Published in final edited form as:

Nat Protoc. 2015 March ; 10(3): 484–494. doi:10.1038/nprot.2015.011.

Supercooling Preservation Of The Rat Liver For Transplantation

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Abstract

The current standard for liver preservation is limited in duration. Employing a novel subzero preservation technique that includes supercooling and machine perfusion can significantly improve preservation and prolong storage times. By loading rat livers with cryoprotectants to prevent both intra- and extracellular ice formation and protect against hypothermic injury, livers can be cooled to -6°C without freezing and kept viable for up to 96 hours. Here, we describe the procedures of loading cryoprotectants by means of subnormothermic machine perfusion (SNMP), controlled cooling to a supercooled state, followed by SNMP recovery and orthotopic liver transplantation.

Introduction

Liver preservation aims to minimize injury to the organ while outside the body and provide the highest quality organ for transplantation. For decades liver preservation has been limited to static cold storage (SCS) of the organ on ice, where the reduced temperature ($0-4^{\circ}\text{C}$) results in hypometabolism and a slower deterioration of organ function and structural integrity. SCS is facilitated by specialized organ preservation solutions that support a reduction in metabolic activity. Although SCS has enabled transplantation for many years, it

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Author contributions: B.G.B., T.A.B., M.L.I., M.L.Y. & K.U. optimized and developed the rat liver supercooling technique. T.A.B. & B.G.B. performed the rat transplantations and gathered data from the supercooling and the static cold storage control experiments. K.U. oversaw the analysis and final presentation of the data. B.G.B. & T.A.B. contributed to the manuscript and B.G.B., T.A.B., M.L.I., H.Y., M.L.Y. & K.U. to the review.

Competing financial interests: K.U. and M.L.Y. are inventors on a pending international patent application that involves some of the perfusion technology used in this work (WO/2011/002926); T.A.B., M.L.Y. and K.U. are inventors on a pending international patent application that describes the supercooling protocol employed in this work (WO/2011/35223); and B.G.B., and K.U. have a provisional patent application related to this work that describes scale-up of the perfusion protocol to human livers. K.U. has a financial interest in Organ Solutions LLC, a company focused on developing organ preservation technology. K.U.'s interests are managed by the Massachusetts General Hospital and Partners HealthCare in accordance with their conflict-of-interest policies.

is limited in both the duration of preservation and the number of livers that are of sufficiently high quality to endure additional injury sustained during SCS. On ice, the liver still suffers a relatively fast deterioration, evidenced by a substantial loss of energy stores and viable cells¹. As a result, livers are best transplanted within 12 hours clinically².

Demands to extend the preservation time and improve post-preservation organ quality led to the development of an alternative storage technique based on supercooling, or subzero non-freezing³. The additional reduction of liver metabolism at subzero temperatures further slows down the deterioration of the liver, importantly preserving energy stores and cellular homeostasis^{4,5}. Improved preservation allowed longer viable preservation times and may result in the expansion of the criteria for clinically transplantable donor livers by reducing additional injury to currently discarded grafts. We anticipate that supercooling will be applicable to biopreservation throughout both experimental biomedical and clinical fields, ranging from the preservation of isolated cells to the transplantation of other solid organs and tissues.

Subzero preservation has been a desirable, yet elusive feat for many years. The main impediment was the formation of ice in the tissue, which thus far has been insurmountably detrimental⁶. Despite numerous attempts to prevent or control ice crystal growth, substantial postthaw function, let alone transplantation survival have yet to be realized. Supercooling offers an alternative by avoiding ice formation altogether. By enabling subzero preservation, we have achieved a substantial improvement over current preservation times, extending viable preservation time threefold³.

Alternative liver preservation techniques

Optimal preservation has been a major focus for as long as organ transplantation has been a reality. The first liver transplants were performed incorporating pump-driven perfusion of the organ in an attempt to maintain oxygenation of the liver⁷. The relatively high complexity of these machine perfusion modalities resulted in a quick replacement by simpler static hypothermic preservation when solutions like the University of Wisconsin (UW) solution greatly improved the results of SCS^{8,9}. For many years, SCS was the standard and provided very good outcome. Only more recently, as a consequence of increasingly severe donor liver shortages, alternative storage techniques are being explored to improve the preservation of marginal grafts that yield inferior results when conventionally preserved¹⁰.

Ex vivo machine perfusion techniques are being developed over a range of different temperatures¹¹. Hypothermic machine perfusion at ± 4 °C has already made its clinical introduction and results have been promising, even after just an hour of perfusion^{12,13}. Alternatives, such as normothermic (37 °C) and subnormothermic (± 21 °C) machine perfusion are also making a clinical translation following reports showing that human livers can be metabolically supported at warm temperatures^{14,15}. Although increasing evidence supports machine perfusion as a superior preservation method, the complexity of sustained *ex vivo* organ metabolism may conceptually limit machine perfusion to shorter preservation periods. However, it can certainly be envisioned that *ex vivo* machine perfusion will play a cooperative role with other preservation techniques.

