, CS preservation. These improvements are indicated by better flow rate, physiologically balanced perfusion pH and bicarbonate during reperfusion. Better bicarbonate concentration is a result of liver using up the lactate, so better lactate clearance results in more balanced bicarbonate and pH, these findings also explain the significantly reduced lactate in the rewarm&HBOC group during reperfusion. Increased bile production in the rewarm&HBOC group suggests improved liver function as bile production is an early indicator of liver function.

The use of HBOC in patients has been reported to induce vasoconstriction and lead to systemic hypertension (18) . In contrast to earlier patient studies finding, however, no evidence of hypertension was detected in our rewarming model and we even experienced positive effects highlighted by significantly higher flow rate and lower trend of vascular resistance in the rewarm&HBOC group. This outcome is in line with the use of HBOC in sub-normothermic and normothermic liver perfusion studies in which they reported no negative effect of HBOC on perfusion pressure and resistance (19,20). In the rewarm&HBOC group, lactate concentration declined and glucose level in the perfusate remained significantly lower which demonstrates that the livers in this group had superior lactate and glucose metabolism and better liver function. The trend of low ALT concentration (p=.056) in the perfusate samples in the rewarm&HBOC group may indicate lower liver parenchyma injury, parallel to this finding, the histological examination showed higher liver congestion in the CS group. It was shown that hepatic congestion could increase liver enzymes and lead to liver injury (21).

Oxygen consumption was found to be higher towards the end of reperfusion in the CS group compared to the rewarming group with no significant difference in ATP production. This finding is in line with previous results in which the investigators found higher oxygen consumption during normothermic machine perfusion after prolonged CS preservation, compared to well preserved liver grafts (22,23). This difference in oxygen consumption was explained previously by referring to respiratory burst and oxygen debt in severely injured post-ischemic livers with no meaningful increase in ATP production (23), and our results are in concurrence.

A number of novel oxygen carriers such as Perfluorocarbons (PFCs) and Hemarina (M-101) have been developed and used in different organ preservation protocols like hypothermic perfusion and cold storage (24). Early outcome of adding PFC in kidney machine perfusion showed instability and adverse effect of PFC on renal function which limits further use of this oxygen carrier in perfusion experiment (25). M101 is another novel oxygen carrier introduced

recently and has a very high affinity for oxygen. Thuillieret al. have showed adding Hemarina during cold storage to the preservation saluting improves renal function, however there is very limited evidence showing the beneficiary effect of M101 during machine perfusion (26). HBOC so far is the only available hemoglobin based artificial oxygen carrier which has been used in a number of different perfusion protocols and showed stability with no adverse effect at different temperatures (19,27,28). Notably, the use of HBOC in extended normothermic liver perfusion has shown improved perfusion parameters and low lactate level (20,27). Our findings are in accordance with the previous studies regarding low lactate level at the end of perfusion (27). HBOC is also the only available oxygen carrier that has been used clinically before (29).

In this study we miss the validation in a transplantation model. However, normothermic ex vivo reperfusion has been often used as a simulated transplant model, and the findings in this study compare favorably to other studies in literature, including ours, that indicate the gradual rewarming group with HBOC proposed improved graft that are viable for transplant, and are functionally better than CS livers.

In conclusion, this study shows the feasibility of gradual rewarm&HBOC, and increased efficacy in recovery of DCD liver grafts compared to CS controls in a DCD rat model. Further, our results indicate the safety of using HBOC in gradual rewarming perfusion.

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DISCLOSURES

The authors of this manuscript have conflicts of interest to disclose: Dr. Uygun is inventor on pending patents relevant to this study (WO2011002926; US20140030231). Dr. Uygun has a financial interest in Organ Solutions, a company focused on developing organ preservation technology. Dr. Uygun's interests are managed by the MGH and Partners HealthCare in accordance with their conflict of interest policies.

Drs. Tessier and Uygun have several IP disclosures on extended organ preservation that may be relevant to this study.
The Hemoglobin Based Oxygen Carrier used in this study (Hemopure) was provided by HBO2 Therapeutics LLC.

REFERENCES

1. Mahboub P, Bozorgzadeh A, Martins PN. Potential approaches to improve the outcomes of donation after cardiac death liver grafts. World J Transplant. 2016 Jun 24;6(2):314–20.
2. Pine JK, Aldouri A, Young AL, Davies MH, Attia M, Toogood GJ, et al. Liver transplantation following donation after cardiac death: an analysis using matched pairs. Liver Transplant Off Publ Am Assoc Study Liver Dis Int Liver Transplant Soc. 2009 Sep;15(9):1072–82.
3. Monbaliu D, Liu Q, Vekemans K, Roskams T, Pirenne J. Potentiation of adverse effects of cold by warm ischemia in circulatory death donors for porcine liver transplantation. Transplant Proc. 2012 Nov;44(9):2874–9.
4. Sutherland AI, Oniscu GC. Challenges and advances in optimizing liver allografts from donation after circulatory death donors. J Nat Sci Biol Med. 2016 Jun;7(1):10–5.
5. Ravikumar R, Jassem W, Mergental H, Heaton N, Mirza D, Perera MTPR, et al. Liver Transplantation After Ex Vivo Normothermic Machine Preservation: A Phase 1 (First-in-Man) Clinical Trial. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2016;16(6):1779–87.
6. Westerkamp AC, Karimian N, Matton APM, Mahboub P, van Rijn R, Wiersema-Buist J, et al. Oxygenated Hypothermic Machine Perfusion After Static Cold Storage Improves Hepatobiliary Function of Extended Criteria Donor Livers. Transplantation. 2016 Apr;100(4):825–35.
7. Bruinsma BG, Avruch JH, Weeder PD, Sridharan GV, Uygun BE, Karimian NG, et al. Functional human liver preservation and recovery by means of subnormothermic machine perfusion. J Vis Exp JoVE. 2015 Apr 27;(98).
8. Vogel T, Brockmann JG, Quaglia A, Morovat A, Jassem W, Heaton ND, et al. The 24-hour normothermic machine perfusion of discarded human liver grafts. Liver Transplant Off Publ Am Assoc Study Liver Dis Int Liver Transplant Soc. 2017 Feb;23(2):207–20.
9. Nasralla D, Coussios CC, Mergental H, Akhtar MZ, Butler AJ, Ceresa CDL, et al. A randomized trial of normothermic preservation in liver transplantation. Nature. 2018 May;557(7703):50–6.
10. Bral M, Gala-Lopez B, Bigam D, Kneteman N, Malcolm A, Livingstone S, et al. Preliminary Single-Center Canadian Experience of Human Normothermic Ex Vivo Liver Perfusion: Results of a Clinical Trial. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2017;17(4):1071–80.
11. von Horn C, Baba HA, Hannaert P, Hauet T, Leuvenink H, Paul A, et al. Controlled oxygenated rewarming up to normothermia for pretransplant reconditioning of liver grafts. Clin Transplant. 2017 Nov;31(11).
12. Minor T, Efferz P, Fox M, Wohlschlaeger J, Lüer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2013 Jun;13(6):1450–60.
13. Hoyer DP, Paul A, Luer S, Reis H, Efferz P, Minor T. End-ischemic reconditioning of liver allografts: Controlling the rewarming. Liver Transplant Off Publ Am Assoc Study Liver Dis Int Liver Transplant Soc. 2016;22(9):1223–30.
14. Izamis M-L, Calhoun C, Uygun BE, Guzzardi MA, Price G, Luitje M, et al. SIMPLE MACHINE PERFUSION SIGNIFICANTLY ENHANCES HEPATOCYTE YIELDS OF ISCHEMIC AND FRESH RAT LIVERS. Cell Med. 2013;4(3):109–23.
15. Lorenz MA, El Azzouny MA, Kennedy RT, Burant CF. Metabolome response to glucose in the β-cell line INS-1 832/13. J Biol Chem. 2013 Apr 12;288(15):10923–35.
16. Bruinsma BG, Avruch JH, Sridharan GV, Weeder PD, Jacobs ML, Crisalli K, et al. Peritransplant Energy Changes and Their Correlation to Outcome After Human Liver Transplantation. Transplantation. 2017;101(7):1637–44.

17. Op den Dries S, Karimian N, Westerkamp AC, Sutton ME, Kuipers M, Wiersema-Buist J, et al. Normothermic machine perfusion reduces bile duct injury and improves biliary epithelial function in rat donor livers. Liver Transplant Off Publ Am Assoc Study Liver Dis Int Liver Transplant Soc. 2016;22(7):994–1005.
18. Taverne YJ, de Wijs-Meijler D, Te Lintel Hekkert M, Moon-Massat PF, Dubé GP, Duncker DJ, et al. Normalization of hemoglobin-based oxygen carrier-201 induced vasoconstriction: targeting nitric oxide and endothelin. J Appl Physiol Bethesda Md 1985. 2017 May 1;122(5):1227–37.
19. Fontes P, Lopez R, van der Plaats A, Vodovotz Y, Minervini M, Scott V, et al. Liver preservation with machine perfusion and a newly developed cell-free oxygen carrier solution under subnormothermic conditions. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2015 Feb;15(2):381–94.
20. Matton APM, Burlage LC, van Rijn R, de Vries Y, Karangwa SA, Nijsten MW, et al. Normothermic Machine Perfusion of Donor Livers Without the Need for Human Blood Products. Liver Transplant Off Publ Am Assoc Study Liver Dis Int Liver Transplant Soc. 2017 Dec 27;
21. Alvarez AM, Mukherjee D. Liver Abnormalities in Cardiac Diseases and Heart Failure. Int J Angiol Off Publ Int Coll Angiol Inc. 2011 Sep;20(3):135–42.
22. Sutton ME, op den Dries S, Karimian N, Weeder PD, de Boer MT, Wiersema-Buist J, et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. PloS One. 2014;9(11):e110642.
23. Imber CJ, St Peter SD, Lopez de Cenarruzabeitia I, Pigott D, James T, Taylor R, et al. Advantages of normothermic perfusion over cold storage in liver preservation. Transplantation. 2002 Mar 15;73(5):701–9.
24. al HS et. Oxygenated kidney preservation techniques. - PubMed - NCBI [Internet]. [cited 2018 Sep 3]. Available from: https://phstwlp2.partners.org:2052/pubmed/22217529
25. Hosgood SA, Mohamed IH, Nicholson ML. The two layer method does not improve the preservation of porcine kidneys. Med Sci Monit Int Med J Exp Clin Res. 2011 Jan;17(1):BR27-33.
26. Thuillier R, Dutheil D, Trieu MTN, Mallet V, Allain G, Rousselot M, et al. Supplementation with a new therapeutic oxygen carrier reduces chronic fibrosis and organ dysfunction in kidney static preservation. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2011 Sep;11(9):1845–60.
27. Laing RW, Bhogal RH, Wallace L, Boteon Y, Neil DAH, Smith A, et al. The Use of an Acellular Oxygen Carrier in a Human Liver Model of Normothermic Machine Perfusion. Transplantation. 2017 Nov;101(11):2746–56.
28. Sadowsky D, Zamora R, Barclay D, Yin J, Fontes P, Vodovotz Y. Machine Perfusion of Porcine Livers with Oxygen-Carrying Solution Results in Reprogramming of Dynamic Inflammation Networks. Front Pharmacol. 2016;7:413.
29. Mer M, Hodgson E, Wallis L, Jacobson B, Levien L, Snyman J, et al. Hemoglobin glutamer-250 (bovine) in South Africa: consensus usage guidelines from clinician experts who have treated patients. Transfusion (Paris). 2016 Oct;56(10):2631–6.

