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SUBZERO ORGAN PRESERVATION; THE DAWN OF A NEW ICE AGE?

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Abstract

Purpose of review—Herein, we review the field of subzero organ preservation with a focus on recent developments in hepatic supercooling.

Recent findings—Organ preservation is making a rapid shift from the decade-old standard of storage on ice towards techniques that improve organ availability as well as preservation time. Long-term organ preservation would have tremendous benefits to the organ transplantation field, including better organ allocation, donor-recipient matching, as well as reduced preservation injury and subsequent improvement of donor organ use. The formation of ice has proven an important limiting factor and novel techniques attempt to control or prevent freezing using cryoprotective agents, and highly-controlled cooling regimens. Various techniques have been employed over the previous decades including true organ freezing, vitrification and subzero non-freezing, or supercooling. For most techniques, successful transplantation following long-term subzero preservation has remained elusive. Supercooling however recently delivered the first promising results, yielding survival after up to 4 days of supercooled preservation at -6°C .

Summary—As the field of organ preservation undergoes significant development, the field of subzero preservation also receives renewed interest. While many obstacles remain to be overcome to make subzero preservation feasible, novel techniques are beginning to show their potential in achieving long-term preservation.

Keywords

cryopreservation; supercooling; liver transplantation; vitrification

INTRODUCTION

Since the pioneering days of organ transplantation, organ cooling has been the means to keeping a graft sufficiently viable to transplant successfully. The organ is rapidly cold-flushed with a cold preservation solution and stored on ice for the remainder of the ex situ

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CONFLICT OF INTEREST

The authors of this manuscript have conflicts of interest to disclose. 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.

period. For livers the general consensus gives us a maximum of approximately 12–16 hours (1), while kidneys can be kept for longer and hearts for quite a bit shorter. This method of static cold storage (SCS) allows transplantable organs to be kept viable for sufficiently long to accommodate the logistics of transplantation within a organ-sharing network. While the success of SCS is commendable, the inevitable decay of the graft under these circumstances limits the time we have to transplant, distance that can be traveled, as well as the number of livers that are of sufficiently high quality to survive this additional ischemic injury. As the deficit in donor organs presses the transplant community to increase graft availability, we look to alternative preservation methods, including ex situ machine preservation (2) and coveted subzero preservation techniques to improve organ availability. While success in the latter has proven arduous, the importance of this field was emphasized in a recent Organ Banking summit (3) and recent innovations have renewed opportunities for the field to move forward. In this review, we discuss the field of subzero organ preservation with a focus on the liver. We describe the natural adaptations that lend lessons to this field, and review the various approaches taken to achieve subzero organ preservation and the dawn of a new ice age.

Lessons from nature

Various observations from the natural world are of noteworthy relevance to subzero organ preservation. The finding that several species of frogs, including the wood frog *Rana sylvatica*, freeze solid during the cold winter was remarkable since both intra- and extracellular freezing cause substantial lethality (4,5). Thorough investigation of these species with a natural tolerance to subzero external temperatures have indicated two adaptive approaches to subzero life; 1) accommodating ice formation and ensuring it is limited to the extracellular space and preferably outside of the vital organs (freeze-tolerance), and 2) preventing freezing by taking advantage of freezing point depression and supercooling (freeze-avoidance) (6).

It was observed in *Rana sylvatica* that ice formation was selective, beginning in the extremities and if freezing persisted surrounding the abdominal organs in the peritoneal cavity. The entire process needed to be very carefully orchestrated to avoid crystallization or dehydration in vital parts of the body. Endogenous small-molecular cryoprotectants including glucose and glycerol are produced to prevent cryoinjury and freezing in key body parts (7,8). This complex coordination of controlling ice formation, cryoprotectant distribution and order may be difficult to reproduce in solid organ preservation, as isolated organs have very little heterogeneity and sacrificial extracellular volume to allow a coordinated freezing scheme.

