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## Whole-liver supercooling for extended storage in transplantation

--Manuscript Draft--

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| **Abstract:** | Optimal preservation of an organ is required for successful transplantation. Using methods available today, liver preservation is very limited in time. Efforts to extend the viable preservation time have not been successful. Cryopreservation of organs remains an elusive feat, as ice crystal formation is injurious to tissue and cells. Supercooling is an alternative to cryopreservation and involves a reduction of the temperature to below a solution's freezing point, without the formation of ice. We recently described a method of liver preservation that makes use of a supercooled temperature, which is achieved by use of cryoprotectants, as well a machine perfusion. Using this novel technique rat livers could be successfully transplanted after 96 hours of preservation.<br>Here we describe this supercooling technique in detail. Rat livers are loaded with 3-O-methyl glucose (3-OMG) as an intracellular cryoprotectant by means of machine perfusion. Polyethylene glycol (PEG) is flushed into vasculature, protecting the extracellular space. Livers are subsequently stored for up to 96 hours at -6 oC, after which they are recovered during 3 hours of machine perfusion and orthotopically transplanted. |
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**TITLE:**
Whole-liver supercooling for extended storage in transplantation

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**KEYWORDS:**
Liver, transplantation, organ preservation, supercooling, subnormothermic, machine perfusion, cryoprotectants, cryopreservation.

**SHORT ABSTRACT:**
We describe methods to achieve successful transplantation of rat livers following 72–96 hours of preservation, using supercooling.

**LONG ABSTRACT:**
Optimal preservation of an organ is required for successful transplantation. Using methods available today, liver preservation is very limited in time. Efforts to extend the viable preservation time have not been successful. Cryopreservation of organs remains an elusive feat, as ice crystal formation is injurious to tissue and cells. Supercooling is an alternative to cryopreservation and involves a reduction of the temperature to below a solution's freezing point, without the formation of ice. We recently described a method of liver preservation that makes use of a supercooled temperature, which is achieved by use of cryoprotectants, as well a machine perfusion. Using this novel technique rat liver could be successfully transplanted after 96 hours of preservation. Here we describe this supercooling technique in detail. Rat livers are loaded with 3–O–methyl glucose (3–OMG) as an intracellular cryoprotectant by means of machine perfusion. Polyethylene glycol (PEG) is flushed into vasculature, protecting the extracellular space. Livers are subsequently stored for up to 96 hours at –6 $^{o}$C, after which they are recovered during 3 hours of machine perfusion and orthotopically transplanted.

**INTRODUCTION:**
Transplantation of a viable liver is the only definitive treatment option for tens of thousands of patients suffering from liver disease. To facilitate successful transplantation, optimal preservation of the liver outside of the body is necessary to prevent rapid deterioration of the graft. The current standard for liver preservation involves cooling the liver on ice, thereby reducing the metabolism of the liver and slowing down the effects of ischemia. Although this cold storage technique has allowed for successful transplantation, it is only useful for a short preservation time. Moreover, this cold storage is not ideal for livers that are already in marginal condition [1]. During cold storage the organ deteriorates slowly with a gradual loss of tissue energy stores, resulting in loss of viable cells [2].

Machine perfusion techniques have been explored to potentially extend and improve preservation [3-5]. Although, machine perfusion seems to be advantageous for short durations of preservation, it is less practical for truly extended durations. Machine perfusion has been applied at the end of preservation to recover livers injured by cold preservation [6]. We have attempted to recover livers at the end of extended cold storage using a subnormothermic (21 $^{o}$C) machine perfusion (SNMP) system. After 48 hours of cold storage survival could be increased from 50 to 100% using 3 hours of cold storage, but after 72 hours liver had sustained too much injury and could no longer be recovered by SNMP [7]. Importantly, after 72 hours of cold storage,

ATP content could not be recovered during SNMP.

Cryopreservation of organs is an attractive alternative, but remains elusive. Ice formation and phase changes in both cooling and warming significantly injure cells and tissue and prevent cryopreservation from being a viable option to date [8]. Preventing ice formation however, by lowering the nucleation point of a solution allows us to supercool the solution to far below the freezing point without the formation of hazardous ice crystals.

