

Cryopreservation of a whole liver

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Preservation of vascularized organs such as the liver is limited to 24hrs. before destructive processes disqualify it for transplantation. This narrow time window prevents surgeons from performing optimal pathogen screening and matching tests which often lead to re-transplantation. Numerous problems are associated with viably freezing and thawing a whole liver: complicated geometry, poor heat transfer, release of latent heat and the difficulty of generating a uniform cooling rate. Our past success led us to apply our novel freezing technique to a larger solid organ, the liver. Whole livers were frozen/thawed using a directional solidification apparatuses; viability was tested by means of integrity and functionality in vitro and in auxiliary liver transplantation. Thawed livers were intact with over 80% viability; histology revealed normal architecture, bile production and blood flow following auxiliary transplantation where normal. Our results suggest a novel cryopreservation method and may enable better organ donor-recipient matching in the future.

The ever-growing need for organs and the narrow time-window surgeons have between harvesting and transplanting the donated organ have led many laboratories to research the field of organ preservation. To date, solid organs such as the liver are preserved ex-vivo in hypothermic conditions under which the organ remains viable for a short period of time, and thus ischemic storage of donated organs is normally limited to 12-24h. Broadening this time-window would enable better pathogenic screening and organ matching, which is the leading cause for rejection and consequent retransplantation. Donated organs such as the liver that are harvested from brain-dead donors (or in some cases live donors) and preserved ex-vivo in hypothermic conditions must be transplanted almost immediately. Hypothermia does not stop metabolism but it slows biochemical reaction rates and decreases the rate at which intracellular enzymes degrade essential cellular components necessary for organ viability.

Hypothermic storage of livers is done in one of two manners: continuous perfusion and cold storage. Continuous perfusion storage involves the continuous infusion of a cold preservation fluid through the vasculature of the harvested organ [1, 2]. In cold storage, the organ is normally suffused with a preservation solution before, or immediately after, being excised, and then placed in a cold chamber (ca. 4-8°C), without further manipulation until its preparation for use. Using continuous perfusion, canine kidneys were hypothermically preserved for 72h [3] and rat livers were preserved at a temperature of -3°C, for a period of 6 h [4]. The best results to date in liver

preservation by continuous machine perfusion were obtained with canine livers that were preserved for 72 h at 5°C [5].

Since hypothermic preservation suggested no solution for the problem of long-term preservation of livers a different approach had to be taken. One option that was, therefore, suggested is storage at subzero conditions, either by freezing or vitrification. Vitrification is an "ice-free" cryopreservation method which enables the overcoming of many of the problems mentioned above however [6], the method of vitrifying large solid organs suffers from several disadvantages such as the high toxicity of the vitrification solution, fractures caused to the vitrified organ by the vitrification process, problems engendered by the warming procedures [7, 8] and the fact that due to a limited capability of heat transfer this method is limited to only very small organs or tissue samples.

There are also numerous problems associated with freezing and thawing large vascularized organs such as the liver including the difficulty of generating heat transfer within a large thermal mass with a geometry that has little malleability, the packing density of cells within the organ and the presence of many different cell types, each with its own requirements for optimal freezing and thawing conditions. Another problem associated with freezing large biological samples is the isothermal period caused by the massive release of latent heat during the crystallization stage of the process [9-11]. In addition, the cooling and warming rates have a direct effect on the post thaw viability of biological samples. During slow and conventional freezing, the cooling rate should be slow enough to allow dehydration and to avoid intracellular ice formation (IIF), but should be fast enough to avoid excessive exposure to the concentrated solutes and cryopreservatives (CP's) in the partially frozen solution [11-13]. The results of recrystallization are that as cooling continues the cells may be exposed to supercooling and sub-physiological temperatures for an extended period (i.e. isotherm). This prevents the resumption of the sample's temperature decline, the outflow of water from the cells by diffusion and IIF, which are damaging to the cells [11, 12, 14].

In previous reports we described the successful freezing, using directional solidification, and subsequent thawing and transplantation of intact sheep ovaries [15], as well as the freezing of a rat heart [16], both of which are well vascularized organs with physical dimensions larger than any other solid organ ever preserved by freezing. Following these successes, we decided to try other even larger organs with the goal in mind to make cryopreservation available as a useful tool for transplantation centers with which to overcome the problems associated with pre-transplantation tests and better donor/recipient matching. In the present study we report the freezing to low temperature, the long term storage and the successful auxiliary transplantation of a whole Liver.

