Review Article

Transfusion Medicine and Hemotherapy

Transfus Med Hemother 2019;46:197–215
DOI: 10.1159/000499453

Received: March 5, 2019
Accepted: March 7, 2019
Published online: June 4, 2019

New Approaches to Cryopreservation of Cells, Tissues, and Organs

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Keywords
Cryopreservation · Vitrification · Nanowarming · Isochoric cryopreservation · Non-Newtonian cryoprotection · Liquidus tracking

Abstract
In this concept article, we outline a variety of new approaches that have been conceived to address some of the remaining challenges for developing improved methods of biopreservation. This recognizes a true renaissance and variety of complimentary, high-potential approaches leveraging inspiration by nature, nanotechnology, the thermodynamics of pressure, and several other key fields. Development of an organ and tissue supply chain that can meet the healthcare demands of the 21st century means overcoming twin challenges of (1) having enough of these lifesaving resources and (2) having the means to store and transport them for a variety of applications. Each has distinct but overlapping logistical limitations affecting transplantation, regenerative medicine, and drug discovery, with challenges shared among major areas of biomedicine including tissue engineering, trauma care, transfusion medicine, and biomedical research. There are several approaches to biopreservation, the optimum choice of which is dictated by the nature and complexity of the tissue and the required length of storage. Short-term hypothermic storage at temperatures a few degrees above the freezing point has provided the basis for nearly all methods of preserving tissues and solid organs that, to date, have proved refractory to cryopreservation techniques successfully developed for single-cell systems. In essence, these short-term techniques have been based on designing solutions for cellular protection against the effects of warm and cold ischemia and basically rely upon the protective effects of reduced temperatures brought about by Arrhenius kinetics of chemical reactions. However, further optimization of such preservation strategies is now seen to be restricted. Long-term preservation calls for much lower temperatures and requires the tissue to withstand the rigors of heat and mass transfer during protocols designed to optimize cooling and warming in the presence of cryoprotective agents. It is now accepted that with current methods of cryopreservation, uncontrolled ice formation in structured tissues and organs at subzero temperatures is the single most critical factor that severely restricts the extent to which tissues can survive procedures involving freezing and thawing. In recent years, this major problem has been effectively circumvented in some tissues by using ice-free cryopreservation techniques based upon vitrification. Nevertheless, despite these promising advances there remain several recognized hurdles to be overcome before deep-subzero cryopreservation, either by classic freezing and thawing or by vitrification, can provide the much-needed means for biobanking complex tissues and organs for extended periods of weeks, months, or even years. In many cases, the approaches outlined here, including new underexplored paradigms of high-subzero preservation, are novel and inspired by mechanisms of freeze tolerance, or freeze avoidance, in nature. Others apply new bioengineering techniques such as nanotechnology, isochoric pressure preservation, and non-Newtonian fluids to circumvent currently intractable problems in cryopreservation.

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Introduction

The ability to replace organs and tissues on demand could save or improve millions of lives each year globally and create public health benefits on par with curing cancer [1, 2]. Unmet needs for organ and tissue preservation place enormous logistical limitations on transplantation, regenerative medicine, drug discovery, and a variety of rapidly advancing areas spanning biomedicine. In a recent commentary in *Nature Biotechnology* [1], we, together with a cohort of experts in these fields, have outlined the unrealized potential of organ transplantation and the key role preservation plays in harnessing this potential.

Here, we outline some new approaches to cryopreservation with the potential to reach these goals for the first time. In some cases, these novel approaches are inspired by nature with a focus on high-subzero preservation, including attempts to harness thermodynamic equilibrium pressure-enabled (isochoric) techniques. In addition, we highlight some new bioengineering applications that enhance biopreservation technology using nanotechnology, as well as non-Newtonian and rheomagnetic fluids.

Developing an organ and tissue supply chain that can meet the healthcare demands of the 21st century means overcoming twin challenges: (1) having enough of these lifesaving resources and (2) having the means to store and transport them for a variety of applications, each with distinct but overlapping logistical needs. The first and conceptually more straightforward challenge has been the subject of heroic efforts in science, medicine, and public policy, ranging from increasing organ donation and utilization to engineering laboratory-grown tissues, bioartificial organs, and "humanized" porcine organs for transplantation. These efforts are intertwined with, and heavily dependent on, meeting the other challenge: preserving organs and tissues during procurement (or manufacturing), storage, transport, and other steps of the supply chain. The latter challenge is the subject of this article, in which we summarize some new and varied approaches to improving the cryobanking of cells, tissues, and organs. Almost all of these novel approaches are conceived and designed to circumvent the barriers that have hindered the long-anticipated goal of biobanking to alleviate the global shortage of organs for transplantation.

Short organ preservation times impose numerous constraints on transplantation, contributing to the organ shortage, exacerbating graft rejection, and limiting the length and quality of life of transplant recipients [1, 3–13]. In addition to their role in helping to address organ shortage, advances in organ banking stand to greatly expand options for donor-recipient matching [1, 3, 6, 7] and equitable allocation; enhance screening for transmissible diseases and malignancies [1, 8–10]; decrease costs and enable flexible scheduling of surgeries [1, 14]; and allow assessment of organ quality before transplantation [3, 11–13]. Advanced organ preservation modalities could even provide the opportunity for cutting-edge pretransplant interventions with the potential to improve transplant outcomes, including immunomodulation [15, 16] or gene therapy [17] and other approaches for functional augmentation [3, 18] in specialized laboratories [1, 4, 5]. Importantly, extending organ preservation times to just 5–7 days could allow current clinical immune tolerance induction protocols [19–22], which are showing promise in live kidney donation at several centers [23, 24] to be used in the context of deceased organ donation – reducing or eliminating the need for lifelong immunosuppression, thereby improving the lives of transplant recipients and saving the healthcare system in the USA roughly USD 100 million each year in costs for immunosuppression [25]. Recently, the goal of developing new approaches to organ preservation has become an increasing national priority in the USA [1, 2, 26–34] due to the recognition that preservation challenges are shared among major areas of biomedicine, including tissue engineering, trauma care, transfusion medicine, and biomedical research.

Despite much progress over the last decades, many cell types (stem cells [35, 36], hepatocytes [37], and granulocytes [38]), important tissues (e.g., skin, blood vessels, and cartilage), and reproductive organs (ovaries and testes in oncofertility contexts) remain difficult to effectively preserve with good viability and function. The difficulties are even more severe for solid organs and vascularized composite allograft tissues (e.g., limbs), which currently are limited to hours of viability.

