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An exploratory study on isochoric supercooling preservation of the pig liver

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ABSTRACT

This study was motivated by the increasing interest in finding ways to preserve organs in a supercooled state for transplantation. Previous research with small volumes suggests that the isochoric (constant volume) thermodynamic state enhances the stability of supercooled solutions.

The primary objective of this study was to investigate the feasibility of storing a large organ, such as the pig liver, in a metastable isochoric supercooled state for clinically relevant durations. To achieve this, we designed a new isochoric technology that employs a system consisting of two domains separated by an interior boundary that can transfer heat and pressure, but not mass. The liver is preserved in one of these domains in a solution with an intracellular composition, which is in osmotic equilibrium with the liver. Pressure is used to monitor the thermodynamic state of the isochoric chamber. In this feasibility study, two pig livers were preserved in the device in an isochoric supercooled state at -2°C. The experiments were terminated voluntarily, one after 24 h and the other after 48 h of supercooling preservation. Pressure measurements indicated that the livers did not freeze during the isochoric supercooling preservation. This is the first proof that organs as large as the pig liver can remain supercooled for extended periods of time in an isotonic solution in an isochoric system, despite an increased probability of ice nucleation with larger volumes. To serve as controls and to test the ability of pressure monitoring to detect freezing in the isochoric chamber, an experiment was designed in which two pig livers were frozen at -2°C for 24 h and the pressure monitored. Histological examination with H&E stains revealed that the supercooled liver maintained a normal appearance, even after 48 h of supercooling, while tissues in livers frozen to -2°C were severely disrupted by freezing after 24 h.

1. Introduction

Liver transplantation offers treatment for patients with end-stage liver disease. In liver transplantation, graft quality is of utmost importance and is predictive of short-term and long-term outcomes. This depends on organ graft's intrinsic performance and on the duration and quality of the organ's preservation until implantation. Liver grafts do not tolerate periods of static cold storage, which is the current standard of liver graft preservation [1].

Currently, static cold storage at +4 °C, under isobaric (constant pressure) atmospheric conditions remains the standard for static cold storage liver preservation, prior to transplant. This storage modality is based on the fact that lower temperatures allow for a decrease in the metabolic rate and thereby prolong *ex-corpore* survival. Static cold storage at +4 °C can preserve livers for less than 12 h [2], which is often too short to facilitate adequate transplantation or long-distance transportation. While lower temperatures would further reduce the metabolism and extend the period of storage, this is hampered by the fact that

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tissues freeze at temperatures below 0 °C (more precisely – 0.56 °C). At this time, no effective method for preserving a whole organ as large as the pig liver in a frozen state exists.

There is growing interest in developing new sub-0°C preservation techniques for the liver that avoid freezing, with the goal of enlarging the pool of donors for liver transplantation. Sub-0°C preservation techniques that avoid freezing, have employed: hyperbaric pressure [3,4] perfusion with antifreeze proteins from Arctic fish [5–9], perfusion with a combination of antifreeze proteins and glycerol [10,11]. A series of recent publications report a technological breakthrough in preservation of large human livers at subfreezing temperatures while avoiding the formation of ice [2,12–14]. A perfusion machine is used to inject and, after preservation remove, a cryoprotective perfusion cocktail comprised of 3-OMG (a glucose compound), PEG -35 (polyethylene), trehalose and glycerol [2,12–14]. All the sub-0°C preservation technologies mentioned above require the use of a cryoprotective agent for the process and, consequently, means to introduce and, after preservation, remove the cryoprotective agent from the tissue or hyperbaric pressure. There is interest in developing sub-0°C for organ preservation using thermodynamic supercooling. Two thermodynamic supercooling techniques have recently emerged. One technology employs the avoidance of contact between the preserving solution and air, with an immiscible layer of fluid on the top surface of the supercooled aqueous solution, to prevent nucleation at the air/fluid interface [15,16]. The second is the use of an isochoric (constant volume) chamber [17–23] to reduce the probability of ice nucleation through the Le Chatelier principle, which will be discussed later. Both techniques have advantages and disadvantages. The isochoric supercooling technique requires the use of a specialized constant volume chamber, but provides greater stability to the metastable supercooling state [22–24].

Theoretical and experimental studies have found that isochoric (constant volume) systems enhance the stability of metastable supercooled aqueous solutions in the isochoric system, without the need for cryoprotective additives or pressure elevation and in a very simple device, a constant volume chamber (22; 23; 24; 25; 17). The mechanism by which isochoric systems enhance the stability of metastable cooled solutions also draws from the Le Chatelier principle. When in a supercooled system, an ice nucleus forms by random nucleation, the local increase in density in a constant volume system yields an increase in the hydrostatic pressure. This is another force that is in opposition to the growth of the ice crystal, in addition to the change in the Helmholtz free energy due to the ice crystal surface tension [17,21].

The design of the experiments in this study has benefited from our research on aqueous, isochoric (constant volume) systems at subfreezing temperature. Simplistic, the phenomena which occur during freezing of an aqueous solution in a constant volume system can be attributed to the change in density of water upon freezing to ice. Since ice has a lower density than water, a system bound by isochoric walls will experience an increase in pressure upon freezing. As the temperature of an aqueous isochoric system is reduced to below freezing, two phases form: ice and solution. The thermodynamic state of a specific constant volume system, containing two phases in thermodynamic equilibrium, is found along the thermodynamic liquidus line between ice and water [25–28] An advanced thermodynamic analysis of an aqueous system in an isochoric system at subfreezing temperatures, derived from the Helmholtz equations, can be found in Ref. [29]. It is possible to completely characterize the thermodynamic state of an aqueous isochoric system at subfreezing temperatures by measuring only the pressure in the system [30,31].

The pressure in an isochoric system is hydrostatic and the use of a simple pressure transducer can provide complete information on the thermodynamic state of the isochoric system [20]. Measuring the pressure in an isochoric chamber is a simple way to detect that freezing has occurred and the amount of ice in such a system [30,31] [25,26,30–32], [24,33,34]. This concept was used to control and analyze the experiments in this study. Measuring the pressure in an isochoric chamber can tell if a metastable supercooled system has frozen. For example, if a supercooled system at – 2 °C freezes, equilibrium thermodynamic predicts that the internal pressure in the isochoric system will increase from 0.1 MPa to 20 MPa [26].

Our group has successfully used isochoric supercooling, for variety of applications. For example,

Human cardiac microtissues were successfully revived after supercooling preservation in an isochoric system [35]. Isochoric supercooling was also successfully used by for preservation of pomegranate juice [36]. However, supercooling is a metastable thermodynamic state, and the probability for ice nucleation increases with volume [37]. Our previous research on isochoric supercooling dealt with small volumes. To the best of our knowledge, there is no report in the literature, on the preservation of an organ as large as the pig liver, in a metastable supercooled state in an isotonic solution, for long periods of time. The previous work on sub -0°C preservation of large livers, without freezing, employed perfusion and the use of cryoprotective additives [2,12,14].

