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Sub-zero non frozen preservation of human livers in the supercooled state

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Abstract

Subzero preservation of human organs has been an elusive goal for many decades. The major complication hindering successful subzero preservation is the formation of ice at temperatures below freezing. Supercooling, or subzero non-freezing, preservation completely avoids ice formation at subzero temperatures. We previously showed that rat livers can be viably preserved three times longer by supercooling compared to hypothermic preservation at +4°C. Scalability of supercooling preservation to human organs was intrinsically limited due to volume dependent stochastic ice formation at subzero temperatures however we adapted the rat preservation approach so it could be applied to larger organs. Here we describe a supercooling protocol that averts freezing of human livers by minimizing air liquid interfaces as favourable sites of ice nucleation and preconditioning with cryoprotective agents to depress the freezing point of the liver tissue. Human livers are homogeneously preconditioned during multiple machine perfusion steps at different temperatures. Including preparations, the protocol takes 31 hours to complete. Using this protocol human livers can be stored free of ice at –4°C which substantially extends the *ex vivo*

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AUTHOR CONTRIBUTIONS

R.J.V., S.N.T. and K.U. conceived and designed the supercooling protocol for human livers; R.J.V., S.N.T. and S.O. designed and constructed the perfusion and supercooling system; R.J.V., S.N.T., P.B., S.N., S.E.J.C. and S.O. performed supercooling experiment and acquisition of experimental data; R.J.V., S.N.T., E.O.A.H., H.Y., M.L.Y., J.F.M., M.T., and K.U. analyzed and interpreted data; R.J.V. wrote the manuscript; R.J.V., S.N.T., E.O.A.H., T.M.G., M.L.Y., J.F.M., M.T., H.Y. and K.U. participated in critical revision of the manuscript for intellectual content; R.J.V., S.N.T. and K.U. performed statistical analysis. All authors contributed to the preparation of the manuscript.

The authors declare competing financial interests. Drs. Toner, Yarmush, de Vries, Uygun and Tessier have provisional patent applications relevant to this study. Dr. Uygun has a financial interest in Organ Solutions, a company focused on developing organ preservation technology. Author's interests are managed by the MGH and Partners HealthCare in accordance with their conflict of interest policies.

life of the organ. To our knowledge, this is the first detailed protocol describing how to perform subzero preservation of human organs.

EDITORIAL SUMMARY:

The formation of ice at temperatures below freezing has hindered attempts at subzero organ preservation prior to transplantation. In this protocol human livers are preconditioned with cryoprotective agents during machine perfusion and then supercooled to avoid ice formation.

TWEET

Prolonged preservation at subzero temperatures of human organs prior to transplant.

COVER TEASER

Supercool transplant preparation.

INTRODUCTION

The limited time that organs can be kept viable outside the body puts huge constraints on transplantation. The short preservation durations result in organ discard, unscheduled emergency surgery, suboptimal donor-recipient matching, and prevents long distance organ sharing^{1–3}. In addition, only the highest quality organs can withstand the injury that occurs during hypothermic preservation, which limits the supply of donor organs. The short preservation times also form a bottleneck for the clinical realization of new breakthroughs such as immune tolerance induction that require enough time to precondition the recipient before transplantation. Given the current donor organ shortage, these preservation restrictions indirectly result in death of patients awaiting transplantation.

In current clinical practice, a human donor liver is kept up to 12 hours outside the body during which time the organ is stored on ice in a preservative solution at 4 °C^{1,4}. Although deterioration of the grafts is slowed because metabolic processes are depressed by hypothermia, metabolism is not completely halted^{3,4}. As vital cell processes slowly continue during storage, every hour of cold ischemia contributes to negative transplant outcomes⁵. By reducing the storage temperature to temperatures below freezing, the metabolic rate can be further depressed, and the preservation duration substantially extended. A 10°C reduction in temperature slows down the metabolic rate by a factor 2 to 3³. However, ice formation at subzero temperatures can be extremely injurious to organs and has made subzero organ preservation an elusive goal for decades^{3,4}.

One advantage of supercooling is that the liquid phase of water is maintained below freezing which prevents injury from ice formation. After initial success using supercooling to preserve primary hepatocytes⁶, our group applied supercooling to subzero preservation of rat livers^{7,8}. In addition to ice free storage at subzero temperatures, the supercooling preservation protocol relied on machine perfusion to recover the livers from the supercooled storage. Also, cryoprotective agents were used to prevent ice formation during supercooling and alleviate hypodermic injury to cell membranes during supercooling. This combination

successfully tripled the preservation duration of rat livers which was confirmed by 100% long-term survival after orthotopic transplantation.

However, ice formation during supercooling is a stochastic process that is dependent on volume and this complicates the supercooling of larger organs such as those found in humans⁹. Freezing of supercooled water at high subzero temperatures (above -20°C) is initiated by a heterogeneous ice nucleation event whereby the ice crystal grows throughout the entire volume⁹. The chance that this nucleation event happens somewhere in the supercooled volume is higher for larger volumes, longer durations and lower storage temperatures. Since human livers are about 200 times larger than rat livers, this substantially increases the likelihood that freezing will occur during supercooled storage.

Additionally, for successful supercooling preservation, livers must be homogeneously preconditioned with agents that protect it from freezing and hypothermic injury. This is much harder to achieve in large sized organs. To develop an improved supercooling protocol that averts freezing of human livers, we minimized favourable sites of ice nucleation and composed a preservative cocktail with additional cryoprotective agents that prevent ice formation during subzero storage. To precondition the human livers with the preservative cocktail, we developed a multi-temperature perfusion modality for homogenous loading of the protective agents in the large size organs. Using this protocol, human livers can be stored free of ice at -4°C , which substantially extends the *ex vivo* life of the graft to 27 hours.^{10,11} Here we present a detailed protocol for this preservation method. To our knowledge this is the first protocol for successful subzero human organ preservation.

Comparison with other organ preservation methods

As machine perfusion plays an essential role in the preconditioning and recovery of grafts from supercooled storage, the latest developments in machine perfusion technology impact supercooling preservation. Machine perfusion has seen a remarkable development over the last two decades which has resulted in multiple perfusion modalities that are currently under investigation in clinical trials.