Supercooling requires the use of cryoprotectants, to protect cells from cold-induced injury as well as to prevent ice formations. In nature, glucose is used to protect various amphibious species from freezing [9]. Analogously we used a non-metabolizable glucose derivative called 3-O-methyl glucose, which is internalized by cells and accumulates intracellularly [10]. Polyethylene glycol is also know to prevent ice formation and has membrane-stabilizing effects and was used in this work to protect the extracellular space. With SNMP, supercooling and the use of cryoprotectants were combined to develop a new preservation scheme to significantly extend the preservation time of the liver. In this work we describe the methodology to achieve successful transplantation up to 96 hours of preservation (Figure 1) (Berendsen et al, Nat Med *in press)*

[Place figure 1 here]

**PROTOCOL:**

Approval must be obtained from an animal care and ethics committee for use of animals in the protocol described here. In our work 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).

**1. Preparation of solutions and apparatus**

1.1) Prepare 3–OMG loading solution by aseptically supplementing 500 mL phenol red–free Williams' medium E as described in table 1. Prepare this just prior to use.

1.2) Prepare supercooling solution by aseptically supplementing 85 mL of University of Wisconsin (UW) solution as described in table 1. Prepare this solution in advance, aliquot and stored at 4 $^{o}$C.

**NOTE:** The expiration date of the UW solution should be upheld.

1.3) Prepare recovery solution by aseptically supplementing 500 mL phenol red–free Williams' medium E as described in table 1. Prepare this just prior to use.

[place table 1 here]

1.4) Set up a recirculating perfusion system that includes an jacketed organ chamber, membrane

oxygenator, and bubble trap and a roller pump. Attach an 18G I.V. catheter to connect to the liver portal vein (PV) just before the organ chamber (figure 2). Gas the oxygenator with 95% $O_2$/5% $CO_2$. Add a divergent tube, just proximal to the PV inflow, which serves as a manometer. Prime the system with the 3–OMG loading solution just before beginning the liver procurement

[place figure 2 here]

1.5) Ensure that the controlled rate chiller is filled sufficiently with antifreeze and is pre-set to 21 $^{o}$C.

1.6) Modify a 14G and 16G I.V catheter to serve as the infrahepatic inferior vena cava (IHIVC) and PV cuff, respectively. Cut a 5 mm section of catheter and make a hemicircumferential cut midway the length of the section. Cut from one side in to meet the hemicicumferential cut, removing one side of half of the section to create a tail. Using a cautery, make a groove in the complete half.

**2. Liver procurement and 3–OMG loading phase**

2.1) Induce anesthesia in a rat of 180-200 grams, with 5% isoflurane/95% oxygen gas inhalation and maintain with 2-3% isoflurane. Confirm adequate anesthesia by tail pinch and monitoring respiratory rate. Procure the liver in preparation for orthotopic transplantation described by Kamada et al. [11], elongating the PV and IHIVC by ligating the supplying veins. Cannulate the bile duct with a 2 cm section of 52G tubing

2.2) Flush the liver *in situ* through the portal vein with 10 mL 3–OMG loading solution (21 $^{o}$C)

2.3) Cuff the portal vein and infrahepatic vena cava on a backtable with a modified 14G and 16G I.V catheter respectively.

2.4) Connect the liver to the perfusion system by inserting the 18G catheter of the system into the PV cuff and begin perfusion at 8 mL/min. Adjust the flow to maintain a portal vein pressure around 10 cm$H_2O$.

**NOTE:** The resistance of the liver can be estimated by measuring the pressure on the portal vein and dividing by the set flow rate.

2.5) Remove the first 150 mL of outflow and then close the system and allow the remaining ± 350 mL solution to recirculate.

2.6) Perfuse for 1 hour and take regular samples of the solution for analysis. Sample both the inflow and the outflow and analyze for dissolved gas and calculate the oxygen consumption as described elsewhere[4]. Optionally, sample the perfusion solution (1 mL), store at –80 $^{o}$C until further use. Analyze the samples for markers of liver injury (e.g. alanine/aspartate aminotransferase; ALT/AST) using a commercial reagent. Collect the bile and quantify in a 1.5

mL tube.

**3. Supercooling**

3.1) At the end of 3–OMG loading phase, gradually lower the temperature of the controlled rate chiller to 4 $^{o}$C at a rate of 1 $^{o}$C min$^{-1}$.

3.2) Remove the liver from the perfusion system and flush with 10 mL of supercooling solution (ice cold) and subsequently transfer the liver to a sealable, sterile bag containing 75 mL of supercooling solution and place the bag in the controlled rate chiller.

3.3) Reduce the temperature of the controlled rate chiller to –6 $^{o}$C (at a rate of 0.1 $^{o}$C min$^{-1}$).

3.4) Supercool the liver for 72–96 hours, or the desired duration.

**4. Recovery phase**

4.1) Prior to the end of the supercooling period, prime the perfusion system with the recovery solution.