CHAPTER 6

The Efficacy of HBOC-201 in Ex-Situ Gradual Rewarming Kidney Perfusion in a Rat Model

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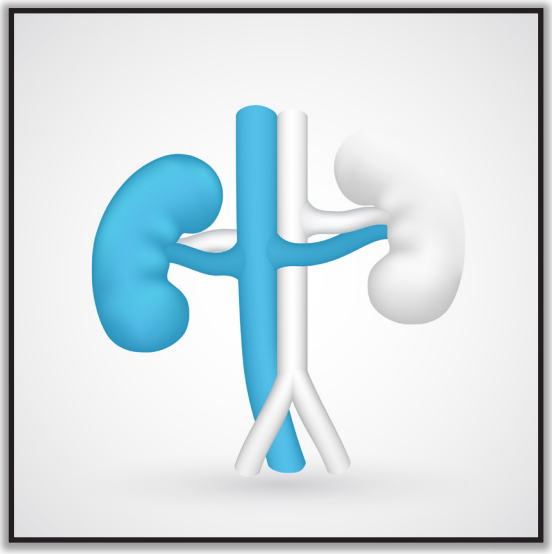


ABSTRACT

Gradual rewarming from hypothermic to normothermic is a novel perfusion modality with superior outcome to sudden rewarming to normothermic. However, the identification of an oxygen carrier that could function at a temperature range from 4-37°C or whether it is necessary to use oxygen carrier during kidney rewarming, remains unresolved. This study was designed to test the use of a hemoglobin-based oxygen carrier (HBOC) during gradual kidney rewarming as an alternative to simple dissolved oxygen. In this study 10 rat kidneys were randomly divided into the control and the HBOC group. In the control group no oxygen carrier was used during rewarming perfusion and the perfusion solution was oxygenated only by applying diffused Carbogen flow. The protocol mimicked a donor after circulatory death (DCD) kidney transplantation, where after 30 minutes warm ischemia and 120 minutes cold storage (CS) in UW, the DCD kidneys underwent gradual rewarming from (10-37°C) during 90 minutes with or without HBOC. This was followed by 30 minutes of warm ischemia in room temperature to mimic the anastomosis time and 120 minutes of reperfusion at 37°C to mimic the early post-transplant state of the graft. The HBOC group demonstrated superior kidney function which was highlighted by higher ultrafiltrate production, better Glomerular filtration rate (GFR) and improved sodium reabsorption. There was no significant difference between the two groups regarding the hemodynamic, tissue injury and adenosine triphosphate (ATP) levels. In conclusion, this study suggests better renal function recovery in DCD kidneys after rewarming with HBOC compared to rewarming without an oxygen carrier.

INTRODUCTION

One of the challenges in organ transplant is improving the organ preservation method especially in the grafts with inferior quality such as DCD (1,2). Machine perfusion has been developed as an alternative preservation method to CS with promising results in organ quality improvement (3–5). Different perfusion protocols have been studied by many groups and among the emerged perfusion protocols, normothermic machine perfusion has gained more attention as it provides the feasibility of assessing organ viability and function before transplant (6,7). It has also been shown that gradual rewarming from hypothermic to normothermic is superior to sudden normothermic perfusion after CS (4,8). Although gradual rewarming solves the problem strategically, it remains to be established whether there is a need for oxygen carrier during gradual rewarming for providing a sufficient amount of oxygen. Red blood cells (RBCs) have been mainly used in normothermic perfusion, however there is major biophysical limits such as hemolysis and rheological complications when submitted to temperature below normothermic (37°C) (9). Artificial oxygen carriers either with hemoglobin based or perfluorocarbon based are the relatively new solutions to this problem. After disappointing results with perfluorocarbons (10,11) HBOC with the ability of functioning in wide range of temperature from hypothermic to normothermic seem to be an option for gradual rewarming and a potential alternative to blood in normothermic perfusion (12). HBOC-201(Hemopure®) is a second-generation glutaraldehyde-polymer of bovine hemoglobin, which can function as the oxygen bridge in order to preserve oxygen carrying capacity in the lack of RBCs (12). HBOC have been used in subnormothermic and normothermic perfusion and the data suggested improved organ quality (12–14). In this study, our aim was to investigate the viability and efficacy of HBOC based perfusion solution in gradual rewarming perfusion in a rat kidney model.

MATERIAL AND METHODS

Experimental Animals and Kidney Procurement

Male Lewis rats weighing 290-350 g were used in this study. Animals received care according to the national research council guidelines on animal experiments. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the Massachusetts General Hospital. Anesthesia and surgical details of recovery are provided elsewhere (15). Briefly, the rats were anesthetized using 5% isoflurane and heparin administration was performed by injection of 1 ml 0.9% NaCl with 500 IU of heparin via the dorsal penile vein. Next, the ureter was cannulated with regard to monitor ultrafiltrate production during gradual rewarming and reperfusion. For controlled warm ischemia, during

cessation of circulation the animal was kept at 37°C for 30 minutes, then renal artery was cannulated using a 20-gauge intravenous catheter and the kidney was flushed through the renal artery with 10 mL 0.9% NaCl at room temperature followed by 10 mL University of Wisconsin (UW) preservation solution at 4°C. Afterwards, the kidneys were removed and stored in cold UW (4°C) during CS preservation for 120 minutes.

Experimental Design and Perfusion Solutions

Rewarming

A total number of 10 rat kidneys were divided into 2 experimental groups (1-Control, 2-HBOC). In both groups, after DCD procurement (n = 5 per group), grafts were preserved for 120 minutes during CS in UW media. Then, grafts underwent gradual rewarming perfusion from 10°C to 37°C for 90 minutes: Temperature of the perfusion solution was set at 10°C at the beginning of rewarming in both groups, minimum allowed by the equipment. Temperature was stable at 10°C for the first 15 minutes of the rewarming phase, and was then gradually increased to 37°C through 60 minutes by gradually increasing the temperature of thermostat and arterial flow. The temperature was kept stable at 37°C for the final 30 minutes of rewarming perfusion. Pressure was also observed during rewarming phase, with the range of 20-40 mm Hg in the hypothermic phase, 40-80 mm Hg in transaction from hypothermia to normothermia and 80-100 mm Hg in the normothermic phase of the rewarming protocol. Afterwards, the grafts were flushed with 10 mL of cold saline and stored in a petri dish covered with wet gauze at room temperature for 30 minutes, in order to mimic surgical implantation period. Subsequently, the kidney grafts were reperfused at 37°C for 120 minutes. The flow was set at 8 mL/min in the beginning of reperfusion and afterwards, was gradually increased with keeping the pressure in physiological range (80-110 mm Hg) (Figure 1).

Gradual Rewarming Perfusion Media

HBOC Group

The perfusion solution consisted of Williams Medium E (Sigma-Aldrich, St Louis, MO, USA) supplemented with heparin (1,000 U/L APP pharmaceuticals, Schaumberg, IL, USA), Albumin 1.5% (Sigma-Aldrich, St Louis, MO, USA), creatinine (1000μmol/L) and 25% HBOC-201 v/v (provided by Hemoglobin Oxygen Therapeutics, Souderton, PA). The solution was oxygenated with a carbogen mixture of 95% O_2 and 5% CO_2 in the arterial flow and saturation SaO_2 > 97% (Table 1). In all the experiments before connecting the kidney, the perfusion solutions were checked to be in physiological osmolarity, oncotic pressure range and contain adequate oxygen amount (>400 mmHg).

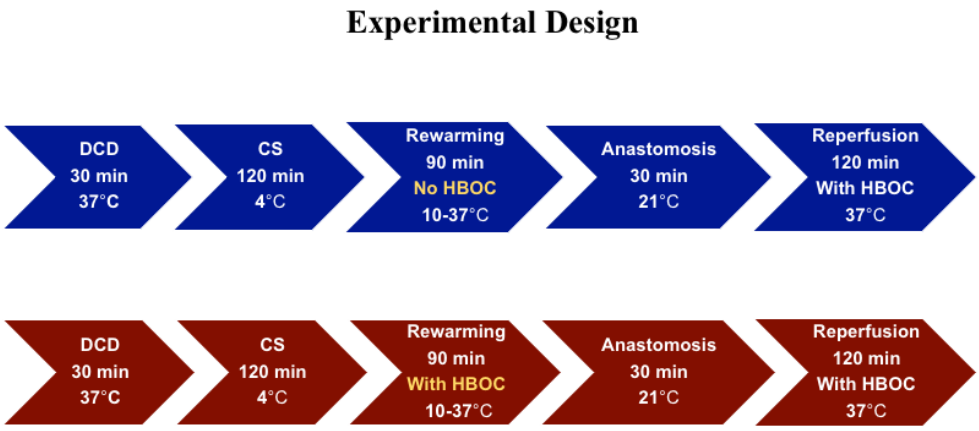


Figure 1. experimental design | Illustrates the experimental design of the study and the duration in each section of the experiments.

Control Group

The same media was used in the control group, except, **No HBOC** was used during rewarming perfusion and the HBOC volume was replaced with Williams Medium E solution. The composition of both perfusion medias is available on (Table 1).

For reperfusion assessment of the simulated transplant, the perfusion formula with HBOC was used in both groups.

Table 1. Represents and compares the biochemical composition of the perfusion solution in the control and HBOC groups, used in rewarming phase.

	Rewarming Media		
	Control	**HBOC**	**p value**
pH	7.5 ± 0.07	7.42 ± 0.14	0.886
PCO2 (mm Hg)	36.2 ± 31.4	32.2 ± 12.5	0.629
PO2 (mm Hg)	590 ± 96.0	452 ± 5.0	0.200
BE (mmol/L)	2 ± 5.0	-3 ± 5.0	0.057
HCO3- (mmol/L)	26.2 ± 4.1	25.8 ± 2.6	1.000
Na+ (mmol/L)	147 ± 0.75	149 ± 2.0	0.016
K+ (mmol/L)	5.0 ± 0.2	5 ± 0.1	0.730
Cl- (mmol/L)	115 ± 2.5	115 ± 3	0.690
HGB (g/dL)	--	3.0	--
SO2 (%)	--	100%	--
Lactate (mmol/L)	0.03 ± 0.0	2.6 ± 0.1	0.016

The perfusion device was flushed, cleaned and the system was primed with fresh HBOC media for reperfusion during the 30 min simulated anastomosis time.

HBOC

This acellular hemoglobin was produced by purifying bovine hemoglobin and was polymerized to decrease the potential side effects and alleviate the risk of toxicities. HBOC with a molecular weight of 250 kDa and in vivo half-life time of 20 hours, can be stored at 2-30°C for up to 3 years (12,13). The oxygen affinity of HBOC is regulated by chloride ion concentration which means that oxygen-HBOC dissociation curve shifts to right and HBOC releases oxygen to tissue more freely compared to other carriers like human hemoglobin with HBOC P50 around 40 mm Hg (±6 mm Hg) at 37 °C compared to 27 mm Hg at human hemoglobin (13). In general, the effects of temperature on oxygen affinity of HBOC are similar to the effects of temperature on native (corpuscular) Hb, which means that oxygen affinity increases by temperature decrease (low in hypothermic and higher in normothermic temperature). However, across all temperatures HBOC demonstrates high tendency to release oxygen which is still better than Hb in erythrocytes (16).