Organ preservation has recently been identified as a key priority by the NIH and the Multi-Agency Tissue Engineering Science working group [39], the Obama White House [34], the American Society of Transplantation [27], and other major transplant societies [1], which have noted its critical importance to efforts to expand the organ donor pool [1–5, 13, 40–42] and biomanufactured tissues and organs [32, 34, 39, 43] and to develop an infrastructure for widespread clinical xenotransplantation (increasingly considered to be years, not decades, away) [1, 44, 45].

Current Status of Cryopreservation and Constraints on the Biobanking of Tissues and Organs

There are several approaches to biopreservation, the optimum choice of which is dictated by the nature and complexity of the tissue and the required length of storage. With the exception of normothermic organ culture, and more recently normothermic organ perfusion [3, 41,

46], all approaches to biopreservation aim to stabilize biological tissues by inhibiting metabolism and significantly retarding the chemical and biochemical processes responsible for degradation during ex vivo storage [47–49].

Short-term preservation of tissues and organs that cannot yet be successfully cryopreserved because they sustain too much injury at deep-subzero temperatures can be achieved using hypothermic storage at temperatures a few degrees above the freezing point. This has provided the basis for all methods of preserving tissues and solid organs that, to date, have proved refractory to cryopreservation techniques successfully developed for single-cell systems. Hypothermic preservation has developed over decades into a dedicated science, and some general principles have emerged and been reviewed extensively [47–54]. In essence, hypothermic preservation has relied principally on the protective effects of reduced temperatures brought about by Arrhenius kinetics of chemical reactions [48, 52]. The focus over several decades has been to design solutions for cellular protection against the effects of warm and cold ischemia [48–50, 55, 56]. However, it is generally accepted that the era of further optimization of preservation strategies based upon these principles is at an end and further advances in the quality and length of storage call for new ideas and approaches to tackle the remaining problems that hinder the biobanking of tissues and organs.

Long-term preservation calls for much lower temperatures than does short-term hypothermic storage, and it requires the tissue to withstand the rigors of heat and mass transfer during protocols designed to optimize cooling and warming in the presence of cryoprotective agents (CPAs). Classic cryopreservation involving freezing, while successful for many isolated cells in suspension, has failed to provide the means of banking more complex tissues and organs. While composed of individual cells, which in themselves may (or may not) be readily cryopreserved, the unique physical properties of tissues and organs have been shown to significantly affect the biological response to freezing and thawing [reviewed in 57–59]. The diversity of cell types and cell densities, as well as the morphological differences between constituent cells, significantly affects the osmotic and thermal state of tissues and organs. This has dramatic implications for the cooling and thawing rates that can be attained and, hence, for the response of tissues and organs to freezing and thawing. In addition, the requisite cell-cell and cell-matrix interactions in a tissue have been implicated in the poor survival of tissues following freezing [60–62]. In tissue and organ systems, ice crystallization becomes much more complicated and difficult to balance through the use of conventional cryoprotectants and cooling/thawing rates, due to thermal and mass transfer limitations creating damaged cellular zones within the tissue or organ. It is now accepted that with current methods, uncontrolled ice formation in structured tissues during cryopreservation is the single most critical factor that severely restricts the extent to which tissues can survive cryopreservation procedures involving freezing and thawing [57, 58, 63, 64, 221].

In recent years, this major problem has been effectively circumvented in some tissues by using ice-free cryopreservation techniques based upon vitrification [65–69]. Nevertheless, despite these promising advances there remain a number of recognized hurdles to be overcome before deep-subzero cryopreservation, either by classic freezing and thawing or by vitrification, can provide the much-needed means for biobanking complex tissues and organs for extended periods of weeks, months, or even years. These remaining challenges were the subject of a symposium (global summit) which served to examine the state of the art of biopreservation and to identify a roadmap for progress towards this ultimate goal [2, 28, 70]. Leaders in the field gathered to consider the remaining challenges, which they collectively identified as:

1. Control excessive ice formation
2. Hold cryoprotectant toxicity at acceptable levels
3. Limit disproportionate mechanical/thermodynamic stress
4. Control excessive injury from chilling
5. Avoid unacceptable levels of ischemic injury
6. Ensure acceptable repair and revival protocols

What has emerged from the 70 years of research into the cryobiological response of tissues and organs is that there is an interconnectedness between the biophysical, thermal, mechanical, and biological behaviors of the system which must be considered when developing strategies for low-temperature preservation [2, 28, 70]. The multidisciplinary summits and the National Science Foundation roadmap summarized how new approaches and their convergence can be brought in from at least 20 different fields to address these remaining challenges. In this concept article we outline a variety of new approaches that have been conceived to address some of the remaining challenges for developing improved methods of biopreservation. In many cases, these approaches are novel and inspired by mechanisms of freeze tolerance, or freeze avoidance, in nature, and others apply new bioengineering techniques such as nanotechnology, isochoric pressure preservation, and non-Newtonian fluids to circumvent currently intractable problems in cryopreservation. Although it will not be a focus in this review, it should be mentioned that machine perfusion technology has found a central role in the application of many of the new approaches to cryopreservation. Vascularized tissues and organs require perfusion for the various stages of preservation, such as preconditioning (including preequilibration with CPAs), preservation, and resuscita-