Growing clinical evidence demonstrates that both hypothermic machine perfusion (HMP)^{12–14} and normothermic machine perfusion (NMP)^{15–19} are safe and effective, and enables liver grafts of lower quality to be used due to the reduction of ischemia reperfusion injury. . An additional important benefit of normothermic perfusion temperatures is the opportunity for *ex vivo* assessment of liver viability because the liver is metabolically active during perfusion. Gradual rewarming from hypothermic to normothermic temperatures is also under clinical investigation in efforts to combine benefits of HMP with viability assessment during NMP^{20,21}. Subnormothermic machine perfusion (SNMP) also aims to provide viability assessment at a lower perfusion temperature (21°C). It is currently in pre-clinical phase of development and has been shown to provide detailed viability assessment^{22–24}. Besides the difference in temperature, an important distinction between perfusion strategies is in the timing of the perfusion during preservation. Machine perfusion is either performed at the end of preservation to recover grafts from ischemia or started directly after procurement to replace the hypothermic preservation period on ice²⁵. The continuous application of NMP from procurement to transplantation showed it is possible

to extend the preservation duration to approximately 24 hours in a recent clinical trial¹⁹. However, the increasing complexity and costs of sustaining *ex vivo* organ metabolism may limit the (cost) effectiveness of extended perfusions if organs can also be stored in suspended animation at subzero temperatures^{1,3}.

Classical cryopreservation (i.e. below -80°C) is the most explored form of subzero biopreservation. The storage temperatures during classic cryopreservation are much lower than the subzero storage temperatures during supercooling^{1,26}. Therefore, the metabolic rate is more depressed and theoretically storage durations in the order of years could be achieved²⁷. However, this lower temperature comes at the cost of ice injury, thermomechanical stresses, and toxicity of the required cryoprotective agents^{4,27–30}. Together these have seriously limited the success of organ cryopreservation in animal models. Nonetheless, parts of this protocol may be adapted to further improve classical cryopreservation of human organs such as the preconditioning with cryoprotective agents during machine perfusion, or the experimental design that controls for the substantial viability differences between human grafts from different donors.

Experimental design

Organs.—Experimental research that involves donor organs that are rejected for transplantation is unique as it enables real human organs to be tested in a preclinical setting. However, there are several aspects that challenge the experimental design when working with these organs. It is important to consider and account for substantial differences in quality of the grafts that are available for research. In addition, as a result of ethical and logistical constraints, the organs endure injury during procurement, preservation, and transportation to the research facility. To overcome these challenges, we leveraged machine perfusion to partially recover the grafts from this injury and to provide a viability measurement at the start and end of the preservation protocol. This results in a well-controlled experimental design that provides an internal control for each replicate, allows powerful paired statistical analysis, and therefore can be used to account for the unavoidable donor-to-donor variability.

Preconditioning.—The supercooling protocol for human livers requires multiple steps, as shown in Figure 1a. On arrival, the first stage (marked 1 on Fig. 1a) is to recover the grafts from the incurred warm and cold ischemia during 3 hours of subnormothermic machine perfusion (SNMP). Viability parameters can be measured during this period of machine perfusion to assess the baseline viability of the graft. (Fig. 1a; 2) During the last hour of perfusion, the graft is preconditioned with 3-O-methyl-d-glucose (3-OMG). 3-OMG is a non-metabolizable glucose analogue that accumulates intracellularly and helps to prevent intracellular ice formation⁷. (Fig. 1a; 3) At the end of SNMP, the perfusion temperature is gradually lowered, and the livers are further preconditioned during hypothermic machine perfusion (HMP) with University of Wisconsin solution (UW) that is supplemented with polyethylene glycol (PEG), trehalose dihydrate and glycerol. PEG and trehalose help to prevent extracellular ice formation and stabilize cell membranes at sub-zero temperatures^{31,32}. Glycerol is freely permeable over the cell membranes and provides both intra- and extracellular freeze protection.

Supercooling.—(Fig. 1a; 4) Following preconditioning with the protective agents, the grafts are placed in a storage bag from which all air is removed. Minimizing air-liquid interfaces is critical since they are favourable sites of ice nucleation during supercooling⁹. (Fig. 1a; 5) The graft is then supercooled and stored free of ice at -4°C for 20 hours.

Rewarming.—(Fig. 1a; 6) After supercooling, the protective agents are washed out during a brief period of hypothermic machine perfusion to minimize osmotic injury. This is followed by gradual rewarming to subnormothermic machine perfusion. (Fig. 1a; 7) Next, the livers are recovered from supercooled storage during SNMP. The conditions during SNMP are identical to pre-supercooling, except for the addition of Trolox to the perfusate, and the absence of 3-OMG and cooling at the end of SNMP. Viability parameters can be collected during SNMP after supercooling and compared to their baseline pre-supercooling values.

Limitations and adaptations of the protocol

The supercooled storage duration of 20 hours was estimated based on our previous results in rat livers. These results showed we could safely triple the preservation duration from 24 hours with hypothermic preservation to 72 hours with a combination of supercooled storage and recovery by machine perfusion². Because the clinically used maximum hypothermic duration at our center is 9 hours, we estimated that the total protocol could be 27 hours. As the protocol includes 7 hours of perfusion, we arrived at a supercooling duration of 20 hours. However, the supercooling protocol was applied on marginal human livers that were acquired after they were rejected for transplantation. Therefore, these livers have already endured cold ischemic injury during conventional hypothermic preservation on ice. This necessitated an additional SNMP phase prior to preconditioning to recover energy reserves after hypothermic preservation before these grafts underwent supercooling preservation. This recovery phase can most likely be omitted when supercooling preservation is initiated directly after procurement, as schematically depicted in Figure 1b. Additionally, we anticipate that the duration of supercooled storage can be increased when grafts are directly supercooled after preservation since there would be no injury from prior conventional hypothermic preservation. This will result in a higher baseline viability at the start of the protocol and may enable a prolonged supercooled storage duration.

To initiate supercooling at the donor hospital, the supercooling chiller will have to be scaled down to ease transportation. Also, vibrations may influence ice formation during transport whilst the organ is supercooled; this will have to be evaluated and could potentially be eliminated with adequate suspension. Additionally, adaptations to the machine perfusion system are required to perform the preconditioning off-site. In this capacity, a great deal can be learnt from other commercial liver machine perfusion devices that are clinically used for HMP and NMP^{12–19}. Hypothermic or normothermic perfusion – or a combination in the form of gradual rewarming – may be tested as an alternative to the SNMP recovery phase after supercooling to potentially improve the supercooling protocol.