Supercooling and experimental design

The supercooling technique described here is based on three pillars, the primary of which is supercooling of the organ. The liver is cooled to -6°C and is kept in an unfrozen state through the application of various cryoprotectants. Since freezing can occur in both the intracellular liquid phase as well as the extracellular space, a cryoprotectant was chosen to protect each. Learning from freeze-resistant amphibious species, a non-metabolizable form of glucose is loaded into the cells. This 3-O-methyl glucose (3-OMG) is internalized by cellular glucose transporters, but its chemical variation prevents further metabolism, resulting in intracellular accumulation¹⁶. For the intravascular space, high-molecular weight polyethylene glycol (PEG) proved to be advantageous. PEG is a known ice-modulator, directly inhibiting ice crystallization and has added benefits to cellular membranes. The tandem application of 3-OMG and PEG allowed for 100% freeze-avoidance in rat livers stored for multiple days at -6°C . PEG. Moreover, PEG was employed to protect against hypothermic injury that is independent of ischemia, such as lipid peroxidation and cell membrane injury^{17,18,19}. Machine perfusion proved to be instrumental in completing this protocol and is the final pillar of the protocol. A subnormothermic machine perfusion (SNMP) protocol at 21°C was used both before and after supercooling. This temperature was chosen to provide a temperature conducive to both loading of 3-OMG and functional testing. This SNMP system perfuses the liver with a nutrient-rich, oxygenated perfusion solution (perfusate) that has been shown to recover rat livers after both warm and cold ischemia^{20,21}. Pre-supercooling, the perfusate is supplemented with 3-OMG and an hour of SNMP facilitates loading of the cryoprotectant. The SNMP system has been applied to the recovery of livers at the end of preservation, significantly improving post-transplant survival²¹. Herein, 3 hours of SNMP was performed at the end of supercooling, presenting a recovery period for the liver prior to transplantation.

This proof-of-concept design aims to show that supercooling can improve the preservation of healthy livers that have not sustained (warm) ischemic injury. Conventional SCS serves as the control group for preservation experiments and involves an *in situ* flush of cold preservation solution, e.g. UW solution or Histidine-Tryptophan-Ketoglutarate (HTK) solution. Our experiments demonstrated that all components of the supercooling protocol proved essential for successful outcome. Omission of either of the cryoprotectants, pre- or post-supercooling SNMP, or supercooling did not effectively preserve the liver. Successful orthotopic rat liver transplantation begins and ends with proficient surgical procedures, the essentials of which are outlined in this protocol.

Limitations and adaptations to this protocol

The supercooling technique is applied in a brain dead donation model of healthy small animals, offering the best organ condition as there is no warm ischemia. We have yet to test the robustness of supercooling in more ischemic liver models or models of diseased organs, such as fatty liver disease or grafts from older donors. Surgical adaptations incorporating a warm-ischemic period can be applied to mimic cardiac death donation. Various cardiac death models exist, including pharmacological cardiac arrest²², phrenotomy^{23,24}, diaphragmatic incision²⁵, exsanguination^{26,27}, ventilator withdrawal²⁸, arterial clamping²⁹, and *ex vivo* warm ischemia³⁰. An important consideration is that rodents cool down rapidly

after cardiac death, reducing the severity of warm ischemia. It is important to adequately control temperature during *in situ* warm ischemia to ensure a reproducible model.

This supercooling protocol, in particular the temperature development, was optimized for small organs (± 10 g). Since temperature changes throughout the process are critical, we anticipate various adaptations will be required to scale this method to larger organs. It is our impression that homogeneous cooling and warming are key features of the protocol. The gradual changes in temperature that have been outlined here were optimized for small livers and it is to be expected that human-sized livers (± 1.5 – 2 kg) will require a more prolonged cooling and rewarming phase. We have recently translated the SNMP elements of the supercooling protocol to a human liver scale and demonstrating the feasibility of this human SNMP system in supporting human liver function and is presented elsewhere¹⁵.