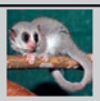
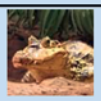
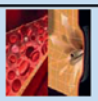
	Classical	New Approaches							Classical	
	Hypothermic	High Sub-Zero				Vitrification			Vitrification	Cryopreservation
	Cold Storage	Supercooling	Partial Freezing	Equilibrium Non-Frozen	Isochoric Sub-Cooling	Isochoric Vitrification	Vitrification and Nano-warming	Non-Newtonian & Rheo-magnetic	Traditional Vitrification	Classical Cryo-preservation (freezing)
Nature Inspiration	 LEMUR (vertebrate and Invertebrate)	 CAIMAN (vertebrate and Invertebrate)	 FROG (vertebrate and Invertebrate)	 SQUIRREL N/A	 DOLPHIN (and deep sea animals)	 GAS GIANT MOONS (and deep sea animals)	 ELECTRO-MAGNETIC N/A	 BLOOD quicksand, blood flow, catsup	 BEETLE Alaskan beetle larvae	 LEECH Leech
Description	Static storage & perfusion in cold solution	Storage below the freezing point, no ice	Storage below the freezing point, controled ice	Storage above the freezing point, no possible ice	Pressure in constant volume prevents ice formation below the freezing point	Constant volume prevents ice when cooling to glass	Rapid warming with biocompatible nanoparticles from glass prevents ice	Instant control of viscosity prevents ice	Rapid cooling, transformation into a glass	Storage below the freezing point, with ice
Temp. Range	+4°C to 8°C	-4°C to -6°C	-5°C to -20°C	-5°C to -20°C	-5°C to -30°C	-80°C to -196°C	-120°C to -196°C		-120°C to -196°C	
Storage Time	Hours	Days	Days, Weeks, Months			Years - Decades			Years - Decades	
Scalability	Practically unlimited	Limited	Practically unlimited			Practically unlimited			Limited	
Thermodynamic State	Equilibrium	Non-Equilibrium	Equilibrium			Equilibrium	Non-Equilibrium	Pseudo-Equilibrium	Non-Equilibrium	Equilibrium
Role of Ice	None	Moderate Risk	Controlled & limited	None	None	None	Very Low Risk	None	Moderate Risk	controlled & extensive
Key Advantages	Clinically established, low-cost, simple	No ice damage, extended storage	Less-toxic CPA, limited ice, stable extended storage	No ice damage, stable extended storage period	Low-cost, simple, practical, reduced CPA toxicity	Low-cost, simple, practical storage temperatures, indefinite storage	No ice damage, less-toxic CPA indefinite storage	No ice damage, less-toxic CPA indefinite storage	No ice damage, indefinite storage	Simple, low-CPA needed, indefinite storage
Main Disadvanteges	Limited Storage Duration	Potentially Unstable +Limited Storage Duration	Potential for ice damage	Technically challenging	No unresolvable obstacles identified	No unresolvable obstacles identified	Technically Challenging	Technically Challenging	Toxic levels of CPA, fracturing, instability, devitrification (possible ice)	Thermal stress, ice damage, recrystallization, LN2

Fig. 1. New nature-inspired and bioengineering approaches to cryopreservation compared with classic methods.

tion/recovery. In addition, ex vivo machine perfusion provides a platform for post-preservation viability assessment and quality control prior to transplantation [49, 50, 53, 71–73].

With the recognition of the limits of conventional hypothermic storage and the variety of challenges that still remain for the cryopreservation of organs, attention is now being given to preservation in the intermediate temperature zone (+20 to –20 °C) at normal atmospheric pressure, which we define as the "viginti zone" (Latin for *twenty*). At increased pressures and constant volume (isochoric), extended preservation is anticipated below –20 °C in an equilibrium state, as discussed below. Figure 1 summarizes the variety of approaches that fall within this zone, all of which have been inspired to a greater or lesser degree by species in nature.

High-Subzero Preservation

Historically, organ preservation strategies have focused on using the passive effects of cold – either hypothermic preservation (around +4 °C) for short-term preservation or vitrification and cryopreservation strategies at cryogenic temperatures (–120 to –195 °C) for indefinite storage durations. In contrast to these extremes of preservation, either by hypothermic storage above the freezing point or by cryopreservation at deep subzero temperatures, there is an opportunity to consider preservation at intermediate temperatures, which potentially offer extended durations of storage compared with current practices of hypothermic preservation. This could eliminate some of the logistical constraints on organ transplantation outlined above, and also allow time for immune tolerance induction, which would be transformational for organ transplantation [1].

Cryopreservation and vitrification hold promise, but the major challenges we have summarized with respect to ice formation, scalability to large systems, mechanical and thermal stresses, cryoprotectant toxicity, etc., have so far proven difficult to overcome. Yet, in nature, at least 45 vertebrate species (including mammals) can survive days, weeks, or even months at high-subzero body temperatures in a state of "suspended animation," without tissue damage [74–76]. Numerous mechanisms that adapt tissues to this temperature range have been uncovered, including upregulation of stress response pathways [77], programmed suppression of metabolism [78], and synthesis of antifreeze proteins that prevent ice nucleation [79, 80]. However, extending hypothermic storage to high-subzero temperatures by implementing strategies employed in nature is an essentially unexplored domain with high potential to markedly impact clinical organ preservation. For convenience, we will define these new approaches to preservation at intermediate temperatures as "high-subzero preservation," and we will discuss three specific aspects, namely: (a) supercooling, (b) partial freezing, and (c) equilibrium nonfrozen subzero preser-

Table 1. Comprehensive cryostasis and revival cocktails: targets and other candidate components

Strategy	Description	Primary and secondary compounds	Previously applied in:	Comments	Support in nature and literature [Ref.]
Nature-inspired low-molecular-weight protectants augmented by the best of classic CPAs	Colligative resistance to decreases in cell volume Stabilize the phospholipid bilayer Inhibit intracellular ice	Glucose analog 3-OMG trehalose DMSO[1] Ethylene glycol[1] Polyethylene glycol[1]	Supercooling Dehydration	Innovative strategy that enhances cell survival using a two-step approach: priming the cells first with nature-inspired compounds, notably 3-OMG, followed by classic CPAs at lower, less toxic temperatures	[73, 81–86, 89, 109, 112–114, 198–201]
Metabolic inhibitors	Actively depress the metabolic rate Minimize ischemic injury	2-Deoxyglucose 5-AMP Hydrogen sulfide Glucose analog 3-OMG	Hypothermia Dehydration/ desiccation	Deeper hypometabolic depression than via passive temperature alone	[48, 52, 78, 81–83, 100–104, 114, 202–205]
Stress tolerance enhancers	Minimize cryoinjury Maintain cellular integrity during the transitions to and from the frozen state and avoid IR injury	Antioxidants: e.g., glutathione, curcumin, acetylcysteine, superoxide dismutase, and catalase Derivatives of antioxidants: glutathione reduced ethyl ester, GSH-MEE Prosurvival, apoptosis inhibitors, broad: e.g., Q-VD-OPh, Z-VAD-FMK Prosurvival, cell-permeable apoptosis inhibitors, specific: e.g., ivachtin, pifithrin, Bax inhibitor peptide P5	Hypothermia Supercooling Dehydration/ desiccation Vitrification	Enhanced stress tolerance is a fundamental characteristic of most forms of naturally occurring metabolic rate depression Preservation of cellular macromolecules is of particular importance, since there is a reduced capacity to replace damaged macromolecules	[77, 105–107, 206, 207]
Endothelial stabilizers	Protect against endothelial damage	Polymers: e.g., dextran, hydroxyethyl starch, polyethylene glycol Membrane-permeable cAMP analogs	Vascular homeostasis Plasma expanders Our supercooling work	Plasma expanders such as dextran and HES stabilize microvessels via physical mechanisms Prevent leukocyte adhesion to the endothelium, maintain the endothelial barrier, and inhibit thrombosis	[86, 88, 208–212]
Revival-recovery-repair cocktails	Reduce time for biologics to return to full function	Many of the compounds above + trophic factors: e.g., BNP-1, NGF, Substance, EGF, and IGF-1	Hypothermic kidney storage Regenerative medicine	Replenish essential trophic factors Positively affect cell signaling pathways	[213–220]

