Video Article

Technique of Subnormothermic *Ex Vivo* Liver Perfusion for the Storage, Assessment, and Repair of Marginal Liver Grafts

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Abstract

The success of liver transplantation has resulted in a dramatic organ shortage. In most transplant regions 20-30% of patients on the waiting list for liver transplantation die without receiving an organ transplant or are delisted for disease progression. One strategy to increase the donor pool is the utilization of marginal grafts, such as fatty livers, grafts from older donors, or donation after cardiac death (DCD). The current preservation technique of cold static storage is only poorly tolerated by marginal livers resulting in significant organ damage. In addition, cold static organ storage does not allow graft assessment or repair prior to transplantation.

These shortcomings of cold static preservation have triggered an interest in warm perfused organ preservation to reduce cold ischemic injury, assess liver grafts during preservation, and explore the opportunity to repair marginal livers prior to transplantation. The optimal pressure and flow conditions, perfusion temperature, composition of the perfusion solution and the need for an oxygen carrier has been controversial in the past.

In spite of promising results in several animal studies, the complexity and the costs have prevented a broader clinical application so far. Recently, with enhanced technology and a better understanding of liver physiology during *ex vivo* perfusion the outcome of warm liver perfusion has improved and consistently good results can be achieved.

This paper will provide information about liver retrieval, storage techniques, and isolated liver perfusion in pigs. We will illustrate a) the requirements to ensure sufficient oxygen supply to the organ, b) technical considerations about the perfusion machine and the perfusion solution, and c) biochemical aspects of isolated organs.

Video Link

The video component of this article can be found at https://www.jove.com/video/51419/

Introduction

Liver transplantation is the only treatment option for patients with end stage liver disease or advanced hepatocellular carcinoma. For the last 25 years, the number of waiting list candidates has gradually increased and exceeded the number of available grafts. The number of heart beating donors has decreased over the last decade. At the same time, numbers of marginal grafts, such as donation after cardiac death (DCD), as well as old and fatty livers have increased[1,2].

Marginal grafts are often declined for liver transplantation because of the higher chance of primary graft non- or delayed function. In DCD grafts, development of ischemic type biliary strictures (ITBS) is of special concern. With the conventional static cold preservation technique, ITBS occur in about 10-40% of DCD grafts. In the majority of patients, ITBS leads to re-transplantation or patient death. Especially prolonged warm and cold ischemic times are risk factors for ITBS[3-7]. Donor age, genetic predispositions (such as CCR5 delta 32), and the choice of preservation solution have also been discussed as additional risk factors[7-10]. Partial microthrombosis of the peribiliary vessels has been suggested as potential mechanism for ITBS after liver transplantation with DCD grafts[11].

Prior to the clinical introduction of liver transplantation, *ex vivo* liver perfusions have been used to study hepatic metabolism and physiology[12,13]. After liver transplantation found its way into the clinical setting in the 1960s, innumerable attempts have been made to use *ex vivo* liver perfusion as a preservation method by mimicking physiological nutrition and oxygenation conditions. Its utility for preservation of marginal grafts has been investigated in the last decade, but it did not reach standard clinical care. We recently described a reduction in bile duct injury in DCD liver transplantation by *ex vivo* perfused preservation[14]. Different approaches regarding the perfusion solution were made. The selection ranges from

cellular solutions like whole blood from the donor animal or packed red cells in combination with human plasma, to acellular approaches like machine University of Wisconsin solution, IGL solution, or Steen solution[14-19].

The temperature ranges from 4-37 °C[20]. The nomenclature in hypothermic, subnormothermic, and normothermic is very variable and inconsistent. All different techniques, solutions, and temperature settings aim at 1) stable perfusion conditions, 2) sufficient oxygenation, and 3) re-establishment of organ function. An enhanced preservation capacity as well as the ability of organ assessment and treatment during normothermic and subnormothermic perfusion faces higher technical complexity and costs compared to hypothermic perfusion[20,21].

We have developed a subnormothermic *ex vivo* liver perfusion system over the last 4 years. The system can be used to 1) "recharge" the hepatic energy content, 2) to assess the graft's quality, and to 3) repair marginal livers prior to transplantation. The following protocol contains all information for a stable hepatic perfusion.

Protocol

A schematic overview of the protocol is presented in **Figure 1**.