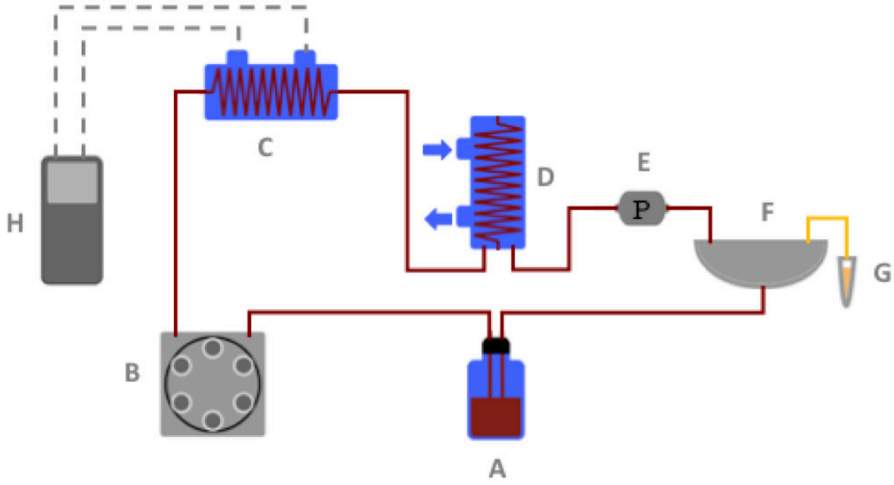
Perfusion System

The perfusion device is a flow-controlled system for rodent organ perfusion. The device consists of a Roller pump (Cole Parmer, Cat. No. HV-07522-20), Oxygenator containing silicon tubing and providing Carbogen and heat exchanger (Radnoti, Cat. No. 130144), Bubble trap (Radnoti, Cat. No. 130149), Pressure probe (Living Systems, Cat. No. PM-P-1), Circulating controlled rate chiller (Neslab, Cat. No. RTE-111) and Organ chamber (Radnoti, Cat. no 158360) (Figure 2, Part I). (Figure 2, Part II) shows a cannulated kidney graft in the organ chamber during perfusion.

Perfusion Profile

During rewarming and reperfusion phase in both groups, flow and pressure were recorded at 30 minutes intervals and subsequently the resistance was calculated. pH and pO_2 in the perfusate samples were measured every 30 minutes. Lactate concentration in the perfusate samples was measured and recorded every 30 minutes during 120 minutes of reperfusion. I-Stat analyzer (Abbott, USA) was used to measure pO_2 in the arterial and venous sides, pH and Lactate levels and in general to monitor hemodynamic, chemistry and electrolytes profile in the perfusate and ultrafiltrate samples. In order to monitor potential edema formation in the organs, the kidney grafts were weighted before, after both rewarming and reperfusion phases. Consequently, the weight gain percentage was calculated (increase / original weight*100).

Part I



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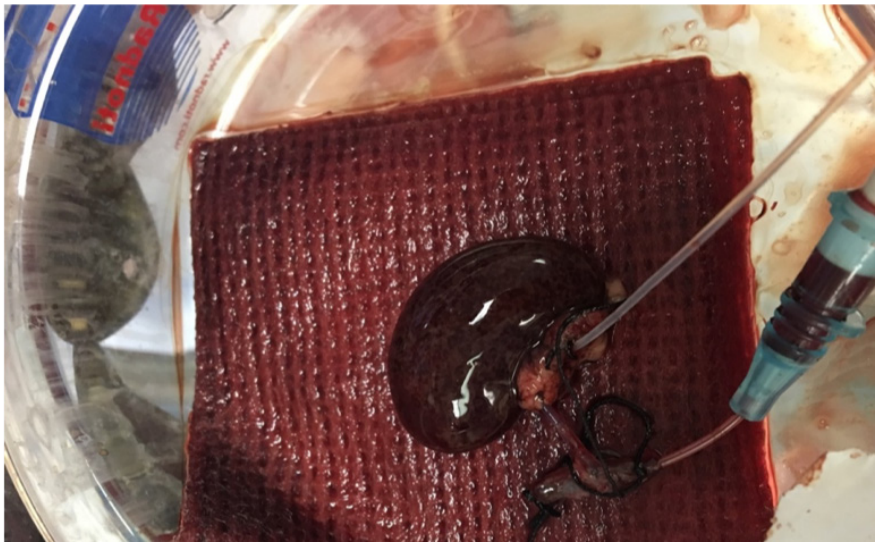

Figure 2 | Graphic representation of the rodent liver perfusion system
Part I) (A)Solution reservoir; (B) Roller pump; (C) Heat Exchanger and Oxygenator containing silicon tubing and providing Carbogen; (D) Bubble Trapper; (E) Pressure probe; (F) Organ chamber; (G) Urine Eppendorf; (H) thermostat which regulates the temperature.
Part II) This picture shows a cannulated rat kidney during machine perfusion.

To measure Methemoglobin (Met-Hb) level in the perfusate samples, we used RAPIDPoint 500 (Siemens, USA). Met-Hb was measured in the samples before, during and after perfusion.

Ultrafiltrate Production and Renal Function

The produced ultrafiltrate was collected in Eppendorf tubes and was measured in milliliter (mL) in a 30 minutes interval during reperfusion phase in the both HBOC and control groups. GFR (ultrafiltrate creatinine × ultrafiltrate volume/perfusate creatinine) and fractional Sodium re-absorption ((perfusate sodium-ultrafiltrate sodium) / (perfusate sodium) ×100) were calculated accordingly (4).

Oxygen Consumption

Oxygen consumption during reperfusion was calculated in the HBOC group during rewarming and in the both groups during reperfusion using the following formula:
([{ApO_2−VpO_2} × K /760] × total flow) + ([{AsO_2−VsO_2} × Hb × c × 0.0001] × flow) / Kidney weight × 100. In which pO_2 was in mm Hg, sO_2 in %, Hb in g/dL, Renal artery flow in mL/min and kidney weight in g. c the oxygen binding capacity of HBOC (1.26) and K was a constant (0.0225) (16).

Tissue Energy State

The tissue samples to determine energy cofactors adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP) were taken at the end of reperfusion (t=120 minutes) in order to prevent inducing injury to the renal grafts during rewarming and reperfusion. Method for the extraction and measurement has been described previously (17). Briefly, after extracting the metabolites using a mixture of methanol/chloroform and 3 freeze-thaw cycles, cold water (4°C) was added to the extracts and the extracts were centrifuged. Then, the analysis was started by transferring the top phase of the mixture into the sample vial. The frozen tissue samples were analyzed using a chromatography-mass spectrometry system (AB Sciex, Foster City, CA).
Energy charge was calculated as:
Energy charge = [2ATP + ADP] / [ATP + ADP + AMP]

Renal Injury & Histological evaluation

Lactic Acid Dehydrogenase (LDH) was measured in the perfusate samples using Elisa kit (# MBS041480 MyBioSource, Inc., San Diego, CA) during reperfusion in the both HBOC and control groups. Of note we did not measure lactate and LDH in the rewarming phase as HBOC interference with these assays and subsequently the HBOC group data would include false positive data. We did the measurements in the reperfusion as both groups

had the same perfusion environment regarding HBOC level. Biopsies were obtained from the renal tissues at the end of 120 minutes reperfusion and stored in 10% formalin for histological evaluation. Paraffin-embedded slides of kidney biopsies were prepared for hematoxylin and eosin (H&E).

Statistical Analysis

Continuous data were presented as the median and interquartile range (IQR). Mann-Whitney U test was used to compare groups. A p-value of less than 0.05 was considered significant. Analyses were performed using SPSS software version 25.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Rewarming

Perfusion Profile and Injury during Rewarming Phase

Temperature profile in the control and HBOC groups during rewarming is shown in (Fig 3A). Renal resistance slightly decreased towards the end of rewarming perfusion with no significant difference between the HBOC and control groups (Fig 3B). Both groups showed normal pH within physiological range (7.34-7.45) through the 90 minutes of rewarming perfusion with no significant difference (Fig 3C).

Perfusion Profile during Reperfusion

The resistance in renal artery was higher at the beginning of reperfusion in the HBOC group. It decreased gradually and there was no significant difference during the rest of reperfusion between the two groups (Fig 4A). pH was within physiological range in the both HBOC and control groups with no significant difference (Fig 4B). No significant difference was found in the lactate concentration in both groups during 120 minutes of reperfusion (Fig 4C). The kidney grafts weigh scan revealed no significant edema level in both groups with a median of (10.5 %versus 12.5%) in the rewarming and (2.5% versus 7.4%) in the reperfusion phase. The comparison between groups (control & HBOC) and the phases (rewarming & reperfusion) did not reveal a significant difference (Fig 4D).

We observed gradual rise in Met-Hb level during rewarming (Fig 5A) and more drastically in normothermic reperfusion, with no significant difference between control and HBOC group during reperfusion phase (Fig 5B).

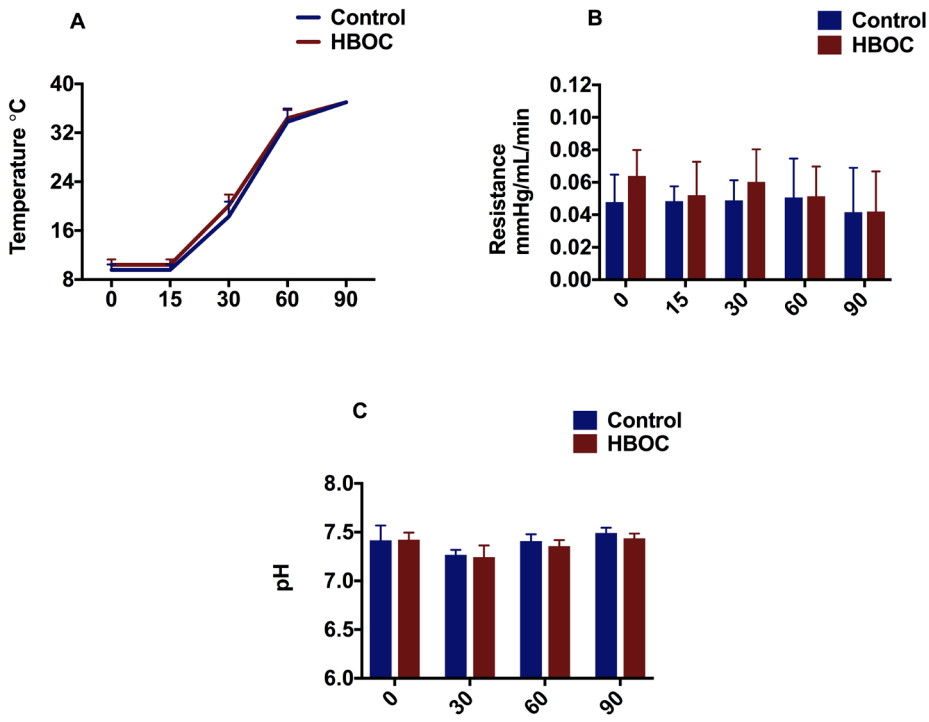

Figure 3. Kidney profile during gradual rewarming
A) Temperature. B) Renal Resistance, which slightly reduced during gradual rewarming in the both HBOC and control groups. C) pH was in the physiologic range during gradual rewarming. D) Oxygen consumption. *p ≤ 0.05

Functional Parameters during Reperfusion

Ultrafiltrate production gradually decreased in the control group during 120 minutes of reperfusion while it gradually increased in the HBOC group with the statistical difference at t=90 (p=0.050) (Fig 6A). GFR rate was higher in the HBOC group during the 120 minutes of reperfusion with the statistical difference at t=90 (p=0.032) compared to the control group (Fig 6B). Fractional sodium re-absorption was improved in the HBOC group compared to the control group during 120 minutes of reperfusion and this reached statistical significance at t=90 (p=0.017) and t=120 (p=0.032) (Fig 6C). It was notable that re-absorption completely ceased in the control group after 90 minutes of reperfusion, but was stable throughout in the HBOC group.