CPAs, cryoprotective agents; IR, ischemia-reperfusion. [1] Used in classic cryostasis and vitrification.

vation (liquidus tracking). Each of these can be augmented with nature-inspired stress tolerance and/or metabolic suppression, as desired. For example, Table 1 summarizes a variety of classes of compounds now considered to be candidate components for cryostasis and revival cocktails supported by nature and the literature. Several of these strategies warrant brief mention here:

1. Nonmetabolizable glucose derivative 3-O-methyl-D-glucose (3-OMG): 3-OMG has been proposed and studied as an additive to preserve the intracellular compartment. 3-OMG is taken up naturally through glucose transporters including GLUT-1, GLUT-2, and muscle-specific glucose transporter GLUT-4 [81–84]. 3-OMG is metabolically inert, ensuring it can accumulate in the intracellular environment and achieve the desired resistance to decreases in cell volume while negating lactic acidosis. 3-OMG is nontoxic, and it has been reported that it improves the viability and quality

of hepatocytes preserved in vitro [85] and, as outlined below, enables supercooling of whole livers [86]
2. Polyethylene glycol (PEG): this polymer has historically been used as a cryoprotectant, as it disrupts hydrogen bonding in aqueous solutions and depresses freezing temperatures [87]. Studies have shown that it provides a range of other protective benefits during preservation, such as membrane and tight junction stabilization, preventing edema and acting as an antioxidant, thereby enhancing cellular stress tolerance by preventing lipid peroxidation [88, 89]. Low-molecular-weight PEG is nontoxic at high-subzero temperatures, and its benefits for hepatocyte storage have recently been reported [89], as well as for whole liver banking in a supercooled state [86]
3. Synthetic ice modulators: these are compounds that influence the formation and growth of ice nuclei and crystals by various purported mechanisms [90, 91]. This general classification embraces several categories of molecules that have been shown to modulate ice formation and growth. The synthetic polymers polyvinyl alcohol (X-1000) and polyglycerol (Z-1000) have been shown to effectively suppress ice nucleation events in aqueous systems even at concentrations as low as 1 ppm, much lower than most other ice control agents, by selectively binding surfaces of molecules that would otherwise promote the formation of ice nuclei [79, 80]. These synthetic ice blockers are inspired by natural antifreeze proteins found in polar fish and insects that can remain in a supercooled state for extended periods without damage [80]. Antifreeze proteins have shown the ability to protect rat hearts during supercooling [92–94]. X- and Z-1000 are nontoxic, readily permeate cell membranes, and remain active at temperatures ranging from 0 ° C all the way to glass transition temperatures (below –120 ° C) [79, 80]. They have been employed to preserve a variety of cell types with no reported toxicity [67, 79, 80, 95, 96]. New nature-inspired approaches in this area of research are focused on synthesizing novel chemically defined molecules with greater potency, less toxicity, and high stability [97–99]
4. Metabolic rate depression: naturally occurring hypometabolic states involve molecular and biochemical strategies that actively suppress metabolism [78]. While cooling roughly halves the rate of biological reactions for every change of 10°C [52], not all cellular metabolic reactions are completely impaired [48, 52]. In nature, decreases in metabolism precede changes in body temperature and synergize with passive cooling effects [100]. Conditioning cells to achieve suppressed metabolism promotes greater tolerance to ischemia and stress during high-subzero preservation. 3-OMG has an inhibitory effect on glycolysis; studies in animal models have shown that blunting mitochondrial respiration can prevent the pathological consequences of ischemia-reperfusion injury [101–103] and inhibit cell death [81, 104]
5. Enhancement of stress tolerance: activation of cytoprotective pathways in nature is core to most forms of metabolic depression [105]. This includes the active regulation of antioxidant defenses (e.g., the increased expression of reactive oxygen species-scavenging enzymes [77]), heat shock proteins involved in the maintenance of protein stability/folding, and the prevention of protein aggregation [106] and prosurvival signals (e.g., Akt signaling) [107], each with functions that serve to maintain cellular integrity during the stressful temperature transitions. This heightened stress tolerance extends the range of stressors that preserved cells/tissues can cope with

Supercooling

Mammals like the arctic ground squirrel hibernate/supercool with body temperatures of –3 °C (–8 °C experimentally) for up to 3 weeks, with every organ "banked" without injury. Even *Caiman crocodilus*, which can grow to over 2.5 m (over 58 kg), can supercool to below –5 °C [74]. Many species in nature, including mammals, can sustain subfreezing body temperatures for weeks or longer, supercooling to avoid ice formation [74–76, 108]. Two of the most prominent strategies involve (1) synthesis of high amounts of low-molecular-weight carbohydrates, which provide colligative resistance to detrimental decreases in cell volume, stabilize phospholipid bilayers, and restrict the formation of intracellular ice, and (2) synthesis of ice-blocking molecules that bind molecular surfaces around which ice would otherwise form. It is anticipated that an effective translation of these strategies to human organs may be achieved through the application of low-molecular-weight cryoprotectants and synthetic ice blockers.