Freeze-avoidance at subzero temperatures is a fundamentally different approach, employed by various insect and fish species (6,9,10). Increased concentrations of cryoprotectants such as glucose and glycerol increase the molality in bodily fluids resulting in freezing-point depression in these species, similar to the manner that *Rana sylvatica* selectively protects vital body parts. Other species employ a high concentration of blood colloids, such as peptides (11). These antifreeze peptides (AFP) exert a noncolligative antifreezing effect many times greater than what can be expected based on their molality, which results from a

direct inhibition of ice crystal formation (12). Five types of AFPs have been identified in fish and two types in insects (Table 1).

In nature, freeze-avoidance comes with certain risk, as the temperature cannot fall below the depressed freezing point. However, in controlled-temperature settings, such as organ preservation, it may be highly applicable.

Subzero preservation of organs (as well as cells and tissues) shares many principles with cold-tolerant species, making use of cryoprotectants and controlled rates of freezing and rewarming to mitigate the effects of cryoinjury. Despite extensive research, attempts to achieve frozen organ preservation have been mostly unsuccessful. Both the mechanical injury from the formation of ice crystals as well as osmotic stress result in direct tissue damage and membrane injury during freezing and thawing (13). In response, more successful alternatives have been explored where ice formation is avoided altogether.

Vitrification, allows solutions to reach cryogenic temperatures without ice crystallization. Alternatively, organs can be kept in a *supercooled* condition below their freezing point. As long as ice nucleation can be prevented, solutions can also be maintained in a liquid state at subzero temperatures (14), benefiting from a substantial reduction in metabolism.

Cryoprotectants

Freezing, vitrification and supercooling all rely greatly on cryoprotectants to protect tissue from the formation of ice and cold-induced injury. Numerous low-molecular-weight additives have yielded promising results for cryoprotection, many of which are derived from natural freeze-tolerant and freeze-avoidant mechanisms. For some however, use is limited by toxicity of the molecule counteracting the benefit of the subzero temperature (ethylene glycol, dimethyl sulfoxide (Me₂SO), 2,3-butanediol). Glycerol, sorbitol, 2,3-butanediol, 1,4-butanediol have been used to facilitate supercooling and mitigate cryopreservation injury (15–18). Various types of anti-freeze proteins have been identified that modify ice structure into a less injurious configuration and prevent ice propagation by binding directly to crystals (19). Moreover, these proteins were also found to have a direct protective effect by reducing membrane injury (20).

ORGAN FREEZING

Freezing of whole organs is many times more complex than cryopreservation of single cell suspension (13). In the latter, optimum freezing and thawing conditions can be determined to satisfy the requirements of the cell, while in whole organs a multitude of cell types are spatially arranged in a dense configuration involving not only cell junctions, but also matrix structure and cell-matrix interactions. Even minimal intracellular ice formation is destructive, but can often be avoided by controlled cooling and adequate use of cryoprotectants (21). The work of Pegg et al. elucidated the importance of the site of ice formation in the extracellular space (13). In various models including smooth muscle tissue, cartilage and in the more complex kidney, extracellular ice formation at <20 °C is directly damaging to the microstructure of tissue (22,23). In particular, the intravascular space in large solid organs is an important target of ice formation leading to vessel rupture (24).

Attempts at frozen preservation of the liver have been sparse and generally with poor outcome. Early attempts followed very shortly after the first liver transplantation in 1963. Using 33% glycerol as a cryoprotectant, Moss et al. froze canine livers at -20°C ranging from 24 hours to 2 weeks (17). Freezing resulted in substantial sinusoidal injury and matrix fragmentation, which resulted in a survival of only a few hours, severe coagulopathy and various metabolic disturbances when transplanted heterotopically.

Only many years later was liver freezing attempted again by Rubinsky et al., closely mimicking the adaptive strategies of freeze-tolerant frogs and freeze-avoiding fish. At a higher subzero temperature of -3°C , rat livers were frozen by introducing ice crystals in a controlled fashion following infusion of 0.5 M glycerol and 1 mg/mL AFP isolated from North Atlantic Cod, and kept for 6 hours (25). On subsequent *ex vivo* reperfusion livers produced bile, and had a well-preserved histological morphology, compared to liver frozen in the absence of cryoprotectants. However, detached endothelium was observed and was likely the cause of high resistance during reperfusion. In a follow-up study, the group showed that endothelial cell detachment with good preservation of hepatocytes occurred as a result of freezing (26). At this temperature, less than 60% of the water in the liver is frozen, preventing substantial injury to cells as a result of dehydration, which is supported by minimal hepatocyte damage. Interestingly, endothelial injury was absent in control experiments at the same temperature that were not frozen, but rather were preserved in a supercooled state, suggesting that it is freezing that damages the microvasculature directly, and not hypothermia (26). On orthotopic transplantation, these livers produced bile, but survival was poor, which was attributed again to microvascular injury (27). A single recipient survived 5 days and histology revealed an intact endothelium.