4.2) Increase the temperature to 4 $^{o}$C (at a rate of 0.1 $^{o}$C min$^{-1}$) and subsequently remove the liver from the chiller and flush with 10 mL of Williams' medium E (21 $^{o}$C) on a backtable.

4.3) Connect the liver to the perfusion system and begin perfusion at 8 mL min$^{-1}$ and adjust the flow to maintain a portal vein pressure around 10 cm$H_2O$. Remove the first 150 mL of outflow and then close the system and allow the remaining solution to recirculate.

4.4) Perfuse for 3 hours and take regular samples for biochemical and blood gas analysis. Collect and quantify bile production and calculate resistance of the liver. Sample the perfusion solution (1 mL), store at –80 $^{o}$C until further use. Analyze the samples for markers of liver injury (e.g. alanine/aspartate aminotransferase; ALT/AST) using a commercial reagent.

**5. Orthotopic liver transplantation and follow-up**

5.1) Induce anesthesia with 5% isoflurane/95% oxygen gas inhalation and maintain with 2-3% isoflurane. Confirm adequate anesthesia by tail pinch and monitoring respiratory rate. Administer 0.1 mg/kg buprenorphine subcutaneously just before beginning the recipient procedure. Begin hepatectomy of the recipient as described elsewhere[12] 20–30 minutes before the end of the recovery phase.

5.2) At the end of the recovery phase, disconnect the liver from the perfusion system and flush with 10 mL or Lactated ringers solution (21 $^{o}$C) on a back table.

5.3) Complete hepatectomy, mark the beginning of the anhepatic time, and orthotopically

transplant the supercooled liver by sutured anastomosis of the suprahepatic inferior vena cava and cuffed anastomosis of the PV and IHIVC as described by Delrivière et al.[12]. Reperfuse the liver, and note the end anhepatic time, this should be < 20 minutes.

**NOTE**: The anheptic time markes the time between ligation of the portal vein and reperfusion of the donor liver, where there is no flow through the liver.

5.4) Monitor the animal continuously for a minimum of 3 hours after transplantation to ensure recovery of consciousness and sternal recumbency. Administer 0.1 mg/kg buprenorphine subcutaneously for a minimum of 72 hours post-transplantation. Monitor recipients daily for signs of liver failure and weight loss. Do not return the recipient to the company of other animals until full recovery.

5.5) Collect 0.5 mL of venous blood daily by tail vein sampling for biochemical analysis with a liver panel in a biochemistry analyzer

5.6) At the end of the follow-up period, induce anesthesia with 5% isoflurane/95% oxygen gas inhalation and maintain with 2-3%. Confirm adequate anesthesia by tail pinch and monitoring respiratory rate. Euthanize the animal by exsanguination and perform necropsy to collect samples for histological and tissue analysis.

**REPRESENTATIVE RESULTS:**

Livers can be preserved at -6 $^{o}$C with 100% non-freezing rate for 96 hours. During the recovery phase liver preserved for 96 hours produce 8–70 µl g liver$^{-1}$ of bile and 26–91 µl g liver$^{-1}$ after 72 hours of supercooling. Oxygen uptake remains constant during recovery perfusion and ranges from 2.8–8.6 mL $O_2$ min$^{-1}$. Resistance will generally remain constant at around 1.0 cm$H_2O$ min mL$^{-1}$ and an increasing resistance may be suggestive of poor survival following transplantation. ATP content is reduced to 9.0% following 96 hours of supercooling, but is reconstituted significantly during the recovery phase (figure 3).

After 72 hours of supercooling, 100% post–transplantation survival can be expected. Increasing preservation time to 96 hours reduces survival rates to 58% (figure 4). In control groups that have been preserved in UW solution at 0–4 $^{o}$C, survival is 0%. Controls that omit any component of the supercooling regimen (3–OMG loading, supercooling or recovery phase) result in 0% survival. Post–operative AST/ALT may increase to 700–1000 U L$^{-1}$ in the first week, but continue to decrease thereafter.

**Figure Legends:**

**Figure 1: Graphic representation of supercooling scheme.** The y-axis represents the temperature of the liver and the x-axis the preservation time, not to scale. The varous phases of the preservation scheme are illustrated.

**Figure 2: Schematic of the subnormothermic machine perfusion system.** Continuous black lines represent tubing for the perfusion solution and the interrupted blue lines circulate cold

antifreeze from the controlled rate chiller to the jackets of the organ chamber, bubble trap and oxygenator.

**Figure 3: ATP content.** During the course of a 96 hours of supercooling ATP content decreases to ± 9.0% after supercooling, but is significantly increased during SNMP.