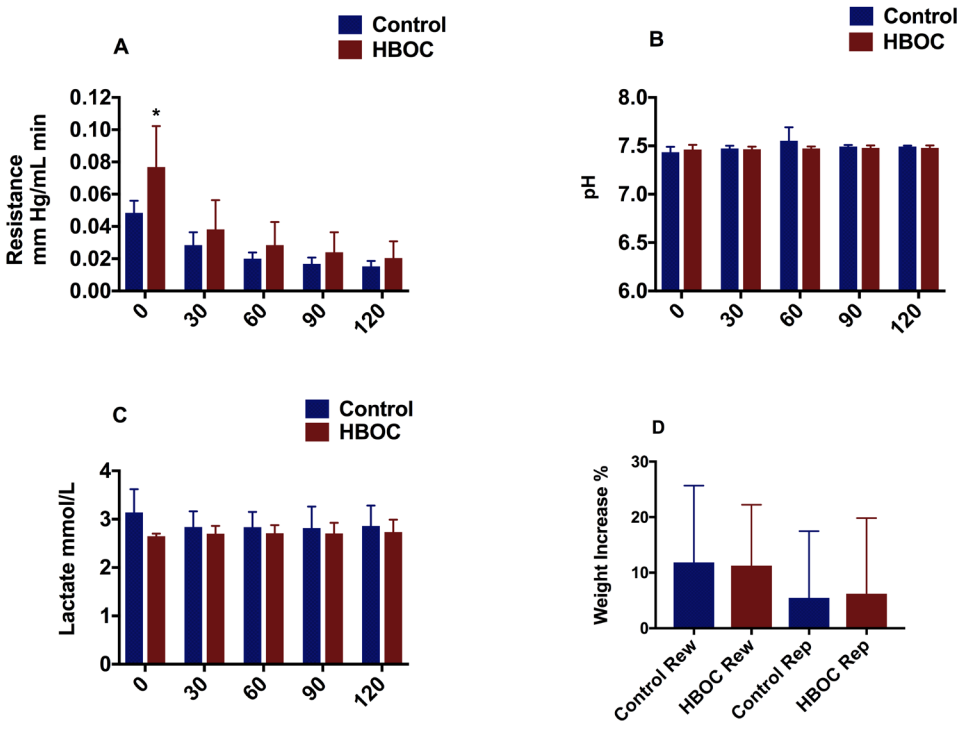

Figure 4. Kidney profile during reperfusion
A) Resistance was high in the both groups in the beginning of the reperfusion with significant difference, and then was reduced in the both groups toward the end of reperfusion with no difference. B) pH was in normal range in the both groups with no significant difference. C) There was no significant difference in the lactate level between both groups. D) This graph indicates the weight gain during rewarming and reperfusion phase in both groups with no significant difference. *p ≤ 0.05

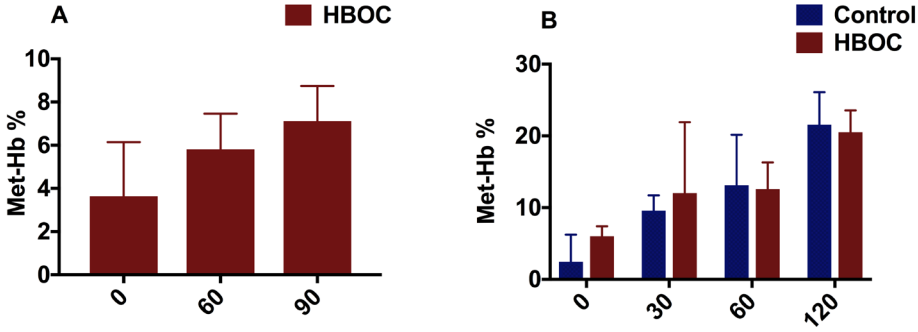

Figure 5 | Kidney Met-Hb during rewarming and reperfusion
A) Represent Met-Hb level in the HBOC group during rewarming. B) Indicates a gradual increase in Met-Hb level in the both HBOC and control groups during 120 minutes of reperfusion.

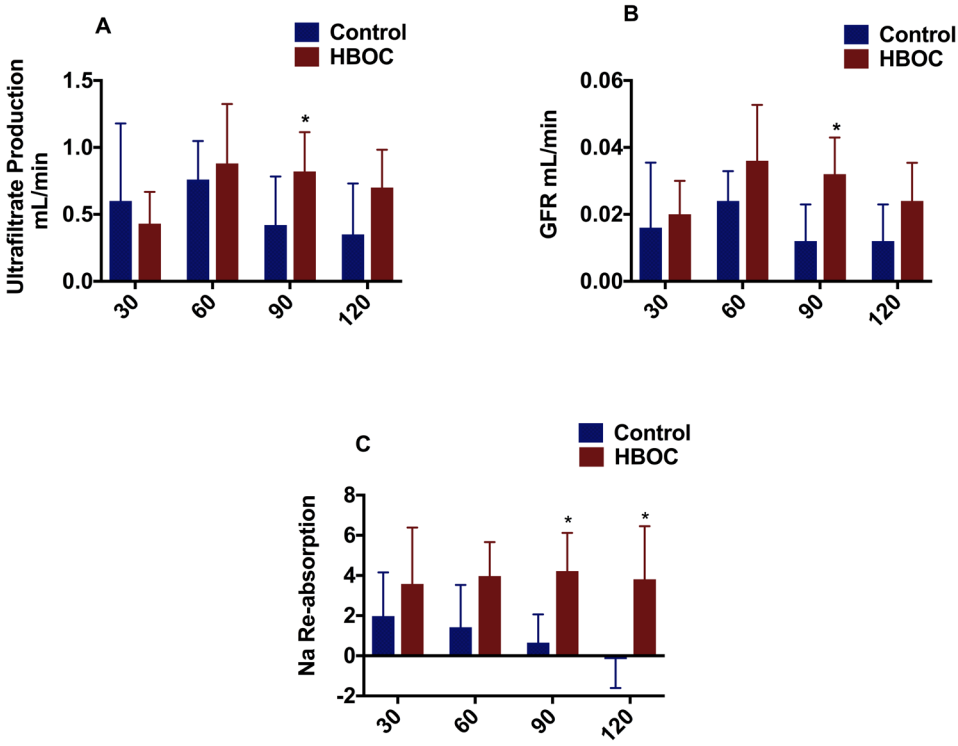

Figure 6 | Kidneys undergoing Gradual Rewarming show improved function and recovery compared to controls during reperfusion
A) The volume of ultrafiltrate production was measured every 30 minutes and was improved better in the HBOC group and this was significant at t=90. B) GFR was superior in the HBOC group with significant differences and t=90. C) Fractional sodium re-absorption was gradually increased in the HBOC groups while it was decreased in the control group with significantly differences at t=90 and t=120. *p ≤ 0.05.

Oxygen Consumption and Energy State

Oxygen consumption was gradually increased parallel to an increase of the temperature over 90 minutes rewarming phase in the group with HBOC (Fig 7A). Oxygen consumption could not be calculated in the control group due to absence of an oxygen carrier, therefore the difference of pO_2 in the arterial and venous was calculated (Fig 7B).

Oxygen consumption was also observed in the both groups during 120 minutes of reperfusion with no difference found between the control and HBOC groups (Fig 7C). After 120 minutes of reperfusion, The HBOC group trended higher in terms of ATP and energy charge however; the difference did not reach statistical difference (Fig 7D, 7E).

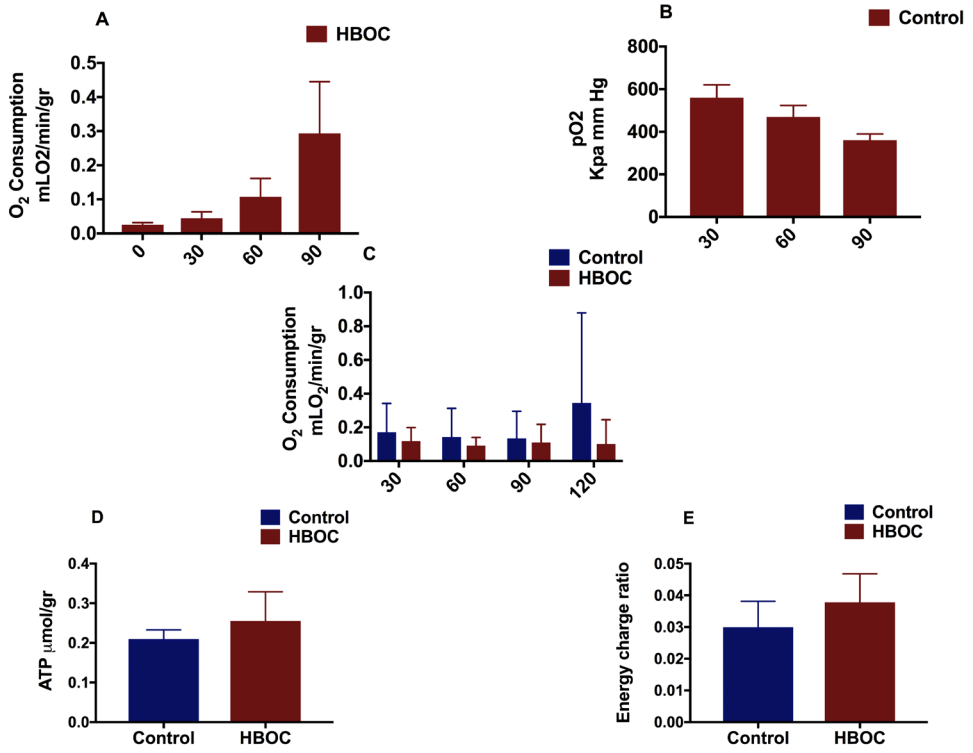

Figure 7. Graphical presentation of oxygen concentration, ATP level and energy charge ratio in the rewarming and control groups

A) This Graph demonstrates oxygen consumption during 90 minutes rewarming in the HBOC group. B) The difference between arterial pO2 and venous pO2 during rewarming phase in the control groups is shown in this graph C) Oxygen Consumption remained the same in the both groups during reperfusion. D&E) ATP and energy charge ratio trended higher in the HBOC group with no significant difference between the groups.

Renal Parenchymal Injury & Histological Evaluation

There was no difference in LDH between the two groups during reperfusion (Fig 8, Part I). Light microscopy performed on tissue samples obtained at the end of the 120 minutes reperfusion also did not show significant differences between the HBOC group and the control group. Overall only slight changes of normal structural appearance like epithelial shedding was observed in both groups (Fig 8, Part II, A & B).

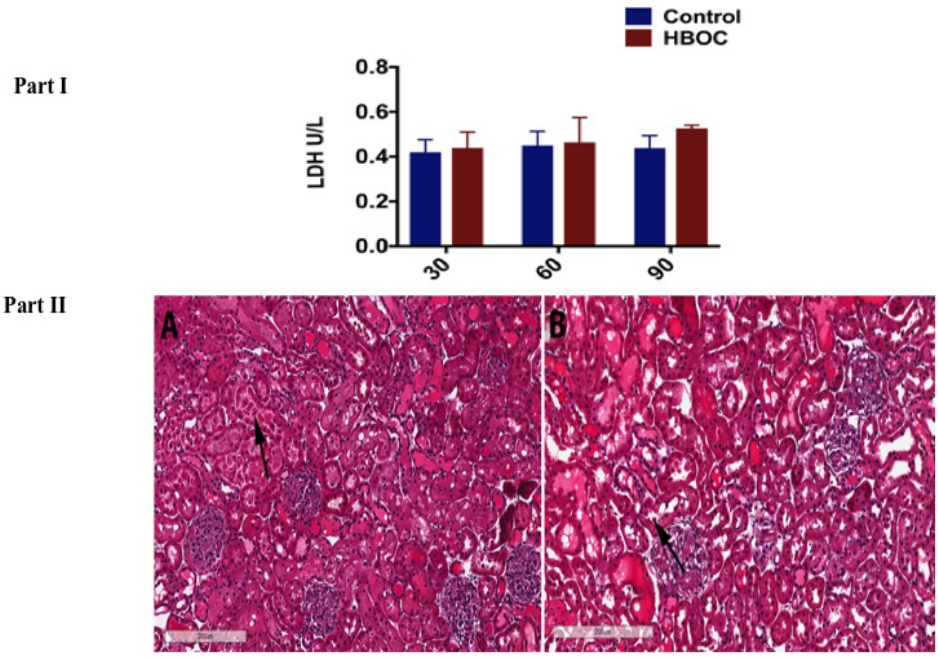

Figure 8. Renal Injury and H & E staining of kidney tissue from HBOC and control groups at the end of reperfusion
Part I) No difference was observed in LDH levels between the two groups.
Part II) A) HBOC. B) Control. The arrow in the both groups shows slight epithelial shredding.