The primary goal of this study was, to explore the feasibility of storing an organ as large as the pig liver in a metastable isochoric supercooled state for periods of time that have significance for organ transplantation. To this end we have designed a new isochoric supercooling system. The new technology employs an isochoric system comprised of two domains separated by an interior boundary that can transfer heat and pressure but not mass. The liver is preserved in one of these domains in a solution with an intracellular composition, in osmotic equilibrium with the liver [38]. The second goal of the study was to describe and demonstrate the feasibility of this new technology. A third goal was to examine the effect of isochoric supercooling preservation on tissue histology in a large organ, a pig liver. To this end we compare the histology of two pig livers preserved for 24h and 48 h in an isochoric supercooled state at -2°C, with that of two pig livers preserved in a frozen state at- 2 °C.

2. Materials and methods

2.1. Animal surgery

The animal study was performed under approval from Project Licenses number 327 from 23.12.20160 granted by ANSVSA - Bucharest (National Veterinary Authority) and approved by “Stefan S Nicolau” Virology Institute Ethical committee no. 1531/14.12.2016. The animals (N = 4) were all females and there is no difference in sexes for this procedure. They were fasted for 12 h prior to surgery with free access to water. Sedation with ketamine/midazolam was followed by endotracheal intubation and mechanical ventilation. The liver harvesting procedure was carried out under general anesthesia. The surgical access was obtained by the midline incision. The liver hilum was dissected exposing the main bile duct, hepatic arteries, and portal vein. The portal vein was catheterized via the splenic vein, and the infrarenal aorta was dissected and cannulated through a direct approach. The descending aorta and the inferior vena cava were exposed intra-thoracically using a right frenotomy. The cross-clamping (clamping of the aorta in the thorax and above the iliac bifurcation, and of the intra-thoracic inferior vena cava) was then performed, the inferior vena cava was sectioned below the liver, and the liver was flushed with 2 L of physiological heparinized saline through the two cannulas at a hydrostatic pressure of 80 mmHg from a pressurized IV drip. The liver was then harvested, and flushing continued with physiological heparinized saline until clean blood-free outflow from the hepatic veins was obtained. Prior to the preservation experiments biopsy samples were taken from each of the four lobes of the liver for histology examination.

2.2. Isochoric chamber

The preservation experiments were performed in an isochoric supercooling chamber designed by us, for large organ isochoric supercooling preservation. A detailed description of the isochoric chamber

was published earlier [34]. Briefly, the isochoric chamber is made is a cylindrical construct made of stainless steel with an internal diameter of 300 mm and a height of 150 mm. The chamber is closed with a stainless-steel lid Fig. 1B and sealed with O-rings (Fig. 1A). The lid is instrumented with two thermocouples to measure the temperature inside the chamber, a pressure transducer to measure the pressure inside the chamber, an inlet port, and an overflow port. As discussed in the introduction, the pressure measurement characterizes the thermodynamic state of the interior of the constant volume chamber. An increase in pressure is an indication that freezing has occurred inside the isochoric chamber [30,31]. The isochoric device was designed to withstand an increase in pressure of 1.5 MPa after which the sealing breaks. The assembled system is shown in Fig. 1C. During a preservation experiment the isochoric chamber is immersed in a fluid whose temperature is controlled by a refrigeration system Fig. 1D.

2.3. Subnormothermic dual oxygen perfusion device and method

It is common clinical practice that when livers are preserved at 4°C, they are prepared for transplantation using a temperature-controlled perfusion and oxygenation system. Our center policy is to do hypothermic machine perfusion for marginal liver grafts and for standard graft (when the static preservation is anticipated to be too long). This is becoming standard in most centers in Europe. This procedure was considered particularly important for our experiment, as the livers were preserved at temperatures below 0 ^{0}C, which is significantly lower than the standard clinical preservation temperature. Consequently, the perfusion part of the experiment was primarily aimed to examine any potential damage during the rewarming period. Therefore, after the preservation stage of the experiment, the livers were connected to a homemade sub-normothermic perfusion device based on the concept and functionalities of LiverAssist® machine perfusion [39,40]. The elements of the device are: isolated dual perfusion of donor livers, pulsatile perfusion of hepatic artery (60 bpm), continuous flow pattern to portal vein, pressure-controlled perfusion, controlled oxygenation of the perfusion solution, dedicated perfusion pressure and flow settings, and temperature adjustable from 10°C to 38 °C. The devices are shown in Fig. 2. They consist of a thermal insulated box (Fig. 2A), a kit for