Figure 1. Study protocol. The porcine study design of liver injury is based on a donation after cardiac death (DCD) model. After dissection of all liver vessels, cardiac death is induced followed by 45 min of warm graft ischemia. To simulate a graft transport in between the donor and recipient hospitals in a clinical setting, the graft is stored on ice for 4 hr after cold, dual flush. After cold storage, the organ is subnormothermic perfused for 6 hr in order to assess the perfusion stability. In a transplant model, the perfusion time could be shorter in order to recharge energy storage and to assess the organ viability. Please click here to view a larger version of this figure.

1. Animals

NOTE: Male Yorkshire pigs, 30-35 kg, were utilized for this study. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care of Laboratory Animals" published by the National Institutes of Health. The Animal Care Committee of the Toronto General Research Institute approved all studies.

2. Organ Retrieval

1. House male Yorkshire pigs in research facilities for 1 week before perfusion/transplantation to reduce the level of stress and to accustom the animals to the housing conditions. Less than 2 days of housing inside the facility will lead to a stress-induced physical reaction, which can alter the perfusion's outcome[22,23].
2. Anesthetize pigs by an intramuscular (i.m.) injection of a mixture of ketamine (25 mg/kg), atropine (0.04 mg/kg), and midazolam (0.15 mg/kg).
3. Prior to intubation, ensure the pig breathes spontaneously 2 L of O_2 dosed with 5% isoflurane. Spray the vocal chords with 2% lidocaine 2 min before intubation to avoid vocal cord spasms. For example, for a 35 kg pig use a 6.5 fr. tracheal tube. Block the tracheal tube with 3-5 ml of room air.
4. After intubation, use capnometry to confirm correct intubation. Lower the isoflurane gas to 2%. Set the ventilator to 14-16 breaths/min and a tidal volume of 10 ml/kg bodyweight.
5. Place a 20 G intravenous (i.v.) catheter in one of the ear veins to allow infusion of Ringer's lactate solution (200 ml per hr). Then scrub the pig and cover it with sterile drapes.
6. Make a midline incision followed by a left lateral extension. Use a towel to cover large and small bowels and move them to the left side.
7. Separate inferior vena cava (IVC) and distal aorta from each other; ligate aorta branches to the back; isolate and free renal arteries from adherent tissue.
8. Divide the falciforme ligament and the triangular ligament using cautery.
9. Release the portal vein by an incision of the peritoneum between pancreas and portal vein. Tie off veins draining from the pancreas to the portal vein.
10. Dissect the coeliac trunk below the portal vein and follow it backwards to the aorta. Surround the mesenteric artery with a 2-0 tie; surround the splenic and left gastric arteries, which branch posteriorly to the coeliac trunk. Dissect the coeliac trunk off the portal vein.
11. Ligate the lymph vessels within the hepatoduodenal ligament to prevent lymphatic leakage. Divide the right gastric artery between ties. Ligate smaller veins. Separate the bile duct from the ligament and divide it distally after ligation.
12. Dissect the aorta behind the diaphragm between heart and coeliac trunk; place a 2-0 tie around the aorta.
13. Release the liver from the lower cava on the right side using electro cautery; use scissors for the upper part between cava and liver.
14. Remove the gallbladder and cauterize bleeders from gallbladder bed.
15. Administer i.v. 1,000 iU/kg donor weight of Heparin. For a DCD model, induce cardiac arrest by intracardial injection of 40 mval KCl 3 min after Heparin administration. Set cardiac arrest as the starting point of warm ischemia.
16. For the perfusion, collect 1.6 L of pig blood in CPDA bags (citrate, phosphate, dextrose, adenosine) immediately after cardiac death. Perform a soft spin (2,000 x g without brake). Remove the plasma and the buffy coat under sterile condition (biosafety cabinet class II) and store the erythrocytes in CPDA bags for transfusion.