Results

Rat liver in vitro model

Following full thawing, out-flow rate was the same as in a fresh, untreated, liver (data not shown). In the livers (n=3), which were stored in liquid nitrogen (LN) (-196° C), bile production, as the indicator of liver function, was 60 ± 15 % (average \pm SD) when compared to fresh liver bile production; and in the group, which was stored in a -80° C freezer (n=3), bile production reached 84 ± 17 %. Integrity of Hepatocyte cell membrane, as an indicator of cell viability, measured using Trypan-Blue exclusion, SYTO/PI and cFDA (fig. 3) fluorescence reached levels of 63 ± 11 % (average \pm SD) when stored in LN, and 85 ± 6 % when stored in -80° C (table 1). General tissue architecture of the post thawed liver biopsies was maintained as revealed by E&H staining when compared to a fresh liver sample. Screening for apoptotic/necrotic cells revealed intact cells with no DNA fragmentation as a sign of apoptosis or necrosis. Immunohistochemistry using α -SMA showed normal expression regardless of the storage conditions (fig. 2).

Porcine Liver in vitro model

Immediately after thawing, the liver was perfused with solution supplemented with collagenase in order to isolate the liver's cells from its surrounding stroma. Isolated hepatocytes from a post-thaw porcine Liver, which was frozen to -40° C using our novel freezing system (n=3) showed viability ranging from 75% to 90%, as measured using a light microscope and Trypan-Blue exclusion assay. The freezing procedure is described in fig. 4.

From the subjective surgeon's report the reperfusion was satisfactory and blood was free-flowing with no observed obstructions. The grafted liver warmed up and developed a homogenous viable color within a few minutes. The parenchymal consistency was adequate without the clinical presentation of overt ischemia or congestion. Surprisingly, we also noted a slow bilious stained secretion at the tip of the graft bile duct.

Discussion

Despite continuing advances in medicine and technology, the demand for organs drastically exceeds the number of organ donors. The waiting list for a liver donation, approximately 17,000 in the USA alone in 2006, is ever-growing and in many cases results in patient deaths. A major goal in the field of organ transplantation is to extend the preservation period of organs while maintaining their functionality and viability at a level that will allow transplantation. Extended time will allow improved tissue typing and histocompatibility matching, better pathogenic screening, adequate preparation and/or treatment of the recipient and the surgical team and will ease other logistical

aspects such as transporting the organs. All these benefits will lead to increased post transplantation survival rate and better quality of life for the organ recipients.

Since the 1950's there have been many attempts to preserve a whole organ by hypothermic storage, freezing or vitrification, starting with the pioneering experiments of A.U. Smith [18] and up to latest achievements, such as the vitrification of kidneys [6] and the freezing of a whole rat heart [16] and sheep's ovary [15].

Vitrification of organs was first proposed by G. Fahy as a method to overcome freezing problems such as the formation of uncontrolled ice crystals [6]. However, this method suffers from several disadvantages, including: (a) the vitrification solution contains high concentrations of cryoprotectants, which are toxic; (b) fractures are caused to the vitrified organ by the vitrification procedure [7] and (c) devitrification problems occurring during the warming step [8].

One of the problems associated with freezing biological material is the release of latent heat during the crystallization stage of the process [9, 19]. The reaction of water molecules rejoining to form an ice crystal releases a large amount of energy in the form of heat (latent heat) causing a rise in the temperature of the surroundings. For example, 500ml of water (as present in our pig liver) will release 165 kilojoules upon crystallization. The heat, in this case, will be transferred mainly to a conductive material, meaning to the ice crystals that were just formed, giving rise to transient thawing followed by recrystallization, a sequence that may damage the cells [10, 11]. Therefore, recrystallization in itself is a damaging process, both in freezing and thawing [20]. Additionally, the results of this recrystallization are that, as cooling continues, the cells may be exposed to a sub-physiologic temperature, an isotherm, for an extended period (see fig. 1), which may cause chilling injury as described by Mazur in the early 90's [14]. In addition, this may lead to intracellular ice crystallization, which may cause further damage [18, 21, 22]. When freezing large volume samples with relatively low ratios of surface to volume, the release of latent heat may cause a long isothermal period (or even heating) in the material being frozen. At the same time, the temperature of the cooling means or the surrounding medium is lowered, thus increasing the temperature difference between the sample and its surroundings. Consequently, when latent heat is no longer released, the temperature of the material being frozen will drop too rapidly to a temperature close to the temperature of the surrounding environment. This might cause a cooling rate, which is higher than the optimal rate, and thus possibly cellular damage due to intracellular crystallization [23-26].