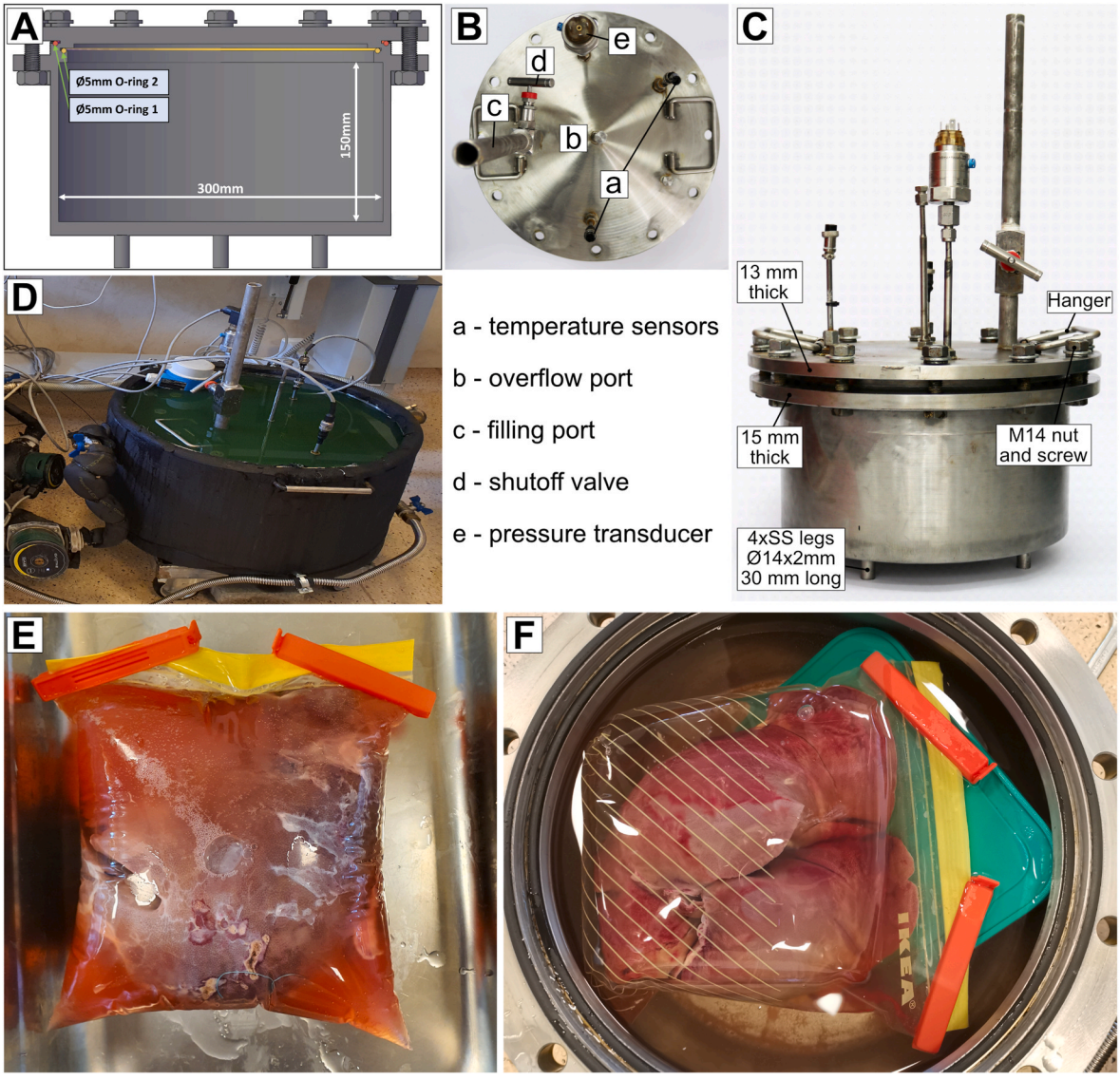

Fig. 1. The main components of the refrigeration and isochoric preservation system. Vertical cross section through the margin of the vessel to observe the two O-rings (A), isochoric chamber's stainless-steel lid – top view (B), the isochoric chamber assembled (C) and the appearance of the isochoric chamber, immersed in the cooling chamber of the refrigeration system (D). (E) The liver inserted in a low-density hydrophobic polyethylene bag, about 30 μm thick and sealed and F) the liver inside the isochoric chamber.

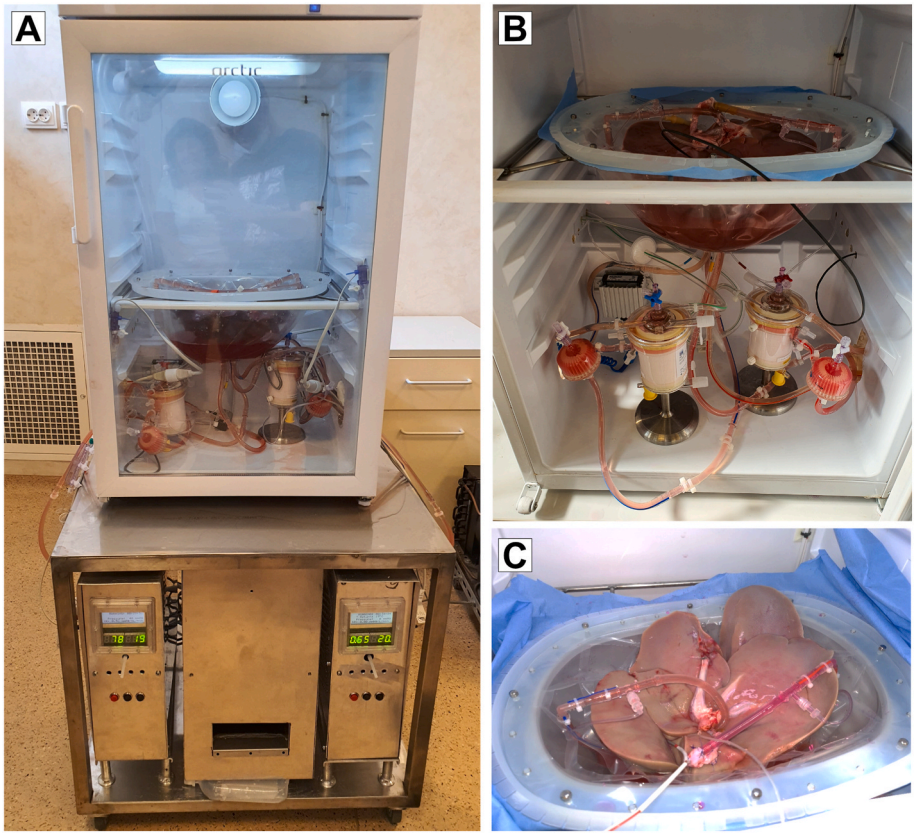

Fig. 2. The main components of the liver perfusion system. A) Front view of the perfusion system, B) detailed view of the interior of the thermal insulated chamber and C) a close view on the liver holder with the liver.

oxygenated perfusion for organs (OrganAssist®). Belzer MPS® UW Machine Perfusion Solution (Bridge to Life Ltd., Northbrook, USA) was used as a perfusate. The following adjuvants were added to the perfusate: insulin (5 U/L Humulin R), penicillin/streptomycin (40,000 U/L/40,000 μg/L), 10 mg/L hydrocortisone, and sodium bicarbonate 8.4%. The temperature of the interior of the chamber was monitored by means of a temperature sensor inside the thermal isolated box. The sensor is attached to a temperature control system that was connected to a resistor heater. Another temperature sensor was placed on the liver.

2.4. Liver treatment post preservation

After preservation at a nominal temperature of – 2°C the temperature of the isochoric chamber was elevated to 0 °C and the chamber was opened. The liver was flushed with a physiological solution, 2 L through the portal vein and 1 L through the artery. Biopsies were taken from each of the four liver lobes. The liver was then placed into the liver holder of the homemade liver perfusion system described above, (Fig. 2C). It was connected via cannulas and disposable tubing sets, to the pumps which pump (and oxygenate) the perfusate into the liver. Dual machine oxygenation and perfusion method was used, through both portal vein and hepatic artery. The procedure was deployed for 2 h. The flow, pressure, and temperature of the perfusate in both circuits (portal and arterial) were controlled throughout the procedure using dedicated cables and sensors. Particularly, the pressure inside the portal system is continuous, while the pressure inside the arterial system is pulsatile, mimicking the physiological perfusion of the liver. The perfusate was oxygenated with 1-L O_2/min. The temperature of the liver gradually increased from 0°C to 21–24 °C within approximately 1 h and maintained around 21–24°C for the remaining time of the procedure. The temperature of the liver was continuously monitored and there were two stages in the perfusion, the hypothermic stage from 0°C to 10–12°C and the subnormothermic phase from 10-12 °C to 21–24 °C. The pressure in the hypothermic phase was maintained at 3–4 mmHg over the portal vein and 25–27 mmHg on the artery. In the sub-normothermic phase the pressure was maintained at 4–7 mmHg over the portal vein and 50–80 mmHg on the artery. The temperature inside the thermo-isolation chamber was monitored throughput the procedure using a thermal sensor. The cooling system was used to maintain a low temperature (4–10°C) needed for the first phase of the machine procedure (hypothermic machine perfusion), while the heating system was used to increase the temperature to 21–24°C, needed for the second phase of the procedure (sub-normothermic machine perfusion. Following the perfusion, biopsies were taken from each lobe of the liver and examined with H&E histology and immunohistochemistry.