17. Cannulate portal vein and aorta with organ flush lines. Tie off the previously set ties around femoral, renal, splenic, mesenteric, and left gastric arteries as well as the upper aorta. For a heart beating donor (HBD) model, perform the cannulation of aorta and portal vein under heart beating conditions.
18. After 45 min warm ischemia, flush the liver with University of Wisconsin (UW) solution using dual perfusion via aorta (pressure bag) and portal vein (gravity driven).
19. Cut the liver out of the pig, leaving all remaining vessels long.
20. During back-table preparation, clamp the upper IVC using a Satinsky clamp and flush the liver a second time with about 0.5 L of UW solution retrogradely via lower IVC until the portal vein outflow is clear.
21. Tie off all arterial branches from the aorta and coeliac trunk. Perform an arterial back-table pressure perfusion with about 0.5 L of UW solution.
22. Flush the bile duct using UW solution.
23. Cannulate the upper and lower parts of the IVC using 1/2" x 3/8" reducers with Luer Lock; cannulate the portal vein and liver artery using 3/8" x 1/4" and 1/4" x 3/8" reducers with Luer Lock. Use the upper and lower vena cava as venous drainage.
24. Place the liver in an organ bag, close the organ bag, and store the liver on ice until the perfusion has started.

3. *Ex vivo* Liver Perfusion

1. Prepare the perfusion solution containing 2,000 ml Steen solution, 400 ml washed erythrocytes, 550 mg sodium pyruvate, 100 ml amino acid solution (10% Travasol), 10 mg calcium gluconate, 1,000 IE rapid acting insulin, 1 g cefazolin, 500 mg metronidazol, and 10,000 iU heparin. Add other molecules for vasodilatation, immunosuppression, scavenging of reactive oxygen species, or liver cell treatment based on the particular study protocol.
2. For the dialysis component, use standard dialysate containing 3.5 mM potassium, 25 mM bicarbonate, 27 mM glucose, as well as 275 mg/L pyruvate.
3. Set up the perfusion circuit (schema see **Figure 2**).