The current method to overcome the problem of cell damage due to release of latent heat is the removal of the excessive heat through adjustment of the cooling rate at specific time points and ensuring uniform freezing by maintaining a high ratio of surface to volume. In other words, the sample to be frozen is made to be as thin as possible, and thereby heat from the inner part of the sample, which is

being released through the surface of the sample, will be removed faster due to the steep temperature gradient. In this way it is possible to apply the optimal cooling rates for each sample and at the same time provide a heat sink for rapid absorption of the released latent heat. The optimal predetermined cooling rate should be at a level that allows water to leave the cell and freeze outside it while the cell is shrinking. In a case of an suboptimal cooling rate, water within the cell will not have enough time to leave it before freezing; and, therefore, the water will freeze within the cell causing intracellular ice formation, which consequently kills the cell [11]. Conventional freezing devices are based on heat transfer by means of convection, i.e. using multi-directional (equiaxial) heat transfer to achieve a rate of temperature change in the sample that is dependent on the thermal conductivity and geometric shape of the container and the biological material that it contains [23, 27]. In contrary to conventional freezers, the freezing apparatus we used is based on directional freezing [28], a method which has been shown successful in freezing bulky systems such as sperm [29] and a whole sheep ovary [15]. This system enables precise adjustment of the temperature gradients in order to achieve an accurate desired cooling rate through the entire tissue. In order to achieve this goal of uniform cooling we have built our freezing devices based on two principals: (a) large mass of conductive material which enables rapid evacuation of latent heat being released due to crystallization in the freezing front, and (b) since the sample is moving through the temperature gradient at a controlled speed we are able to create a very precise and uniform freezing front (the interface), which allows efficient removal of the latent heat. Moreover, the freezing system enabled us to monitor the precise measurement of temperatures along the cooling process. As described in the methods section, a thermocouple is inserted inside the organ enabling changes in the organs temperature to be monitored "on-line". This data gave us the opportunity to test our freezing method with two different approaches: (1) According to former observations the device temperature gradient and all other parameters are preset in order to achieve an optimal freezing procedure. (2) The rate of cooling is monitored on-line, whereby any deviation from a desired temperature or desired rate of cooling is corrected through increasing or decreasing the device blocks' temperatures, thus, controlling the rate of heat transfer.

In a directional freezing device this rate may also be changed by increasing the velocity of the sample movement along a temperature gradient, or by using a different temperature gradient, or both.

Isothermal period, as a damaging process during the freezing procedure, was relatively short when compared to the conventional freezing curve of the rat liver experiments (fig.1) and was totally avoided in the porcine liver experiments (fig. 4).

Applying our freezing methods to a whole liver has demonstrated the possibility of slow and directional freezing to cryopreserve whole organs. The thawed liver was intact and had no fractures on its surface. Thermal characteristics of the freezing process, as monitored using thermocouples in and outside the organ (figs. 1, 4), revealed a uniform cooling gradient with expected release of latent heat. During re-perfusion the spreading change of tissue color indicated the inner integrity of the liver's parenchyma and the perfusate out-flow strengthened this observation. Histological examination of tissue sections revealed normal tissue organization; and screening for apoptotic/necrotic cells revealed intact cells with no DNA fragmentation as a sign of apoptosis or necrosis. Immunohistochemistry using α -SMA showed normal expression regardless of the storage conditions (fig. 2).

One of the strongest pieces of evidence indicating integrity and functionality of a liver is its ability to produce bile [30] and indeed, our thawed livers maintained this ability (table 1). The viability of the isolated hepatocytes, as expressed by exclusion of the Trypan-blue (membrane integrity) and the live/dead staining with SYTO/PI (fig. 3), has strengthened our hypothesis about the efficiency of our freezing method and encourages us to test this method in a model of orthotopic liver transplantation.

Materials and Methods

Cryopreservation apparatuses

For rat liver freezing the "Multi-Thermal-Gradient" (MTG) freezing device applying directional freezing technology was constructed (US 5,873,254 to Arav). In brief, the device is built of 4 temperature domains consisting of 270 mm copper blocks. The tube containing the liver is advanced at a constant velocity (V) through the predetermined temperature gradient ($G = \Delta T/d$ where ΔT indicates temperature differences and d is the distance between temperatures) resulting in a cooling rate (B) according to the equation $B = G * V$.