3. Results and discussion

3.1. Preservation experiments – freezing

In the freezing experiments livers were harvested from two pigs weighing 60–80 kg and flushed with UW solution and biopsied. It should be emphasized that in the freezing studies there was no attempt to induce conditions in which the livers would supercool. The chamber volume is very large (11L) and the inner wall of the isochoric chamber is rough and provides numerous nucleation sites, at welds and asperities. These numerous nucleation sites counter the stabilizing effect of the isochoric conditions and should induce ice nucleation [18,20]. In the freezing experiments the livers were inserted directly in the isochoric chamber which was filled with a physiological saline solution. The isochoric chamber was closed, inserted into the cooling chamber (Fig. 1D) and the temperature reduced to – 2°C. At this temperature physiological saline solution at atmospheric pressure, will freeze completely. As mentioned earlier, the isochoric device was designed to withstand an

increase in pressure of 1.5 MPa after which the sealing breaks. One goal of the freezing experiment was to show that freezing can be detected in an isochoric system by measuring pressure. Indeed, in the experiments in which there was freezing, we detected an increase in pressure (Fig. 3A) after which the pressure decreased to atmospheric pressure. This is evidence that there was freezing in the isochoric chamber after which the sealing was breached at 0.1 MPa and the chamber became open to the atmosphere. Under atmospheric pressure, the liver is completely frozen at – 2 °C. Ice was observed in the isochoric chamber, when it was opened, Fig. 3B. A first primary goal of this part of the study was to verify that the design is sensitive to the onset of freezing and can detect that freezing is occurring in the isochoric chamber. An important conclusion from this part of the study is that pressure measurements are a sensitive indication of the thermodynamic state of the content of the isochoric chamber, either supercooled or frozen. This part of the study also provided the opportunity to study the histology of pig livers frozen at – 2 °C in comparison to supercooled livers preserved in an isochoric system at – 2 °C.

3.2. *Preservation experiments – isochoric supercooling*

The experiments in this section were specially designed to inhibit ice nucleation from exterior sites and the chamber walls. The histology of the next two pig livers was examined after isochoric supercooling, at a nominal preservation temperature of – 2°C, one after 24 h and the second after 48 h. In these experiments the livers were obtained from two pigs at the site of the preservation device. The following steps were taken to avoid the nucleation from the chamber walls reported in the freezing experiments. After removal of the livers as described earlier, the livers were inserted in a low-density hydrophobic polyethylene (LDP) bag, about 30 μm thick and sealed Fig. 1D. The hydrophobic polyethylene minimizes the ice nucleation sites around the liver [38]. The livers were perfused with Custodiol, and care was taken to remove any air from the LDP bag, which was well sealed so as not to allow any mass transfer from and into the bag. The bag with the liver was introduced into the isochoric chamber which was completely filled with a 3 M NaCl solution, Fig. 1E, and the lid closed with care to avoid any air entrapment in the chamber. The use of the 3 M NaCl solution around the bag with the liver, eliminates the possibility for container wall surface nucleation [38]. In this way we obtained a two compartments isochoric chamber, separated by a boundary that can transfer pressure and heat but not mass. This design completely eliminated the possibility for surface nucleation. Only the possibility for volume nucleation is left, for which isochoric conditions improve the stability of the metastable solution. The composition of the solution surrounding the bags, 3 M is high enough so that no freezing can occur in that solution at – 2°C. The bag is also hydrophobic and therefore it does not present any nucleation sites to the solution in the bag. The only possibility for nucleation is random volumetric nucleation in the bag. Isochoric conditions enhance the stability of supercooled solutions from such mode of nucleation [17] [18,20,22] As in the previous set of experiments the pressure and temperature of the system were continuously monitored. No increase in pressure was observed, which indicates that there was no ice formation in the chamber. The temperature of the cooling fluid was – 2°C ± 0.2 °C. The temperature in the isochoric chamber was measured to be, between – 1.78 °C and – 1.97 °C.

As a side note. In isochoric supercooling preservation there is no increase in pressure. As mentioned in the introduction, there is a technology that employs preservation at sub-0°C temperatures without ice, using hyperbaric pressure. Research on the effect of high pressure on the so-called hyperbaric pressure preservation technology shows that elevated pressures are detrimental to the organ. In fact, unfrozen livers under hyperbaric pressure and temperatures below – 4°C cannot survive [41]. However, this is exactly the point of the isochoric supercooling – it avoids high pressures.

4. Results from histology

For histology, samples were taken from all four lobes of the liver before preservation, after preservation and after perfusions. Representative micrographs are shown in Figs. 4 and 5. Fig. 4 shows a comparison between the controls before preservation; livers that experienced freezing at – 2 C, liver that experienced supercooling at – 2C for 24 h and liver that experienced supercooling at – 2C for 48 h.