DISCUSSION

The aim of our study was to assess whether the supplement of HBOC during gradual rewarming in DCD kidneys recovers and improves renal function, as viability testing was evaluated during 120 minutes of reperfusion at 37°C. Our study indicates that 90 minutes gradual rewarming with HBOC after CS could improve renal function compared to gradual rewarming alone. The improvements in renal function are highlighted by a higher trend of ultrafiltrate production, better GFR and improved sodium reabsorption during reperfusion phase. Although the kidneys in the HBOC group showed higher trend of ATP content and elevated energy charge status, these findings did not reach a significant difference.

It has been established that combination of warm and cold ischemia in DCD kidneys lead to severe ischemia reperfusion injury and subsequently increase the rate of delayed graft function after transplant and shorten long term graft survival(18). Urine production is the first sign of graft function after transplant and the urine volume may correlate with graft survival post-transplant(19). Alongside urine volume, the quality of urine also plays an important role,

for instance, GFR as a marker of renal glomerular function is used as a predictor factor of patient survival after kidney transplant (20). HBOC group demonstrated better graft function with higher trend of ultrafiltrate production and improved GFR compared to control group. Fractional sodium re-absorption is another functional parameter as a marker of renal tubular function (21). Tubular function is an energy demanding process and sufficient oxygenation is required to provide adequate energy (21). HBOC group also showed improved tubular function and recovery indicated by superior sodium reabsorption.

HBOC has originally been developed as an alternative to RBCs in emergency care and trauma in the acutely anemic patients; however the use in human trials has been limited due to hypertensive response as a result of nitric oxide (NO) depletion and vasoconstriction (22). We therefore closely observed the resistance during both rewarming and reperfusion by monitoring pressure and flow, HBOC caused higher resistance in the beginning of the both rewarming and reperfusion phases, however it quickly decreased with no sign of sustained hypertension. The use of HBOC in liver normothermic perfusion also did not report higher pressure and resistance (13,14) which is in line with our findings. In the context of machine perfusion, these findings could be useful for translating to clinical studies as HBOC will be flushed out before graft implantation in recipient.

Fontes et al have tested HBOC in a preclinical porcine model during prolonged sunnormothermic perfusion with the outcome indicating consistent oxygen delivery and adequate graft function after transplant (12). The use of HBOC in two discarded human liver studies reported the feasibility and safety of applying HBOC based perfusion media during normothermic machine perfusion (NMP) as an alternative to RBCs (13,14). A recent study by Vries et al described the use of HBOC in a pretransplant combined dual oxygenate hypothermic machine perfusion (D-HOPE) with controlled oxygenated rewarming (COR) to NMP in initially declined human liver grafts (16). The study presenting promising results for further future use of HBOC in clinical studies with the outcome of 100% patient survival in the first 3 months post-transplant.

Although HBOC has been used in a number of liver perfusion studies with improved perfusion parameters, the liver studies were either preclinical or clinical with small population and limited information about HBOC in human kidney perfusion. Hence, further studies could assess the effects of HBOC based perfusion solution in a comprehensive clinical trial model. One potential limitation of HBOC is the high cost compared to RBCs, however the other advantages of HBOC such as no storage cost, long term storage, no need for cross matching and no risk of blood born infections sort it still as a superior product to RBCs (13). Due to the lack of NADH-dependent enzyme (methemoglobin reductase) in HBOC, the possibility of Met-Hb production is a disadvantage associated with HBOC use and the gradual rise in Met_Hb level has been previously reported (16). Our experiments also revealed a slight increase in the Met-Hb during rewarming and more extreme during reperfusion. In

spite of high Met-Hb formation, the risk of Met-Hb transfer after perfusion to the organ recipient's body is minimal as the perfusion solutions gets flushed out of the organ prior to transplantation (14).

This study as a preclinical kidney rewarming model presents supportive information about the feasibility of HBOC use in renal perfusion and the effective outcome on renal function. However, lack of transplantation validation is a limitation of our study and the next phase would be to validate this data in a transplant model. The other limitation is that although positive effect of HBOC on kidney function could be due to better oxygen carrying capacity of HBOC nevertheless, we did not specifically study the O_2 carrying capacity in this study and this could be further investigated in future studies.

In conclusion this study is the first report on using HBOC-201 in the gradual rewarming kidney model with improved kidney function. Altogether, this proposes the possibility of using HBOC in future in different perfusion setups as a potential oxygen carrier in different temperatures.

ACKNOWLEDGEMENTS

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DISCLOSURES

The authors of this manuscript have conflicts of interest to disclose: Dr. Uygun is inventor on pending patents relevant to this study (WO2011002926; US20140030231). Dr. Uygun has a financial interest in Organ Solutions, a company focused on developing organ preservation technology. Dr. Uygun's interests are managed by the MGH and Partners HealthCare in accordance with their conflict of interest policies.

Drs. Tessier and Uygun have several IP disclosures on extended organ preservation that may be relevant to this study.

The (HOBC-201) used in this study was provided by HBO2 Therapeutics LLC.

REFERENCES

1. Mahboub P, Bozorgzadeh A, Martins PN. Potential approaches to improve the outcomes of donation after cardiac death liver grafts. World J Transplant. 2016 Jun 24;6(2):314–20.
2. Anderson PT, Aquil S, McLean K, McAlister VC, Sener A, Luke PP. First Canadian experience with donation after cardiac death simultaneous pancreas and kidney transplants. Can J Surg J Can Chir. 2017 Sep;60(5):323–8.
3. Op den Dries S, Karimian N, Porte RJ. Normothermic machine perfusion of discarded liver grafts. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2013 Sep;13(9):2504.
4. Mahboub P, Ottens P, Seelen M, 't Hart N, Van Goor H, Ploeg R, et al. Correction: Gradual Rewarming with Gradual Increase in Pressure during Machine Perfusion after Cold Static Preservation Reduces Kidney Ischemia Reperfusion Injury. PloS One. 2016;11(3):e0152006.
5. Westerkamp AC, Mahboub P, Meyer SL, Hottenrott M, Ottens PJ, Wiersema-Buist J, et al. End-ischemic machine perfusion reduces bile duct injury in donation after circulatory death rat donor livers independent of the machine perfusion temperature. Liver Transplant Off Publ Am Assoc Study Liver Dis Int Liver Transplant Soc. 2015 Oct;21(10):1300–11.
6. Kaths JM, Hamar M, Echeverri J, Linares I, Urbanellis P, Cen JY, et al. Normothermic ex vivo kidney perfusion for graft quality assessment prior to transplantation. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2018 Mar;18(3):580–9.
7. Hosgood SA, van Heurn E, Nicholson ML. Normothermic machine perfusion of the kidney: better conditioning and repair? Transpl Int Off J Eur Soc Organ Transplant. 2015 Jun;28(6):657–64.
8. Gallinat A, Lu J, von Horn C, Kaths M, Ingenwerth M, Paul A, et al. Transplantation of Cold Stored Porcine Kidneys After Controlled Oxygenated Rewarming. Artif Organs. 2018 Jun;42(6):647–54.
9. Fontes PA. The Evolution of Oxygen Carrier Solutions for Machine Perfusion. Transplantation. 2017;101(11):2657–8.
10. Hosgood SA, Nicholson HFL, Nicholson ML. Oxygenated kidney preservation techniques. Transplantation. 2012 Mar 15;93(5):455–9.
11. Hosgood SA, Nicholson ML. The role of perfluorocarbon in organ preservation. Transplantation. 2010 May 27;89(10):1169–75.
12. Fontes P, Lopez R, van der Plaats A, Vodovotz Y, Minervini M, Scott V, et al. Liver preservation with machine perfusion and a newly developed cell-free oxygen carrier solution under subnormothermic conditions. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2015 Feb;15(2):381–94.
13. Laing RW, Bhogal RH, Wallace L, Boteon Y, Neil DAH, Smith A, et al. The Use of an Acellular Oxygen Carrier in a Human Liver Model of Normothermic Machine Perfusion. Transplantation. 2017 Nov;101(11):2746–56.
14. Matton APM, Burlage LC, van Rijn R, de Vries Y, Karangwa SA, Nijsten MW, et al. Normothermic machine perfusion of donor livers without the need for human blood products. Liver Transplant Off Publ Am Assoc Study Liver Dis Int Liver Transplant Soc. 2018;24(4):528–38.
15. van den Eijnden MMED, Leuvenink HGD, Ottens PJ, 't Hart NA, van Oeveren W, Morariu AM, et al. Effect of brain death and non-heart-beating kidney donation on renal function and injury: an assessment in the isolated perfused rat kidney. Exp Clin Transplant Off J Middle East Soc Organ Transplant. 2003 Dec;1(2):85–95.
16. de Vries Y, Matton APM, Nijsten MWN, Werner MJM, van den Berg AP, de Boer MT, et al. Pretransplant sequential hypo- and normothermic machine perfusion of suboptimal livers donated after circulatory death using a hemoglobin-based oxygen carrier perfusion solution. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2018 Dec 26;

17. Bruinsma BG, Avruch JH, Sridharan GV, Weeder PD, Jacobs ML, Crisalli K, et al. Peritransplant Energy Changes and Their Correlation to Outcome After Human Liver Transplantation. Transplantation. 2017;101(7):1637–44.

18. Zhao H, Alam A, Soo AP, George AJT, Ma D. Ischemia-Reperfusion Injury Reduces Long Term Renal Graft Survival: Mechanism and Beyond. EBioMedicine. 2018 Feb 2;28:31–42.

19. Khosroshahi HT, Oskui R, Shoja MM, Tubbs RS, Ardalan MR. Time-dependent variations in urine output after renal transplantation. Transplant Proc. 2007 May;39(4):932–3.

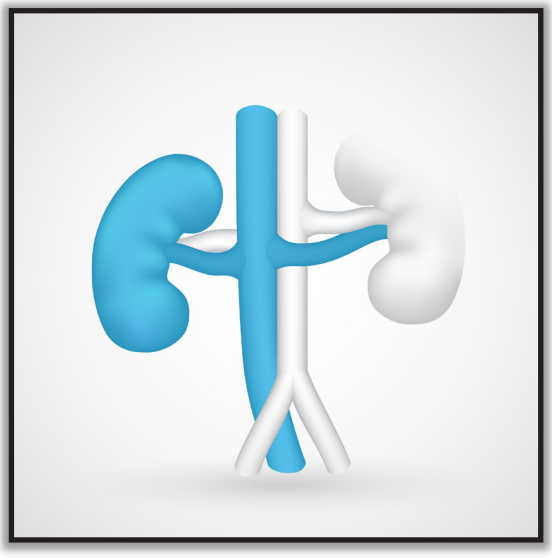
20. Santos J, Martins LS. Estimating glomerular filtration rate in kidney transplantation: Still searching for the best marker. World J Nephrol. 2015 Jul 6;4(3):345–53.

21. von Horn C, Minor T. Isolated kidney perfusion: the influence of pulsatile flow. Scand J Clin Lab Invest. 2018 Apr;78(1–2):131–5.

22. Taverne YJ, de Wijs-Meijler D, Te Lintel Hekkert M, Moon-Massat PF, Dubé GP, Duncker DJ, et al. Normalization of hemoglobin-based oxygen carrier-201 induced vasoconstriction: targeting nitric oxide and endothelin. J Appl Physiol Bethesda Md 1985. 2017 May 1;122(5):1227–37.