Materials

Reagents

- Male rats weighing 250–300g and 65–85 days old. The protocol has been validated in Lewis rats (Charles River Laboratories, Boston, MA, USA) **CRITICAL** Approval must be obtained from appropriate institutional and government agencies and animals must be kept in accordance with relevant guidelines and regulation. Herein, animals were maintained in accordance with National Research Council guidelines, and were approved by the Institutional Animal Care and Use Committee (IACUC) at Massachusetts General Hospital (Boston, MA, USA).
- University of Wisconsin (UW) solution (Preservation Solutions Inc. Cat. No. CoStorSol)
- Dexamethasone, water-soluble (Sigma, Cat. No. D2915)
- Penicillin G (MGH Pharmacy, Cat. No. 7672700)
- Poly(ethylene glycol) BioUltra, 35,000 (Sigma, Cat. No. 94646)
- 3-O-methyl glucose (Sigma, Cat. No. M4879)
- Forane (Isoflurane), (Baxter, Cat. No. 1001936040) **! CAUTION** Isoflurane is an inhalation anesthetic and can be harmful to the investigator after prolonged exposure. Ensure proper maintenance and upkeep of the rodent anesthesia machine and familiarize yourself with proper use and handling.
- Humulin (Insulin), (Eli Lilly & Co., cat. no. Humulin R U-100)
- Penicillin/Streptomycin 5,000 U/ml, 100 ml (Life Technologies, cat. no. 15070-063)
- Williams' Medium E (Sigma, Cat. No W1878-6 $\times$ 500ml)
- L-glutamine, 200 mM (Invitrogen, Cat. No. 25030-156)
- Hydrocortisone (MGH pharmacy, Cat. No. 7750500)
- Buprenex (Buprenorphine)

- Sodium heparin (APP pharmaceuticals, Cat. No. Heparin 10.000 USP)
- Carbogen gas tank 95% O₂/5% CO₂ (Airgas, Cat. No. ZO2OX9522000043)
- Oxygen tank, 100% O₂ (Airgas, Cat. No. OX USP 200)
- Lactated Ringer's solution (Baxter, Cat. No. 2B2324X)
- 10% neutral buffered formalin (Fischer Scientific, Cat. No. 316-155)
- Antifreeze solution
- Dry ice and regular ice

Equipment

- Media bottles, 500 ml/125 ml (Fischer Scientific, Cat. No. 61100 500/125)
- Bottle top filter 0.2 µm pore size, 500 ml capacity (Cole Parmer, Cat. No. EW-06730-43)
- Circulating controlled rate chiller (Neslab, Cat. No. RTE-111)
- Jacketed oxygenator (Radnoti, Cat. No. 130144)
- Jacketed bubble trap, 5 ml volume (Radnoti, Cat. No. 130149)
- Jacketed organ chamber/tissue bath (Radnoti, Cat. no 158360)
- Lab stand (Radnoti, Cat. No. 159951-1)
- Ring clamp for bubble trap (Radnoti, Cat. No. 120149RC)
- Ring clamp for tissue bath (Radnoti, Cat. No. 159953-25)
- Ring clamp for oxygenator (Radnoti, Cat. No. 159953-300)
- Masterflex pumps (Cole Parmer, Cat. No. HV-07522-20)
- Pump head for L/S 16 tubing (Cole Parmer, Cat. No. EW-07016-20)
- Masterflex platinum-cured silicone tubing L/S, 16 (Cole Parmer, Cat. No. EW-96410-16)
- Masterflex platinum-cured silicone tubing L/S, 24 (Cole Parmer, Cat. No. EW-96410-24)
- Tube insulator, Thermacel (Grainger, Cat. No. 2CKE7)
- Membrane oxygenator tubing (Radnoti, Cat. No. 130144-078)
- Luer fitting kit (Cole Parmer, Cat. No. EW-45511-00)
- Barbed elbow/tee/Y-connector kits (Cole Parmer, Cat. No. EW-41514-15)
- Specialty gas regulator (Airgas, Cat. No. Y11244D580)
- Tabletop rodent anesthesia machine (VetEquip, Cat. No. 901806)
- Sterile surgical gloves