**Figure 4: Survival.** Kaplan-Meier curve of survival after orthotopic transplantation. Controls include protocols that omit a single element of the supercooling regimen,

**Table 1:** Composition of the solutions.

**DISCUSSION:**

Current liver preservation is limited to 12 hours, with inferior results if cold ischemic time is extended past this limit due to relatively rapid deterioration of the liver preserved on ice [13]. As a result, organ sharing is limited to small regions and organ viability is lost during storage [2]. To improve preservation, we have attempted to extend preservation times and improve the overall preservation of organ, by slowing down deterioration. By reducing the temperature of the liver from 4 $^{o}$C to –6 $^{o}$C we significantly slow down metabolic processes that result in ischemic injury. True cryopreservation has proven difficult due to the formation of ice crystals, which directly damage tissue as well as dehydrating cells by drawing water from them on nucleation.

This protocol makes use of cyroprotectants and machine perfusion to facilitate a supercooled state of preservation. Cryoprotectants can be used to mitigate cold-induced injury, either by preventing ice formation or alleviating the effects of freezing. In this work we make use of a glucose derivative and polyethylene glycol, which protect the intra– and extracellular space respectively, and are able to cool rat livers to –6 $^{o}$C for up to 4 days without evidence of ice formation

Machine perfusion has proven very effective in recovering livers injured after both warm and cold ischemia. Here we made use of machine perfusion to load the cyroprotectant into the liver as well as recover the liver at the end of supercooling. Using machine perfusion, which supplies the liver with nutrients and oxygen, the liver is recovered prior to transplantation, replenishing ATP stores [14]. This recovery proved to be an essential component in the achieving survival.

Since the liver is cooled to below the freezing point of the preservation solution, freezing of the liver is possible if the temperature is too low. If this occurs, it is recommended that the temperature inside the bag be monitored using a thermocouple to ensure the temperature does not fall below the safe temperature of –6 $^{o}$C.

We previously determined that rat liver cold be preserved on ice for up to 24 hours with 100% survival [7]. Using supercooling, the viable preservation time was extended to 72 hours, tripling the safe preservation time and successful transplantation can even be achieved after 96 h of storage. Further optimization of this technique may allow even longer storage. This protocol will allow other researchers to build on our initial experience.

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**DISCLOSURES:**

The authors of this manuscript have conflicts of interest to disclose. Dr. Uygun and Yarmush are inventors on a pending patent that is relevant to this study (WO/2011/ 002926), and Drs. Berendsen, Uygun and Yarmush are inventors on a pending patent that is relevant to this study (WO/2011/35223). Dr. Uygun has a financial interest in Organ Solutions, a company focused on developing organ preservation technology. Dr Uygun's interests are managed by the MGH and Partners HealthCare in accordance with their conflict of interest policies.