Although this protocol has been validated in an *ex vivo* model with human livers, this model can only suggest the adequacy of supercooling preservation. Therefore, before clinical

transplantation of supercooled human livers, the safety of this protocol must be established in an appropriate large-animal study with long term follow up. This protocol can be adapted to swine and potentially to non human primate livers. However, we note that different volumes of the loading solutions in relation to the volume of the organ may change osmotic shift during preconditioning and alter freezing behavior of the liver tissue³³. Therefore, we recommend scaling the volumes of the preconditioning and storage solutions to the size of the organs. Because freezing during supercooling is dependent on volume and the liver is the largest solid organ in the human body, this protocol could likely be adapted for subzero preservation of other solid organs such as kidneys, or hearts. However, it would be important to evaluate susceptibility of the specific tissues to hypothermic injury and CPA toxicity. Additionally, the preconditioning perfusion will have to be modified to account for the absence of a portal venous system that is unique to the liver.

MATERIALS

Reagents

Human liver [CAUTION: Approval of the institutional review board (IRB) must be obtained before any experiments involving human organs are performed. All research involving human organs should be in line with the declaration of Helsinki and the declaration of Istanbul. Herein, this protocol was approved by the IRB at Massachusetts General Hospital and informed consent was obtained from the donors or their families by the organ procurement organizations (OPO) New England Donor Services Bank (NEDS, Waltham, MA, USA) and LiveOn NY (New York, NY, USA).

Williams' medium E (Sigma-Aldrich, cat. no. W4125)

Sodium bicarbonate (Sigma-Aldrich, cat. no. S5761)

Humulin (insulin) (Eli Lilly, cat. no. Humulin R U-100)

Dexamethasone, water-soluble (Sigma-Aldrich, cat. no. D2915) [CAUTION: The high purity dexamethasone powder can cause local and systemic immunosuppression related health issues. Avoid direct contact with the powder and wear protective gloves, eyewear, and a mask when handling.]

Sodium heparin (MGH Pharmacy, cat. no. 7721000)

Penicillin Streptomycin (Invitrogen, cat. no. 15140163)

25% human albumin (MGH Pharmacy, cat. no. 2221402909)

Polyethylene glycol 35,000 (Sigma-Aldrich, cat. no. 81310)

Trolox (Cayman Chemical Company, cat. no. 10011659)

Glycerol (Thermo Fisher Scientific, cat. no. BP229)

D-(+)- Trehalose dihydrate (Sigma-Aldrich, cat. no. 90210)

Belzer University of Wisconsin cold storage solution (Bridge to Life, cat. no. BUW-001)

Lactated Ringer's solution (Baxter, cat. no. 2B2324X)
Carbogen gas tank, 95% O₂/5% CO₂ (Airgas, cat. no. ZO2OX9522000043)
Propylene glycol refrigerant (Grainger, cat. no. 20LP86)
Mineral oil (Sigma-Aldrich, cat. no. 330760–1L)
3-O-methyl-d-glucose (Sigma-Aldrich, cat. no. M4879–100G)
Mucosol Universal Detergent (Sigma-Aldrich, cat. no. Z637181–2L) [CAUTION:
The high concentration liquid detergent can cause skin and eye injury. Avoid direct
contact and wear protective gloves and eyewear during handling.]

Equipment

Graduated cylinder 2 liter (Fisher Scientific, cat. no. 08–572H)
Erlenmeyer flask (Fisher Scientific, cat. no. 10–040)
Bottle-top filter, 0.22- μ m pore size, 500-ml capacity (Fisher Scientific, cat. no. 09761111)
Steri-drape Isolation Bag (Medline, cat. no. MMM1003)
Sterile drape (Fisher Scientific, cat. no. 19–823)
Inspire 6 Membrane Oxygenator/Heat exchanger (LivaNova, cat. no. 050700)
OTC Series Air Cooled Chiller (OptiTemp, cat. no. O002639AD)
Digital Heating Immersion circulator (Cole Parmer, cat. no. UX-12135–00)
Bubble Trap F/ Porcine (Radnoti, cat. no. 140139)
Perfusion chamber (Radnoti, custom part, see Supplementary Figure S1)
Lab stand (Radnoti, cat. no. 159951–1)
Three-Prong Extension Clamp (Fisher Scientific, cat. no. 05–769)
Connector between lab stand and extension clamp
Needles injection site (quosina, cat. no. 80105)
Masterflex L/S Digital Pump System with Easy-Load® II Pump Head, 600 rpm,
115/230 VAC (Cole-Parmer, cat. no. EW-77921–75)
Masterflex Polyethylene TEE-Connector, L/S (Cole-Parmer, cat. no. EW-30613–13)
Burkle Barked Cross Connector, PVDF, 7–9 Tubing (Cole-Parmer, cat. no. EW-06044–31)
Masterflex Barbed Y-Connector, PVDF (cat. no. EW-30614–24)
Masterflex Polyethylene Straight Connector, L/S (Cole-Parmer, cat. no. SI-30612013)
PendoTech Adapter Tee Hose Barb with Male Luer Lock Fitting (Cole-Parmer, cat. no. UX-19406–34)

Stopcock with 1-way Luer Connection, Male Lock (Cole-Parmer, cat. no. EW-30600-00)

Stopcock with 4-way Luer Connection, Male Lock (Cole-Parmer, cat. no. EW-30600-04)

High Pressure Tubing with Luer Ends (Cole-Parmer, cat. no. EW-30526-16)

Luer Fitting Kit (Cole-Parmer, cat. no. EW-45511-00)

Portable Pressure Monitor (Catamount Research and Development, cat. no. PM-P-1)

Pressure Transducer (Catamount Research and Development, cat. no. PT-F)

Steel bowls (Thomas Scientific, cat. no. BMX3000)

Masterflex Pump Tubing, L/S 24 (Cole-Parmer, cat. no. EW-96419-24)

Masterflex Pump Tubing, L/S 36 (Cole-Parmer, cat. no. EW-96419-36)

Masterflex Pump Head for L/S 24 tubing (Cole-Parmer, cat. no. EW-07024-20)

Syringes, 3 ml (Fisher Scientific, cat. no. 14-823)

Syringes, 60 ml (Fisher Scientific, cat. no. 13-689-8)

Tubing clamps (Cole-Parmer, cat. no. UX-06833-00)

Specialty gas regulator (Airgas, cat. no. Y11244D580)

Nylon wire (Uline, cat. no. S-12867)