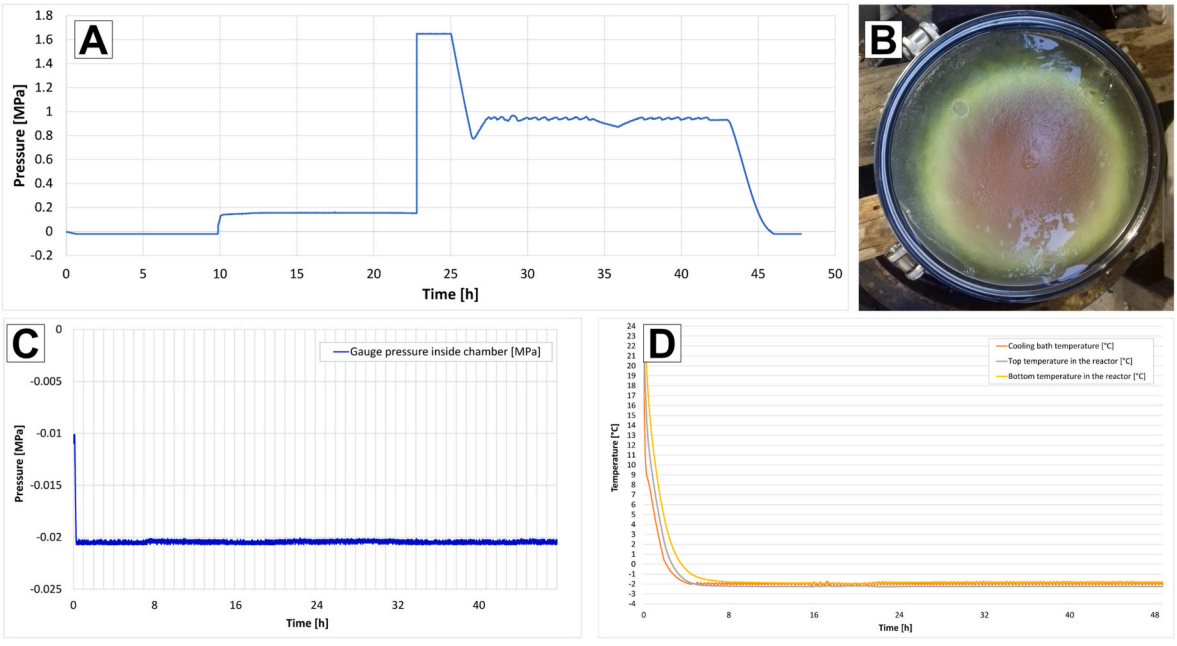

Fig. 3. A) Gauge pressure as a function of time, when the liver freezes inside the isochoric chamber. Note the pressure increases with freezing and at 1.5 MPa the sealing is breached. B) Plot when freezing occurs in an isochoric chamber, B) A photograph of the frozen liver in the isochoric chamber for 48 h preservation. C) Gauge pressure as a function of time in the supercooled isochoric chamber. D) The temperature inside the supercooled isochoric chamber for 48 h preservation. (Note that figures A and C use different scales).

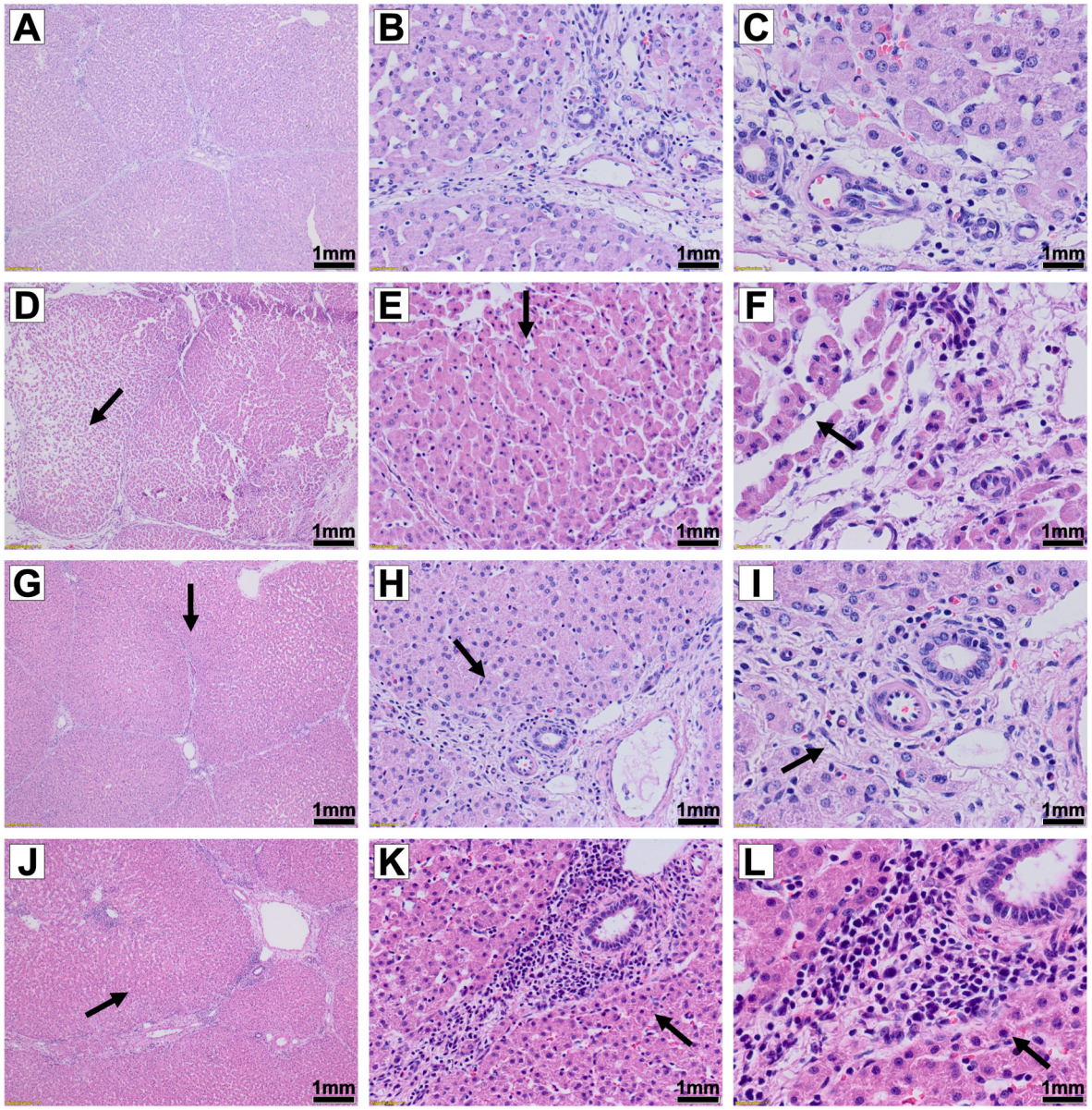

Fig. 4. Comparison: control (A,B,C), after 24h freezing at – 2 C (D,E,F), after 24 h supercooling at – 2C (G,H,I), after 48 h supercooling at – 2 °C (J,K,L), left row x4, middle row x20, right low x40.

The pre-preservation controls were taken from the livers prior to freezing. The slides show preserved architecture. We have observed some inflammation prior to the preservation in the pigs. This was observed in all the controls prior to the preservation and has no relation to the preservation experiment.

Histology of the livers preserved for 24 h in a frozen state at – 2°C showed that the liver tissue architecture was severely damaged by freezing. Cell-cell connections were disrupted and pulled apart. There were fissures, gaps, and voids within the liver parenchyma. Histology showed hepatocyte necrosis (98% nuclear pyknosis and fusion of cytoplasm pyknotic nuclei, granular and focal vacuolized eosinophilic cytoplasm), with focal obliteration of lobular architecture and filiform nuclear material. This is consistent with results by others who have shown that livers preserved by freezing at – 2°C do not survive freezing [11].

In contrast, the livers extracted from the isochoric supercooling chamber had a normal macroscopic appearance and consistency. The histology and immunohistochemistry of the two livers after the supercooling preservation and after the perfusion showed that the livers were undamaged after supercooling preservation. After 24 h of supercooling preservation, images show preserved architecture comparable to pre-preservation controls. Similarly, after 48 h of supercooled preservation, the tissue architecture remained intact without any signs of cryo-injury.