From limited studies in liver freezing it appears that deep-freezing results in an irreparable damage to various hepatic microstructures. A high subzero temperature limits ice formation and better preserves hepatocyte morphology and function, but remaining endothelial injury results in encumbering microvascular dysfunction.

VITRIFICATION

Not surprisingly, various groups came to the similar conclusion that preventing the formation of ice plays a central role in subzero preservation of organs. Organ vitrification gained interest starting around the year 2000, as a method to substantially cool down organs without the formation of ice crystals. Using various cryoprotectants that depress the freezing point and a rapid cooling scheme, the organs' temperature can be reduced to a glass transition temperature where the liquid assumes an amorphous, or glasslike state in which molecular movement effectively ceases (28). To date, successful vitrification has been limited to small structures that can be easily cooled rapidly, like cell suspensions (29), corneas, blood vessels (30), reproductive tissue (31,32), and early developmental structures (33–35).

Liver vitrification remains limited to hepatocyte preservation and preservation of liver slices, used extensively for drug metabolism and toxicity studies, but remains unexplored in whole livers (36,37). Research into vitrification of other vascularized organs has primarily involved

the kidney, in work from Fahy's group (28). Even after achieving successful vitrification, a major challenge is preventing ice nucleation on rewarming, so called devitrification (38). Using a 9.3 molar cryoprotectant solution known as M22, the group was able to prevent devitrification, restore renal function and support life as the sole kidney after autotransplantation (39). This isolated case remains the only report of successful vitrification of large solid organs, but is promising for the future of vitrification on optimization of vitrification solution and cooling-rewarming regimens.

SUPERCOOLING

The most successful results from subzero preservation come from supercooling techniques. The general consensus behind high subzero temperatures is that even a minor reduction in temperature can result in markedly improved liver preservation (40). *In vitro* hepatocyte storage is superior at subzero temperatures, with a better preservation of ATP levels and cell survival (41,42). In 1966 Brown et al. applied vacuum dehydration and 2M glycerol or Me₂SO to permit non-freezing of canine livers at -6 °C. Oxygen consumption ceased after 4–5 days of storage and survival of the 1-day stored grafts after heterotopic and orthotopic transplantation was less than 7 days and 12 hours, respectively (43). In an isolated liver perfusion model, it was shown that rat livers could be preserved unfrozen for 72 h at -4 °C, with similar outcome (bile production, AST/ALT release) to storage at +4 °C in UW solution (44). Toxicity of 2,3-butanediol, which was added to UW solution as a cryoprotectant may counteract the beneficial effect of supercooled temperature. In a similar study, Soltys et al. also demonstrated that rat livers could be supercooled to -4 °C without freezing (15). Again 2,3-butanediol was used and was equally effective for 24 h preservation as 4 °C cold storage in UW solution. Addition of type 1 AFP did not improve hepatic function during isolated liver perfusion, but through an unclear mechanism did increase LDH release. Importantly, adenine nucleotide content including adenine triphosphate (ATP) was significantly higher in supercooled livers. This finding was confirmed in a study by Yoshida et al. who showed that ATP content and energy charge was better preserved in livers stored at -0.8 °C compared to those stored at 4 °C for up to 4 days (45). Although these results were promising, successful transplantation after long-term supercooled preservation is not reported, or even attempted.