Tube insulator, Thermacel (Grainger, cat. no. 2CKE7)

Stainless steel solution basin (PolarWare, cat. no. 136)

IV pole (Grainger, cat. no. 23YH32)

IV administration set (Medsource, cat. no. MS-83250)

Feeding tube 6fr (Vygon, cat. no. 362.062)

Humidity temperature meter (Omega, cat. no. HH314A)

Bucket (Thermo-Scientific 7012-0140PK)

2-0 Silk Black Sutures (eSutures, cat. no. SA75H)

5-0 Prolene Sutures (eSutures, cat. no. 8713H)

Perfusion cannula 25 Fr straight/curved (Organ Assist, cat. no. 11.01.519/11.01.520)

Perfusion cannula 10/12 Fr (Organ Assist, cat. no. 05.01.504/05.01.504)

Metzenbaum scissors (Roboz Surgical Instrument, cat. no. RS-6871SC)

Mayo scissors (Roboz Surgical Instrument, cat. no. RS-6967)

DeBakey Forceps (Roboz Surgical Instrument, cat. no. RS-7561)

Acorn tip vessel cannula 4 mm (Medtronic, cat. no. 30005)

Sterling probe 8" 2mm tips (Roboz Surgical Instrument, cat. no. RS-9490)

Sterile Gauze Sponges (Fisher-Scientific, cat. no. 22037902)

REAGENT SETUP

Pre- and post-supercooling recovery perfusate

Precisely mark all glass ware at 1-liter increments using an accurate graduated cylinder as the standard marks on large glassware may be inaccurate. Prepare 4 liters of Williams' medium E with ultra-pure (milli-Q) water in accordance with the manufacturer's instructions. Take 1 liter and set aside before aseptically adding 40 U of regular insulin, 32 mg of dexamethasone, 20,000 units of sodium heparin, 32 ml of penicillin streptomycin, 200 ml of 25% human albumin, and 80 g of 35kDa polyethylene glycol. Directly start stirring after adding polyethylene glycol because the flakes clump together otherwise and take a long time to dissolve. We prefer to use a strong stir plate with a heavy 5cm stir bar. When preparing the post-supercooling solution also add 2 g of Trolox but omit this for the pre-supercooling solution. Stir until all additives are dissolved and fill the flask back up to the 4-liter mark using the remaining Williams' medium E. Sterile filter the solution using a 0.22-micron filter into another sterile flask. Adjust the pH to 7.35 – 7.45 with NaHCO₃ or HCL. [CRITICAL: L-glutamine in Williams medium E degrades in solution. Therefore, we advise to make the perfusate fresh or alternatively substitute L-glutamine when the perfusate is made more than 24 hours before use.] [CRITICAL: check commercial expiry dates of all reagents before use.]

Loading solution

Aseptically add 40 U of regular insulin, 8 mg of dexamethasone 50 g of 35 kDa polyethylene glycol, 50 ml of glycerol, and 37.83 g of D-(+)-trehalose dihydrate to 800 ml of University of Wisconsin Solution. Stir until all additives are dissolved and exactly fill the flask to the 1-liter mark with University of Wisconsin Solution. Sterile filter the solution using a 0.22-micron filter into another sterile 1-liter flask. Adjust the pH to 7.35 – 7.45 with NaHCO₃ or HCL. [CRITICAL: solution must be kept at 4 °C during preparation as glutathione in University of Wisconsin Solution may oxidize at higher temperatures. The loading solution can be stored up to 90 d, but must be refrigerated at 4 °C] [CRITICAL: check commercial expiry dates of all reagents before use.]

Storage solution

Aseptically add 120 U of regular insulin, 24 mg of dexamethasone 150 g of 35 kDa polyethylene glycol, 300 ml of glycerol, and 113.49 g of D-(+)-trehalose dihydrate to 2.5 liters of University of Wisconsin Solution. Stir until all additives are dissolved and exactly fill the flask to the 3-liter mark with University of Wisconsin Solution. Sterile filter the solution using a 0.22-micron filter into another sterile 1-liter flask. Adjust the pH to 7.35 – 7.45 with NaHCO₃ or HCL. [CRITICAL: solution must be kept at 4 °C during preparation. The storage solution can be stored up to 90 d, but must be refrigerated at 4 °C] [CRITICAL: check commercial expiry dates of all reagents before use.]

Unloading solution

Prepare 1 liter of Williams' medium E with ultra-pure (milli-Q) water in accordance with the manufacturer's instructions. Take 200 ml aside and aseptically add 10 U of regular insulin, 8 mg of dexamethasone, 8 ml of penicillin streptomycin, 50 g of 35 kDa polyethylene glycol, 500 mg of Trolox, 50 ml of 25% human albumin, 50 ml of glycerol, 37.83 g D-(+)-trehalose dihydrate. Stir until all additives are dissolved and fill the flask back up to the 1-liter mark using the remaining 200 ml Williams' medium E. Adjust the pH to 7.35 – 7.45 with NaHCO_3 or HCL. [CRITICAL: L-glutamine in Williams medium E degrades in solution. Therefore, we advise to make the unloading solution fresh or alternatively substitute L-glutamine when the perfusate was made more than 24 hours before use.] [CRITICAL: check commercial expiry dates of all reagents before use.]

EQUIPMENT SETUP

Perfusion and supercooling system:

The perfusion system provides a dual, non-pulsatile, temperature and pressure-controlled circulation and is symmetrical for the portal vein and hepatic artery. The inferior vena cava drains freely into the perfusion chamber from where the perfusate circulates back to the portal vein and hepatic artery through a flow diverter, roller pump, heat exchanger, oxygenator, sample port, and pressure sensor. The flow diverter enables switching from recirculation to single-pass perfusion and has a perfusate inflow and a drain which allows changing perfusates during perfusion. The system has a separate circuit for temperature regulation and glassware are jacketed. Follow the steps below to build the perfusion system, as shown in Figure 2a.

Setup of perfusion system components

1. Ensure you have a level and stable lab bench that is situated in a clean room. [CRITICAL: ensure the room is certified to handle human organs.]
2. Cover the surface of the bench with a sterile drape.
3. Place a lab stand on the bench and firmly secure the two oxygenators/heat exchangers and the two jacketed glass bubble traps to the stand using three pronged clamps. [CAUTION: ensure the lab stand is balanced. The components are heavy when filled with perfusate and refrigerant which can tip over the stand.]
4. Place the two roller pumps on both sides of the stand and position the jacketed glass perfusion bowl in front. [CRITICAL: Use clean and sterile components to build the perfusion system. Between use, clean the non-disposable components such as the glassware with 2 % (v/v) Mucosol detergent solution, then flush thoroughly with milliQ water and autoclave.] [CAUTION: handle the fragile glassware gently during assembly and cleaning.]