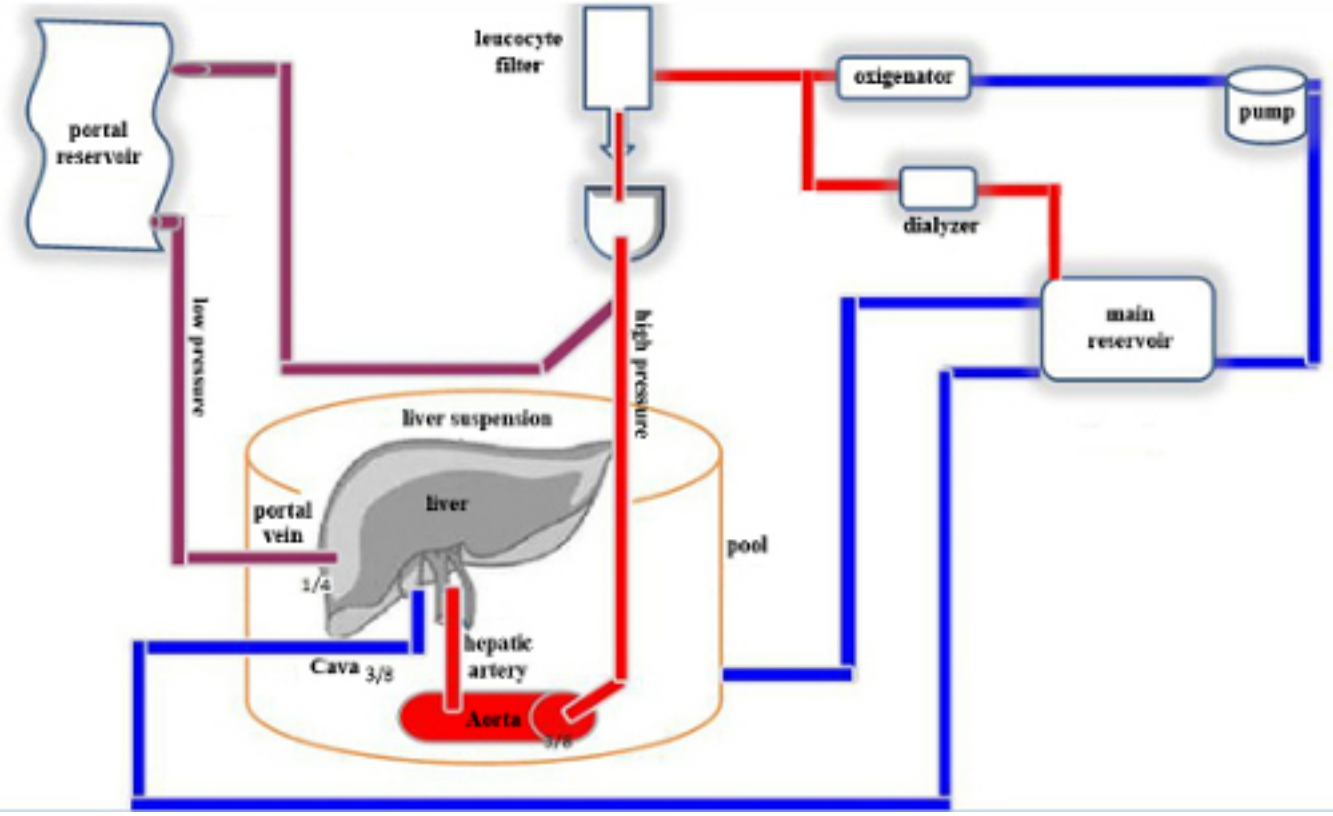

Figure 2. Circuit set up. Coming from the main reservoir as the starting and end point, the perfusion solution is driven by a centrifugal pump through an oxygenator. Right after oxygenation of the solution, the circuit splits into a smaller line running to a dialyzer unit for electrolyte homeostasis and a bigger line running to a leukocyte filter for reduction of remnant white blood cells (WBC). The solution that runs through the dialyzer returns to the main reservoir. After the leukocyte filter, the circuit splits up again into 2 equal lines. One line runs at high pressure (around 60 mmHg) directly into the aorta to perfuse the hepatic artery. The other line drains into a second reservoir. From this reservoir the portal vein is perfused. The pressure of the portal perfusion depends on the elevation energy of the reservoir's solution level (around 2-6 mmHg). All fluids are drained via the infra- and supra-hepatic cava back into the main reservoir. For gravity reduction and homogeneous perfusion, the liver is placed in a pool filled with temperature regulated water. It is separated from the water by an impermeable membrane and it swims in a perfusion suspension. Please click here to view a larger version of this figure.

1. Collect all fluids from the circuit in a 3 L reservoir (main reservoir) and clamp the outflow.
2. Connect the outflow to a centrifugal pump followed by a commercial oxygenator.
3. Behind the oxygenator, split up the tubing into 2 lines. Connect one line to a dialyzer and drain it back into the main reservoir. Connect the second line to a leukocyte reduction filter.

4. Split up the line after the leukocyte reduction filter into an arterial line, which supplies perfusion solution to the hepatic artery, and a portal venous line delivering perfusion solution to a second reservoir, which drains into the portal vein by gravity outflow. Clamp the portal inflow.
5. Connect the arterial line to a vena cava line draining into the main reservoir for fluid recollection.
6. For collection of ascites or leakage of perfusion solution from the liver, prepare a suction line connected to the main reservoir.