CHAPTER 7

General Discussion and Future Perspectives



Cold storage (CS), as a preservation method for organ storage prior to transplantation, continues as the most effective contemporary storage method used in transplant programs worldwide today. The value of CS rests in its role of reducing the active metabolism level of a harvested organ by placing the organ in an appropriate preservation solution and storing the organ at 4°C. While CS has enabled practitioners to preserve organ quality, it is associated with significant limitations, such as reduced adenosine triphosphate (ATP) levels, accumulation of waste products, and no opportunity of organ viability assessment. CS can seriously jeopardize organ quality during preservation specially in organs with low quality like those harvested from extended criteria donors (ECD) and donation after circulatory death (DCD). This is because storing organs at reduced temperatures exposes them to cold ischemic injury which possibly accelerates organ quality deterioration before transplantation and may contribute to severe ischemia reperfusion after transplantation. Therefore, a more elaborate preservation and evaluation method is needed to increase the availability, viability and quality of the existing organ pool.
Recently, machine perfusion use during organ preservation has emerged as a preferred preservation method which could overcome the limitations of the current preservation system by offering viability assessment and organ quality improvement. Hypothermic machine perfusion (HMP), one of the first perfusion preservation methods in kidney preservation, has resulted in faster recovery in patients and reduced delayed graft function after kidney transplant (1). In human liver perfusion, HMP has led to enhanced ATP levels during organ preservation and improved liver function after transplantation (2).There is also a growing interest in using Normothermic machine perfusion (NMP) at body temperature with the possibility of performing viability assessments of organs and providing more information about organ quality before transplantation (3,4). Both HMP and NMP methods are beneficial in organ recovery as they represent a less expensive, safer method with improved energy status during HMP and viability testing during NMP. Therefore, the possibility exists that the combination of HMP and NMP with a gradual increase to NMP might be even more sufficient to improve and maintain the viability of high-risk organs with low quality.

The aim of this thesis was to study the role of gradual rewarming on enhancing organ quality in organs with low quality such as ECD and especially DCD during the organ preservation phase and before implantation. This thesis also addresses an improvement in gradual rewarming protocol by adding a hemoglobin-based oxygen carrier (HBOC) to the perfusion solution in the preclinical kidney and liver rodent models.

In this chapter, the findings of relevant studies are summarized and discussed, followed by a section envisioning future perspectives in gradual rewarming perfusion preservation techniques processes, and protocols.

Chapter 1 provides a general introduction to the thesis components and summarizes subsequent chapters.

In **Chapter 2,** two rat kidney and liver perfusion systems constructed in the surgical lab of the University Medical Center of Groningen (UMCG) are described in detail. Both systems are temperature and pressure controlled with pulsatile flow in the arterial side (liver and kidney system) and continuous flow in the venous side (liver system). Oxygen is provided by two artificial lungs in each system and the thermostat allows perfusion protocols in different temperature ranges such as HMP, subnormothermic (SNP), NMP as well as uniform gradual rewarming.
These two models represent the first rodent perfusion models with the technical innovation of adjustable temperature and pressure systems during perfusion. The technology of these early systems was later transferred to the human kidney and liver perfusion models (kidney and liver organ assist).

Consequently, we used these perfusion systems to study the effect of gradual rewarming on ischemia reperfusion injury in rat kidneys with extended CS time, as discussed in **Chapter 3**. The kidney grafts were preserved in CS solution at 4°C for 24 hours followed by 90 minutes of gradual rewarming perfusion either starting at 10°C and rewarming to 37°C or starting at 20°C and gradually rewarming to 37°C. The results were compared to immediate reperfusion at 37°C. Regardless of the temperature at the start point, gradual rewarming of the kidney grafts provided better renal preservation, reduced reperfusion injury and improved renal function compared to immediate reperfusion at 37°C. Technical feasibility of gradual rewarming was also successfully demonstrated in this study.

In **Chapter 4,** different perfusion temperatures, including gradual rewarming perfusion, were studied in order to find the optimal machine perfusion protocol to protect bile duct injury in DCD rat livers. We tested HMP, SNP and gradual rewarming (8°C to 20°C) liver preservation for 60 minutes after 6 hours of CS, and the results were compared to the control group with 6 hours of CS and no machine perfusion during liver preservation. The livers in all the groups underwent 120 minutes ex vivo reperfusion at 37°C for viability assessment. Irrespective of perfusion temperature, machine perfusion preservation led to enhanced liver quality which was highlighted by reduced hepatobiliary injury markers (ALT, AST, LDH), improved liver function markers (bile production) and better energy preservation indicated by superior mitochondrial function during 120 minutes of reperfusion.

Active metabolism varying between 10%-100% during gradual rewarming from HMP to NMP requires a sophisticated rewarming protocol with a method to ensure sufficient

oxygenation including a proper oxygen carrier. The concept of applying an oxygen carrier in gradual rewarming perfusion has been very challenging due to temperature shift HMP to NMP which could induce lysis in oxygen carriers like human red blood cells (RBCs). The new generation of hemoglobin-based oxygen carriers could be the solution to this problem as they are functional in a temperature range different from hypothermia to normothermia (5,6). Therefore, the feasibility, safety, advantages and disadvantages of using HBOC during gradual rewarming in rat liver and kidney models were studied to establish whether HBOC could be used during a gradual rewarming perfusion.

In chapter 4, the liver study data demonstrated that gradual rewarming, along with other perfusion protocols, would enhance liver graft quality compared to immediate reperfusion. Another study, performed by Minor et al., demonstrated significant superiority of rewarming liver grafts compared to immediate reperfusion (4). In both studies, rewarming began from HMP until the temperature reached and remained stable at the target SNP temperature. An NMP phase was excluded due to a lack of a proper oxygen carrier for continuous gradual rewarming protocol from HMP to NMP. Therefore, in **Chapter 5,** we performed gradual rewarming from 8-37 °C using HBOC as the oxygen carriers and focused on feasibility and safety of using HBOC in the previously discussed gradual rewarming protocol. The DCD rat livers underwent 90 minutes of gradual rewarming with HBOC after 270 minutes of CS in the rewarming group. The results were compared to a control group with 360 minutes of CS. After 30 minutes of room temperature ischemia that represents surgical implantation time, a viability assessment was performed by applying reperfusion at 37°C for 120 minutes in both groups. The liver grafts in the gradual rewarming with HBOC group demonstrated an absence of side effects from the use of HBOC. The rewarming grafts displayed even better recovery as indicated by enhanced lactate clearance, superior bile production, relatively lower vascular resistance and a reduced liver injury marker (ALT).

In **Chapter 6**, the beneficial effect of using HBOC in a gradual kidney rewarming perfusion was tested and the results were compared to a group of gradual rewarming without an oxygen carrier. In this study, DCD rat kidneys were cold-stored for 120 minutes. In the control group, the kidneys underwent 90 minutes of gradual rewarming after CS with dissolved Carbogen and no oxygen carrier in the perfusion solution. In the group with HBOC, the kidneys underwent 90 minutes of rewarming with added HBOC as the oxygen carrier in the perfusion solution. After 30 minutes of ischemia in room temperature that represents surgical anastomoses time, the kidneys in both groups were reperfused for viability and renal function assessment at 37°C for 120 minutes. While no difference in ATP level or cellular and tissue injury could be detected between the two groups, gradual rewarming with HBOC demonstrated better renal function recovery indicated by superior urine production, higher glomerular filtration rate (GFR) and enhanced sodium reabsorption during reperfusion.

FUTURE PERSPECTIVES AND CONCLUSION

The aim of this thesis was to provide a better understanding of gradual organ rewarming from hypothermia to normothermia during organ preservation. This thesis also investigated the advantages of improved organ oxygenation protocol by adding HBOC during gradual rewarming.

Although the included data in this thesis answered our questions about the benefits of gradual rewarming and functionality of HBOC during this relatively new perfusion protocol, new research questions and challenges surfaced in parallel with the continued research underway in this field. The final segment of this Chapter offers a discussion regarding new approaches for future research and innovation. Areas for future investigation include the following:

- Determine best gradual rewarming protocol for human organs.
- Investigate the role of a gradual rewarming perfusion protocol to increase the quality of steatotic livers and expand the use of these organs for transplantation.
- Determine the applicability and sufficiency of gradual rewarming perfusion in further studies such as those involved in the use of organs after tissue engineering or the use of critically compromised organs like HCV positive and older organs.

Clinical Gradual Rewarming Protocol

Rodent rewarming studies have been performed by using homemade systems. The basic principles used in developing these systems were presented in detail in Chapter 2. These same principles have been used to develop human perfusion devices. From commercially available human perfusion devices, *organ assist* was used in a number of liver (*liver assist*) and kidney (*kidney assist*) clinical experiments and clinical trials in HMP and NMP perfusion (7–9). The temperature and pressure adjustable system in the organ assist devices makes them a preferred option for testing gradual organ rewarming in human organs. In the initial organ gradual rewarming study, Minor et al used liver assist for gradual rewarming of porcine liver grafts (10) and later it was utilized in a number of human liver rewarming perfusion experiments. It was also recently used in the very first combined HMP and NMP study that researched gradual rewarming to NMP in a human liver (6,11). The next envisioned step would be to confirm the liver findings in a clinical trial study with an adequate number of patients and initiate a gradual rewarming protocol for the human kidney.
RBCs are the only source of oxygen carriers used in the clinical NMP trials (12). However, the use of RBCs is associated with challenges such as the need for blood matching and the

risk of transmitting blood borne infections to the recipients (13). Furthermore, the biophysical limitations of RBCs when applied to prolonged perfusion or perfusion below 37°C, restricts the use of RBCs in perfusion protocols like HMP, SNP and gradual rewarming perfusion. HBOC, used in a number of liver NMP models, was applied in rodent gradual rewarming models included in this thesis (13,14) and recently de Vries et al. published a study presenting the use of HBOC during machine perfusion in a human liver transplantation model with 100% 3-month post-transplantation patient survival rate (6).

Studies thus far have demonstrated the safety of using HBOC in perfusion models with positive results of HBOC on organ quality and function during machine perfusion. However, more research is needed to focus on this field as there is no human renal transplantation study available with HBOC, and in a most recent liver study, the patient population was very small as it included only 5 livers (6). Therefore, a randomized clinical trial is an essential step towards establishing HBOC as a safe and functional oxygen carrier, first for use in perfusion studies, and then for gradual rewarming in human organs.

Gradual Rewarming Perfusion in Steatotic Livers

The use of steatotic livers in liver transplantation is associated with potential poor graft function and primary non-function after transplantation (15). Although there are studies reporting relative safety as a result of carefully choosing steatotic livers for transplantation, there is still a high decline rate in accepting these organs for transplantation especially if the steatosis level in the organ is moderate or severe (≥30%) (15,16).

In an attempt to elevate the quality of steatotic livers, HMP and SNP have been performed on these organs, and the results demonstrate an improved ATP level and improved liver function as indicated by better bile production (17,18). However regardless of the enhanced organ quality, transplant centers remain hesitant to use steatotic livers based on the perception that these organs are of lessor or low-quality.

A potential approach to improving organ quality to an optimal and acceptable level includes applying a pharmacological preconditioning for defatting, using a defatting cocktail during liver perfusion. In an attempt to do this, Liu et al perfused steatotic rat livers with a defat cocktail using SNP for 6 hours (17). Although triglyceride concentration was increased in the perfusate during the perfusion, no significant change was observed in intracellular triglyceride levels. This data raised the conjecture that physiologic temperature might be needed for increasing the effectiveness of the defatting process as two in vitro studies demonstrated reduced fat level in human hepatocytes and hepatoma cells (17,19,20).