In a recent study, our group demonstrated that rat livers could be successfully transplanted after 96 h of supercooling (-6 °C) preservation with long-term survival (46**). Supercooling was combined with subnormothermic machine perfusion (SNMP) (described here (47)) for the loading of cryoprotectants into the liver as well as post-supercooling recovery. A nonmetabolizable glucose analogue (3-O-methyl glucose, 3-OMG) was used as an intracellular cryoprotectant and 35 kDa-polyethylene glycol (PEG) was used to protect the extracellular compartment including the vasculature (48*). Previous *in vitro* work has demonstrated that 3-OMG improved cryopreservation of primary hepatocytes, without evident toxicity (49). With this protocol 100% survival after was achieved (3 month follow-up) after 72 hours of supercooling, triple the 4 °C preservation time in UW solution (50). After 96 hours of storage, ATP content could be recovered to just under 50% of fresh values using SNMP – a level that corresponds to 48 h of cold storage in UW solution (51). This correlation translates to transplant survival as well; after 96 h of supercooling survival was

58%, while survival is only 50% after just 2 days of cold storage (50). Interestingly, survivors and nonsurvivors segregated over perfusion parameters during recovery SNMP; vascular resistance to perfusion flow as well as oxygen uptake may be useful in determining liver viability post-supercooling. It appears that even a minor supercooled state is able to significantly improve key aspects of preservation. Further experimentation with additional freezing point depressors and cryoprotectants may allow a further extension of preservation time as well as a more stable situation, better prepared for clinical translation. Figure 1 shows the set-up of the presupercooling perfusion in the rat liver (Fig. 1).

Alternative methods of achieving supercooled temperatures in the liver have been explored. Takahashi et al. describes a short period of -2°C supercooling by applying 10 MPa of pressure, which enabled 2-week survival after 5 hours of preservation (52), however slightly higher pressurization appeared to be damaging and some grafts were lost to freezing at even slightly lower temperatures (53). Monzen et al. used an 100–500 V electrostatic field to achieve a temperature of -4°C in livers, hearts and kidneys preserved in UW solution, but post-preservation assessment was limited (18).

The importance of preserving the organ's energy status, and the use of ATP have been previously established (54, 55*, 56). Slowing down metabolism and reducing high ATP consumption appears to be a key mechanism of cold storage and appears to be improved during supercooling. Preservation of organ energy status during supercooling has been confirmed in lungs (57) and hearts (58,59), where short-term supercooling preservation has been successfully performed, but transplantation has not been attempted.

Recent results in rat livers are encouraging, but successful scaling to larger-volume organs remains to be demonstrated. Since ice nucleation is a stochastic process, a larger volume of water in human organs may be more challenging to keep unfrozen.

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References

1. Porte RJ, Ploeg RJ, Hansen B, van Bockel JH, Thorogood J, Persijn GG, et al. Long-term graft survival after liver transplantation in the UW era: Late effects of cold ischemia and primary dysfunction. European multicentre study group. *Transpl Int*. 1998; 11(Suppl 1):S164–7. [PubMed: 9664970]
2. Ravikumar R, Jassem W, Mergental H, Heaton N, Mirza D, Perera MT, et al. Liver transplantation after ex vivo normothermic machine preservation: A phase 1 (first-in-man) clinical trial. *Am J Transplant*. 2016 Jan 11.
3. Lewis JK, Bischof JC, Braslavsky I, Brockbank KG, Fahy GM, Fuller BJ, et al. The grand challenges of organ banking: Proceedings from the first global summit on complex tissue cryopreservation. *Cryobiology*. 2016 Apr; 72(2):169–82. [PubMed: 26687388]