4. Release the outflow clamp from the main reservoir and fill the circuit with the perfusion solution. Start the centrifugal pump at 1,500 rounds/min. The perfusion solution will drive through the arterial line into the vena cava line back into the main reservoir. Make sure all air is driven out the circuit.
5. Turn on the gas supply to the oxygenator.
6. Take the liver off the ice. Flush out the UW solution using saline.
7. Place the liver, with its convex side down to facilitate the access to the vessels, ideally in a gravity-free environment to avoid organ compression at the contact surface. Use a heat- and coolable water bath. Set the starting temperature of the circuit and water bath to 20 °C. Cover the water bath with an impermeable membrane and place the liver onto that membrane. Reduce gravity driven compression by submerging the liver with perfusion solution.
8. Reduce the speed of the centrifugal pump to 1,000 rounds/min and place two clamps at the connection of arterial and vena cava lines. Then, cut the tubing in between the clamps. Using a 3-way connector, join both cava outflows and connect them to the vena cava line.
9. Release the clamp from the arterial line, pour perfusion solution into the arterial cannula to get rid of bubbles, and connect the line to the cannula. Increase the centrifugal pump to 1,500 rounds/min. Release the second clamp from the vena cava line.
10. Release the clamp from the portal venous reservoir in order to fill it up. Let perfusion solution pour into the portal cannula and connect it. Take special care of stable fluid levels in the portal reservoir.
11. Connect pressure lines to the Luer Locks of the arterial, portal, and vena cava cannulas.
12. To mimic physiological conditions, apply treatments into the right vessel. Inject glucose into the portal vein and not into the arterial line in order to establish a gradient mimicking an increased portal venous glucose gradient and inducing glycogen synthesis[24,25].
13. After connecting the liver to the circuit, raise the temperature to 33 °C within 60 min.
14. Aim for an arterial starting flow at about 250 ml/min at 40 mmHg. This may reach 700 ml/min during perfusion once the pressure is increased up to 70 mmHg.
15. At starting temperature, aim for a portal vein flow of 500-600 ml/min at 3-5 mmHg. After raising the temperature, monitor the portal venous flow, which will increase up to 1,100 ml/min at 4-6 mmHg. Avoid exceeding portal pressure above physiological values (around 8 mmHg) to protect sinusoidal fenestrations[26]. Avoid exceeding total flow above 2,000 ml/min in order to prevent damaging the organ. Set the outflow to -2 mmHg by lowering the main reservoir to prevent liver congestion by functional outflow obstruction.
16. Add the dialysis component to the circuit in order to equilibrate the perfusion solution to predetermined values[27]. Set the dialysate flow to 500 ml/hr. Take special attention to adjust the dialysis outflow so that the perfusion solution is neither diluted nor concentrated. Within the first hour of perfusion the main reservoir must be watched carefully!
17. Ensure homogeneous oxygenation of the tissue to recover and maintain organ function by using a main gas mixture component of O_2 (95-98%) and CO_2 (2-5%). Use variable gas during perfusion since the liver changes its metabolism and its pH demand during perfusion.
18. Maintain a low pH during the start of perfusion to protect the organ using the paradox pH concept[28] and avoid severe tissue damage that can result from fast connections to physiological pH under reoxygenation, since after storage in UW solution, the organ has an acidotic pH below 7. Adjust the partial pressure of CO_2 continuously down to 25-30 mmHg so that the pH will reach a physiologic level within 1 hr.
19. Add sodium- or potassium bicarbonate to the circuit to achieve a physiological concentration of standard bicarbonate in the perfusion solution. Inject it carefully under repetitive blood gas and electrolyte control.
20. Monitor the perfusion by periodical venous and arterial blood gas and AST analyses. The venous PO_2 remains above 175 mmHg during the perfusion. Monitor vascular flow and pressure and note a stable perfusion by a constant vascular resistance.
21. Keep the perfusion system stable for up to 8 hr. At the end of the *ex vivo* perfusion period, cool down the perfusion system to 20 °C and, after disconnecting the circuit's tubing from the liver, flush the perfusion solution out the liver dually with ice cold UW solution. Store the liver once again placed on ice in a sterile organ bag.

Representative Results

Below, we present the results of 5 perfusion experiments with DCD-grafts after 45 min warm- and 4 hr cold ischemia prior to the start of the subnormothermic *ex vivo* perfusion.

The main goal for an *ex vivo* liver perfusion is to ensure a sufficient oxygen supply to the organ. Ischemia causes vasoconstriction, thus increasing the perfusion resistance. Achieving constant vascular flows with stable pressures is a good indicator of adequate oxygenation. During an induction period of 1-2 hr the perfusion solution and the organ are warmed up to 33 °C, which deceases the vascular resistance of the liver. Once the target temperature of 33 °C is achieved, flow values level at a constant, nearly physiological range for the rest of the 6 hr perfusion time (**Figures 3A-3D**).

At the same time, the organ becomes metabolically active. **Figure 4A** shows the venous pO_2, a marker of oxygen consumption. Within the initial 2 hr the venous pO_2 declines to a constant plateau. At this metabolically active state, the liver starts producing bile (**Figure 4B**). The dialyzer provides a balanced electrolyte homeostasis (**Figures 4C-4D**). An initial hyperkalemia is quickly leveled out. Online AST measurement serves as monitoring of hepatocellular damage. **Figure 5** displays only a shallow linear AST increase over the entire perfusion period. H&E staining after 6 hr of perfusion reveals hepatocyte necrosis <5 % with an intact lobular and sinusoidal structure (**Figure 6**). PAS staining at the same time point shows replenished cellular glycogen storage compared to exhausted storage in cold preserved DCD-grafts (**Figure 7**).