As mentioned earlier, the perfusion was done to mimic a clinical transplantation procedure. The micrographs of the tissue following perfusion are shown in Fig. 5. The controls were taken from the livers that underwent 24 h supercooling, prior to the preservation. The controls show preserved architecture, slight portal and sinus inflammation and foci of hematopoiesis. As mentioned earlier, we have observed a certain degree of inflammation prior to the preservation in the pigs. This was observed in the controls prior to the preservation and has no relation to the preservation experiment. Histology of the livers preserved for 24 h in a frozen state at – 2°C showed, after perfusion, preserved architecture, centrilobular and zonal hepatocytes atrophy (70%), preserved periportal hepatocytes minimal portal inflammation. After cryopreservation by freezing, the organ exhibits a measure of damage and after perfusion cells become necrotic. We see preserved

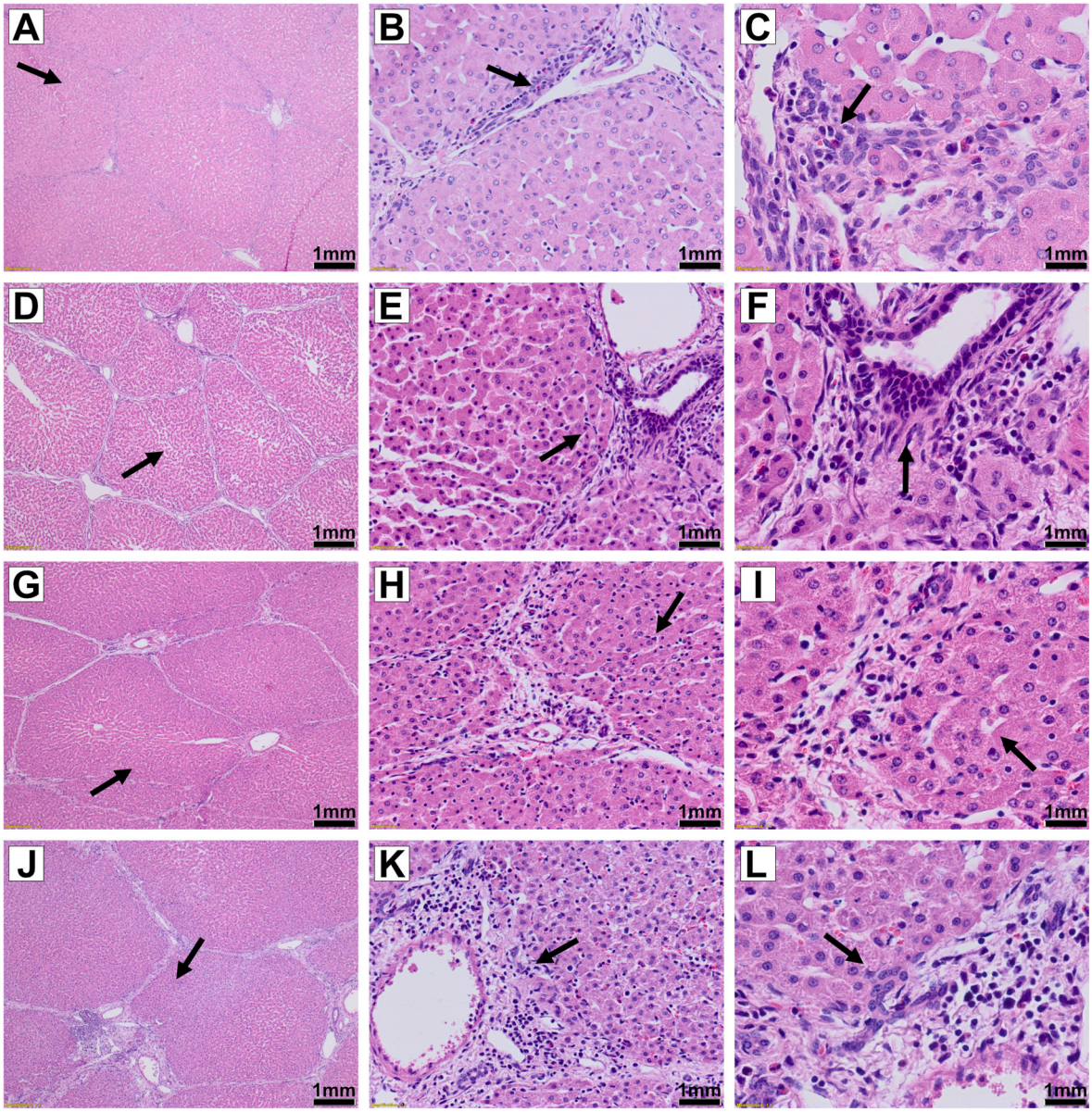

Fig. 5. Comparison: control (A, B, C), after 24h freezing at – 2 Cand perfusion (D, E, F), after 24 h supercooling at – 2Cand perfusion (G, H, I), after 48 h supercooling at – 2 °C and perfusion (J, K, L), left row x4, middle row x20, right low x40.

architecture, centrilobular and zonal hepatocytes atrophy (70%), preserved periportal hepatocytes. Minimal portal inflammation.

The livers extracted from the isochoric supercooling chamber had a normal macroscopic appearance and consistency. After 24 h preservation in a supercooled state at – 2 C followed by perfusion the slides show preserved architecture, slight portal and sinus inflammation and foci of hematopoiesis. After 48 h preservation in a supercooled state at – 2 C followed by perfusion, we see preserved architecture, slight portal and sinus inflammation and moderate lobular inflammation.

We believe that our group was the first to introduce the concept of isochoric supercooling for organ preservation and to patent the concept [18,19,21,24,36]. While preservation of biological matter by supercooling is well known [42–44], it is the isochoric effect on supercooling that is novel. This paper demonstrates the potential of isochoric supercooling for preservation of large organs. Supercooling is a metastable thermodynamic state and the probability for nucleation increases with the volume of the system [18,45]. Most research on preservation of biological matter by supercooling alone is done in small volumes. Supercooled systems under atmospheric pressure freeze even when the volume is very small (1.5 ml) [16,45]. Previous work on supercooling of large volumes, such a human liver, was done with perfusion of the organ with chemical species that depress the freezing temperature [2,13]. To the best of our knowledge this paper is the first publication which shows that a large organ such as the pig liver can be preserved in a supercooled "metastable" state at – 2°C in an isochoric system without the use of hyperbaric pressure or perfusion with cryoprotecting chemical, for 48 h without ice nucleation.

5. Conclusions

This feasibility study demonstrates that a large organ, such as the pig liver, can be preserved in a supercooled state in an isochoric system, for periods of time of clinical significance. The study also demonstrates the value of a new isochoric chamber design that employs pressure monitoring to detect freezing and which employs a two compartments design, to eliminate the probability for nucleation from the isochoric chamber walls. As expected, histology shows that pig livers preserved for up to 48 h at -2 ^{a}C in a supercooled state are superior to pig liver exposed to

freezing at the same temperature. This is the first evidence of the potential value of liver preservation by isochoric supercooling and suggests the value of further research in the field. It is important to emphasize that the isochoric supercooling preservation does not require any addition of cryoprotectant or machine perfusion during the preservation. Obviously, this is a first feasibility study and much more research is needed in this area.