- Heating pad (Braintree Scientific, Cat. No. HP-1M)
- Sterile surgical instruments
 - Sterile Scalpel blade, #15 (Roboz, Cat. No. RS-9801-15)
 - Scalpel handle # 3 solid 4" (Roboz, Cat. No. RS-9843)
 - Curved-tip Rhoton forceps (Roboz, Cat. No. RS-5264)
 - Toothed Adson forceps (Roboz, Cat. No. RS-5234)
 - Micro Dissecting scissors, 4.5" (Roboz, Cat. No. RS-5983)
 - Metzenbaum scissors, 7" curved (Roboz, Cat. No. RS-6953)
 - Castroviejo Needle holder 5.5–7"
 - Mini dissecting scissors (WPI, Cat. No. 503667)
 - Vannas 3" spring scissors curved, 3mm (Roboz, Cat. No. RS-5611)
 - Micro clips, 0.75 mm (Roboz, Cat. No. RS-5422)
 - Micro clip applying forceps (Roboz, Cat. No. RS-5410)
 - Dumont forceps (Roboz, Cat. No. RS-4962)
 - Micro mosquito forceps (Roboz, Cat. No. RS-7116)
 - Electrocautery set (World Precision Instruments, Cat. No. 500392)
 - Satinsky clamp (surgical123.com, Cat. No. 44-1480S)
- Sterile cotton-tipped applicators (Fischer Scientific, Cat. No. 14-959-91)
- Syringe 10 ml Luer lock
- BD Insulin syringe U-100 28G 1/2" 1.0 ml (Owens & Minor, Cat. No. 0723-329461)
- 18G, 16G, 14G catheters; Insyte Autoguard (BD Cat. Nos. 381705/381707/381709) modified for vascular cuff, see equipment setup
- 7-0 prolene suture, BV1 Taper Point needle (Ethicon, Cat. no. M8703)
- 6-0 silk suture spool (Harvard Apparatus, Cat. No. 723288)
- 4-0 nylon suture, Nurolon RB1 Taper Point needle (Ethicon, Cat. no. C554D)
- Sterile gauze sponges (Fischer Scientific, Cat. No. NC9114188)
- 28G PTFE tubing (Small Parts Inc, Cat. No. STT-28-50)
- 3 × 6' leakproof polyethylene bags (ULINE, Cat. no S-17703)
- Blood gas analysis machine (Siemens, Cat. No. RapidPoint 500)
- Balance scale (Cole Parmer, Cat. No. EW-10000-12)
- Handheld thermocouple thermometer and probe (Cole Parmer, Cat. No. EW-91500-04 and EW-08516-55)

- Male-female luer adapter 1/8"; (Cole Parmer, Cat. No. EW-30800-24)
- Male-male luer adapter (Cole Parmer, Cat. No. EW-45504-72)
- Sterilized 10×10 cm sheet of parafilm wrap (Cole Parmer, Cat No. EW-06720-40)

Reagent Setup

3-OMG loading solution—Aseptically add to a 500 ml bottle of phenol-red free Williams' Medium E, 5 ml of 200 mM L-glutamine (0.292 mg/L), 4 ml of penicillin-streptomycin (5,000 U/mL), 5 mg of hydrocortisone, 5000 U of sodium heparin and 375 U of insulin. Add 19.42 g of 3-O-methyl glucose (3-OMG; 0.2 M) and gently stir to solubilize. The final osmolality of the solution should be 290–310 mOsm. Check the pH of the solution and correct to a pH of 7.35–7.45 using NaHCO₃ base or HCL acid. Filter the solution through 0.22 µm bottle top filters to sterilize. **CRITICAL** Prepare the solution just before use and keep the solution on ice before priming the perfusion system. L-glutamine is not stable in solution and insulin must be kept cold before use.

Supercooling solution—Aseptically add to 1 bag (1 l) of UW solution, 200,000 units of penicillin G, 40 U of insulin and 16 mg of dexamethasone. Solubilize 50 g of polyethylene glycol, mol. mass 35 kDa (PEG 35 kDA) in 1 L (5% w/v). Divide into 100 ml aliquots in 125 ml bottles, filtered through 0.22 µm bottle top filters. **CRITICAL** Supercooling solution can be stored, but must be refrigerated (4 °C) ! **CAUTION** Long-term storage of UW solution results in gradual oxidation of glutathione and must be avoided, or alternatively glutathione can be supplemented.

Recovery solution—The recovery solution differs from the 3-OMG loading solution in the supplementation of 3-OMG and the concentration of insulin. To prepare the recovery solution aseptically add to a 500 ml bottle of phenol-red free Williams' Medium E, 5 ml of 200 mM L-glutamine (0.292 mg/L), 4 ml of penicillin-streptomycin (5,000 U/mL), 5 mg of hydrocortisone, 5000 U of sodium heparin and 1 U of insulin. The final osmolality of the solution should be 290–310 mOsm. Check the pH of the solution and correct to a pH of 7.35–7.45 using NaHCO₃ base or HCL acid. Filter the solution through 0.22 µm bottle top filters to sterilize. **CRITICAL** L-glutamine is not stable in solution and insulin must be kept cold. Prepare the solution just before use and keep the solution on ice before priming the perfusion system.

Warm saline—Heat 100 ml of normal saline to 37 °C in a water bath prior to the liver procurement and transplantation. Warm saline is used to keep tissues moist during the procedure as well as maintain body temperature. Use warm saline to irrigate the abdomen of the recipient before closing.