Setup of the flow diverter

5. To create the flow diverter (as shown in Figure 2b) slide two 7 cm sections of size 24 tubing over opposite sides of a barbed T-fitting.
6. Slide a Dura-clamp over one of the tubing sections.
7. Slide a 150 cm tubing section with a Dura-clamp over the perpendicular side of the T-fitting - this forms the perfusate drain off the system.
8. Connect a barbed cross-fitting to one of the 7cm tubing section with the Dura-clamp.
9. Slide another 7cm tubing section with a Dura-clamp over the opposite side of the cross-fitting and slide the other side over a straight barbed fitting with a Luer-lock sampling port.
10. To finish the flow diverter, slide another 150 cm tubing section over the other side of this straight fitting with sampling port - this forms the inflow of perfusate to the system.

Connection of the perfusion system components

11. Attach the bare 7cm tubing section of the flow diverter to the outlet of the perfusion chamber. [CAUTION: handle the glass components with care as the in and outflow ports can break easily]
12. For both the portal and arterial circulation, run an 80 cm section of tubing from the two exposed barbed fittings of the flow diverter through the pump head to the inlet of the oxygenator/heat exchanger.
13. Close all Luer-lock ports of the oxygenators with stopcocks.
14. Attach the outflow of the oxygenator to the inflow of the bubble trap with a 25 cm tubing section.
15. Close the top and side ports of the bubble trap with two 7 cm tubing sections and Dura-clamps.
16. Attach a 10 cm tubing section to the outflow of the bubble trap and slide the other side over a straight barb fitting with Luer-lock side port.
17. Slide a 45 cm tubing section over a straight barb fitting and place the open end in the organ chamber.
18. Connect an extension line to the Luer-lock side port of this straight barb fitting and use a Luer-lock T-fitting to connect the far end of the extension line to a pressure transducer and a sampling port, as shown in Figure 2c.
19. Close the other side of the pressure sensor with a Luer-lock stopcock and connect the pressure transducer to the pressure monitor. [CRITICAL: ensure that position of the pressure transducer is secured at the same level as perfusate outflow from the inferior vena cava.]

20. Connect the oxygenators/ heat exchangers to a gas tank with a mixture of 95% O₂ and 5% CO₂ with oxygen tubing and a gas regulator. [CAUTION: Gas tanks must be secured to prevent injury].

Setup of the temperature regulation circuit

21. For the temperature regulation circuit, connect a 70 cm length of size 36 tubing via a barbed Y-fitting and two additional 30 cm tubing sections to the two inlets of the oxygenator/heat exchangers.
22. Connect the outlets of the oxygenator/heat exchangers to the bottom inlets the jacket of the bubble trap using 30 cm tubing sections.
23. Connect the outlets with three 30 cm tubing sections, via a barbed Y-fitting to the bottom inlet of the perfusion chamber's jacket, and attach a 100 cm tubing section to the outflow.
24. Place the supercooling chiller and the warm water bath in close proximity (1 m) of the perfusion system.
25. Insulate the tubing sections with pipe insulation.
26. Connect the inlet tubing of the temperature regulation circuit to the circulation pumps of the supercooling chiller or the warm water bath to cool or warm the system respectively. [CRITICAL: the order of flow through the components is important to generate a counter flow heat exchange, to minimize heat loss, and to prevent buildup of air into the temperature regulation system.]

Setup of the supercooling chiller

27. Fill the basin of the chiller with 85 liters of refrigerant (ethylene glycol, 30% v/v).
28. Ensure adequate circulation of refrigerant in the basin using the chiller's circulation pump outlet.
29. Secure a strong nylon wire over the top of the basin, as shown in Figure 1e. This wire will be used to later to suspend the liver in the chiller during supercooling. [CAUTION: wear appropriate personal protective equipment to avoid direct contact with the refrigerant]. [CRITICAL: adequate circulation is required for a homogeneous temperature throughout the chiller basin.] [PAUSEPOINT: The supercooling chiller can be turned off when not in use. Ensure the lid is closed to prevent evaporation of the refrigerant and check the refrigerant before use. The liver is sealed in a plastic bag during supercooling and therefore not in direct contact with the chiller. However, in case of accidental spillage, the chiller basin needs to be drained, cleaned, and refilled with fresh refrigerant.]

Back table:

To prepare the back table, ensure a level and stable lab bench that is situated in the same room as the supercooling system. Cover the surface with a surgical drape and place the surgical instruments, two stainless steel bowls, perfusion cannulas, an acorn cannula,

sutures, and syringes on the table. Place an infusion pole close to the back table and properly light the working area with a surgical light. [CRITICAL: all instruments must be clean and sterile.]

PROCEDURE

Priming of the perfusion system:

1. Set the flow diverter to single pass perfusion (See Figure 3a) and put the perfusion inlet tube into the flask with 4L recovery perfusate.
2. Put the drain tube of the system in an empty 4-liter graded cylinder.
3. Start the portal pump at 500 ml/min.
4. Prime the portal perfusion circuit with perfusate, including the extension line to the pressure sensor and sample port.
5. Fill the bubble trap to a level of 5 cm to trap bubbles while the compliance of air damps pressure pulses from the pump (See Figure 1d)
6. Stop the pump immediately when the perfusate reaches the portal outlet.
[CRITICAL: ensure there are no air bubbles in the tubing or the oxygenator/heat exchanger]
7. Open the Luer-lock stopcock and zero the pressure sensor to ambient pressure and close the stopcock after zeroing.
8. Repeat steps 3 to 7 for the arterial side of the perfusion circuit.
9. Set the temperature to 21 °C and confirm the temperature with a thermal couple. Connect the temperature regulation circuit to the warm water bath and ensure all the components of the circuit are filled.
10. Set the supercooling chiller to 0 °C
11. Open the flow of gas to the oxygenators/ heat exchangers at combined flow of 2 liter per minute.
[CRITICAL: Confirm gas flow from the oxygenator/heat exchangers vents]

Graft preparation:

[CAUTION: wear appropriate personal protective equipment to prevent potential disease transmission from human grafts.]