This has inspired researchers, notably Toner and Uygun's group at Massachusetts General Hospital/Harvard, to apply knowledge of supercooling to develop a novel protocol enabling the viable preservation of rat livers at –6 °C for 3–4 days [86, 109] (Fig. 2). This is significant, since 72 h (3 days) represents a more than 5- to 10-fold increase over current typical clinical practice. Berendsen et al. [86] and Bruinsma et al. [109] demonstrated that a simple first-generation supercooling protocol dramatically extends the preservation of rat livers. The method relies on several crucial components: (1) the use of subzero temperatures to slow metabolism during static storage while avoiding injury from freezing; (2) preservative agents (PEG and 3-OMG); and (3) a recovery step with subnormothermic machine perfusion, which mitigates cold ischemic injury. Using the protocol in Figure 2a, su-

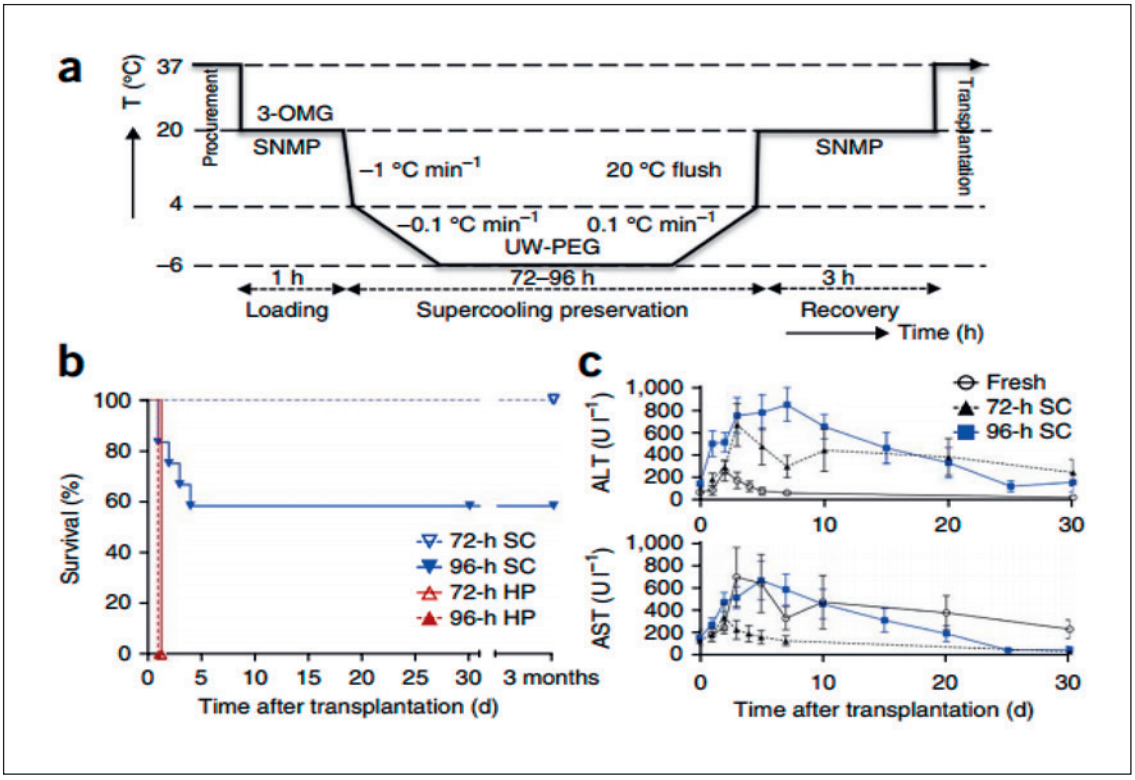


Fig. 2. Supercooled rat livers successfully transplanted after 3–4 days of storage. The 30-day survival rate was 100% for livers stored 3 days, and 58% for livers stored for 4 days. By comparison, recipients of livers stored in UW medium for 3 or 4 days perished within 48 h. 3-OMG, 3-*O*-methyl-D-glucose; HP, hypothermic preservation in UW medium at 4 °C; SNMP, subnormothermic machine perfusion (21 °C); UW, University of Wisconsin. **a** Schematic of the supercooling temperature profile. Loading of 3-OMG, as an additive to Williams' E-based medium, is performed by SNMP for 1 h through the portal vein. Cooling to 4 °C (1 °C/min) is carried out during perfusion. The liver is then flushed with 4 °C-cold UW solution containing 5% w/v 35 kDa PEG, and slowly cooled to −6 °C (1 °C/10 min). Storage is maintained for up to 96 h, after which rewarming to 4 °C takes place (1 °C/10 min). The liver is then flushed with 21 °C-warm, oxygenated SNMP medium, recovered with 3 h of SNMP, and transplanted orthotopically. **b** Kaplan-Meier curve of transplantation recipients ($n > 6$ in all groups shown). **c** Posttransplantation trends in transaminase output that normalize in a month; 3 months after transplantation, the differences had completely vanished (data not shown). Adapted from Berendsen et al. [86].

percooled whole rat livers were stored for 3 days retaining full viability (Fig. 2b, c) [86]. Recipients appeared completely healthy and did not display any signs of jaundice. Liver enzymes (ALT and AST) had fallen to normal levels by week 3, and all animals eventually regained their normal weight. Without optimization, storage was extended by supercooling to 4 days, but at a reduced survival of 58%. By comparison, posttransplantation survival with classic hypothermic preservation was 0% at 3 or 4 days. These studies included control transplants to identify which steps of the developed protocol were critical. No survival was achieved after 4 days if (1) supercooling was replaced with ice-cold storage at +4 °C, (2) final machine perfusion was skipped, (3) preloading 3-OMG was skipped, or (4) preservative PEG was not added (i.e., storage in standard University of Wisconsin solution), indicating that all four components were critical for 3- to 4-day preservation.

These recent achievements represent significant advances in establishing the feasibility of supercooling preservation of mammalian organs and pave the way for translation to organs with even shorter ischemic tolerance times, such as hearts and lungs. The feasibility of optimizing and translating supercooling preservation to hearts is supported by the fact that Amir et al. [92–94] have reported rat heart preservation 7 times longer than with conventional methods using a solution containing only antifreeze proteins and no other cryoprotectants, as well as by our own results with nonfrozen heart preservation under pressure discussed further below. These early achievements in supercooling preservation also highlight some of the nature-inspired strategies that contributed to the demonstrated successes outlined here.