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Figure 1
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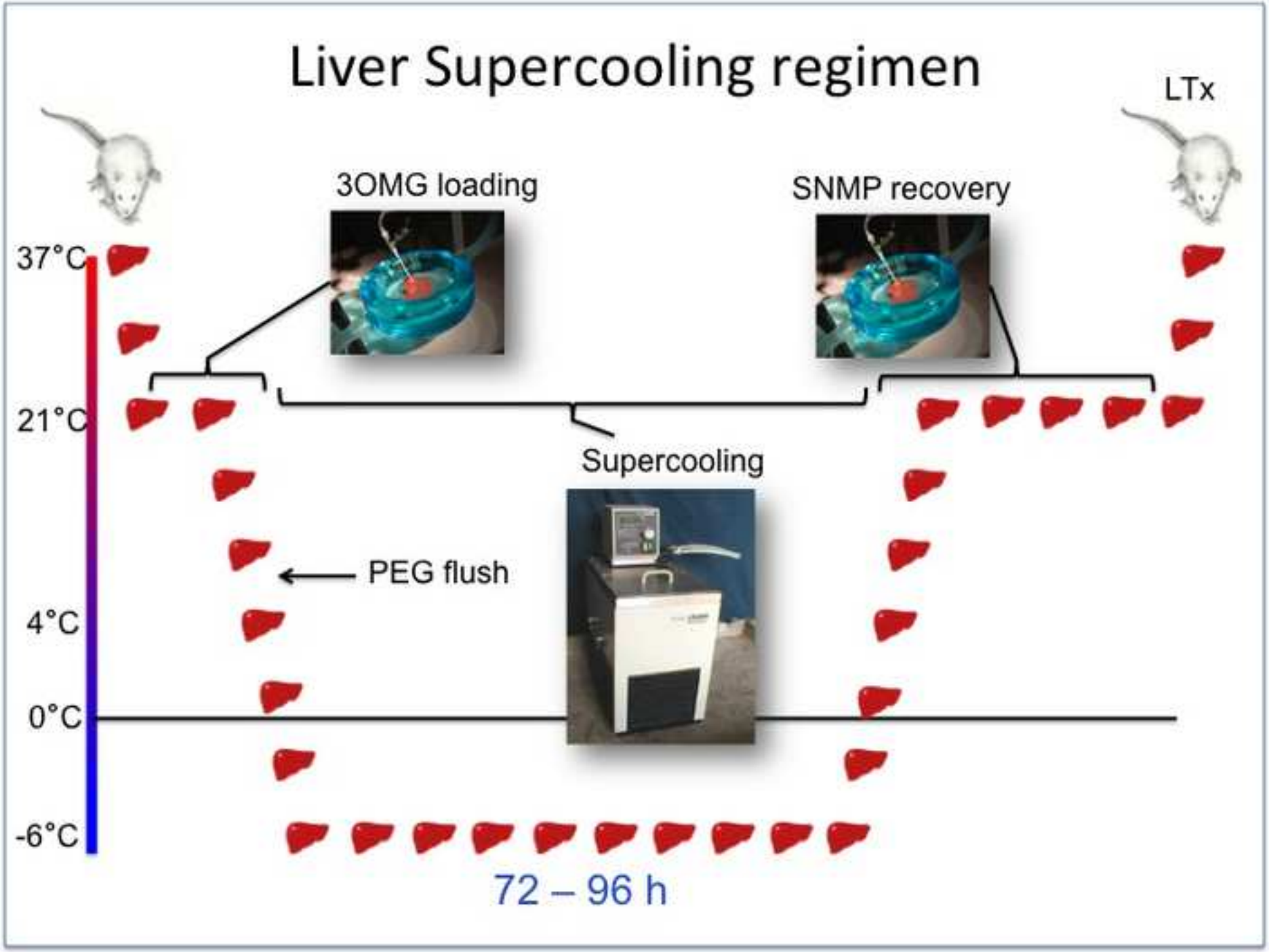

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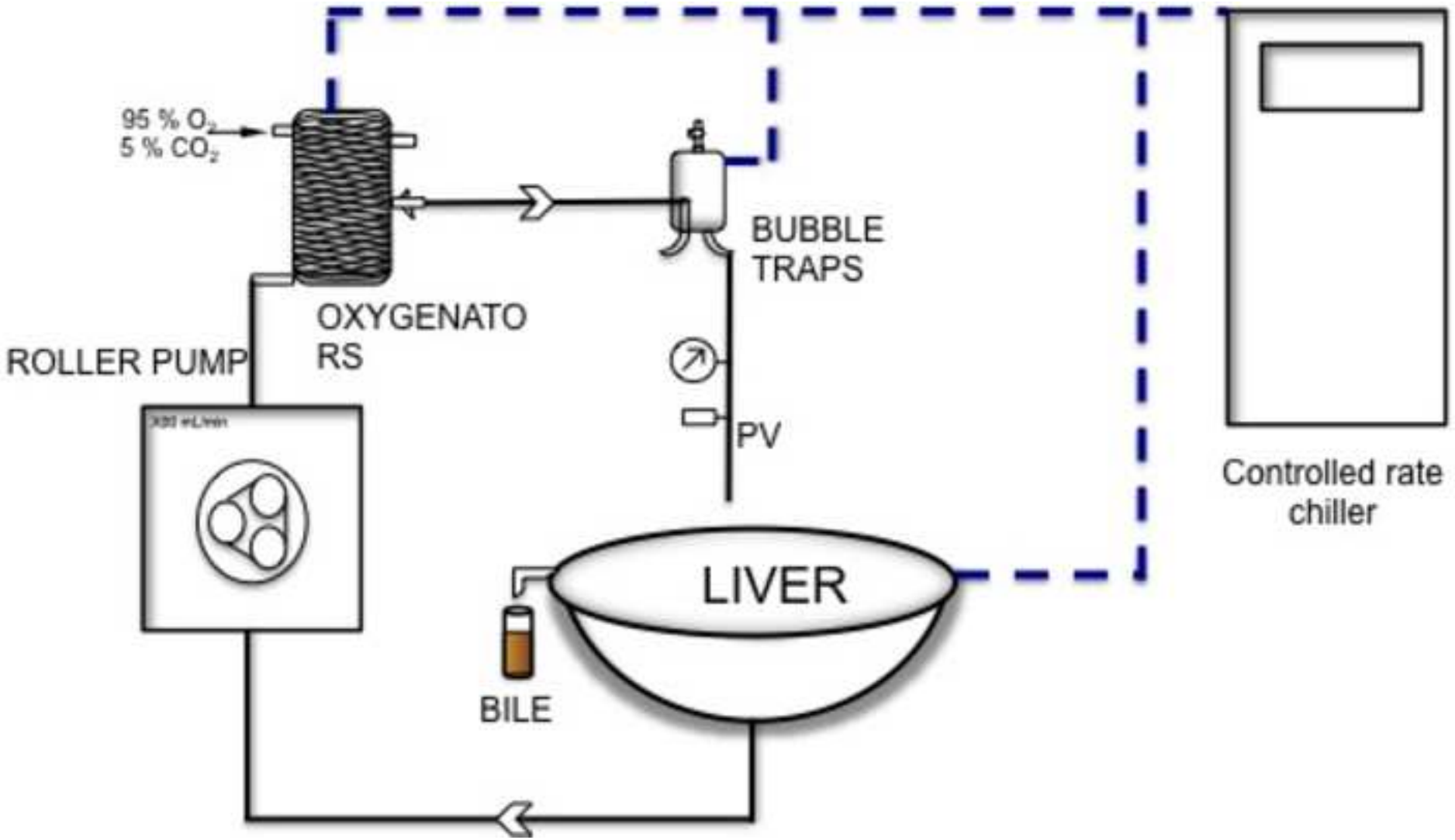