Declaration of competing interest

The authors whose names are listed above certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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References

[1] V.E. de M, M. F, R.J. P, Ex situ machine perfusion strategies in liver transplantation, J. Hepatol. 70 (2019) 203–205, https://doi.org/10.1016/j.jhep.2018.09.019.

[2] R.J. de Vries, S.N. Tessier, P.D. Banik, S. Nagpal, S.E.J. Cronin, S. Ozer, E.O. A. Hafiz, T.M. van Gulik, M.L. Yarmush, J.F. Markmann, M. Toner, H. Yeh, K. Uygun, Supercooling extends preservation time of human livers, Nat. Biotechnol. 37 (2019) 1131–1136, https://doi.org/10.1038/s41587-019-0223-y.

[3] A. Hirsh, T. Takahashi, Deep supercooling and high pressure as approaches to cryopreservation, Cryobiology 19 (1982) 674–675, https://doi.org/10.1016/0011-2240(82)90276-0.

[4] T. Ueno, T. Omura, T. Takahashi, H. Matsumoto, Y. Takahashi, A. Kakita, S. Yamashina, Liver transplantation using liver grafts preserved under high pressure, Artif. Organs 29 (2005) 849–855, https://doi.org/10.1111/j.1525-1594.2005.00139.x.

[5] G. Amir, B. Rubinsky, A.K. Smolinsky, J. Lavee, Successful use of ocean pout thermal hysteresis protein (antifreeze protein III) in cryopreservation of transplanted mammalian heart at subzero temperature, J. Heart Lung Transplant. 21 (2002) 137, https://doi.org/10.1016/s1053-2498(01)00662-3.

[6] G. Amir, L. Horowitz, B. Rubinsky, B.S. Yousif, J. Lavee, A.K. Smolinsky, Subzero nonfreezing cryopresevation of rat hearts using antifreeze protein I and antifreeze protein III, Cryobiology 48 (2004) 273–282, https://doi.org/10.1016/j.cryobiol.2004.02.009.

[7] G. Amir, B. Rubinsky, L. Horovitz, B.S. Yousif, J. Leor, A.K. Smolinsky, J. Lavee, Improved viability and reduced apoptosis in subzero 21 hours preservation of transplanted rat hearts using antifreeze proteins, J. Heart Lung Transplant. 23 (2004) S171–S172.

[8] G. Amir, B. Rubinsky, L. Horowitz, L. Miller, J. Leor, Y. Kassif, D. Mishaly, A. K. Smolinsky, J. Lavee, Prolonged 24-hour subzero preservation of heterotopically transplanted rat hearts using antifreeze proteins derived from arctic fish, Ann. Thorac. Surg. 77 (2004) 1648–1655, https://doi.org/10.1016/j.athoracsur.2003.04.004.

[9] G. Amir, B. Rubinsky, Y. Kassif, L. Horowitz, A.K. Smolinsky, J. Lavee, Preservation of myocyte structure and mitochondrial integrity in subzero cryopreservation of mammalian hearts for transplantation using antifreeze proteins - an electron microscopy study, Eur. J. Cardio. Thorac. Surg. 24 (2003), https://doi.org/10.1016/S1010-7940(03)00306-3.

[10] N. Ishine, B. Rubinsky, C.Y. Lee, Transplantation of mammalian livers following freezing: vascular damage and functional recovery, Cryobiology 40 (2000) 84–89, https://doi.org/10.1006/cryo.1999.2225.

[11] N. Ishine, B. Rubinsky, C.Y. Lee, A histological analysis of liver injury in freezing storage, Cryobiology 39 (1999) 271–277, https://doi.org/10.1006/cryo.1999.2205.

[12] T.A. Berendsen, B.G. Bruinsma, C.F. Puts, N. Saeidi, O.B. Usta, B.E. Uygun, M. L. Izamis, M. Toner, M.L. Yarmush, K. Uygun, Supercooling enables long-term transplantation survival following 4 days of liver preservation, Nat Med 20 (2014) 790–793, https://doi.org/10.1038/nm.3588.

[13] R. de Vries, S.N. Tessier, P.D. Banik, S. Ozer, S.E.J. Crorin, S. Nagpal, H. Yeh, K. Uygun, Extending the human liver preservation time for transplantation by supercooling, Transplantation 102 (2018) S396, https://doi.org/10.1097/01.tp.0000543168.17458.55.

[14] R.J. de Vries, S.N. Tessier, P.D. Banik, S. Nagpal, S.E.J. Cronin, S. Ozer, E.O. A. Hafiz, T.M. van Gulik, M.L. Yarmush, J.F. Markmann, M. Toner, H. Yeh, K. Uygun, Subzero non-frozen preservation of human livers in the supercooled state, Nat. Protoc. 15 (2020) 2024–2040, https://doi.org/10.1038/s41596-020-0319-3.

[15] H. Huang, M.L. Yarmush, O.B. Usta, Long-term deep-supercooling of large-volume water and red cell suspensions via surface sealing with immiscible liquids, Nat. Commun. 9 (2018) 3201, https://doi.org/10.1038/s41467-018-05636-0.

[16] O.B. Usta, Y. Kim, S. Ozer, B.B. Bruinsma, J. Lee, E. Demir, C.F. Berendsenm, T. A. Puts, M.-L. Izamis, K. Uygun, B.E. Uygun, M.L. Yarmush, Supercooling as a viable non-freezing cell preservation method of rat hepatocytes, PLoS One 8 (2013), e69334, https://doi.org/10.1371/journal.pone.0069334.

[17] S.A. Szobota, B. Rubinsky, Analysis of isochoric subcooling, Cryobiology 53 (2006) 139–142, https://doi.org/10.1016/j.cryobiol.2006.04.001.

[18] A.N. Consiglio, D. Lilley, R. Prasher, B. Rubinsky, M.J. Powell-Palm, Methods to stabilize aqueous supercooling identified by use of an isochoric nucleation detection (INDe) device, Cryobiology 106 (2022) 91–101, https://doi.org/10.1016/j.cryobiol.2022.03.003.

[19] B. Rubinsky, S. Szobota, Cryopreservation of a Biological Sample Comprises Placing the Sample in Aqueous Solution in a Chamber, Supercooling the Fluid, and Adding Cryoprotective Compound, Vitrification Promoter and Nucleation Inhibitor to the Solution or the Sample, (n.d)..

[20] A. Consiglio, D. Lilley, R. Preasher, B. Rubinsky, M. Powell-Palm, J, Methods to Stabilize Aqueous Supercooling Identified by Use of an Isochoric Nucleation Detection (INDe) Device, 2021, 10404. ArXiv. 2108.

[21] M.J. Powell-Palm, B. Rubinsky, Devices and Methods for High-Stability Supercooling of Aqueous Media and Biological Matter. US Patent 2022/0325937 A1, (n.d)..