For the porcine liver experiments, a static freezing device was used (WO 2006/016372 to Shacham). The static directional freezing apparatus (known as LSFS) is based on two brass blocks, thermal-conducting masses, which can be cooled or warmed to any desired temperature at any cooling/warming rate. The biological material is placed in tight contact between the two blocks. Dependant on the temperature of the blocks, the contact may allow directional freezing by generation of one or more controlled thermal gradients within the object, without a need to move the object being frozen or warmed.

Rat Liver in vitro model

All chemicals were purchased from Sigma, Israel, unless otherwise indicated. This research was approved by the Animal Research Ethics Committee. Lewis Rats, 220-300gr., (n=6) were anaesthetized with Ketamine-xylasin (intra peritoneal). The liver was exposed by a transverse mid incision. The vena cava caudalis was ligated and a small cut was made. 20G tubing was inserted via the portal vein; then the perfusion was started. For collection of bile fluid a polyethylene tube (length: 5 cm, I.D. 0.28 mm, O.D. 0.61 mm, Becton Dickinson, Sparks, MD, USA) was placed in the common bile duct (ductus choledochus). The liver was first perfused with 1ml of phosphate buffered saline containing 400 units of Heparin, followed by 5 minutes of perfusion using a perfusing solution consisting of Hank's balanced salt solution (Biological industries, Beit Ha'emek, Israel) containing EDTA (0.5 mM). This was followed by a 3 min. perfusion with UW solution (Bristol-Myers Squibb Pharmaceutical, Ireland) supplemented with 10 % EG. Flow rate was maintained at 23 ml/min. After *in situ* perfusion the liver was excised and transferred to a 25mm glass tube that contained the same freezing solution used for perfusion. The tubing of the excised liver ensured continuity between the perfused organ and the surroundings. A thermocouple was inserted through the cannula in order to monitor the temperature inside the liver during the freezing process. A freezing protocol was employed using the MTG freezing apparatus (fig. 1). First, seeding was initiated by plunging the tip of the tube into LN for 10 sec. Then the tube was inserted into the MTG freezing apparatus. The tube was cooled down to -40° C, and when the freezing procedure was completed the frozen tube was transferred either to a -80°C freezer (Thermoforma, U.S.A.), or to a standard -196°C LN tank, for storage. Storage time varied between 1 to 3 days for the LN storage and 3 to 21 days for the tubes that were store at -80° C. For the thawing process, the frozen tube was left at room temperature for 5 min. and 30 sec. then followed by submergence in a turbulent thawing bath containing phosphate buffered saline at 38°C for 2.5 min.

Viability tests

Liver out-flow, bile production and viability of isolated hepatocytes

For viability tests, following full thawing, a cannula was re-connected to the portal vein and the freezing solution was washed out for 15 min. Out-flow rate was measured for 5 min. following washing. Bile production was measured by collecting the bile produced by the thawed liver for 10 min. following washing and was calculated as volume of bile produced per min. divided by the weight of the liver. Following washing, biopsies were taken from the rat liver for histology and Hank's balanced salt solution containing NaHCO₃ (25 mM), CaCl₂ (5 mM), and collagenase (0.2 U/ml, Sigma, Israel) was perfused for 8 min. The liver was then shaken gently in a 37°C bath for 10 min. Then the liver was filtrated through a 100µm mesh and the filtrate was centrifuged 3 times for 4 min at 2000 rpm. The pellet of cells was re-suspended in DMEM supplemented with 10% fetal

calf serum, penicillin (100 U/ml), streptomycin (0.1 mg/ml) and insulin (100 nM). Hepatocyte viability was assessed under a light microscope by Trypan-Blue exclusion using a Hemocytometer and by Microscope equipped with an epi-fluorescence attachment using SYTO/PI (Molecular probe USA).