12. Place the organ procurement bag with the liver submerged in ice-cold University of Wisconsin Solution in a stainless-steel bowl with 1 liter of crushed ice. Open the bag and fold the edge over the bowl's rim.
[CRITICAL: ensure the liver stays submerged and therefore cold during preparation]

13. Dissect the liver hilum to identify the common bile duct, the portal vein, the proper hepatic artery, and their branches (Fig. 4).
14. Ligate any side branches of the portal vein and the hepatic artery with 2–0 silk sutures.
15. Dissect the gallbladder from the liver. Tie off the cystic duct and artery with two 2–0 silk sutures, and finally cut both structures to remove the gall bladder.
16. Dissect the diaphragm from the suprahepatic inferior vena cava.
17. Remove the diaphragm, and other connective, adipose, pancreatic, and adrenal tissues.
18. Insert a 25 Fr perfusion cannula in the portal vein and secure its position with two 2.0 silk sutures.
19. Insert a 10 or 12 Fr perfusion cannula in the proper hepatic artery or celiac trunk and secure its position with two 2.0 silk sutures.
20. Insert an acorn tip canula in the common bile duct and secure its position with a 2.0 silk sutures.
21. Flush the liver using 1.5 liters of ice-cold Ringer's lactate through the portal vein, and 0.5 liters through the hepatic artery using an IV administration set. [CRITICAL: de-air the tubing and cannulas to ensure no air gets in the liver during the flush.]
22. Check the vasculature for leaks during the flush. Ligate side branches with 2.0 silk sutures and repair holes with 5.0 prolene sutures.
23. Move the liver to an empty sterile stainless-steel bowl.

Pre-supercooling SNMP:

24. Place the liver in the perfusion chamber with hilum and canula's facing up.
25. Start the portal vein pump at 100 ml/min.
26. Connect the portal vein cannula to the portal outlet of the perfusion system.
27. Start the hepatic artery pump at 50 ml/min.
28. Connect the hepatic artery cannula to the hepatic artery outlet of the perfusion system.
[CRITICAL: de-air tubing and the cannulas to ensure no air gets in the liver.]
29. Attach a 50 cm long, 6 Fr tube to the bile duct canula and place the open end in a bile collection reservoir. When bile pH and HCO_3^- are used as viability metrics, collect bile under a 2 mm layer of mineral oil to limit CO_2 equilibration with ambient air.
30. Wet a sterile gauze with perfusate and cover the portal vein and hepatic artery to prevent them from drying.

31. Place the lid on the perfusion chamber.
32. Slowly increase the flow rates of the pumps to reach a perfusion pressure of 5 mmHg and 60 mmHg in the portal vein and hepatic artery respectively.
33. Adjust the flow rates throughout perfusion to maintain the targeted perfusion pressures. [Critical: change flow rates in small increments – approx. 25 ml/min the portal vein and 10 ml/min for the hepatic artery – and wait 1 minute in between adjustments.] [TROUBLESHOOTING]
34. Draw perfusate samples from the portal and arterial sample port to confirm adequate oxygenation and pH of the perfusate by running the sample in a blood gas analysis machine. If needed, correct the pH to 7.35 – 7.45 with NaHCO₃ or HCL. Additional perfusate and tissue samples can be collected during perfusion if desired.
35. Switch the flow diverter from single pass to recirculation perfusion (see Figure 3b) when exactly 2 liters of perfusate has drained into the graded cylinder and continue recirculation perfusion with the remaining 2 liters of perfusate.
36. After 30 minutes of perfusion, confirm that the liver has warmed to 21°C by measuring the outflow temperature from the inferior vena cava with a thermocouple. [TROUBLESHOOTING]
37. After 90 minutes of perfusion, take 100 ml perfusate from the perfusion chamber and dissolve 77.68 gram of 3-OMG and 380 U of regular insulin.
38. Add this solution to back to the perfusate chamber at a rate of 10 ml/min.
39. Perfuse the liver for 60 minutes with the 3-OMG enriched perfusate

Perfusion Cooling phase:

40. After 150 minutes of perfusion, switch the cooling circuit from the warm water bath to the supercooling chiller that is pre-cooled to 0 °C. [CAUTION: briefly stop the circulation pumps and clamp the tubing during switching to avoid spilling]
41. During cooling, change the flow rates to achieve a pressure of 3 mmHg and 30 mmHg for the portal vein and hepatic artery respectively.
42. Adjust the flow rates throughout hypothermic perfusion to maintain the targeted perfusion pressures.
43. Cool the liver during 30 minutes of perfusion and confirm that the perfusate outflow temperature from the inferior vena cava is 4 °C at the end of cooling. [TROUBLESHOOTING]

Hypothermic machine perfusion: TIMING 60 min.

44. Put the perfusion inlet tube in the flask with 1 liter of loading solution and put the drain tube in an empty 4-liter flask.

45. De-air the perfusion inlet tube. This can be done by removing the air through the Luer-lock side port with a 50 ml syringe.
46. Set the flow diverter from recirculation to single pass perfusion (See Figure 2a).
47. Lower the perfusate level in the bubble traps to 1 cm. To do so, open the bubble trap's side port and directly pull up two 50 ml syringes from the perfusion systems sampling port, and close the bubble trap's side port.

[CRITICAL: check the perfusion pressures that are likely to change due to this handling and adjust flow rates of the pumps if needed.]
48. When the loading solution reaches the liver (this can be seen due to the color difference with the SNMP perfusate) substantially lower the perfusion flow rates to 50 ml/min and 25 ml/min for the portal vein and hepatic artery, respectively, to compensate for the anticipated rising perfusion pressures as result of the high viscosity of the loading solution.
49. Maintain a pressure of 3 mmHg in the portal vein and 30 mmHg in the hepatic artery during hypothermic perfusion.
50. When 1 liter of loading solution has entered the perfusion system, quickly place the perfusion inlet tube in the flask with 3 liter of storage solution. [CRITICAL: make sure to switch flasks in time because otherwise a substantial amount of air can enter the system.]
51. Perfuse the liver with the 3 liters of storage solution.