Notwithstanding the recent developments in supercooling preservation of organs, the approach is nonetheless a non-equilibrium process with inherent risks of destabilization in the form of random ice nucleation. More-

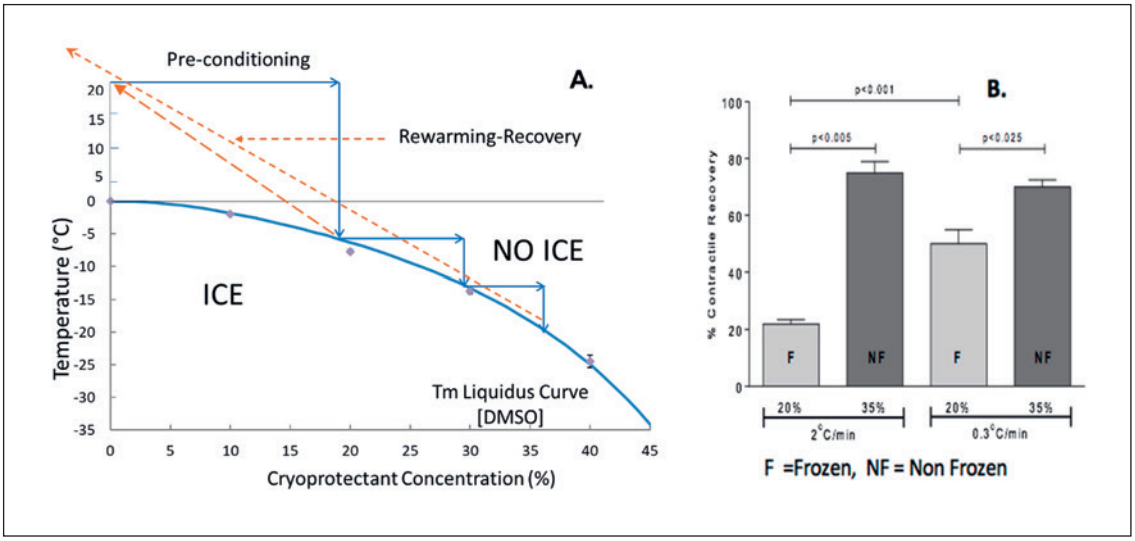


Fig. 3. A Section of the phase diagram for DMSO-H_2O showing the equilibrium melting curve in blue (Tm liquidus). The stepped line above the curve represents a scheme for incremental equilibration of a tissue with sufficient cryoprotectant such that the system does not freeze during cooling. **B** Function of smooth muscle tissue after cooling to –21 °C in either the frozen (F) or unfrozen (NF) state. Histograms of post-warming contractility (mean ± SEM) normalized to the control responses derived prior to cooling. Avoidance of ice is critical for good survival with high function, and liquidus tracking ensures that the system remains in equilibrium and ice-free during subzero cooling [63].

over, since ice nucleation is a stochastic event, the probability of ice formation increases with both the size of the compartment/system and the degree of undercooling. Most recently, advances have been reported in the use of surface sealing of water with an oil phase to significantly diminish the primary heterogeneous nucleation at the water/air interface. Huang et al. [110] achieved deep supercooling (down to –20 °C) of large volumes of water (up to 100 mL) for long periods (up to 100 days) simultaneously via this approach. Furthermore, in these preliminary studies they demonstrated the utility of deep supercooling in extended (100-day) preservation of human red blood cells [110]. Apart from the inherent risk of uncontrolled nucleation, a further hazard in non-equilibrium undercooling is related to the fact that the morphology of the ice crystals forming in highly supercooled solutions presents a sharper dendritic – and potentially more damaging – shape when compared with equilibrium freezing processes [111]. These constraints on high-subzero preservation by supercooling have encouraged the pursuit of alternative nature-inspired strategies involving thermodynamic equilibrium in the presence or absence of ice.

Controlled, Partial-Ice Freezing

One good example from the at least 45 animals that can survive long periods of time at high-subzero temperatures in a state of "suspended animation" is the wood frog (*Rana sylvatica*), surviving with 65–70% of the total body water as extracellular ice [112]. One of the most critical strategies for freeze tolerance involves the synthesis of high amounts of low-molecular-weight carbohydrates (glucose in wood frogs), which provide colligative resistance to detrimental decreases in cell volume while also serving to stabilize the phospholipid bilayer of membranes and to restrict the formation of intracellular ice [112–114]. Importantly, research has shown that simple augmentation strategies such as increasing endogenous levels of cryoprotectants (via injection) prior to freezing have the capacity to improve survival and extend the time in a frozen state in a certain context from 2 weeks to 49 days [113]. A translation of nature's cryostasis strategies to human systems may be achieved through low-molecular-weight CPAs, in particular the aforementioned non-metabolizable glucose derivative 3-OMG and oligosaccharides, of which trehalose has received notable attention in recent years [54, 115, 116].

Studies on diverse freeze-tolerant species have shown that ice-nucleating agents play a critical role in survival of freezing. Ice-nucleating agents in the blood and in the gut/skin induce controlled freezing of extracellular water at multiple nucleation sites. In this way, secure and protective extracellular freezing occurs before any nucleating components present in cells trigger injurious freezing. It is generally accepted that minimization of cryoinjury may be achieved when ice nucleation occurs as near as possible to the equilibrium freezing point – i.e., the highest temperature which promotes ice crystallization and propagation [117, 118]. It therefore has been hypothesized that through the application of biocompatible ice nucleators,

controlled, slow propagation of freezing at relatively high subzero temperatures can be achieved and thereby cryodamage be minimized. Preliminary support for this approach in a simple blood vessel model has recently been provided [116].

Equilibrium Nonfrozen Subzero Preservation (Liquidus Tracking)

Notwithstanding the strategy adopted by the arctic wood frog for high-subzero survival in a partially frozen state, there is extensive evidence that mammalian organ banking might be better served by freeze avoidance rather than freeze tolerance [108, 112], which is also employed in nature. A third option is to leverage the strengths of these two approaches for nonfrozen subzero storage in thermodynamic equilibrium.

To this end, an alternative cryopreservation strategy has been conceived that relies upon incorporation of classic cryoprotectants into the cryostasis cocktail to maintain the system above the equilibrium freezing point at all stages during cooling. This approach is based upon the same principles of equilibrium cryopreservation as originally proposed by Farrant [119] (reviewed by Taylor et al. [69]) and more recently studied and referred to in the literature as "liquidus tracking" [120–123] – a process used to sequentially increase the concentration of CPAs in a stepwise manner during cooling to minimize solute toxicity and osmotic shock at each temperature and to ensure an ultrastable equilibrium state where it is impossible for ice to form.

