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Successful Supercooled Liver Storage for 4 Days

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Abstract

The realization of long–term human organ preservation will have groundbreaking effects on the current practice of transplantation. Herein we present a novel technique based on sub–zero non–freezing tissue preservation and extracorporeal machine perfusion that allows transplantation of rat livers preserved for up to 4 days, thereby tripling the viable preservation duration.

Introduction

With 119,000 patients waiting to receive a donor organ today, the field of transplantation is facing a serious donor shortage crisis. The introduction of University of Wisconsin (UW) solution by Belzer and Southard in 1983[1] represented a pivotal breakthrough in hypothermic organ preservation (HP). It substantially extended the viable preservation time of donor organs[2,3], which led to the first intercontinental kidney transplantation and provided a major thrust that led to the current success of solid organ transplantation. To this day, donor livers are preserved in ice–cold UW solution, which offers a maximum cold preservation time of 6–12 h. Extension of this storage time, to a hypothetical 24 h, would allow a larger donation territory, reduce pressure on procedural logistics, and optimize recipient preparation. Together, such advances could contribute towards intercontinental liver sharing, which would greatly reduce the donor shortage[4].

Cryopreservation has been successful in several cell and tissue types[5] and investigated to achieve long–term solid organ storage. However, success remains elusive due to adverse processes brought on by these extreme temperature and processes necessary to reach them,

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which can disrupt tissues at the cellular level[6,7]. Experimental endeavors to achieve viable long–term preservation of whole organs range from vitrification at −196 °C[4,8,9] to supercooling (sub–zero non–freezing) at 0 °C to −5 °C[10–16] but have yet to yield substantial success, especially demonstrated by transplantation. These challenges are augmented when preserving liver tissue, due to the delicate hepatic anatomy comprising multiple cell types with variable preservation properties and functions: for instance it has been shown that frozen rat livers could be transplanted with apparently healthy hepatocytes and a functioning biliary system, but would fail due the impaired vasculature and endothelial cell death[17]. Machine perfusion (organ support through extra–corporeal artificial circulation) is one technique to have shown a substantial advantage over conventional hypothermic storage, and has since been implemented into routine clinical practice for kidneys[18,19]. While this method could hypothetically extend viable preservation times, with few exceptions[20], most studies focus on alleviation of the donor shortage through recovery of (warm ischemic) organs that would otherwise have been discarded[19,21,22].

In this report we present a novel method for extended liver storage that combines supercooling (SC) and machine perfusion. Our central hypothesis was that since HP primarily works through deceleration of cellular metabolism at lowered temperatures, supercooling would allow further extension of the viable preservation time. However, supercooling poses several challenges, a) ice nucleation can occur during storage and needs to be avoided; b) extended storage at this temperature along with the rewarming process can result in irreversible plasma membrane injuries[23,24] and eventual osmotic imbalance that follows; and c) cold temperatures and subsequent rewarming, in general, increase susceptibility of cells to produce free radicals while reducing their natural defense ability against such damaging reactive species[25]. This particularly affects the hepatic sinusoid, which is the functional unit of the liver that directly interacts with the exterior milieu[26]. The sinusoidal endothelial cells (SECs) are extremely sensitive to hypothermia, which causes cellular swelling and disruption of the microcirculation[27,28]. Moreover, extension of the storage duration is likely to exacerbate the ischemia reperfusion injury observed. Therefore we sought to create a novel protocol and media to dramatically extend liver preservation duration as described below.

Study Design

For HP, the UW preservation solution has been a remarkable success. However for the purposes of supercooling we sought to supplement the solution to reduce cold–induced membrane injury. As traditional freezing point depressors such polyglycols are toxic and may lead to issues in clinical translation, we avoided their use at least in these first studies. Based on a literature survey, we chose PEG–35kD (5%), which is shown to protect the epithelial cell membrane[29,30] and has been previously used as a colloid for liver machine perfusion as well[31].

Since PEG is limited to extracellular media, we also considered employing an intracellular cytoprotectant that is nontoxic and usable for the liver: Inspired by freeze–tolerant species that produce high concentrations of glucose as a cryoprotectant[32], a non–metabolizable glucose derivative (3-O-methyl-D-glucose, 3–OMG) was tested as an intracellular

protectant. 3–OMG is taken up naturally by hepatocytes through the GLUT–1 and −2 transporters, is non–metabolizable therefore accumulates internally, and is non–toxic and used in clinical glucose uptake studies[33,34]; it was therefore chosen over typical preservatives such as glycerol. Our group has previously shown that primary hepatocytes *in vitro* show superior post–thaw quality when cryopreserved with 3–OMG[33] although to our knowledge this study is the first in testing 3–OMG for supercooling.