Equipment Setup

Subnormothermic Machine Perfusion system—The SNMP system consists of a single circulation providing portal venous perfusion through the rat liver and passive outflow of the solution from two sides of the vena cava, into the organ chamber (Fig. 1a–c). An outflow pump returns the perfusion solution to a reservoir bottle. The system can be

opened, stopping recirculation by draining the outflow tubing into a waste container, rather than returning it to the reservoir. An inflow pump drives the solution around various components of the system ending in the liver. Begin the setup by ensuring a clean and stable workplace, preferably a clean lab bench, table top or flow hood. Place a sterile drape on the surface. Attach various ring clamps for the oxygenator, bubble trap and organ chamber to the lab stand and install the components securely in the rings. The bubble trap should be placed at a level higher than the organ chamber. Next, measure lengths of size 16 tubing to run 1) between the perfusion solution reservoir and the oxygenator, through the pump head, 2) from the oxygenator to the bubble trap. Use a 15 cm section of membrane oxygenator tubing to run from the bubble trap to an 18 G catheter (inflow catheter), which can be connected through a 1/8" male-male luer adapter. **CRITICAL** Ensure that the in- and outflow of the bubble trap are connected to the correct side. Attach the pieces of tubing to the system components using 1/8" male-female luer adapters. Suspend the inflow catheter over the organ chamber. Cut the section of oxygenator tubing between the bubble trap and the inflow catheter 2 cm above the catheter and insert a small three-way connector. Attach a 20 cm length of tubing to the third connection running up, to serve as a hydrostatic manometer (Fig. 1d). Fix the gas regulator to a tank of 95% O₂/5% CO₂ gas. **! CAUTION** Tanks must be tightly secured to prevent injury. Run tubing from the tank to the inflow of the oxygenator and confirm adequate movement of air from the oxygenator outflow tubing.

CRITICAL Sterilize the components of the system before assembly.

Supercooling device—A controlled rate chiller is used the supercooling device to control the temperature internally during the supercooling period as well as control the temperature of the machine perfusion system. A second antifreeze circuit of tubing runs from the supercooling device through the jacketed components of the system independent from the perfusion solution circuit (Figure 1a, blue lines). To set this up, run size 24 tubing from 1) the outflow port of the chiller to the organ chamber, 2) the organ chamber to the bubble trap 3) from the bubble trap to the oxygenator and 4) from the oxygenator back to the inflow of the chiller. **CRITICAL** The order of flow is important since minimal equilibration of the antifreeze temperature occurs with the atmosphere, while passing through the tubing. The order reflects priority of cooling, which decreases with distance from the liver. Fill the inner chamber of the supercooling device with antifreeze. Wrap long segments of antifreeze tubing in tube insulator to further minimize temperature equilibration. **! CAUTION** Antifreeze solutions can be toxic, always wear protective equipment and avoid contact of the antifreeze with skin and eyes. Begin recirculation of the antifreeze through the system, filling the tubing and component jackets. Remove air from the system.

Sterile surgical table—In preparation for liver procurement and liver transplantation a clean surgical table must be prepared. It is important to prepare the table with a clear presentation of instruments to maintain sterility and facilitate a smooth procedure. Disinfect the surface with an alcohol spray and cover the surgical field with sterile drapes. Position a heating pad and cover with a sterile drape. Place sterile instruments in preferred locations. Cut sutures to size. Cut a 6 cm section of 28G polyethylene tubing for cannulation of the

bile duct and sterilize. Attach a 100% oxygen tank to the rodent anesthesia machine and fill the machine with isoflurane.

Vascular cuffs—Cuffs, fashioned from catheters, significantly shorten the vascular anastomosis time during transplantation²⁷. Cut a 4 mm section off the catheter and secure one end in a mosquito clamp (Fig. 2a,b). Make a hemicircumferential cut through in the middle of the section and remove one side of the top half by making two cuts inward from the side joining with the hemicircumferential cut (Fig 2c). Remove and discard the loose quarter section. **CRITICAL** Use the cautery to make a shallow full-circle groove half way down the body of the cuff (Fig. 2d). This groove will fixate the suture during cuffing. Two cuffs are required per liver, from a 14 and 16G for the infrahepatic vena cava and portal vein respectively. Sterilize cuffs before use.

Procedure

System priming and rat liver procurement

1 Before beginning the surgical procedure prime the SNMP system.

A. Set the pump speed to 8.0 ml/min

! CAUTION Confirm correct settings on the pump, including direction of flow and tubing size. Load the system with 500 ml of 3-OMG loading solution and allow the solution to recirculate. **CRITICAL** Ensure that the bubble trap is sufficiently filled and the tubing is bubble-free.

B. Set the temperature of the supercooling device to 21 °C and confirm the temperature with the thermocouple.

C. Open the flow of gas (95% O₂ and 5% CO₂) to the oxygenator at 1 l/min.

D. Confirm adequate oxygenation and pH of the solution by drawing a 0.4 ml inflow sample and running it in a blood gas analysis machine. Adjust the pH by titrating NaHCO₃ to correct acidosis and HCL to correct alkalosis.