Equilibrium phase diagrams, such as that illustrated in Figure 3, provide a useful tool for designing cryopreservation protocols based upon the principle of "liquidus tracking." Figure 3 also illustrates how these equilibrium phase diagrams have been used to design experiments to specifically investigate the role of ice formation in cryoinjury to multicellular tissues [63]. Such studies have provided compelling evidence for the profound effect of avoiding (uncontrolled and nonlimited) ice during cryopreservation. Moreover, the amount of ice and its location within the tissue has been shown to impact the structural and functional integrity of tissues [63, 64, 69].

Bioengineering Applications

Vitrification, the transformation of liquid water into a glass instead of crystalline ice during cooling, is one of the more promising technologies for avoiding ice damage to biological systems during cryopreservation [68, 69]. In fact, in the years since Rall and Fahy's seminal publication on the successful vitreous preservation of murine embryos [65], there has been a phenomenal increase in the number of publications related to vitrification as the most promising approach to avoiding ice damage during the cryopreservation of multicellular tissues and organs [67–69, 124–128]. Early studies of vitrification were conducted by Luyet in the 1930s. He reported successful vitrification with moss [129], frog sperm [130], chick embryo heart [131], vinegar eels [132], and other materials. Luyet also studied the effect of pressure on cells [133–136], presumably as a means to improve the probability for vitrification, and he reported a detrimental effect of elevated pressures on the systems he was studying. Nevertheless, vitrification has emerged as a primary approach to cryopreservation, underpinned by the extensive foundational work by Fahy and his colleagues. Of particular note is the fundamental work by Fahy and McFarlane [126], who established that several factors affect the probability for vitrification. Luyet's original approach used high cooling rates during cooling to cryogenic temperatures in order to reduce the probability for ice nucleation. However, while rapid cooling is effective for achieving vitrification of small volumes, it is not feasible for large organs or tissues due to limitations to heat transfer. Fahy's group took another approach and focused on developing vitrification solutions, which replace part of the water in the organ with glass-promoting solutes and thereby facilitate vitrification at lower cooling rates [66]. This has been established to be a more effective approach for use in tissues and organs.

Until recently [137], these advances in vitrification had not been matched by advances in rewarming of vitrified tissues without ice growth and fracturing or in addressing the inherent toxicity of the high concentrations of CPAs required. The CPA concentration necessary for vitrification, and the associated toxicity, can be minimized by cooling and warming at the fastest rate possible. Rapid warming is especially important, as the critical warming rates (CWRs) needed to avoid devitrification (the process of crystallization during warming) are typically an order of magnitude higher than the corresponding critical cooling rates needed to achieve a vitrified state [138]. Successful rewarming depends on two factors: (1) the warming rate and (2) uniform warming. Heating must be uniform to avoid stress on the tissue that often results in fractures or cracks [138]. Hence, it is well established in the field of vitreous cryopreservation that the critical cooling rate of large biosamples, tissues, and organs is not a constraint using state-of-the-art vitrification cocktails, but achieving a CWR to avoid devitrification and fracturing during rewarming remains one of the principal remaining challenges [33].

Nanowarming

The conflicting requirements to accomplish both rapid and uniform warming in large systems can be satisfied by applying heat transfer methods capable of warming

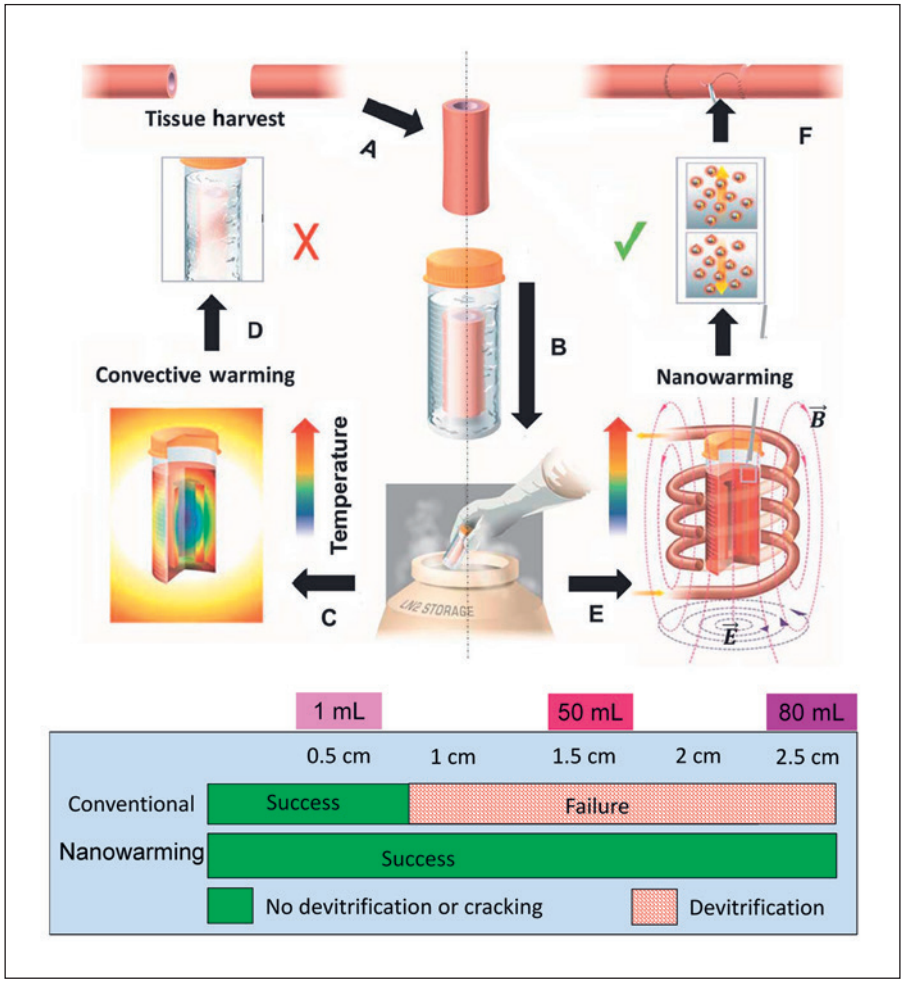


Fig. 4. Schematic illustrating a tissue nanowarming system [137]. Nanowarming scale: up from 1 to 15 kW inductive heating system. The 15-kW systems enable heating up to 80 mL. The illustration shows the limitations of convective cooling and rewarming (C, D) compared to nanowarming (E, F) of vitrification (success and failure) of 0.5- to 2.5-cm-radius cylinders. Failure and success (red and green shading) is defined by the critical minimum cooling and warming rates for VS55 vitrification solution and a thermal stress <3.2 MPa [137].

tissue from within, rather than relying exclusively on warming through surface conduction. Electromagnetic warming (or "microwave" [139, 140] or "dielectric" [141, 142] warming) and, more recently, magnetic nanoparticle warming [137, 143, 144] (nanowarming) and warming with metal forms [145] have been proposed and studied for faster and more uniform heating of tissue during recovery from the vitrified state.