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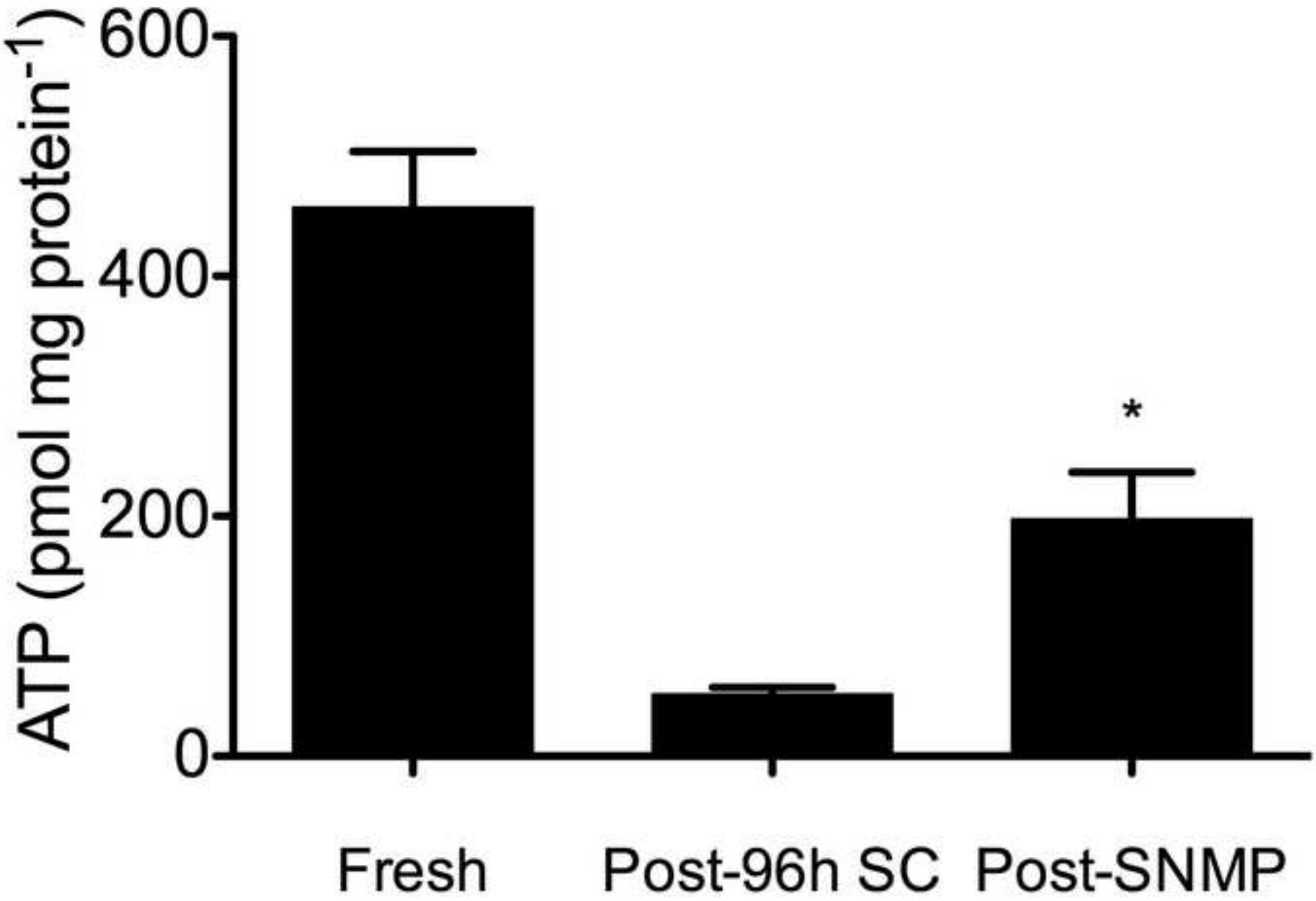