Given the overall increase in obesity worldwide, the possibility of using steatotic livers could considerably increase the donor organ pool and consequently, the number of available fatty livers. Fatty livers are extremely sensitive and vulnerable to ischemia-reperfusion

injury. However, the potential exists to reverse or ameliorate ischemia-reperfusion injury and improve organ quality to make the organ suitable for transplantation. As already demonstrated, the gradual re-warming process decreases reperfusion injuries and increases transplanted organ performance.
Applied to fatty livers, the potential exists that gradual rewarming perfusion could represent an exciting perfusion protocol during the defatting process. For instance, in the early stage of re-warming perfusion, HMP could play a preconditioning role and improve organ ATP levels, and effectively reduce ischemia reperfusion injury. This, in turn, could further improve organ quality and enhance the uptake and effectiveness of a defatting cocktail. The defatting cocktail would then be infused into the perfusion solution during the NMP phase of the gradual rewarming perfusion. Clearly, the effectiveness of gradual rewarming on elevation of fatty livers quality as viable transplantable organs requires further research that focuses on investigating the potential benefits of gradual rewarming during defatting treatment.

Gradual Rewarming in Future Research

Tissue engineering such as cell therapy, gene therapy and SiRNA treatment is a new method that could be used during machine perfusion to improve organ quality from ECD donors (21–23). The idea of applying tissue engineering during machine perfusion for targeting and modifying injurious biological pathways represents a very innovative approach in organ transplantation therapy. The organ transplantation field could benefit from tissue engineering in manipulating and preventing ischemia reperfusion injury or even down-regulating of the immune system and thereby eliminate organ rejection. Although, the optimal temperature for the mentioned therapies is not known yet, Gradual rewarming, the combined method of HMP and gradual rewarming to NMP and NMP, with the potential to reduce reperfusion injury could be an effective perfusion protocol.

Gradual rewarming perfusion could be similarly valuable in preserving hepatitis C virus (HCV) positive organs and organs retrieved from elder donors as these organs are more disposed to ischemia reperfusion injury and consequently inferior organ function after transplantation (24,25). Gradual rewarming with the benefit of reducing ischemia reperfusion injury could be efficient in increasing the organ quality and therefore boosting the use of more mentioned organs. Future studies should focus on defining a proper gradual rewarming protocol and optimizing the system to avoid the technical issues in continuous gradual rewarming. Finally, investigations should include researching potential advantages or disadvantages of applying gradual rewarming treatment and the outcome on organ quality in the vast majority of the organs with inferior quality.

CONCLUSION

In summary, this thesis offers a contemporary, thorough overview of gradual rewarming perfusion protocol. In addition, it highlights the positive effect of this approach on organ quality along with the improvement in oxygenation protocol during gradual rewarming by applying HBOC as the oxygen carrier. Further, the discussion addresses the expanded benefits of rewarming protocol up to 37°C. Finally, this thesis outlines a comprehensive rewarming protocol that could be utilized in developing a safe and functional rewarming method for human organ use in the clinical arena.

My hope is that this thesis provides sufficient data to launch the first stage of clinical utilization, and by broadly applying its findings, increase the quality and quantity of available organs for human transplantation.

REFERENCES

1. Moers C, Smits JM, Maathuis M-HJ, Treckmann J, van Gelder F, Napieralski BP, et al. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med. 2009 Jan 1;360(1):7–19.
2. Westerkamp AC, Karimian N, Matton APM, Mahboub P, van Rijn R, Wiersema-Buist J, et al. Oxygenated Hypothermic Machine Perfusion After Static Cold Storage Improves Hepatobiliary Function of Extended Criteria Donor Livers. Transplantation. 2016 Apr;100(4):825–35.
3. Weissenbacher A, Lo Faro L, Boubriak O, Soares MF, Roberts IS, Hunter JP, et al. Twenty-four-hour normothermic perfusion of discarded human kidneys with urine recirculation. Am J Transplant 2019 Jan;19(1):178-192.
4. op den Dries S, Karimian N, Sutton ME, Westerkamp AC, Nijsten MWN, Gouw ASH, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2013 May;13(5):1327–35.
5. Fontes PA. The Evolution of Oxygen Carrier Solutions for Machine Perfusion. Transplantation. 2017;101(11):2657–8.
6. de Vries Y, Matton APM, Nijsten MWN, Werner MJM, van den Berg AP, de Boer MT, et al. Pretransplant sequential hypo- and normothermic machine perfusion of suboptimal livers donated after circulatory death using a hemoglobin-based oxygen carrier perfusion solution. Am J Transplant. 2019 Apr;19(4):1202-1211.
7. Op den Dries S, Karimian N, Westerkamp AC, Sutton ME, Kuipers M, Wiersema-Buist J, et al. Normothermic machine perfusion reduces bile duct injury and improves biliary epithelial function in rat donor livers. Liver Transplant Off Publ Am Assoc Study Liver Dis Int Liver Transplant Soc. 2016;22(7):994–1005.
8. Dutkowski P, Schlegel A, de Oliveira M, Müllhaupt B, Neff F, Clavien P-A. HOPE for human liver grafts obtained from donors after cardiac death. J Hepatol. 2014 Apr;60(4):765–72.
9. Bruinsma BG, Avruch JH, Weeder PD, Sridharan GV, Uygun BE, Karimian NG, et al. Functional human liver preservation and recovery by means of subnormothermic machine perfusion. J Vis Exp JoVE. 2015 Apr 27;(98).
10. Minor T, Efferz P, Fox M, Wohlschlaeger J, Lüer B. Controlled oxygenated rewarming of cold stored liver grafts by thermally graduated machine perfusion prior to reperfusion. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2013 Jun;13(6):1450–60.
11. Hoyer DP, Mathé Z, Gallinat A, Canbay AC, Treckmann JW, Rauen U, et al. Controlled Oxygenated Rewarming of Cold Stored Livers Prior to Transplantation: First Clinical Application of a New Concept. Transplantation. 2016 Jan;100(1):147–52.
12. Nasralla D, Coussios CC, Mergental H, Akhtar MZ, Butler AJ, Ceresa CDL, et al. A randomized trial of normothermic preservation in liver transplantation. Nature. 2018 May;557(7703):50–6.
13. Laing RW, Bhogal RH, Wallace L, Boteon Y, Neil DAH, Smith A, et al. The Use of an Acellular Oxygen Carrier in a Human Liver Model of Normothermic Machine Perfusion. Transplantation. 2017 Nov;101(11):2746–56.
14. Matton APM, Burlage LC, van Rijn R, de Vries Y, Karangwa SA, Nijsten MW, et al. Normothermic Machine Perfusion of Donor Livers Without the Need for Human Blood Products. Liver Transpl. 2018 Apr;24(4):528-538.
15. Boteon YL, Boteon APCS, Attard J, Mergental H, Mirza DF, Bhogal RH, et al. Ex situ machine perfusion as a tool to recondition steatotic donor livers: Troublesome features of fatty livers and the role of defatting therapies. A systematic review. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2018 Oct;18(10):2384–99.

16. Westerkamp AC, de Boer MT, van den Berg AP, Gouw ASH, Porte RJ. Similar outcome after transplantation of moderate macrovesicular steatotic and nonsteatotic livers when the cold ischemia time is kept very short. Transpl Int Off J Eur Soc Organ Transplant. 2015 Mar;28(3):319–29.
17. Liu Q, Berendsen T, Izamis M-L, Uygun B, Yarmush M, Uygun K. Perfusion defatting at sub-normothermic temperatures in steatotic rat livers. Transplant Proc. 2013 Nov;45(9):3209-13.
18. Bessems M, Doorschodt BM, Kolkert JLP, Vetelainen RL, van Vliet AK, Vreeling H, et al. Preservation of steatotic livers: a comparison between cold storage and machine perfusion preservation. Liver Transplant Off Publ Am Assoc Study Liver Dis Int Liver Transplant Soc. 2007 Apr;13(4):497–504.
19. Yarmush G, Santos L, Yarmush J, Koundinyan S, Saleem M, Nativ NI, et al. Metabolic Flux Distribution during Defatting of Steatotic Human Hepatoma (HepG2) Cells. Metabolites. 2016 Jan 4:6(1).
20. Boteon YL, Wallace L, Boteon APCS, Mirza DF, Mergental H, Bhogal RH, et al. An effective protocol for pharmacological defatting of primary human hepatocytes which is non-toxic to cholangiocytes or intrahepatic endothelial cells. PLOS ONE. 2018 Jul 25;13(7):e0201419.
21. Brasile L, Stubenitsky BM, Booster MH, Arenada D, Haisch C, Kootstra G. Transfection and transgene expression in a human kidney during ex vivo warm perfusion. Transplant Proc. 2002 Nov;34(7):2624.
22. Yang B, Hosgood SA, Nicholson ML. Naked small interfering RNA of caspase-3 in preservation solution and autologous blood perfusate protects isolated ischemic porcine kidneys. Transplantation. 2011 Mar 15;91(5):501–7.
23. Thijssen MF, Brüggenwirth IMA, Gillooly A, Khvorova A, Kowalik TF, Martins PN. Gene Silencing With siRNA (RNA Interference): A New Therapeutic Option During Ex Vivo Machine Liver Perfusion Preservation. Liver Transplant Off Publ Am Assoc Study Liver Dis Int Liver Transplant Soc. 2019;25(1):140–51.
24. Park Y, Hirose R, Coatney JL, Ferrell L, Behrends M, Roberts JP, et al. Ischemia-reperfusion injury is more severe in older versus young rat livers. J Surg Res. 2007 Jan;137(1):96–102.
25. Berenguer M. Risk of extended criteria donors in hepatitis C virus–positive recipients. Liver Transpl. 2008;14(S2):S45–50.

Nederlandse Samenvatting

Koude preservatie ‘cold storage’, CS is wereldwijd de meest gebruikte vorm van preservatie van donor organen voor transplantaties. Afkoeling van het transplantaat tot 4°C in een geschikte preservatie oplossing omdat het zorgt voor een verlaging van het celmetabolisme. Hoewel koude preservatie effectief is voor het preserveren van organen, kent het echter significante beperkingen, zoals een verlaging in de hoeveelheid adenosinetrifosfaat (ATP), accumulatie van afvalstoffen en het ontbreken van de mogelijkheid tot beoordeling van de vitaliteit van het orgaan tijdens de preservatie periode. Koude preservatie kan daarnaast leiden tot een aanzienlijke verlaging van de kwaliteit van organen, met name bij minder vitale organen verkregen van *extended criteria donors* (ECD), en organen verkregen via donatie na circulatiestilstand, *donation after circulatory death* (DCD). Dit komt doordat het bewaren van organen op lage temperatuur kan leiden tot ischemische schade, met een versnelling van de achteruitgang van het te transplanteren orgaan tot gevolg. Dit kan leiden tot ernstige ischemie-reperfusie schade na de transplantatie. Voor het verhogen van de beschikbaarheid en de kwaliteitsverbetering van beschikbare organen is daarom een verbeterde methode voor preservatie en evaluatie nodig.
Machine perfusie, een recent ontwikkelde en momenteel de geprefereerde methode voor orgaanpreservatie, biedt de mogelijkheid tot beoordeling van de vitaliteit van organen en een verbetering van de orgaankwaliteit. Het is daarmee een mogelijke oplossing voor de tekortkomingen van het koude preservatie. Hypothermische machine perfusie (HMP), één van de eerst ontwikkelde perfusie methoden gebruikt voor preservatie van donornieren, heeft geleid tot een korter postoperatief herstel alsmede het verminderd voorkomen van het vertraagd aanslaan van de donornieren na transplantatie (*delayed graft function*) (1). Bij menselijke levertransplantaties leidt HMP tot een verbetering van de hoeveelheid ATP in het donor orgaan en een verbetering in leverfunctie na transplantatie (2). Daarnaast is er toenemende belangstelling voor normothermische machinale perfusie (NMP), perfusie op lichaamstemperatuur, waarbij het beter mogelijk is de orgaanfunctie en vitaliteit vast te stellen voorafgaand aan transplantatie (3,4). De veiligheid alsmede de lagere kosten van HMP en NMP, de verbeterde energiestatus met HMP en de mogelijkheid voor het testen van vitaliteit tijdens NMP, bieden voordelen voor het herstel van organen na de transplantatie. Het is daarom mogelijk dat een combinatie van HMP en NMP, waarbij de temperatuur tijdens perfusie geleidelijk tot lichaamstempratuur wordt verhoogd, een nóg grotere bijdrage levert aan de levensvatbaarheid van met name hoog risico organen van lagere kwaliteit, zoals verkregen van ECD en na DCD.