4. Storey KB. Life in a frozen state: Adaptive strategies for natural freeze tolerance in amphibians and reptiles. *Am J Physiol.* 1990 Mar; 258(3 Pt 2):R559–68. [PubMed: 2180324]
5. Mazur P. Freezing of living cells: Mechanisms and implications. *Am J Physiol.* 1984 Sep; 247(3 Pt 1):C125–42. [PubMed: 6383068]
6. Lee RE Jr. Insect cold-hardiness: To freeze or not to freeze. *Bioscience.* 1989; 39(5):308–13.
7. Storey KB. Organ-specific metabolism during freezing and thawing in a freeze-tolerant frog. *Am J Physiol.* 1987 Aug; 253(2 Pt 2):R292–7. [PubMed: 3618830]
8. Schmid WD. Survival of frogs in low temperature. *Science.* 1982 Feb 5; 215(4533):697–8. [PubMed: 7058335]
9. McMullen DC, Storey KB. Mitochondria of cold hardy insects: Responses to cold and hypoxia assessed at enzymatic, mrna and DNA levels. *Insect Biochem Mol Biol.* 2008 Mar; 38(3):367–73. [PubMed: 18252250]
10. DeVries AL, Wohlschlag DE. Freezing resistance in some antarctic fishes. *Science.* 1969 Mar 7; 163(3871):1073–5. [PubMed: 5764871]
11. Jin Y, DeVries AL. Antifreeze glycoprotein levels in antarctic notothenioid fishes inhabiting different thermal environments and the effect of warm acclimation. *Comp Biochem Physiol B Biochem Mol Biol.* 2006 Jul; 144(3):290–300. [PubMed: 16725360]
12. DeVries AL. Antifreeze peptides and glycopeptides in cold-water fishes. *Annu Rev Physiol.* 1983; 45:245–60. [PubMed: 6342516]
13. Pegg DE. The relevance of ice crystal formation for the cryopreservation of tissues and organs. *Cryobiology.* 2010 Jul; 60(3 Suppl):S36–44. [PubMed: 20159009]
14. Moore EB, Molinero V. Structural transformation in supercooled water controls the crystallization rate of ice. *Nature.* 2011; 479(7374):506–8. [PubMed: 22113691]
15. Soltys KA, Batta AK, Koneru B. Successful nonfreezing, subzero preservation of rat liver with 2,3-butanediol and type I antifreeze protein. *J Surg Res.* 2001 Mar; 96(1):30–4. [PubMed: 11180993]
16. Guibert EE, Almada LL, Mamprin ME, Bellarosa C, Pizarro MD, Tiribelli C, Rodriguez JV. Subzero nonfreezing storage of rat hepatocytes using UW solution and 1,4-butanediol. II- functional testing on rewarming and gene expression of urea cycle enzymes. *Ann Hepatol.* 2009; 8(2):129–33. [PubMed: 19502656]
17. Moss GS, Reed P, Riddell AG. Observations on the effects of glycerol on the cold storage of the canine liver. *J Surg Res.* 1966 Apr; 6(4):147–51. [PubMed: 5324811]
18. Monzen K, Hosoda T, Hayashi D, Imai Y, Okawa Y, Kohro T, et al. The use of a supercooling refrigerator improves the preservation of organ grafts. *Biochem Biophys Res Commun.* 2005 Nov 18; 337(2):534–9. [PubMed: 16202974]
19. Rubinsky B, Cogger R, Ewart KV, Fletcher GL. Ice-crystal growth and lectins. *Nature.* 1992 Nov 12; 360(6400):113–4.
20. Rubinsky B, Arav A, Fletcher GL. Hypothermic protection--a fundamental property of "antifreeze" proteins. *Biochem Biophys Res Commun.* 1991 Oct 31; 180(2):566–71. [PubMed: 1953726]
21. Mazur P. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *J Gen Physiol.* 1963 Nov; 47:347–69. [PubMed: 14085017]
22. Pegg DE, Wang L, Vaughan D, Hunt CJ. Cryopreservation of articular cartilage. Part 2: Mechanisms of cryoinjury. *Cryobiology.* 2006 Jun; 52(3):347–59. [PubMed: 16527262]
23. Taylor MJ, Pegg DE. The effect of ice formation on the function of smooth muscle tissue stored at –21 or –60 degrees C. *Cryobiology.* 1983 Feb; 20(1):36–40. [PubMed: 6831909]
24. Rubinsky B, Pegg DE. A mathematical model for the freezing process in biological tissue. *Proc R Soc Lond B Biol Sci.* 1988 Aug 23; 234(1276):343–58. [PubMed: 2906129]
25. Rubinsky B, Arav A, Hong JS, Lee CY. Freezing of mammalian livers with glycerol and antifreeze proteins. *Biochem Biophys Res Commun.* 1994 Apr 29; 200(2):732–41. [PubMed: 8179606]
26. Ishine N, Rubinsky B, Lee CY. A histological analysis of liver injury in freezing storage. *Cryobiology.* 1999 Nov; 39(3):271–7. [PubMed: 10600261]
27. Ishine N, Rubinsky B, Lee CY. Transplantation of mammalian livers following freezing: Vascular damage and functional recovery. *Cryobiology.* 2000 Feb; 40(1):84–9. [PubMed: 10679152]