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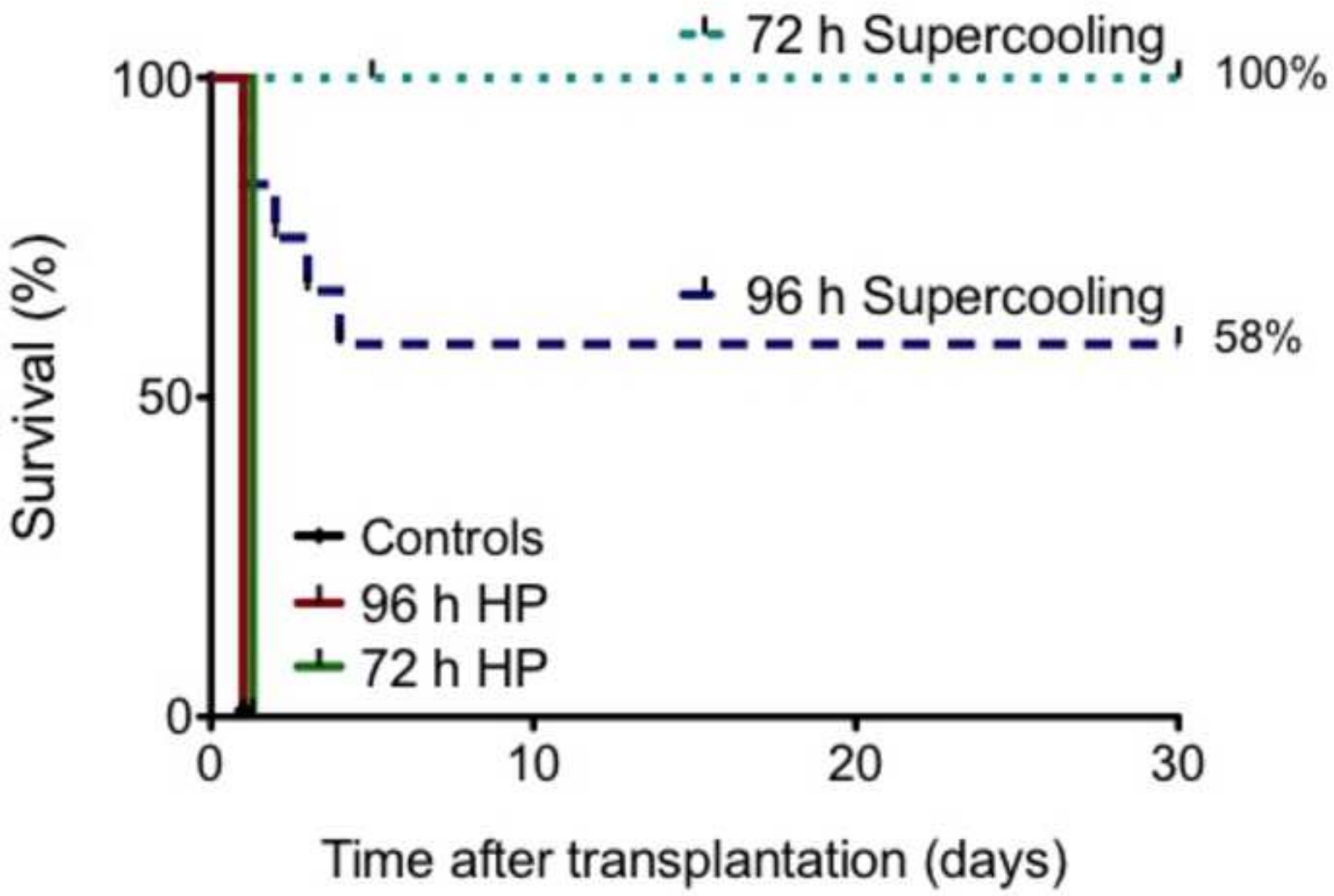