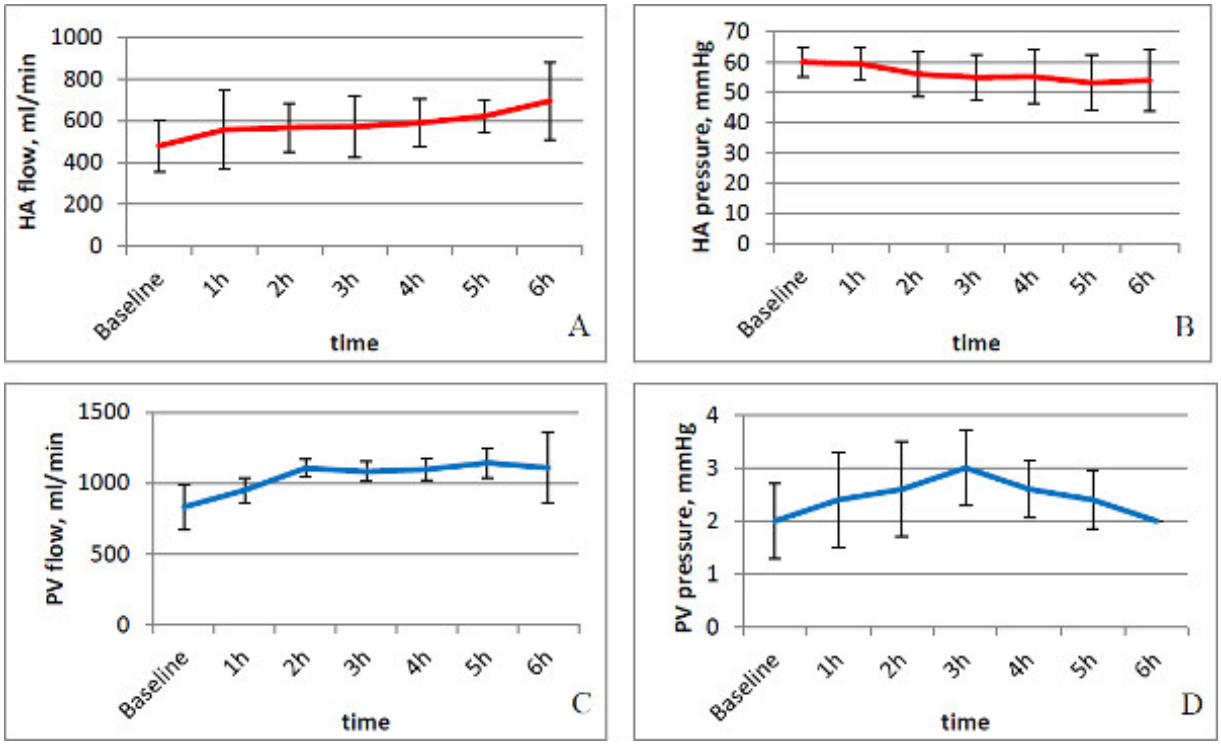

Figure 3. Perfusion flows and pressure (n = 5, error bars show standard deviation). (A,B) Hepatic artery (HA) flow and pressure: During the warming phase in the first 1-2 hr, the flow increases at stable pressures and is constant afterwards. Looking at the decreasing portal venous pressure **(C)**, the increase of HA flow towards the end of the perfusion might be an autoregulatory reaction of the liver. **(C,D)** The portal venous (PV) flow increases corresponding to the HA flow during the first 2 hr of warming. The pressures remain relatively stable.