28. Fahy GM, MacFarlane DR, Angell CA, Meryman HT. Vitrification as an approach to cryopreservation. *Cryobiology*. 1984 Aug; 21(4):407–26. [PubMed: 6467964]
29. Spurrier RG, Speer AL, Grant CN, Levin DE, Grikscheit TC. Vitrification preserves murine and human donor cells for generation of tissue-engineered intestine. *J Surg Res*. 2014 May 1.
30. Song YC, Khirabadi BS, Lightfoot F, Brockbank KG, Taylor MJ. Vitreous cryopreservation maintains the function of vascular grafts. *Nat Biotechnol*. 2000 Mar; 18(3):296–9. [PubMed: 10700144]
31. Song Y, Sharp R, Lu F, Hassan M. The future potential of cryopreservation for assisted reproduction. *Cryobiology*. 2010 Jul; 60(3 Suppl):S60–5. [PubMed: 19818745]
32. Zhang H, Yang Y, Ma W, Wu H, Zheng X, Hei C, et al. The revascularization and follicular survival of mouse ovarian grafts treated with FSH during cryopreservation by vitrification. *Cryo Letters*. 2016; 37(2):88–102. [PubMed: 27224529]
33. Garcia-Dominguez X, Vera-Donoso CD, Jimenez-Trigos E, Vicente JS, Marco-Jimenez F. First Steps Towards Organ Banks: Vitrification Of Renal Primordial. *Cryo Letters*. 2016; 37(1):47–52. [PubMed: 26964025]
34. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at –196 degrees C by vitrification. *Nature*. 1985; 313(6003):573–5. [PubMed: 3969158]
35. Kader A, Falcone T, Sharma RK, Mangrola D, Agarwal A. Slow and ultrarapid cryopreservation of biopsied mouse blastocysts and its effect on DNA integrity index. *J Assist Reprod Genet*. 2010 Aug; 27(8):509–15. [PubMed: 20512616]
36. Fuller BJ, Petrenko AY, Rodriguez JV, Somov AY, Balaban CL, Guibert EE. Biopreservation of hepatocytes: Current concepts on hypothermic preservation, cryopreservation, and vitrification. *Cryo Letters*. 2013; 34(4):432–52. [PubMed: 23995411]
37. de Graaf IA, Draaisma AL, Schoeman O, Fahy GM, Groothuis GM, Koster HJ. Cryopreservation of rat precision-cut liver and kidney slices by rapid freezing and vitrification. *Cryobiology*. 2007 Feb; 54(1):1–12. [PubMed: 17166492]
38. Fahy GM, Wowk B, Pagotan R, Chang A, Phan J, Thomson B, Phan L. Physical and biological aspects of renal vitrification. *Organogenesis*. 2009 Jul; 5(3):167–75. [PubMed: 20046680]
39. Fahy GM, Wowk B, Wu J, Phan J, Rasch C, Chang A, Zendejas E. Cryopreservation of organs by vitrification: Perspectives and recent advances. *Cryobiology*. 2004 Apr; 48(2):157–78. [PubMed: 15094092]
40. Hertl M, Chartrand PB, West DD, Harvey PR, Strasberg SM. The effects of hepatic preservation at 0 degrees C compared to 5 degrees C: Influence of antiproteases and periodic flushing. *Cryobiology*. 1994 Oct; 31(5):434–40. [PubMed: 7988152]
41. Usta OB, He X, Kim Y, Ozer S, Bruinsma BG, Lee J, et al. Supercooling as a viable non-freezing cell preservation method of rat hepatocytes. *PLoS One*. 2013 Jul 16; 8(7):e69334. [PubMed: 23874947]
42. Matsuda H, Yagi T, Matsuoka J, Yamamura H, Tanaka N. Subzero nonfreezing storage of isolated rat hepatocytes in university of wisconsin solution. *Transplantation*. 1999 Jan 15; 67(1):186–91. [PubMed: 9921819]
43. Brown H, Patel J, Blair DW, Brown ME. Biochemical studies with preserved transplanted canine liver. *JAMA*. 1966 May 30; 196(9):775–9. [PubMed: 5326280]
44. Scotte M, Eschwege P, Cherruau C, Fontaliran F, Moreau F, Houssin D. Liver preservation below 0 degrees C with UW solution and 2,3-butanediol. *Cryobiology*. 1996 Feb; 33(1):54–61. [PubMed: 8812085]
45. Yoshida K, Matsui Y, Wei T, Kaibori M, Kwon AH, Yamane A, Kamiyama Y. A novel conception for liver preservation at a temperature just above freezing point. *J Surg Res*. 1999 Feb; 81(2):216–23. [PubMed: 9927543]
- 46*. Berendsen TA, Bruinsma BG, Puts CF, Saeidi N, Usta OB, Uygun BE, et al. Supercooling enables long-term transplantation survival following 4 days of liver preservation. *Nat Med*. 2014 Jul; 20(7):790–3. this study describes the first successful results using supercooling to extend the preservation time of the rat liver significantly. Using a combination of subnormothermic machine perfusion, various cryoprotectants and a storage temperature of –6 ° Celsius a viable preservation time of 72 hours was achieved with 100% survival, and 58% after 96 hours. [PubMed: 24973919]