2 Procure a rat liver in preparation for orthotopic transplantation. A full protocol for Rat liver procurement can be found in the Supplementary Information. Briefly,

A. Ligate branches of the PV and IHVC, the diaphragmatic vein and the hepatic artery.

B. Cannulate the bile duct with a 6 cm section of 28 G tubing.

C. Clamp the IHVC and flush the PV with 10 ml of 3-OMG loading solution (21 °C), interrupted by opening of the SHVC.

D. Excise the liver and cuff the PV and IHVC using the prepared vascular cuffs for fast anastomosis. Flush with an additional 10 ml of 3-OMG loading solution (21 °C).

CRITICAL Monitor anesthesia continuously by assessing the depth of respiration and check for a response to tail or toe pinching every 5 minutes.

3 Weigh the liver before perfusion.

Loading SNMP phase

4 Connect the liver the SNMP system by inserting the 18G catheter on the system into the PV cuff. Begin flow at 8 ml/min. Cover the organ chamber with a section of sterile parafilm wrap.

CRITICAL Closely monitor the pressure during the first minutes of perfusion to ensure that it does not exceed 10 cmH₂O.

? TROUBLESHOOTING

5 Let the outflow drain into a waste container, interrupting the recirculation of the solution. This prevents the recirculation of remaining blood flushing out of the liver.

6 Allow drainage of the bile duct cannula into a microcentrifuge tube for quantification and analysis of bile. Ensure that the bile duct is not kinked or in any way obstructing the outflow of bile.

7 After the first 150 mL of solution has passed through the liver into a waste container, close the tubing and continue recirculation of the remaining 350 ml of solution.

8 In 1 ml/min increments, increase the flow to 12 ml/min after the system has been closed in the previous step. Ensure that the pressure does not exceed 15 cmH₂O.

9 Sample the perfusion solution from the organ bowl every 15 minutes and store samples at – 80 °C for further analysis.

10 Take 0.4 ml samples in a 1 ml syringe from both in- and outflow for blood gas analysis. Inflow samples can be taken from the manometer tube proximal to the PV inflow. Outflow samples can be taken by inserting the syringe with an 18G catheter into the vena cava and gently drawing a sample.

! CAUTION Allow the dead volume in the manometer tube to be renewed to obtain a fresh sample for inflow. While sampling from the vena cava, extreme care must be taken to not injure the endothelium or collapse the venous circulation by pulling up too fast.

11 After 60 minutes of loading SNMP lower the temperature on the controlled rate chiller at a rate of 1 °C min⁻¹. After 17 minutes, when the system has been cooled to 4 °C the liver can be disconnected from the SNMP system.

12 Weigh the liver.

Supercooling Phase

13 Flush the liver through the PV with 10 ml of supercooling solution (4 °C) over the course of 120 seconds (5 ml/min).

14 Place the liver in a sealable sterile bag filled with 75 mL of supercooling solution. Remove air from the bag before fully sealing (Fig. 1e).

CRITICAL Ensure that the bag is waterproof by very gently squeezing the bag and checking for leaks.

15 Transfer the bag to the controlled-rate chiller, which is still set to 4 °C. Make sure the bag is fully submerged in the antifreeze and that the bag is not in contact with the sides of the device.

16 Gradually lower the temperature at a rate of 0.1 °C min⁻¹. After 100 minutes the temperature will reach the supercooling set point of -6 °C. Confirm the temperature inside the chiller with a thermocouple.

17 Leave the liver in the controlled rat chiller for 72–96 hours. Regularly confirm that the temperature inside the chiller remains stable at -6 °C.

Recovery phase

18 At the end of the supercooling period increase the temperature of the supercooling device to 4 °C at a rate of 0.1 °C min⁻¹, while the liver remains inside the device.

19 When the temperature has reached 0 °C begin to prepare the SNMP system similarly to the procedure outlined in **step 3**, but priming with the tubing with recovery solution instead of 3–OMG loading solution, and a flow of 3 ml/min. The temperature of the system will assume the settings of the supercooling device.

20 When the temperature has reached 4 °C open the supercooling device and remove the sealed bag. Open the bag and weigh the liver.

? TROUBLESHOOTING

21 Flush the liver with 10 ml of recovery solution (4 °C) at a rate of 3 ml/min.

22 Connect the liver to the SNMP system. Cover the organ chamber with a section of sterile parafilm wrap.

CRITICAL Closely monitor the pressure during the first minutes of perfusion to ensure that it does not exceed 5 cmH₂O at the beginning of perfusion. If the pressure is too high, the flow rate can be reduced.

? TROUBLESHOOTING

23 Allow drainage of the bile duct cannula into a microcentrifuge tube for quantification and analysis of bile.

24 Begin increasing the temperature to 21 °C at a rate of 1 °C min⁻¹.

25 Increase the flow by 1 ml/min every three minutes to a maximum of 12 ml/min.

CRITICAL The pressure that is tolerated by the liver greatly depends on the temperature and should be controlled by regulating the flow. Fig. 3 illustrates the temperature and flow regimen and recommended maximum pressures during the first 30 minutes of recovery SNMP. If the pressure permits, the flow can be increased to a maximum of 12 ml/min.