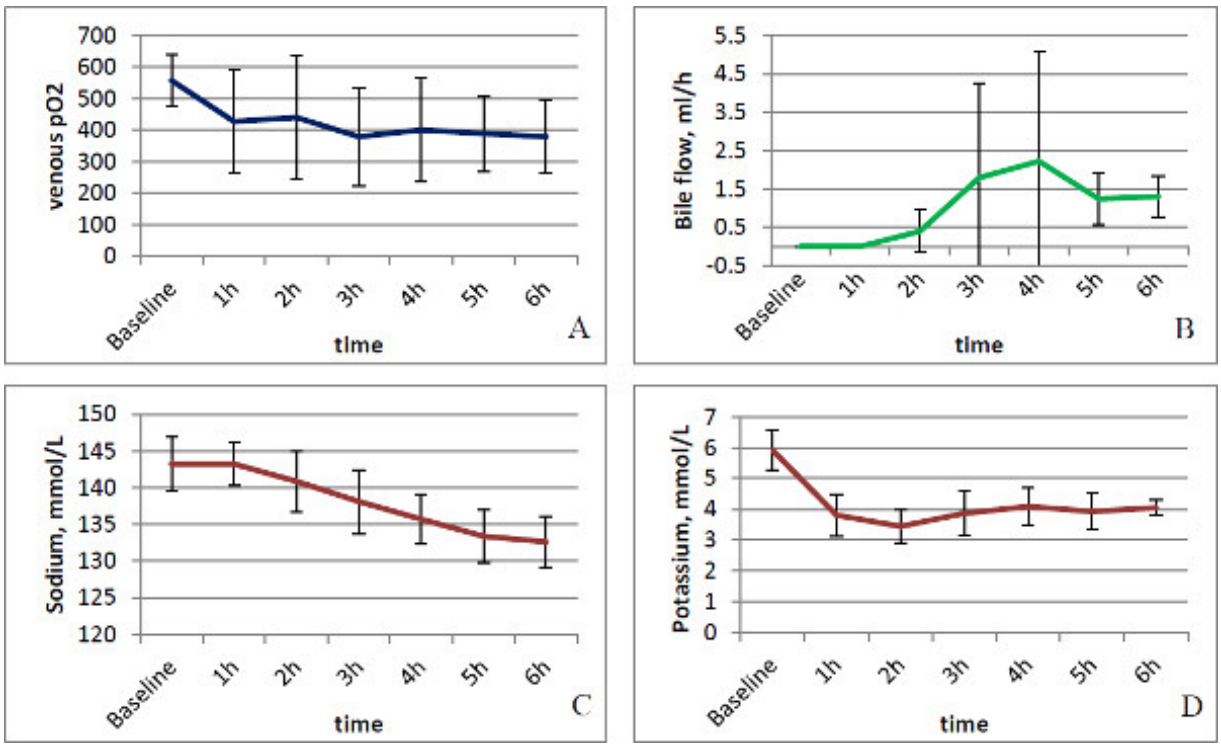

Figure 4. Monitoring parameters (n = 5, error bars show standard deviation). (A) The venous pO_2 as a marker of oxygen demand and metabolic activity decreases within the initial phase of warming due to activated cellular metabolism; it remains stable afterwards. **(B)** Bile production as a marker of metabolic activity starts at temperatures around 30 °C and, thus, between the first and second hour of perfusion. **(C,D)** The dialyzer assures electrolyte homeostasis; an initial hyperkalemia is quickly balanced.

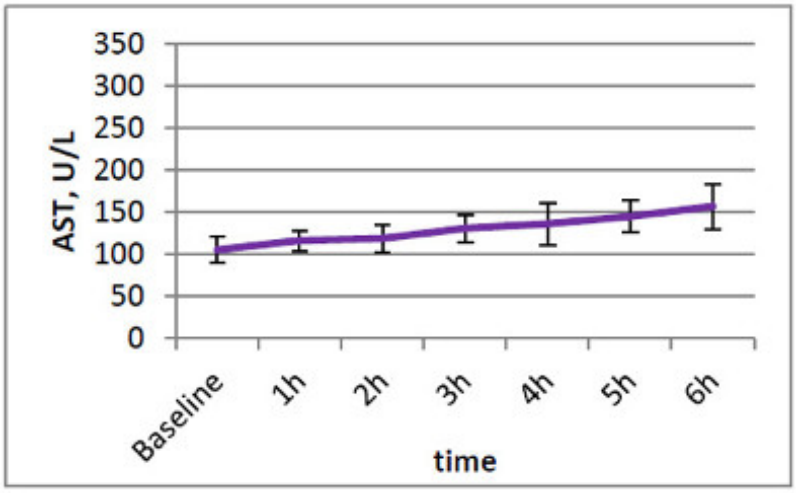

Figure 5. AST (n = 5, error bars show standard deviation). AST is a sensitive marker of hepatocellular injury; the shallow increase suggests no significant injury during *ex vivo* perfusion.