Finally, to counter the effects of extended ischemia we employed machine perfusion, which has been demonstrated to alleviate hypothermic endothelial injury[28], reinitialize metabolic activity, replenish ATP, and mechanically prime the vasculature for reperfusion[19,35–37]. We chose subnormothermic machine perfusion (SNMP) based on our success in using this method for recovering 1 h warm ischemic rat livers for transplantation[38–40].

The method, as displayed in Fig. 1a entails storage of rat livers for 3 and 4 days at −6 °C, more than thrice the maximum preservation time achievable by HP, and testing by transplantation. The protocol (for comprehensive details, see Supplementary Material) includes the following: First, prior to supercooling, we used SNMP with modified Williams medium E to load isolated rat livers with 0.2 M 3–OMG. The dosage and loading characteristics of 3–OMG were derived from *in vitro* experiments using isolated rat hepatocytes[33]. Next, we cooled the organ during SNMP to 4 °C (1 °C min^{-1}), and flushed it with 10 mL, 4 °C UW solution containing 5% PEG–35kD (UW–PEG). The liver was submerged in 75 mL of 4 °C UW–PEG. We then placed the liver inside a controlled–rate freezer, and lowered the temperature further to −6 °C (1 °C 10 min^{-1}), initiating the supercooling phase that was maintained for 72 h (n=6) or 96 h (n=12). Following supercooling we gradually increased the temperature to 4 °C, after which we flushed the liver with room temperature, oxygenated Williams E medium and subjected it to SNMP (3 h, 21 °C), previously shown in our laboratory to recover ischemic rat livers for transplantation[38], while we recorded multiple viability parameters. We finally transplanted the liver orthotopically[38]. We obtained blood samples for up to a month and monitored the recipient for survival and clinical signs of cirrhosis for up to 3 months.

Results

Post–transplantation

Three–month survival in the 72 h SC group was 100%, while no survival was achieved after an equal duration of HP (Table 1). This result triples the achievable storage duration by HP, which is limited to 24 h in rat transplants. Increasing the duration of supercooling to 96 h resulted in 58% survival, which is comparable to 48 h of HP. Negative controls in which either the SNMP loading phase, 3–OMG and PEG supplementation were individually omitted succumbed within 6 days (Table 1), whereas negative controls omitting the SNMP recovery succumbed within 1 h post–transplantation. Despite a prolonged post–surgical recovery period and increased postoperative cellular damage parameters up to 14 days post–surgery (Fig. 1b), recipients of 72 h and 96 h SC livers thrived past 3 months post–transplantation without any signs of organ failure. Post–operative blood levels of albumin, bilirubin, alkaline phosphatase, and blood urea normalized within 1 month post–op, and coagulation times were normal in all animals (data not shown). There were no signs (either

histological, hematological, or morphological) of postoperative cholangiopathy up to 3 months post–surgery in any of the animals except one (72 h SC group), which developed a large biloma 18–20 days post–surgery, likely due to blockage of the bile duct stent.

SNMP recovery phase

SNMP enables real–time evaluation of metabolic and vascular parameters. Tissue ATP levels, that dropped to approximately 10% (51.5 ± 45.7 pmol mg protein^{-1}) of fresh levels (457.2 ± 77.9 pmol mg protein^{-1}) after 96 h supercooling, were replenished to ca. 50% after recovery SNMP (197.5 ± 39.0 pmol mg protein^{-1}). While supercooled livers produced less bile than fresh livers subjected to SNMP (Fig. 2a), bile production was substantial compared to an equal duration of HP where bile production was minimal. Additionally, a significant difference in bile production was observed between livers that led to survival and those that did not ($p=0.0017$). Increased hepatic resistance during SNMP also correlated strongly with transplant survival in the 96 h SC group ($p<0.0001$, Fig. 2b), suggesting that both bile production and a mean resistance <15 cm H_2O min mL^{-1} during SNMP recovery could be predictive of liver viability. Aminotransferase output was increased from fresh livers in both SC groups (Fig. 2c). Oxygen consumption during SNMP was not different between groups. Within livers in the 96 h SC, a significantly higher oxygen uptake was observed in livers that survived ($p<0.0001$; Fig. 2d). Liver weight did not increase significantly during supercooling (+2.6%, $p>0.05$) or during recovery SNMP (+5.6%, $p>0.05$) and there were no significant differences between groups. H&E staining and TEM of supercooled livers showed normal hepatic anatomy in all slides (Fig. 2e). Post–supercooling samples contained intracellular accumulation of glycogen–like intracellular matter, which may be internalized 3–OMG. In the post–transplantation specimens, hepatocyte crowding and biliary hyperplasia were observed, consistent with typical hepatocellular regeneration after liver transplantation.