Table 1
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| Component (volume) | Concentration |
|---|---|
| **3–OMG loading solution (500 mL)** | |
| phenol red–free William's medium E (Sigma) | |
| Insulin (Humulin; Eli Lilly & Co) | 750 U L-1 |
| penicillin (Life technologies) | 40,000 U $L^{-1}$ |
| streptomycin (Life technologies) | 40,000 µg L-1 |
| L-glutamine (Life technologies) | 0.292 g $L^{-1}$ |
| hydrocortisone (Solu-Cortef; Pharmacia & Upjohn/Pfizer) | 10 mg $L^{-1}$ |
| Sodium heparin (APP pharmaceuticals) | 1000 U $L^{-1}$ |
| 3–O–methyl glucose (Sigma) | 0.2 M |
| | |
| **Supercooling solution (85 mL)** | |
| University of Wisconsin (UW) solution | |
| Penicillin | 200.000 U L-1 |
| Insulin | 40 U $L^{-1}$ |
| Polyethylene glycol, PEG (35 kDa) | 5% w/v |
| | |
| **Recovery solution (500 mL)** | |
| phenol red–free William's medium E (Sigma) | |
| Insulin (Humulin; Eli Lilly & Co) | 2 U L-1 |
| penicillin (Life technologies) | 40,000 U $L^{-1}$ |
| streptomycin (Life technologies) | 40,000 µg L-1 |
| L-glutamine (Life technologies) | 0.292 g $L^{-1}$ |
| hydrocortisone (Solu-Cortef; Pharmacia & Upjohn/Pfizer) | 10 mg $L^{-1}$ |
| sodium heparin (APP pharmaceuticals) | 1000 U $L^{-1}$ |
| | |
| **Lactated Ringers solution (10 mL)** | |

Excel Spreadsheet- Table of Materials/Equipment

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| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| **Solutions and supplements** | | | |
| Williams' Medium E | Sigma | W1878-6x500mL | phenol red free |
| University of Wisconsin solution | Preservation Solutions Inc. | CoStorSol | |
| Poly(ethylene glycol) BioUltra, 35,000 | Sigma | 94646 | |
| Insulin | Eli Lilly & Co | Humulin R U-100 | |
| Penicillin-Streptomycin | Life Technologies | 15070-063 | |
| Hydrocortisone | Pharmacia & Upjohn/Pfizer | Solu-Cortef 100 mg | |
| L-glutamine | Life technologies | 25030-081 | |
| Sodium heparin | APP pharmaceuticals | Heparin 10.000 USP | |
| 3–O–methyl glucose | Sigma | M4879 | |
| Lactated Ringers solution | | | |
| **Machine perfusion system** | | | |
| Masterflex L/S Digital Drive | Cole-Palmer | WU-07522-20 | |
| Masterflex platinum-cured silicone tubing, L/S 16 | Cole-Palmer | EW-96410-16 | |
| Membrane oxygenator | Radnoti | 130144-001 | Jacketed |
| Tissue bath | Radnoti | 158360 | Jacketed |
| Bubble trap 5 mL | Radnoti | 130149 | Jacketed |
| Carbogen gas tank | Air gas | Z02OX9522000043 | |
| I.V. catheters (14/16/18G) | BD | Insyte 381705/381707/381709 | |
| Controlled rate chiller | Neslab | RTE–111 | |
| Surgical instruments | various | various | |
| Cautery kit | World Precision Instruments | 500392 | |
| Syringes | various | various | |
| Sutures (6-0, 7-0) | various | various | |
| ALT/AST reagent | Sigma | TR70121/TR71121 | |

| | | |
|---|---|---|
| Isoflrane anesthetic gas (Forane) | Baxter | Forane (isoflurane, USP) |
| Buprenorphine | various | various |
| Piccolo biochemistry analyzer | Abaxis | Piccolo Xpress |
| Piccolo Liver panel plus | Abaxis | 400-0003 |

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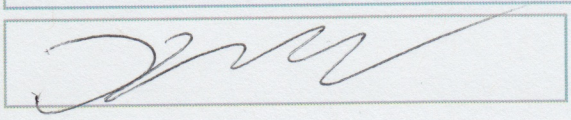
| Signature: |  Date: 5/22/14 |

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