26 Follow **steps 7, 9 and 10**.

27 After 2.5 hours of perfusion, begin the recipient hepatectomy. Administer 0.05 mg/kg buprenorphine before beginning. Briefly, isolate the right and left PV branches, ligate the bile duct, diaphragmatic vein, adrenal vein, lumbar venous plexus and the hepatic artery.

28 After 3 hours of perfusion, disconnect from the SNMP system and weigh the liver.

29 Prior to implantation, flush the liver with 10 ml of Lactated Ringer's solution (4 °C) (10 ml/min). ! **CAUTION** An adequate flush of the liver is required to remove the hyperkalemic supercooling solution from the liver. Insufficient flushing can lead to potassium-induced cardiac arrhythmias on reperfusion.

30 Orthotopically transplant the liver into a weight-matched syngeneic recipient. A detailed protocol for orthotopic rat liver transplantation can be found in the Supplementary Information. Continue where left off in step 27 and complete the recipient hepatectomy and following the following key steps.

- A.** Tie of the left and right PV branches, marking the beginning of the anhepatic time.
- B.** Implant the donor liver, placing a sutured anastomosis of the SHVC, cuffed anastomosis of the PV and IHVC and cannulating the recipient bile duct.

CRITICAL Monitor anesthesia continuously by assessing the depth of respiration and check for a response to tail or toe pinching every 5 minutes.

31 Allow the animal to recover under continuous monitoring under a heat lamp and perform routine health checks as mandated by an approved animal protocol.

CRITICAL Provide adequate analgesia for a minimum of 72 hours post-transplantation. A regimen of buprenorphine 0.05 mg/kg subcutaneously, every 12 hours is recommended.

32 Monitor the animal for a desired follow-up period. Routine health and liver function tests include inspecting for clinical signs of liver failure (e.g. jaundice, scratching), weight measurements and blood tests

Timing

Rat liver procurement, steps 1–3 and supplementary steps 1–19: 90–120 minutes

Loading SNMP, steps 4–12: 80 minutes

Supercooling, steps 13–17: 3–4 days

Recovery SNMP, steps 18–29: 5 hours

Orthotopic liver transplantation, steps 30–32 and supplementary steps 20–36: 120–150 minutes + follow-up period

Static cold storage control, Box 1, steps 1–5: 1–4 days + follow-up period

Troubleshooting

(TABLE 1)

Anticipated Results

While the clinical standard for liver preservation secludes the liver in a closed box between procurement and transplantation, supercooling involves a number of steps that allow direct and indirect observation of the liver and liver function. Besides its purpose for loading and recovery of the liver, machine perfusion has the additional benefit of enabling functional testing of the organ pre-transplantation. Particularly post-preservation, the viability of the organ, reflected as perfusion parameters, can be highly valuable in assessing whether a liver is suitable for transplantation.

Real-time perfusion measurements

While running the perfusion, various perfusion parameters can be measured and observed in real time, including flow and pressure and bile production. Vascular resistance can be approximated from the quotient of pressure over flow and reflect the ease of perfusion. Fresh rat livers with minimal preservation injury should exhibit very similar perfusion hydrodynamics. In our experiments, after 30 min of perfusion and once a flow rate of 10 ml/min has been reached the resistance remains very stable throughout the remainder of perfusion and pressures between 7.5–10 are typically observed (resistance 0.75–1.0)(Fig. 4a). Particularly in fresh livers, pressures that exceed the maximum values outlined in figure 5 are cause for technical troubleshooting. In fresh rat livers, bile production should be seen within 15 minutes of perfusion and will increase over the first hour, after which a steady bile production will be seen. Increasing preservation injury will result in an absolute change in perfusion parameters as well increased variation between livers of a single group. Typically, an increase in resistance and decrease in bile production will be observed with longer cold ischemic time (Fig. 4b, c).

Transplant survival

Successful long-term survival following transplantation is the primary endpoint of preservation research. The maximum permissible warm and cold ischemic times have been established in various models, following both warm ischemia²¹, cold ischemia²⁰ and the combination thereof³¹. The SCS control group is important, since it represents the clinical standard. Using UW solution as a cold preservation solution in the SCS group survival is 100% up to 24 hours of preservation (Fig. 4d). When we extend the preservation time to 48 hours post-transplantation survival drops to 50%. Further extending cold preservation time to 72 hours, yielded no survival in our experience. Successful supercooling can be expected to prolong preservation time significantly. By following this protocol we were able to transplant supercooled livers with 58% long-term survival after 96 hours of preservation, while 100% survival was limited to 72 hours. Directly following transplantation, recipients of supercooled livers or livers preserved for extended SCS periods (>12 h) will recover less quickly from the surgery, more slowly regaining consciousness and mobility. Moreover, the recipients of 96-h supercooled livers lose weight during the first week after transplantation

(Fig. 4e). Weight should begin to increase as the appetite of the animal returns, but remains less than control animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding from the National Institutes of Health (R00DK080942, R01DK096075, R01EB008678) and the Shriners Hospitals for Children are gratefully acknowledged.