Discussion

Supercooling is the first preservation technique capable of producing transplantable livers after 4 days of storage. The protocol comprises four essential components: 1) supercooling; 2) 3–OMG; 3) PEG–35kD; and 4) machine perfusion loading of 3–OMG and for recovering viability and energy stores prior to transplantation. We have shown that each of these components is individually required to achieve viable supercooling preservation in our model, as evidenced by long–term recipient survival.

We show here that clinically acceptable survival is limited to 72 h of storage, dropping considerably to 58% if the storage time is extended to 96 h. As extensive screening of different additives or variations in protocol is still ongoing, additional improvements may be achieved from future experimentation. In addition, continued investigation of the individual protocol components should provide a better understanding of the mechanisms at play.

During SNMP recovery of the 96 h SC group, we retrospectively observed segregation of survivor and nonsurvivor recipients in terms of hepatic resistance, after just 30 min of SNMP. Hence, observation of elevated hepatic resistance after supercooling can be interpreted as a marker for hepatic damage, which is similar to that observed in non–supercooling SNMP studies. This conclusion would suggest the sinusoid, the crucial

interface between the liver and the outside milieu, as the element of major focus for future work.

As a proof–of–concept small animal study, our current model has limitations and requires validation in a large animal model. The size, robustness, and preservation properties of human hepatocytes and livers differ from rodent, presenting translational challenges not only regarding preservation biology, but also engineering and cost. For example, our model is not suited to investigate ischemic cholangiopathy, as this type of complication does not mimic clinical situations in rat models. Therefore, while histological analysis, morphological examination, and hematology did not reveal any indication of severe cholangiopathy in supercooled livers, this must be examined further using a higher order species model that utilizes clinically relevant biliary anastomosis that includes reconstruction of the hepatic artery. A second size–related issue is that in a human liver the amount of liquid volume subject to freezing will be much larger and therefore the probability of freezing may increase; this may require tuning the preservation media or supercooling process.

As determine feasibility, we only focused on fresh livers in this work. However a major use for a superior preservation protocol would be enhancing the utilization of marginal donor organs, such as ischemically injured livers donated after cardiac death. Therefore a profitable next step is to test and optimize the protocol described here to enable transplantation of such currently untransplantable livers.

The achievement of long–term survival after supercooling preservation, with more than a threefold increase in the currently achievable preservation time, signifies the potential of this novel modality. The eventual goal of extending the viable preservation time of human organs will contribute towards global organ sharing, increased organ availability, and reduction of overall cost.

Methods

Brief description of the experimental protocol

The liver was recovered from inbred male Lewis rats as described in detail previously[41,42] (see *Liver Recovery* below for surgical details). Livers were subjected to subnormothermic machine perfusion (SNMP) at room temperature[38,43] (see below for detailed description), through the portal vein, for 60 min; the PV cuff attached to a 20G catheter in the perfusion system. The perfusate consisted of supplemented Williams medium E (Sigma-Aldrich, St Louis, MO, USA) and contained the non–metabolizable glucose 3–OMG (Sigma-Aldrich, St Louis, MO, USA). After 60 min of loading, the temperature of the perfusate was lowered gradually (1 °C min^{-1}) to 4 °C under continuous perfusion. Then, the liver was flushed briefly with 20 mL of UW solution containing 5% 35kD–PEG, transferred to a sterile bag filled with the same solution, and moved to a controlled–rate freezer. The temperature was lowered to −6 °C (1 °C 10 min^{-1}) and preservation was continued for up to 4 days. The temperature was then raised to 4 °C (1 °C 10 min^{-1}), and the liver was flushed with supplemented with Williams E medium, and subjected to 3 h of recovery SNMP (see *Supercooling Procedure* below for more operational details). During SNMP, measurements included liver weight, blood gas analysis, perfusate AST and ALT, bile flow, and hepatic

vascular resistance. The liver was then transplanted orthotopically in a recipient animal[38]. We collected and analyzed blood, weighed the recipients and assessed the clinical condition for 30 days. All animals were observed for up to 3 months clinically.