Supercooled storage:

52. Open the lid of the perfusion chamber to disconnect the perfusion cannulas from the perfusion system and remove the bile collection tube from the bile duct cannula.
53. Place the liver in the sterile organ procurement bag.
54. Submerge the organ procurement bag in a bucket with ice-cold water to help remove all air from the bag. Make sure no water gets into the bag and create an airtight seal using two zip ties. [CRITICAL: Ensure all air is removed from the bag and the bag is sealed airtight.]
55. Attach a 250 g weight to the bag which will keep the liver submerged in the basin of the supercooling chiller.
56. Suspend the liver in the basin of the supercooling chiller that is pre-cooled to 0 °C.

[CRITICAL: Ensure the liver is completely submerged in the circulating refrigerant.]
57. Set the supercooling chiller to -4 °C and confirm the temperature of the chiller after 30 minutes.

58. Store the liver in the chiller at -4°C for 20 hours. During this period, proceed with steps 59–62.

Intermediate cleaning of the perfusion system:

CRITICAL This needs to be completed prior to the liver being removed from the chiller in step 63.

59. Completely fill the perfusion system with 2 liters of 2 % (v/v) Mucosol detergent solution in milliQ water.
60. Set the flow diverter to recirculation perfusion and recirculate the cleaning solution for 30 minutes at a flow rate of 1000 ml/min per side.
61. Set the flow diverter to single pass perfusion and flush the system, including components and side ports with 12 liters of milliQ water.

Priming of the perfusion system:

CRITICAL This section must be completed prior to the liver being removed from the chiller in step 63.

62. Prepare the perfusion system as outlined in steps 1 to 11 except for two differences: prime the system with 1 liter of ice-cold unloading solution; and connect the temperature regulation circuit to the supercooling chiller, instead of the warm water bath.

CPA unloading:

63. At the end of supercooled storage (step 58), set the supercooling chiller to 0°C and wait 15 minutes after the chiller reaches 0°C before removing the organ procurement bag containing the liver from the chiller basin.
64. Open the organ procurement bag and transfer the liver from the bag into the perfusion chamber. [TROUBLESHOOTING]
65. Start perfusion as outlined in steps 24 to 34 of this protocol. [TROUBLESHOOTING]
66. When 1 liter of unloading solution has entered the perfusion system, quickly place the perfusion inlet tube into the flask with 4 liters of post-supercooling recovery perfusate.
[CRITICAL: make sure to switch flasks in time because otherwise a substantial amount of air can enter the system.]
67. As described in step 35, flush the liver with 2 liters of perfusate to remove the protective agents from the liver.

Post supercooling SNMP recovery:

68. Switch the flow diverter from single pass to recirculation perfusion when exactly 3 liters (i.e. 1 liter of unloading solution plus 2 liters of post-supercooling recovery perfusate) have drained into the graded cylinder.

69. Continue recirculation perfusion with the remaining two liters of perfusate for 3 hours. [TROUBLESHOOTING]

TIMING

When working with human organs that are rejected for transplantation it is important to be prepared as organs may be offered for research by the organ procurement organization at any time of the day. In our experience, there is a time window of at least 3 hours between notification by the organ procumbent organization and arrival of the graft at our facility. Although the all preparations for this protocol could be done during this time, we advise to have the system setup and ready. Also, we prefer to aliquot all reagents in advance as this substantially reduces efforts and time in preparing the solutions, which is especially appreciated outside office hours.

Part of the protocol	Steps	Duration
Preparation of the solutions	Reagent setup	60 min.
Perfusion and supercooling system setup	Equipment setup	75 min.
Priming of the perfusion system	Steps 1–11	15 min.
Graft preparation	Steps 12–23	45 min.
Pre-supercooling SNMP	Steps 24–39	150 min.
Perfusion Cooling phase	Steps 40–43	30 min.
Hypothermic machine perfusion	Steps 44–51	60 min.
Supercooled storage	Steps 52–58	20 hrs.
Intermediate cleaning of the perfusion system	Steps 59–61	45 min.
Priming of the perfusion system	Step 62	15 min.
CPA unloading	Steps 63–67	20 min.
Post supercooling SNMP recovery	Steps 68–69	3 hrs.

TROUBLESHOOTING

Troubleshooting guidance can be found in Table 1.

ANTICIPATED RESULTS

The viability of human livers that are rejected for transplantation can substantially differ between grafts and this is a challenge that needs to be accounted for in research. One of the advantages of this protocol is that it provides the opportunity for detailed viability assessment during SNMP^{7,22–24} before and after supercooling, which can be used to control for donor-to-donor variability. We compared important parameters of metabolic function before and after supercooling, including the adenylate energy charge, bile production and oxygen uptake in 5 organs¹⁰. The adenylate energy charge is considered one of the most representative metrics for liver viability^{4,23,34–36}. The energy charge is expected to be low after both hypothermic preservation and supercooling but should recover during SNMP. While over 40% differences in energy content have been observed between successful and

unsuccessful transplanted human livers^{36–38}, the difference in energy charge at the end of SNMP before and after supercooling was less than 20% (Fig. 5a, **n=5**).

Bile production and oxygen uptake were correlated to transplant survival of 4-day supercooled rat livers⁷. All but one of the tested human livers produced the same amount of bile before and after supercooling and no significant difference in cumulative bile production before and after supercooling was found (Fig. 5b). The oxygen uptake rate, as well as the cumulative oxygen uptake, was not significantly different between SNMP before and after supercooling (Fig. 5c). In addition to bile production, lactate metabolism (Fig. 5d) is an important parameter of liver function. The perfusate lactate levels were significantly lower during the first hour of SNMP after supercooling than during the first hour of SNMP before supercooling ($P=0.0044$ and $P=0.0164$ at $T=30$ and 60 min. respectively. Repeated measures two-way ANOVA, followed by the Sidak multiple comparison test, $n=5$ independent biological replicates). We hypothesised that this may be the result of a lower metabolic rate and therefore less lactate build up during supercooling as compared to static cold storage¹⁰.

Increased vascular resistance may reflect endothelial cell injury whereas increased transaminase levels are specific to hepatocellular injury. In our rat model, 4-day supercooled livers that failed after transplantation had significantly higher portal resistance during SNMP recovery after supercooling than successfully transplanted livers^{7,8}. The supercooled human livers had the same portal and arterial resistance during SNMP before and after supercooling (Fig. 5e). The transaminase levels during perfusion in this protocol are anticipated to be relatively low compared to reported values for perfused discarded grafts^{18,39–41}. This could be a result of the 2-liter flush with perfusate at the start of SNMP both before and after supercooling. Although this flush is necessary to wash out the preservative cocktail after supercooling, this flush is also performed before supercooling to match the post-supercooling perfusion conditions and thus to allow for comparison of pre- and post-supercooling transaminase levels. The observed levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) during SNMP before and after supercooling were the same which indicates limited hepatocellular injury after supercooling preservation (Fig. 5f).