[22] A. Consiglio, G. Ukpai, B. Rubinsky, M.J. Powell-Palm, Suppression of cavitation-induced nucleation in systems under isochoric confinement, Phys Rev Res 2 (2020), 023350, https://doi.org/10.1103/physrevresearch.2.023350.

[23] M.J. Powell-Palm, A. Koh-Bell, B. Rubinsky, Isochoric conditions enhance stability of metastable supercooled water, Appl. Phys. Lett. 123702 (2020), https://doi.org/10.1063/1.5145334.

[24] S.-I. Campean, G.-A. Beschea, A. Serban, M.J. Powell-Palm, B. Rubinsky, G. Nastase, Analysis of the relative supercooling enhancement of two emerging supercooling techniques, AIP Adv. 11 (2021), 055125.

[25] A. Pedro Perez, Thermodynamic and Heat Transfer Analysis for Isochoric Cryopreservation, PhD Thesis, Department of Mechanical Engineering, University of California Berkeley. (OCoLC)945, 2006. OCLC Number: 892833675.

[26] B. Rubinsky, P.A.P.A. Perez, M.E.M.E. Carlson, The thermodynamic principles of isochoric cryopreservation, Cryobiology 50 (2005) 121–138, https://doi.org/10.1016/j.cryobiol.2004.12.002.

[27] J. Preciado, The Fundamentals of Isochoric Freezing and its Role in the Cryopreservation of Biological Materials, 2007.

[28] J.A. Preciado, B. Rubinsky, Isochoric preservation: a novel characterization method, Cryobiology 60 (2010) 23–29, https://doi.org/10.1016/j.cryobiol.2009.06.010.

[29] M.J. Powell-Palm, B. Rubinsky, W. Sun, Freezing water at constant volume and under confinement, Commun. Phys. 3 (2020), https://doi.org/10.1038/s42005-020-0303-9, 10.1038/s42005-020-0303-9.

[30] B. Rubinsky, G. Nastase, G. Ukpai, Process and Devices for Temperature and Pressure Controlled Cryopreservation, US 2020/0178518 A1, (n.d)..

[31] G. Ukpai, G. Năstase, A. Şerban, B. Rubinsky, Pressure in isochoric systems containing aqueous solutions at subzero Centigrade temperatures, PLoS One 12 (2017), e0183353, https://doi.org/10.1371/journal.pone.0183353.

[32] Y. Zhang, G. Ukpai, A. Grigoropoulos, M.J. Powell-Palm, B.P. Weegman, M. J. Taylor, B. Rubinsky, Isochoric vitrification: an experimental study to establish proof of concept, Cryobiology 83 (2018) 48–55, https://doi.org/10.1016/j.cryobiol.2018.06.005.

[33] G.A. Beschea, T.I. Campena, M.-B. Tabacaru, A. Serban, B. Rubinsky, G. Nastase, Glucose and glycerol temperature-pressure correlations for the design of cryopreservation protocols in an isochoric system at subfreezing temperature, Biochem. Biophys. Commun 559 (2021) 42–47.

[34] G. Nastase, F. Botea, G. Beschea, S. Campean, A. Barcu, I. Popescu, B. Rubinsky, A. Serban, A two-compartment system for subfreezing temperatures preservation of large volumes of organic matter in an isochoric system, BioRxib (2022), https://doi.org/10.1101/2022.08.31.506083.

[35] M.J. Powell-Palm, V. Charwat, B. Charrez, B. Siemons, K.E. Healy, B. Rubinsky, M. Powell-Palm, J.V. Charwat, B. Charrez, B. Siemons, E.K. Healy, B. Rubinsky, M. J. Powell-Palm, V. Charwat, B. Charrez, B. Siemons, K.E. Healy, B. Rubinsky, Isochoric supercooled preservation and revival of human cardiac microtissues, Commun Biol 4 (2021) (2021) 1118, https://doi.org/10.1038/s42003-021-02650-9.

[36] C. Bilbao-Sainz, B.-S. Chiou, G. Takeoka, T. Williams, D. Wood, M. Powell-Palm, B. Rubinsky, T. McHugh, Isochoric freezing and isochoric supercooling as innovative postharvest technologies for pomegranate preservation, Postharvest Biol. Technol. 194 (2022), 112072.

[37] A.N. Cansiglio, Yu Ouyang, M.J. Powell-Palm, B. Rubinsky, An Extreme Value Statistics Model of Heterogeneous Ice Nucleation for Quantifying the Stability of Supercooled Aqueous Systems, 2023, https://doi.org/10.48550/arXiv.2304.10455. Https://Arxiv.Org/Abs/2304.10455.

[38] B. Rubinsky, Device and method to reduce the probability of ice nucleation during preservation of biological matter in isochoric (constant volume) systems., US Patent Application 63351825, 2022..
[39] D.P. Schlegel A, X. Muller, Hypothermic machine preservation of the liver: state of the art, Curr Transplant Rep 5 (2018) 93–102, https://doi.org/10.1007/s40472-018-0183-z.
[40] D.P. Schlegel A, X. Muller, Erratum: hypothermic machine preservation of the liver: state of the art, Curr Transplant Rep 5 (2018) 103.
[41] T. Takahashi, A. Kakita, Y. Takashi, K. Yokoyama, I. Sakamoto, S. Yamashina, Y. Takahashi, K. Yokoyama, I. Sakamoto, S. Yamashina, Preservation of rat livers by supercooling under high pressure, Transplant. Proc. 33 (2001) 916–919, https://doi.org/10.1016/s0041-1345(00)02268-5.
[42] E.P.M.A.D. Beljakov, N.A. Rojdestvenskaja, Comparative study of conserved blood stored at a temperature below 0 °C in a liquid supercooling state and in a frozen state, Bibl. Haematol. 38 (1969) 336–338.
[43] T. Okamoto, T. Nakamura, J. Zhang, A. Aoyama, F. Chen, T. Fujinaga, T. Shoji, H. Hamakawa, H. Sakai, T. Manabe, H. Wada, H. Date, T. Bando, Successful sub-zero non-freezing preservation of rat lungs at-2 degrees C utilizing a new supercooling technology, J. Heart Lung Transplant. 27 (2008) 1150–1157, https://doi.org/10.1016/j.healun.2008.07.008.
[44] R. Monzen, K, T. Hosoda, R. Nagai, Koshiro Monzen, Toru Hosoda, Doubun Hayashi, Yasushi Imai, Yasuhiro Okawa, Takahide Kohro, Hiroshi Uozaki, Tomoki Nishiyama, Masashi Fukayama, K. Monzen Nagai, T. Hosoda, D. Hayashi, Y. Imai, Y. Okawa, T. Kohro, H. Uozaki, T. Nishiyama, M. Fukayama, R. Nagai, The use of a supercooling refrigerator improves the preservation of organ grafts, Biochem. Biophys. Res. Commun. 337 (2005) 534–539, https://doi.org/10.1016/j.bbrc.2005.09.082.
[45] B. Luyet, On the supercooling of water, Phys. Rev. 85 (1952) 746, 746.