Liver Recovery—Inbred male Lewis rats weighing 250 to 300 g (Charles River Laboratories, Boston, MA, USA) were used for transplantation. The animals were maintained in accordance with National Research Council guidelines, and the experimental protocols were approved by the IACUC at Massachusetts General Hospital (Boston, MA, USA). After dissection of the ligaments surrounding the liver, the intrahepatic inferior vena cava (IHIVC) and portal vein (PV) were elongated by dissecting their distributive veins (right renal and adrenal veins, lumbar venous plexus, gastroduodenal and splenic veins). The left diaphragmatic vein was ligated. The hepatic artery was ligated and cut. The common bile duct was cannulated for bile collection and dissected. The portal vein was cannulated and the liver was perfused with 10 mL, 21 °C oxygenated Williams medium E, excised from its recess and transferred to the subnormothermic machine perfusion (SNMP) system. The portal vein was cuffed using a modified 22G intravenous catheter.

Subnormothermic Machine Perfusion (SNMP)—The liver was flushed in situ with room temperature Williams Medium E and immediately connected to the SNMP system. Machine perfusion took place in a circuit comprising a perfusion chamber, a peristaltic pump, a membrane oxygenator, and a bubble trap. Details can be found elsewhere[38,43]. Temperature within the system equilibrated to room temperature at 21 °C and was not controlled otherwise. The perfusate volume was 350 mL, consisting of Williams Medium E supplemented with insulin (2 U L^{-1} Humulin; Eli Lilly & Co, Indianapolis, IN, USA*)*, penicillin (40,000 U L^{-1}) / streptomycin (40,000 μg L^{-1}; Gibco/Invitrogen, Camarillo, CA, USA), L–glutamine (0.292 g L^{-1}; *Gibco/Invitrogen)*, hydrocortisone (10 mg L^{-1} Solu-Cortef; Pharmacia & Upjohn/Pfizer, New York, NY, USA) and heparin (1000 U L^{-1}; APP pharmaceuticals, Schaumberg, IL, USA) (note that during preloading this was supplemented with 3–OMG and insulin as noted below; otherwise the loading and recovery perfusions are identical). The oxygenator was gassed with a mixture of 95% O_2 and 5% CO_2. Flow–rate was started at 8.0 mL min^{-1}, and adjusted according to the portal resistance, which was kept between 0.5 and 1.5 cmH_2O min mL^{-1}. Aspartate aminotransferase and alanine aminotransferase were measured using an Infinity AST (GOT) and ALT liquid stable kit (Cellomics/Thermo Electron, Pittsburgh, PA, USA). Every 30 min PV inflow and IVC outflow were analyzed using a using a blood gas analyzer (Rapidlab 845 blood gas analyzer, Bayer). Bile was collected continually via a cannula inserted into the bile duct, which drained into a collection tube.

Supercooling Procedure

Loading phase: Livers were subjected to 1 h of SNMP using oxygenated Williams E supplemented with 750 U L^{-1} insulin and 0.2 M 3-O-methyl-D-glucose. The final osmolality of the solution was 290–310 mOsm L^{-1}. The loading time was based partly on *in vitro* work with 3–OMG[33], as well as preliminary trials conducted with our system (data not shown).