Histology and immunohistochemistry

Biopsies were fixed in 4% paraformaldehyde in PBS at 4°C. Then dehydration in graded ethanol solutions, cleared in chloroform and embedded in Paraplast (Sigma, USA) Serial 5 µm sections were prepared and stained with E&H for general tissue architecture. Apoptosis/necrosis was detected by the TumorTACS in situ Apoptosis Detection Kit (R&D Systems, Minneapolis, MN, USA) by using terminal deoxynucleotidyl transferase (TdT) that adds biotinylated nucleotides to the 3'-ends of the DNA fragments. The apoptotic cells were visualized by streptavidin-conjugated horseradish peroxidase specifically bound to biotinylated DNA fragments that generated a brown color in the presence of diaminobenzidine (DAB). Methyl-Green served as a counter stain [17]. Immunohistochemistry was performed using a polyclonal anti α -smooth muscle actin (α -SMA) antibody (1:200, Dako corp., USA) and secondary Ab. from a Histostain plus kit (Zymed laboratories, USA) according to the manufacturer instructions (fig. 2).

Porcine Liver In vitro model

Female Pigs (Lahav Research Institute, Israel. n=3, BW 16-20 Kg) were anaesthetized (intramuscularly) with 10mg/kg ketamine hydrochloride and 4 mg/kg xylazine hydrochloride (Vetmarket, Israel). This research was approved by the Animal Research Ethics Committee. The liver was exposed by a longitudinal midline incision. The hepato-duodenal ligament was dissected by division of the bile duct, ligation and division of the hepatic artery and isolation of the portal vein. The liver was immobilized and the infra- and supra-hepatic inferior vena cava was divided, the portal vein was transected and the liver was extracted en-bloc and was immersed in an icy basin. A Silicon tubing (Teva medical, Israel. I.D. 3mm O.D. 4mm) was inserted via the portal vein, fixed and connected to a peristaltic pump. The liver was perfused with Hanks balanced salts solution (Biological industries, Beit Ha'emek, Israel) supplemented with 370mg/liter Ethylene Diaminetetraacetic Acid (EDTA) and 5 U/ml Heparin at 150ml/min for 5 min. at room temperature in order to flush the blood out of the liver. This was followed by a 5 min. perfusion with freezing solution consisting of University of Wisconsin (UW) solution (Bristol-Myers Squibb Pharmaceutical, Ireland) supplemented with 10 % Ethylene Glycol (EG) at 4°C. Flow rate was maintained at 150 ml/min. After perfusion, the liver (about 25cmX20cmX5cm) with its catheter

attached, was excised, and transferred to a Polyethylene bag (37cmX26.5cm, with Polyethylene thickness of 0.1mm). A thermocouple was inserted through the tubing of the portal vein for monitoring the liver temperature. The bag was transferred to the LSFS Freezing System and an additional 250ml of freezing solution (4°C) was added to the bag. The LSFS was tightened to ensure maximal contact between the bag and the LSFS walls, such that the distance between the device cooling plates was about 5cm. Total time of cold ischemia was 15±2 min.

During the freezing procedure the Freezing System initially cooled the sample to 0°C for 20 min. Then seeding was initiated by lowering the temperatures of system's blocks to -10°C for 20 min. This was followed by lowering the block temperatures to -20°C at a cooling rate of 1° C/min, in order to achieve quick removal of latent heat. This temperature was also maintained followed by a cooling rate of 0.2°C/min to a final temperature of at least -40°C in order to ensure directional growth of the ice crystals inside the liver (fig. 4).

After freezing to -40°C, the liver was thawed as follows: The frozen bag was immersed in a 10 liter bath of 0.9% NaCl in distilled water at 38°C. During the thawing process the liver was rubbed and gently shaken to maximize its surface area in order to quicken the thawing process. Full thawing was achieved after 17 ± 2 min.

Viability tests

For viability tests, following full thawing, a cannula was re-connected to the portal vein and the freezing solution was washed out with Hanks balanced salts solution for 8 min. at 150 ml/min. Hank's balanced salt solution containing NaHCO₃ (25 mM), CaCl₂ (5 mM), and collagenase (0.2 U/ml) was perfused for 8 min. The liver was then shaken gently in a 38°C bath for 10 min., and then filtrated through a 100µm mesh. The filtrate was centrifuged 3 times for 4 min at 2000 rpm. The pellet of cells was re-suspended in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and insulin (100 nM). Hepatocyte viability was assessed under a light microscope by Trypan-Blue exclusion using a Hemocytometer and by Microscope equipped with an epi-fluorescence attachment using cFDA flouorstaining (Table 2).