Het doel van dit proefschrift is het bestuderen van het effect van geleidelijke opwarming op de vitaliteit van lage-kwaliteit organen, zoals ECD en met name DCD, gedurende de

orgaanpreservatie en voorafgaand aan de implantatie. Dit proefschrift behandelt daarnaast ook de verbetering van het protocol voor geleidelijke opwarming, waarbij een op hemoglobine gebaseerde zuurstofdrager wordt toegevoegd aan de perfusieoplossing in preklinische nier- en lever proefdiermodellen.

Nu volgt een beschrijving van de overige hoofdstukken.

Hoofdstuk 1 introduceert de verschillende onderdelen van dit proefschrift en geeft een overzicht van de overige hoofdstukken.

Hoofdstuk 2 bevat een gedetailleerde beschrijving van zowel een lever als een nier perfusiesystemen in ratten, ontwikkeld in het Universitair Medisch Centrum Groningen (UMCG). Beide systemen zijn temperatuur- en drukgereguleerd en voorzien een pulserende stroom van de perfusie vloeistof in de arteriën (voor de lever en de nier) en een continue stroom in de poortader (alleen voor de lever). De zuurstof in beide systemen wordt geleverd door twee artificiële longen en een thermostaat maakt perfusieprotocollen binnen verschillende termparatuurbereiken mogelijk, zoals HMP, subnormothermische perfusie (SNP) en NMP, alsmede uniforme geleidelijke opwarming tijdens perfusie *(uniform gradual rewarming)*. Deze twee modellen representeren de eerste innovatieve perfusie knaagdiermodellen met regelbare temperatuur en druk tijdens de perfusie. De technologie welke is gebruikt voor deze eerste systemen is daarna toegepast op menselijke nier en levelperfusie modellen en gecommercialiseerd door het bedrijf Organ Assist in Groningen.).

Vervolgens zijn deze perfusiesystemen toegepast voor het bestuderen van het effect van geleidelijke opwarming op ischemie-reperfusie in rattenieren met verlengde tijd in CS, beschreven in **Hoofdstuk 3**. De niertransplantaten werden gepreserveerd in een CS-oplossing op 4°C gedurende 24 uur, gevolgd door 90 minuten geleidelijke opwarming en perfusie van zowel 10°C en opwarming tot 37°C, en 20°C met geleidelijke opwarming tot 37°C. De resultaten werden vergeleken met directe opwarming tot 37°C. Onafhankelijk van de starttemperatuur resulteerde geleidelijke opwarming in verbeterde nierpreservatie, verminderde reperfusieschade en verbeterde nierfunctie, vergeleken met directe opwarming op 37°C. Daarnaast bewijst deze studie de technische haalbaarheid van geleidelijke opwarming.

Hoofdstuk 4 beschrijft de studie van verschillende perfusietemperaturen, inclusief perfusie met geleidelijke opwarming, met als doel het vinden van het optimale machinale perfusieprotocol voor het beperken van galwegletsel in DCD-rattenlevers. Zowel HMP, SNP en geleidelijke opwarming (8°C tot 20°C) tijdens leverpreservatie, na 6 uur CS werden getest

en vergeleken met een controlegroep na 6 uur CS zonder machine perfusie gedurende de lever preservatie. Alle levers ondergingen 120 minuten ex vivo reperfusie op 37°C voor de beoordeling van levensvatbaarheid. Ongeacht de perfusietemperatuur leidde machine perfusie tot verbeterde leverfunctie, onderstreept door een reductie van galwegletsel markers (ALT, AST, LDH) en verbeterde leverfunctie markers (galproductie) en verbeterd behoud van energy, geïllustreerd door superieure mitochondriële functie gedurende de 120 minuten reperfusie.

Actief celmetabolisme, variërend van 10%–100% gedurende geleidelijke opwarming van HMP naar NMP vereist een verfijnd opwarmingsprotocol dat voldoende zuurstofvoorziening garandeert, inclusief een adequate zuurstofdrager. Het toepassen van een geschikte drager is gecompliceerd in het licht van de temperatuurverschuiving die plaats vindt van HMP naar NMP en die lyse in zuurstofdragers zoals menselijke rode bloedcellen kan veroorzaken. De huidige generatie zuurstofdragers op basis van hemoglobine (HBOC) kunnen een oplossing bieden, aangezien deze functioneren over een groot temperatuurbereik, van hypo- tot normothermie (5,6). In dit kader werden tevens de haalbaarheid, veiligheid en andere voor- en nadelen van de toepassing van HBOC tijdens de perfusie met geleidelijke opwarming van lever en nier rattenmodellen bestudeerd.

De data in de leverstudie in Hoofdstuk 4 demonstreert dat geleidelijke opwarming, gecombineerd met andere perfusieprotocollen, een verbetering in leverkwaliteit teweeg kan brengen ten opzichte van onmiddellijke opwarming. Een andere studie, uitgevoerd door Minor et al., heeft ook een significante verbetering laten zien bij geleidelijke opwarming van levertransplantaten ten opzichte van directe reperfusie (4). Beide studies startten de opwarming op de temperatuur van HMP, totdat de SNP-temperatuur bereikt was, waarna de temperatuur stabiel werd gehouden. Wegens het ontbreken van een geschikte zuurstofdrager voor opwarming van HMP tot NMP, ontbrak het in deze studies aan een NMP-fase. In **Hoofdstuk 5** werd daarom HBOC gebruikt tijdens geleidelijke opwarming van 8°C naar 37°C, waarbij gefocust werd op de toepasbaarheid en veiligheid van het gebruik van HBOC. De DCD-rattenlevers in de opwarmingsgroep ondergingen geleidelijke opwarming gedurende 90 minuten met HBOC, voorafgegaan door 270 minuten in CS. De resultaten werden vergeleken met een controlegroep waarbij 360 minuten CS was toegepast. Na 30 minuten ischemie op kamertemperatuur, als representatie van de chirurgische implantatietijd, werd de levensvatbaarheid beoordeeld, door middel van reperfusie op 37°C voor een tijd van 120 minuten in zowel de opwarmings- als de controlegroep. De levertransplantaten in de opwarmingsgroep vertoonden geen bijwerkingen als gevolg van de toepassing van HBOC en vertoonden daarnaast een verbeterd herstel, gezien de verbeterde lactaat klaring, hogere galproductie, relatief lagere vasculaire weerstand, alsmede lagere waarden van de leverschade marker ALT.

In **Hoofdstuk 6** werd het effect van HBOC op perfusie met geleidelijke opwarming van nieren getest. De resultaten weren vergeleken met een groep waarbij geen zuurstofdrager was toegepast. Voor deze studie werden DCD-rattennieren voor 120 minuten koud gepreserveerd (cold storage, CS). De nieren in de controlegroep werden na CS met opgelost Carbogen gedurende 90 minuten opgewarmd. Bij de HBOC-groep werd tijdens de 90 minuten opwarming HBOC als zuurstofdrager toegevoegd aan de perfusieoplossing. Na 30 minuten ischemie, dat de chirurgische anastomosetijd representeert, werd gedurende 120 minuten reperfusie van de nieren in beide groepen op 37°C de levensvatbaarheid beoordeeld. Hoewel geen verschil werd gemeten in de hoeveelheid aanwezige ATP alsmede de aanwezigheid van cel- en weefselletsel, werd een verbetering in nierfunctie waargenomen in de HBOC-groep, dat tot uiting kwam in de hogere urineproductie, hogere glomerulaire filtratiesnelheid (GFR) en verbeterde natrium terugresorptie gedurende de reperfusie.

CONCLUSIE

Dit proefschrift geeft een actueel en grondig overzicht van het perfusieprotocol met geleidelijke opwarming. Daarnaast benadrukt het de positieve effecten van deze benadering op de orgaankwaliteit en een verbetering van het zuurstoftoevoer protocol gedurende de geleidelijke opwarming, door middel van het toevoegen van HBOC als zuurstofdrager. De discussiesectie behandeld tevens de uitgebreide voordelen van geleidelijke opwarming tot 37°C. Dit proefschrift beschrijft een alomvattend opwarmingsprotocol dat kan worden toegepast voor de ontwikkeling van een veilig functioneel opwarmingsprotocol voor het preserveren van menselijke organen in de een klinische context. Ik hoop dat dit proefschrift voldoende data bevat voor het starten van de initiële fase voor klinische toepassing en dat de toepassing van de bevindingen leidt tot een verbetering in de kwaliteit en beschikbaarheid van menselijke donororganen.

REFERENCES

1. Moers C, Smits JM, Maathuis M-HJ, Treckmann J, van Gelder F, Napieralski BP, et al. Machine perfusion or cold storage in deceased-donor kidney transplantation. N Engl J Med. 2009 Jan 1;360(1):7–19.
2. Westerkamp AC, Karimian N, Matton APM, Mahboub P, van Rijn R, Wiersema-Buist J, et al. Oxygenated Hypothermic Machine Perfusion After Static Cold Storage Improves Hepatobiliary Function of Extended Criteria Donor Livers. Transplantation. 2016 Apr;100(4):825–35.
3. Weissenbacher A, Lo Faro L, Boubriak O, Soares MF, Roberts IS, Hunter JP, et al. Twenty-four-hour normothermic perfusion of discarded human kidneys with urine recirculation. Am J Transplant 2019 Jan;19(1):178-192.
4. op den Dries S, Karimian N, Sutton ME, Westerkamp AC, Nijsten MWN, Gouw ASH, et al. Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. Am J Transplant Off J Am Soc Transplant Am Soc Transpl Surg. 2013 May;13(5):1327–35.
5. Fontes PA. The Evolution of Oxygen Carrier Solutions for Machine Perfusion. Transplantation. 2017;101(11):2657–8.
6. de Vries Y, Matton APM, Nijsten MWN, Werner MJM, van den Berg AP, de Boer MT, et al. Pretransplant sequential hypo- and normothermic machine perfusion of suboptimal livers donated after circulatory death using a hemoglobin-based oxygen carrier perfusion solution. Am J Transplant. 2019 Apr;19(4):1202-1211.

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Biography

Paria Mahboub was born in Tabriz, Iran in 1981. After finishing high school, she successfully passed the university entrance exam and was accepted in faculty of veterinary medicine, Urmia University in 2001. In 2007, she graduated with her DVM degree from faculty of veterinary medicine. Between 2007-2011, she worked as a licensed veterinarian in a veterinary pharmacy and poultry food production factory in Iran. In November of 2011, she moved to the Netherlands to start her PhD education at the University Medical Center Groningen (UMCG) within the department of surgery. The focus of her research was to study the role of gradual rewarming perfusion preservation in liver and kidney transplantation, and the protective effect of the mentioned treatment on donor grafts with inferior quality. Paria is currently working at UMass Medical Center, transplant division as a decision support specialist and at Mass General Hospital, Harvard Medical School as a research scientist. Paria enjoys cooking, baking and travelling in her free time.