Supercooling phase: The SNMP system is equipped with jacketed components (perfusion chamber, oxygenator, bubble trap, and perfusion reservoir) that allow circulation of antifreeze through the system on a separate circuit, enabling exact temperature regulation during machine perfusion. Liver temperature was measured using a thermocouple inserted into the suprahepatic inferior vena cava. After the 60 min 3–OMG loading phase, the temperature was reduced (1 °C min^{-1}) to 4 °C. The liver was flushed with 10 mL ice–cold UW solution supplemented with polyethylene glycol (35kD PEG, 5% W/V; Sigma-Aldrich) (UW–PEG) and transferred to a sterile bag filled with 4 °C UW–PEG. To prevent thermocouple–induced ice nucleation, the temperature calibrations were performed in separate experiments, however; independent temperature monitoring of the antifreeze and UW–PEG solution was performed throughout. The liver was flushed with 10 mL UW–PEG prior to supercooling to ensure distribution of the preservation solution to the hepatic sinusoid. As the flush is under hypothermic conditions, washout of 3–OMG should be at a minimum, while failure to remove the SNMP perfusate from the liver would likely result in ice formation during supercooling. The sterile bag was sealed and was immersed in ice–cold antifreeze, transferred to a controlled rate freezer, and cooled to −6 °C (1 °C 10 min^{-1}, final intrahepatic temperature: between −5.5 and −6 °C); this is the lowest temperature at which ice formation could be prevented reliably over a period of four days in our system. The liver was preserved for 72–96 h avoiding perturbations and temperature variation.

Recovery phase: The temperature was raised to 4 °C (1 °C min^{-1}), the liver was flushed with Williams E medium, and SNMP recovery was performed for 3 h. The recipient was prepared for surgery during perfusion recovery. Perfusion was stopped, the liver disconnected, and flushed with room temperature Lactated Ringer's solution. The liver was orthotopically transplanted into a weight–matched recipient Lewis rat as described elsewhere[1]. Please note that we specifically avoided the cold flush during the transplant procedure.

Post–Transplantation Analysis—Tail–vein blood samples were taken from transplantation recipients hourly after the procedure for 3 h, then daily for 7 days, and finally after 30 days. The blood samples were analyzed using a Piccolo Blood Chemistry Analyzer (metabolic panel; Abaxis, Union City, CA). At 30 days post–transplant, the liver was recovered for histology.

Histology—Samples from dedicated livers were processed for light and electron microscopy (EM). For light microscopy samples were fixed in 10% buffered formalin until further processing and stained using heamatoxylin and eosin (H&E). For EM samples were fixed in Karnofsky's solution.

Statistics—Sample sizes were selected based on a power analysis based on past transplant experiments with similar variances. There was no blinding or randomization in selection of animals; however experiments were standardized (inbred Lewis rats of the same age and weight, transplantations performed at the same time of day by the same microsurgeon (T.A.B). Two–way (repeated measures) analysis of variance (ANOVA) was performed at α=0.05, with post–hoc Bonferroni correction for comparisons between different preservation

groups over time. An unpaired student's t–test was performed for ATP recovery and bile production between groups. A log–rank (Mantel–Cox) test was performed to compare survival curves. Normality assumption and distributions within groups were tested where appropriate.

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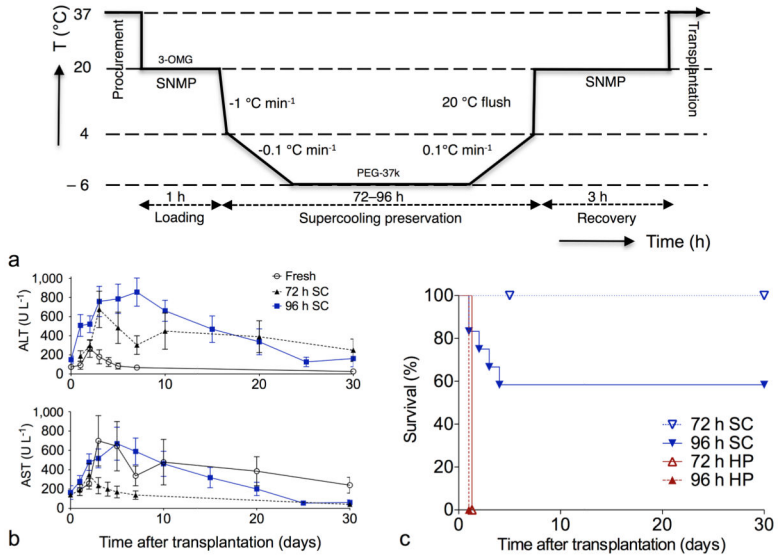

Figure 1.

Transplantation of supercooled livers (**a**) Schematic temperature profile of the supercooling protocol. (**b**) Post-transplantation trends in transaminase output (shown are all recipients, including those that did not survive past day 1). (**c**) 30-day survival post-transplantation for selected groups.