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Box 1**Static Cold Storage (SCS) Controls**

Static cold storage remains the clinical standard for liver preservation and is the most relevant control group for preservation studies. A short period of SCS can be used to verify the surgical technique, as well as determine the maximum SCS preservation time. In our model, we found viable preservation after SCS to be limited to 24 hours (see **ANTICIPATED RESULTS** section).

Procedure

1. Procure the rat liver as described in **steps 2 A–D** and the full procedure in the Supplementary Information. Instead of flushing with 3-OMG loading solution, use ice-cold UW solution.
2. After excising and cuffing the liver, flush the PV with an additional 10 ml of ice-cold UW solution over the course of 120 seconds (5 ml/min) and weigh the liver.
3. Store the liver in a styrofoam ice-box for 24–96 hours.

CRITICAL Ice will need to be replaced during longer preservation periods.

4. Prior to implantation, flush the liver with 10 ml of Lactated Ringer's solution (4 °C) at a rate of 5 ml/min.

CRITICAL An adequate flush of the liver is required to remove the hyperkalemic UW solution from the liver. Insufficient flushing can lead to potassium-induced cardiac arrhythmias on reperfusion.

5. Orthotopically transplant the liver and monitor the animal following **steps 30–32** and the full procedure in the Supplementary Information.

Figure 1.

Rat liver subnormothermic machine perfusion system. Illustration of various components (a); black lines represent the perfusate circuit; blue lines the antifreeze circuit running through the jackets of the organ chamber, bubble trap and oxygenator; green line the bile duct drain cannula. Red * indicates where the circuit can be interrupted to open the system and stop recirculation. Image of the SNMP system (b) and schematic representation (c). Section of tubing that runs between the bubble trap and the liver portal vein, interrupted to include an section of tubing to act as a hydrostatic manometer (d). Liver in a supercooling bag (e).

Figure 2.

Fashioning of vascular anastomosis cuffs. A 4 mm section is cut from a 16 and 14G catheter (**a**, **b**). A quarter section is removed from the piece, leaving a tail and body of the cuff (**c**). A groove is made fully around the body for proper fixation of the suture (**d**).

Figure 3.

Flow and pressure regimen for the first 30 minutes of SNMP recovery. Temperature is slowly increased in the first 17 minutes to 21 °C (grey), while the flow is increased in parallel to a maximum of 12 ml/min (green). The pressure should not exceed the temperature-dependent maximum (black)

Figure 4.

Real time observable perfusion parameters (**a–c**) and transplantation outcome (**d, e**).

Vascular resistance of fresh livers and liver stored for increasing durations of static cold storage (SCS) (**a**). Bile production in fresh livers and liver preserved by SCS and supercooling for 72 or 96 hours (**b**), vascular resistance of livers preserved by supercooling for 72 or 96 hours (**c**). Long-term (30 days) survival for various groups (**d**). Supercooling controls include all groups in which a component of the supercooling protocol was omitted. Body weight of recipients of fresh livers and livers supercooled for 96 hours (**e**).

Table 1

Troubleshooting table.

STEP	PROBLEM	POSSIBLE REASON	SOLUTION
26, 44	High perfusion pressure	Kinked or twisted inflow cannula or portal vein	Disconnect the liver and check that the vein is torsed. Make sure the PV cuff is correctly in place. Reconnect the liver. If needed, confirm patency of the cuff and PV by gently flushing with perfusate.
		Pump setting too high	Confirm that the pump is set to the correct flow rate and tubing size.
		Air emboli	If air is likely, an attempt can be made to remove the air by disconnecting the liver and retrograde flushing through the vena cava.
			Clear any air from the tubing and make sure bubble trap is full.
		Outflow obstruction	Inspect the vena cava and remove any potential blockages
	Low perfusion pressure	Leak in the PV	Inspect the PV for holes and ensure the cuff is correctly placed
		Pump setting too high	Confirm that the pump is set to the correct flow rate and tubing size.
		Opening in the circuit	Check the tubing between the inflow pump and the liver. Ensure that the bubble trap openings are sealed.
42	Liver frozen	Temperature too low	Check the temperature of the antifreeze in the supercooling device.
		Contact with sides	Prevent the liver bag from coming in contact with the sides of the device during supercooling
		Imperfections in bag/solution	Remove air from the bag and make sure there are no unnecessary focus points for nucleation