47. Berendsen TA, Bruinsma BG, Lee J, D'Andrea V, Liu Q, Izamis ML, et al. A simplified subnormothermic machine perfusion system restores ischemically damaged liver grafts in a rat model of orthotopic liver transplantation. *Transplant Res.* 2012; 1(1):6. [PubMed: 23369351]
- 48*. Bruinsma BG, Berendsen TA, Izamis ML, Yeh H, Yarmush ML, Uygun K. Supercooling preservation and transplantation of the rat liver. *Nat Protoc.* 2015 Mar; 10(3):484–94. A successful supercooling method as applied to preservation of the rat liver is outlined, providing step-by-step guidance through the protocol. [PubMed: 25692985]
49. Sugimachi K, Roach KL, Rhoads DB, Tompkins RG, Toner M. Nonmetabolizable glucose compounds impart cryotolerance to primary rat hepatocytes. *Tissue Eng.* 2006 Mar; 12(3):579–88. [PubMed: 16579691]
50. Bruinsma BG, Berendsen TA, Izamis ML, Yarmush ML, Uygun K. Determination and extension of the limits to static cold storage using subnormothermic machine perfusion. *Int J Artif Organs.* 2013; 36(11):775–80. [PubMed: 24338652]
51. Berendsen TA, Izamis ML, Xu H, Liu Q, Hertl M, Berthiaume F, et al. Hepatocyte viability and adenosine triphosphate content decrease linearly over time during conventional cold storage of rat liver grafts. *Transplant Proc.* 2011 Jun; 43(5):1484–8. [PubMed: 21693222]
52. Takahashi T, Kakita A, Takahashi Y, Yokoyama K, Sakamoto I, Yamashina S. Preservation of rat livers by supercooling under high pressure. *Transplant Proc.* 2001; 33(1–2):916–9. [PubMed: 11267128]
53. Ueno T, Omura T, Takahashi T, Matsumoto H, Takahashi Y, Kakita A, Yamashina S. Liver transplantation using liver grafts preserved under high pressure. *Artif Organs.* 2005 Oct; 29(10): 849–55. [PubMed: 16185349]
54. Vajdová K, Graf R, Clavien PA. ATP-supplies in the cold-preserved liver: A long-neglected factor of organ viability. *Hepatology.* 2002 Dec; 36(6):1543–52. [PubMed: 12447882]
- 55*. Bruinsma BG, Sridharan GV, Weeder PD, Avruch JH, Saeidi N, Özer S, et al. Metabolic profiling during ex vivo machine perfusion of the human liver. *Sci Rep.* 2016 Mar 3.6:22415. Herein, the importance of energy status as it pertains to organ preservation is emphasized and studied. [PubMed: 26935866]
56. Bruinsma BG, Wu W, Ozer S, Farmer A, Markmann JF, Yeh H, Uygun K. Warm ischemic injury is reflected in the release of injury markers during cold preservation of the human liver. *PLoS One.* 2015; 10(3):e0123421. [PubMed: 25822248]
57. Okamoto T, Nakamura T, Zhang J, Aoyama A, Chen F, Fujinaga T, et al. Successful sub-zero non-freezing preservation of rat lungs at -2 degrees C utilizing a new supercooling technology. *J Heart Lung Transplant.* 2008 Oct; 27(10):1150–7. [PubMed: 18926408]
58. Sakaguchi H, Kawachi K, Kobayashi S, Yoshida Y, Gojo S, Kitamura S. Effects of subzero nonfreezing storage on the preservation of myocardial energy and function. *Transplant Proc.* 1996 Jun; 28(3):1910–1. [PubMed: 8658942]
59. Kato H, Tomita S, Yamaguchi S, Ohtake H, Watanabe G. Subzero 24-hr nonfreezing rat heart preservation: A novel preservation method in a variable magnetic field. *Transplantation.* 2012 Sep 15; 94(5):473–7. [PubMed: 22885880]
60. Kim HJ, Lee JH, Hur YB, Lee CW, Park SH, Koo BW. Marine Antifreeze Proteins: Structure, Function, and Application to Cryopreservation as a Potential Cryoprotectant. *Mar Drugs.* 2017; 15