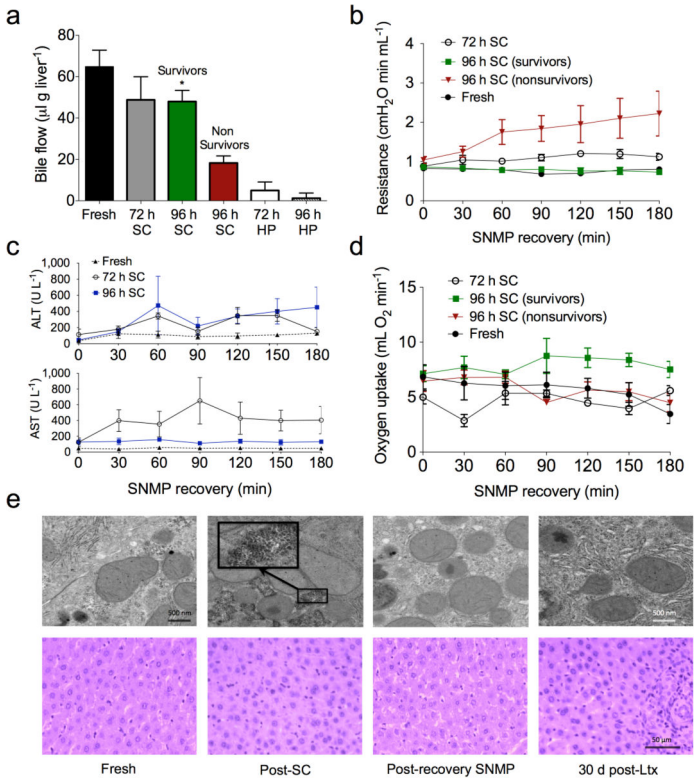

Figure 2.

Subnormothermic machine perfusion recovery and histology (**a**) Bile production per gram of liver during SNMP recovery phase. Survivors and nonsurvivors are separately represented for the 96 h supercooling group. * Indicates a statistically significant difference between survivors and nonsurvivors ($p<0.01$). (**b**) Hepatic resistance during SNMP recovery. (**c**) Hepatic transaminase levels in media during the SNMP recovery. (**d**) Oxygen consumption during SNMP recovery. (**e**) TOP: Transmission electron microscopy images (1500x initial magnification) of different stages of the supercooling protocol; fresh liver tissue, post-supercooling liver, post-SNMP recovery liver, and 30 days post-transplantation. The post-supercooling image contains a close-up of the glycogen-like structure observed in these specimens. BOTTOM: Light microscopy images of H&E stained tissue samples at corresponding stages of study.

Table 1

Experimental groups, positive controls, and negative controls used to verify the individual contributions of components of the supercooling protocol.

Protocol Steps	N	Loading Phase	Static Preservation	Recovery	Survival
Supercooling 72h	6	60 min SNMP + 3-OMG	Supercooling (−6°C, UW-PEG solution)	180min SNMP	100%
Supercooling 96h	12	60 min SNMP + 3-OMG	Supercooling (−6°C, UW-PEG solution)	180min SNMP	58%
Fresh liver Tx	6	-	-	-	100%
24h Hypothermic Preservation	6	-	24 hours UW solution (4°C)	-	100%
48 Hypothermic Preservation	4	-	48 hours UW solution (4°C)	-	50%
72h Hypothermic Preservation	4	-	72 hours UW solution (4°C)	-	0%
96h Hypothermic Preservation	4	-	96 hours UW solution (4°C)	-	0%
Recovery phase	4	60 min SNMP + 3-OMG	Supercooling (−6°C, UW-PEG. 96 hr)	-	0%
Control for recovery +temp	4	60 min SNMP + 3-OMG	Hypothermic Preservation (4°C, UW-PEG, 96 hr)	-	0%
Control for temperature	6	60 min SNMP + 3-OMG	Hypothermic Preservation (4°C, UW-PEG, 96 hr)	180min SNMP	0%
Control for loading	6	-	Supercooling (−6°C, UW-PEG, 96 hr)	180min SNMP	0%
Control for PEG	6	60 min SNMP + 3-OMG	Supercooling (−6°C, UW, 96 hr)	180min SNMP	0%
Control for 3-OMG	6	60 min SNMP	Supercooling (−6°C, UW-PEG, 96 hr)	180min SNMP	0%