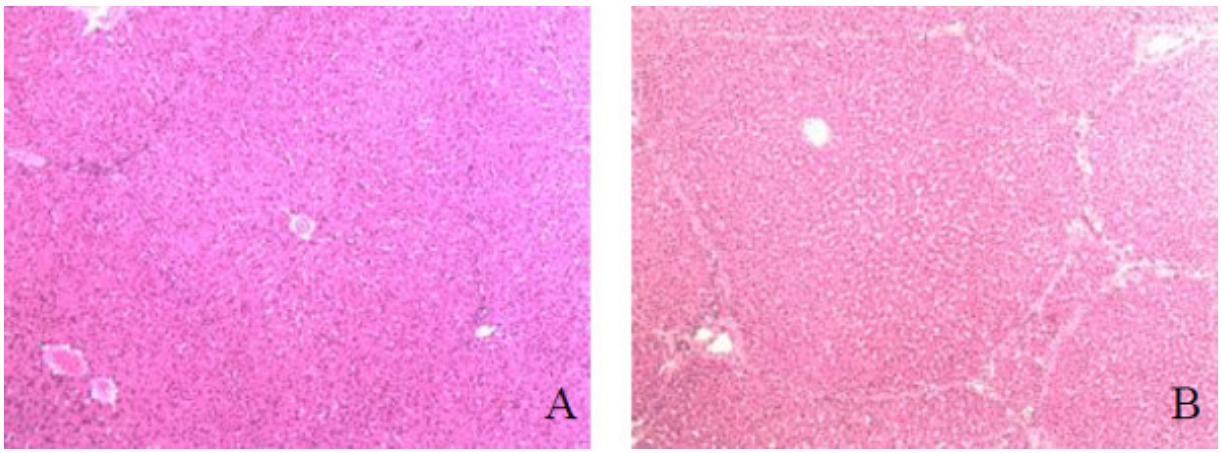

Figure 6. H&E staining (20X magnification). (A) Sham liver sample before warm ischemia, one representative liver lobule with intact architecture. **(B)** Liver sample after 45 min of warm ischemia, 4 hr of cold ischemia, and 6 hr of subnormothermic perfusion, the lobular architecture is intact without necrosis and only minimal cell swelling, the sinusoidal spaces are mildly dilated in comparison to the sham sample.

Discussion

In a pig model that mimics DCD liver transplantation, we demonstrated that subnormothermic liver perfusion with a cellular perfusion solution results in stable perfusion parameters, minimal hepatocyte injury, and active hepatic metabolism. Our subnormothermic perfusion set up has proven to recover a hepatocellular homeostasis and metabolism. Glycogen storage is restored and metabolites are discarded.

Ex vivo liver perfusion as preservation technique offers for the first time the opportunity to assess markers of graft function and injury during organ preservation and prior to transplantation. Beside the macroscopic evaluation of the graft perfusion homogeneity, flow values provide a good indicator of the graft's viability and the extent of the ischemic injury it had suffered earlier[29]. Oxygen consumption and bile production are markers of metabolic function. Levels of hepatic enzymes like AST can be used to assess the degree and dynamics of hepatocellular injury[30]. This thorough graft assessment may allow a reliable discrimination between transplantable and non-transplantable marginal organs.

We chose a subnormothermic temperature of 33 °C in our perfusion system because the temperature is sufficient to allow metabolism as well as ATP and glycogen synthesis. At the same time, it provides a decreased oxygen demand in comparison to normothermic perfusion settings which provides additional safety against ischemic injury. In general, perfusion temperatures above 30 °C have shown to minimize cold ischemic injury and provide sufficient metabolic activity[31].

Contrary to other groups, we did not use whole blood as perfusate, but a normo-osmotic albumin solution (Steen) with washed and filtered red blood cells. By excluding the plasma components as well as thrombocytes and leukocytes, the perfusion solution is designed to minimize pro-inflammatory signaling during the *ex vivo* perfusion.

In addition to the graft assessment, stable perfusion conditions over several hours allow graft treatment. Numerous molecules have shown to attenuate reperfusion injury under experimental conditions[32]. However, almost no treatment regime has made its way into clinical practice, yet. One reason seems to be the lack of opportunity to apply those treatments during cold storage. A metabolically active liver on an *ex vivo* perfusion system is optimal for applying any kind of treatment. In this regard, not only treatments to ameliorate reperfusion conditions like

attenuation of Kupffer cell activity or scavenging of reactive oxygen species are conceivable but also treatments like gene therapy to condition the graft, *e.g.*, against Hepatitis C recurrence. Other potential strategies could include reduction on steatosis during the *ex vivo* perfusion period[33].

In summary, *ex vivo* liver perfusion is a novel strategy to minimize cold ischemic injury and to assess marginal liver grafts prior to liver transplantation. The *ex vivo* perfusion setting provides unique opportunity to repair and condition grafts prior to transplantation.

Disclosures

The authors have nothing to disclose.

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