CLINICAL TRANSLATION AND CONCLUSION

The potential of organ banking remains widely acknowledged, but many challenges remain (3). Both freezing and vitrification of the liver are hurdled by achieving ice-control and freeze-tolerance, while supercooling attempts freeze-avoidance and appears to be the most promising approach for clinical translation in the short term, with successful pre-clinical results indicating feasibility of the technique. Vitrification has shown its potential for true long-term preservation in non-vascularized tissue and expansion to whole organs, such as the liver, would bring tremendous advance to the cryopreservation field.

Amidst a strong pursuit of novel warm preservation techniques, subzero preservation offers a relatively underexplored alternative. In our own work we have found supercooling to be an effective strategy to extend short-term preservation times significantly and efforts by others in the field of vitrification have made a tremendous push towards long-term storage of whole organs. It is our opinion that subzero preservation provides a relatively low complexity storage method for true organ banking to the extent of off-the-shelf organs preserved for extensive periods with minimal structural or metabolic deterioration.

KEY POINTS

- Recent years have shown a push towards novel organ preservation techniques in an attempt to improve organ availability.
- Subzero preservation remains relatively underexplored, yet the potential for achieving organ banking and long-term preservation are great.
- Various approaches, including organ freezing, vitrification and supercooling have been attempted to overcome the challenges associated with subzero preservation.
- Recently supercooling at -6°C was able to render long-term preserved livers transplantable after a world first 96h preservation period.
- Various critical hurdles remain to be overcome to extend preservation time further as well as translate to human application.

Fig. 1. Pre-supercooling liver perfusion set up

Rat livers are perfused for 60 minutes in a subnormothermic machine perfusion (SNMP) system to load the organ with cryoprotectants.

Table 1

Antifreeze protein (AFP) sources and types

	Source species	Size	Studied for (cell, tissue or organ)(60)
<i>Fish</i>			
Type I	Flouders, sculpins	3.3–4.5 kDa	Supercooling, vitrification, freezing
Type I 'maxi'	Winter flounder	32 kDa	Vitrification, freezing
Type II	Sea raven, smelt, herring, and long snout poacher	11–24 kDa	Supercooling, vitrification, freezing
Type III	Antarctic eelpout	6.5 kDa	Vitrification, freezing
Type IV	Longhorn sculpin	12 kDa	Vitrification, freezing
<i>Insect</i>			
Type I	Beetles	8–12.5 kDa	Freezing
Type II	Moths, butterflies	8–12.5 kDa	