Although the first results of this supercooling preservation are promising, several steps must be taken before supercooling preservation can be introduced into the clinic. The protocol should be adapted such that supercooling preservation of discarded human donor livers can be initiated directly after procurement (as shown in Fig. 1b). The viability of these livers after supercooling preservation may be additionally confirmed during *ex vivo* pseudo transplantation or normothermic machine perfusion. Supercooling preservation should also be tested in a large animal transplant model with long term follow up to study potential long-term adverse effects of supercooling preservation. Finally, the perfusion and storage solutions, as well as all the perfusion system and supercooling chiller, should be produced according to good manufacturing practice (GMP) before this preservation method can be translated into the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files. Any additional data if needed will be provided upon request.

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Key reference(s) using this protocol

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Figure 1:

The supercooling protocol. **a.** Schematic temperature profile of the supercooling protocol. The numbers match the steps explained in the introduction. **b.** Adaption of the supercooling protocol that can be used from procurement to transplantation. **c** Machine perfusion system. **d.** Liver during SNMP recovery. **e.** Liver in supercooling basin of the chiller. This research was approved by the IRB at Massachusetts General Hospital. This figure is adapted from reference 10 (Nature Publishing Group)¹⁰.

Figure 2: Human liver perfusion system. **a.** Photo of the perfusion system setup. **b.** Detail of the flow diverter **c.** Schematic of the perfusion system. Note: red = perfusate circuit; blue = temperature regulation circuit; black dashed line = electronic wire; O/H = oxygenator/heat exchanger. **d.** Detail of the pressure transducer and sample port setup.

Figure 3:
Schematic design of the flow diverter. a. Flow diverter set in single pass perfusion. b. Flow diverter set in recirculation perfusion. Blue: tubing. Black: barb fittings. Green: open Dura-clamp. Red: Closed Dura-Clamp. Yellow: Luer-lock samplingport.

Figure 4:

Liver graft after preparation for supercooling preservation. 1: 10 Fr cannula in the hepatic artery (HA) that bifurcates into the left and right hepatic artery (LHA and RHA, respectively). 2: 25 Fr cannula in the portal vein (PV). 3: acorn tip cannula in the common bile duct (CBD) that bifurcates into the hepatic duct (HD) and the ligated cystic duct (CD). The inferior vena cava (IVC) is not cannulated and drains freely during perfusion. (Photo credit: Dr. Siavash Raigani, Massachusetts General Hospital and Harvard Medical School).

Figure 5:

Key *ex vivo* viability parameters during pre- and post-supercooling subnormothermic machine perfusion (SNMP), blue and green respectively. **(a)** Tissue adenylate energy charge showing recovery of energy charge after supercooling. **(b)** Cumulative bile production. **(c)** Oxygen uptake. **(d)** Lactate concentration (top) and pH (below) of the arterial inflow. **(e)** Vascular resistance of the hepatic artery (HA) and the portal vein (PV), top and below respectively. **(f)** Aspartate AminoTransferase (AST) and Alanine AminoTransferase (ALT) concentrations in the perfusate, top and below respectively. Boxes: median and inter quartile range. Whiskers: minimum and maximum. Error bars of line graphs: mean $\pm$ s.e.m. Stars: two-sided P-value < 0.05 (repeated measures two-way ANOVA, followed by the Sidak multiple comparison test, n=5 independent biological replicates). This research was approved by the IRB at Massachusetts General Hospital. This figure is adapted from reference 10 which described the detailed methods through which this data was obtained (Nature Publishing Group)¹⁰.

Table 1:**Troubleshooting**

Problem	Steps	Possible reason	Solution
High or unstable perfusion pressures	Steps 33 and 65	Twisted or kinked portal vein or hepatic artery	Disconnect the perfusion cannula from the system and straighten out the vessel. Reconnect the cannula and secure it in its correct position with 2–0 silk sutures.
		Air emboli	Directly stop perfusion when air is seen in the tubing after the bubble trap, the perfusion cannulas or in the vessels. Disconnect the perfusion cannula, remove the air from the lumen and reconnect. Small air volumes (~10 ml) that enter the graft slowly dissolve during perfusion, however, large air volumes may render the graft unavailable for further use.
		Incorrect pressure sensor level	Ensure that the level of the pressure sensor is horizontal with the inferior vena cava.
		Incorrect pressure sensor calibration	Open the Luer-lock stopcock connected to the pressure transducer and zero the pressure sensor. Wait 5 seconds and close the Luer-lock stopcock.
No bile production	Step 36 and 69	Not enough compliance in system	Lower the perfusate level in the bubble trap to 5cm.
		Hydrostatic counter pressure in bile duct cannula	Place the bile collection tube in horizontal position in the perfusion chamber. When bile reaches the end of the tubing, move the tubing over the rim of the perfusion chamber to the bile reservoir which will create a syphon. Ensure the bile reservoir is lower than the liver
High temperatures during preconditioning	Step 43	Twisted or kinked bile duct	Disconnect the bile duct cannula from the collection tube and straighten out the bile duct. Reconnect the collection tube and secure the cannula in place with 2–0 silk sutures.
		Chiller temperature too high	Check the temperature of the anti-freeze solution in the chiller and adjust the chiller setting accordingly.
		Heat loss in perfusion system	Insulate the tubing of the perfusion system with tube insulator.
Freezing of graft	Step 64	High ambient air temperature	Set the room's thermostat to 21 °C or lower.
		Air in storage bag	Remove all air from the bag before supercooling
		Imperfect seal of storage bag	Seal the bag airtight with two very tightly closed zip ties
		Contamination of graft and or solution	Sterile filter all solutions and rebuild the perfusion system with clean and sterile components. Keep the perfusion chamber lid closed during perfusion.
		Chiller temperature is too low	Check the temperature of the anti-freeze solution in the chiller and adjust the chiller setting accordingly.
		Vibrations due to incorrect suspension of the liver in the chiller basin	Ensure the procurement bag with the liver is suspended in the middle of the basin and that it does not touch the sides of the chiller.
		Incorrect preconditioning	Prepare the loading and storage solution with the correct protective agent concentrations and ensure the livers are preconditioned with the complete solution volumes.