Auxiliary post-thawed porcine liver transplantation model

In order to check the feasibility of our method and to mimic the future use of this method in human trials we preformed a preliminary study using the Auxiliary Porcine Liver transplantation model as approved by the Animal Ethics Committee.

A female donor pig (BW 16 Kg) was anaesthetized using the following protocol: Pre-medication: Ketamine HCl 10mg/kg+Xylazine 1-2 mg/kg+Diazepam 5 mg/kg IM. Induction: Thiopental 1-2

mg/kg (to effect) - I.V. Maintenance: After intubation, Halothane 1- 2% was delivered via positive pressure ventilation utilizing 100% oxygen (2 Liter/min). The excision and preparation of the liver was the same as in the in vitro model described above, but with an emphasis on harvesting the greatest possible lengths of the portal-mesenteric vein and inferior vena-cava. Euthanasia of the donor pig was applied by intraperitoneally injection of Pentothal. During the freezing procedure the freezing system initially cooled the sample to 0°C for 20 min. Then seeding was initiated by lowering the temperatures to -12°C for 15 min. This was followed by lowering the temperatures to -20°C at a cooling rate of 0.3° C/min. Temperature was maintained until the Liver's temperature reached -20°C in order to ensure directional growth of the ice crystals inside the liver. The total freezing time was 1 hour. The release of latent heat was observed about 22 minutes into the protocol.

After freezing to -20°C, the liver was thawed as follows: The frozen bag was immersed in a 10 liter bath of 0.9% NaCl in distilled water at 38°C. During the thawing process the liver was rubbed and gently shaken to maximize its surface area in order to quicken the thawing process. Full thawing was achieved after 17 min. Following full thawing, during the preparation of the recipient pig, the liver was kept on ice for 1 hour. Total storage time (from harvest to inflow resumption) was 2.5 hrs.

The recipient pig (female, BW 18.5 Kg) was anaesthetized using the same protocol as the donor pig. The abdomen was exposed by a midline incision. The portal vein and the inferior vena cava were exposed and isolated with tapes. The thawed liver's cannula was re-connected to the peristaltic-pump and the freezing solution was washed out with Hanks balanced salts solution for 8 min. at 150 ml/min. The thawed liver was then brought and placed in the abdomen of the recipient, positioned perpendicularly and below the native liver. Outflow was re-established, by an end (graft) to side (recipient) anastomosis between the vana-cavas. Anastomosis was done with a pair of 6/0 prolene running sutures. The upper supra hepatic end of the IVC was kept clamped. Inflow was now reconstructed while the recipient's portal vein was partially blocked with a side biting vascular clamp and an end (graft) to side (recipient) anastomosis was created between the portal veins (prolene 6/0 running sutures). Following completion of the anastomosis, the clamps were removed and the Auxiliary liver graft was reperfused.

Viability tests

Visual impression following reperfusion included assessment of blood flow, re-warming, appearance of the grafted liver (color and consistency) and macroscopic bile secretion for 2 hours. All these were judged qualitatively by an experienced liver transplant surgeon.

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Figure Legends

Table 1. Summary of post thaw rat liver viability results.

Figure 1. Freezing process of a Rat Liver using the MTG system. Segmented line represents the programmed cooling curve, black line represents MTG directional solidification, arrow points to where latent heat release was evident and grey line represents equiaxial freezing curve of rat liver. Note the long isotherm (IT) period due to latent heat release.

Figure 2. Histology and Immunohistochemistry of rat liver. Eosin& Hematoxylin staining of fresh (a) and thawed (b), apoptosis detection on samples stored in LN (c, d), α -SMA Immunohistochemistry on sample stored in LN (e, f).

Figure 3. Viable Hepatocytes, isolated from a fresh, untreated liver (a,c) and from a post-thaw rat liver(b,d), cryopreserved to -196°C , fluorostained with SYTO/PI (a,b) and Trypan-Blue (c,d).

Figure 4. Freezing process of a Porcine Liver. The thin solid line represents the liver's temperature as measured during freezing, and the thick segmented line represents the temperature of the freezing device walls. Arrow points to where latent heat release was evident Notice the absence of isothermal periods.

Final Temperature (°C)	n	Post thaw Viability (% ,average \pm SD)	Post thaw Bile Production (% of fresh, average \pm SD)	Time of frozen Storage (Days)
-196	3	63 \pm 11	60 \pm 15	1-3
-80	3	85 \pm 6	84 \pm 17	3-21