There has been limited study of electromagnetic warming for cryopreserved organs, tissues, and blood products since the 1970s and 1980s. The reader is referred to the work of Pegg's and Gao's groups for a review of the status and challenges to this approach, to which uniform heating remains a major obstacle [141, 142, 146, 147].

Generally, the CWR and uniformity requirements have only been fulfilled in small-scale systems of 1–3 mL (Fig. 4) [148] – until recently having used nanowarming, which takes advantage of the ability of metallic nanoparticles to transform a radio frequency or "light energy" into heat [149], as evidenced by a report in *Science Translational Medicine* from the Bischof group [137]. In that article, the authors demonstrated their scalable and biocompatible nanowarming technology using radio frequency-excited iron oxide nanoparticles (IONPs) to uniformly warm large vitrified volumes (up to 80 mL) at over 100 °C/min and avoid (a) ice crystallization and (b) fracturing while (c) decreasing the total concentration of toxic CPAs needed [137]. This technique has also been applied in the past to cancer therapies with IONPs [150, 151]. For tissue and organ heating, Bischof's group also explore the dispersion of the IONPs within the CPA used to preserve tissues. One of the challenges presented by this approach is IONP stability within the strongly ionic environment of CPAs. However, coating the IONPs with mesoporous silica has been shown to improve stability [152, 153]. Moreover, this coating has demonstrated a high stability within biological environments and has maintained that stability within CPAs [154, 155].

By limiting the risk of ice formation during rewarming, rapid nanowarming can also help decrease the concentration of CPAs required and thus toxicity to tissues. With these and other advancements, vitreous cryopreservation now, for the first time, holds promise to be scalable to whole-organ cryobanking and to help transform transplantation in key ways [1–7, 13, 14, 19–22, 40–42, 156].

Isochoric Cryopreservation

High-Subzero Isochoric Preservation

Human life occurs in a relatively constant atmospheric pressure environment (near sea level), and hence most research into cell and tissue preservation has been performed under constant-standard pressure (isobaric) conditions (near 1 atm of pressure). At the same time, alternative thermodynamic systems exist (and, based on nature, are consistent with healthy life). As such, the basic principles of biological thermodynamic preservation under constant-volume (isochoric) conditions with changing pressures have recently been explored [157–160]. This has enabled a novel paradigm for biopreservation that utilizes more thermodynamic degrees of freedom and hence can avoid ice formation while keeping CPA toxicity low – across biologics of any size. Based on this concept, Rubinsky's group have introduced an innovative, but very simple and cost-effective, technology for reducing the temperature of "hypothermic storage" to as low as –20 °C in a thermodynamic nonfrozen equilibrium state. This isochoric process can also be combined with other powerful strategies employed by supercooling and hibernating animals in nature, such as those described above.

Isochoric high-subzero preservation is a good example of a new preservation paradigm that addresses virtually all the remaining shortcomings in long-duration tissue and organ preservation (Fig. 1). In a nutshell, isochoric (constant-volume) cooling provides a means to significantly lower nonfrozen storage temperatures without any – or with only minimum – requirements for CPAs, achieving greater metabolic reduction without injury associated with freezing, CPA toxicity, or increased amounts of osmotic solutes. This is done by taking advantage of the thermodynamics of water and other solutes' pressure temperature phase diagrams to lower the system's temperature and thermodynamically guaranteeing the avoidance of dangerous ice formation in the biologic [158, 159, 161]. In an isochoric system, volumetric expansion, which normally results from ice formation in a solution surrounding the tissue, is prevented by using a pressure-resistant constant-volume chamber (Fig. 5); this instead results in increased pressure, which restricts further ice growth [159]. As the temperature is further decreased, ice crystal growth occurs in a controlled manner within the chamber separately from the biological sample being preserved, so that the sample itself remains ice-free, well below the freezing point of the aqueous solution at atmospheric pressure (Fig. 5). Unlike methods that control ice formation using hyperbaric pressure [126], pressure increases resulting from ice within in an isochoric system are inherently minimized to the lowest required at each temperature level. This eliminates the possibility of applying excess pressure to biologics as they are preserved over a wide temperature range. It also allows nonfrozen biologic systems to be maintained at subzero temperatures in a thermodynamically stable state (in contrast to conventional supercooling, which is a state of nonequilibrium). Furthermore, solute concentrations remain an order of magnitude lower than the toxic levels occurring under traditional isobaric conditions [162], and the concentrations of CPAs required are significantly lower [163]. Crucially, Rubinsky's group have recently shown that isochoric cooling can be accomplished with an inexpensive constant-volume chamber designed, assembled, and proven to accomplish the desired thermodynamic conditions which should scale to isochoric systems of virtually any size [161–163] (Fig. 5), and they have demonstrated the potential of this approach for basic isochoric preservation of fish muscle [164], other food products [157], and an entire organism (the research model *Caenorhabditis elegans*) [165], together with results from a broader collaboration with our group presenting early studies of the preservation of hearts in a rat model [166]. Altogether, equilibrium subcooling in an isochoric system – i.e., isochoric high-subzero preservation – provides a novel method to achieve stable preservation for unprecedented durations, without the drawbacks of traditional cryopreservation paradigms but with the range of benefits outlined in Figure 1.

Life – even in active forms – under pressure finds many proofs of principle throughout nature. As discussed above, many organisms, including mammals, are adapted to tolerate subfreezing temperatures for extended time periods. Similarly, in nature many animal species tolerate hyperbaric pressures greater than those we anticipate will be employed in the isochoric cooling approach, without significant tissue injury (Fig. 6) [167–169].

Isochoric Vitrification

Fahy et al. [126] proposed that another effective way to improve the probability for vitrification is to increase pressure. Using visual inspection, they compared the concentrations of vitrification solutions required for vitrification at 1 atm and at 1,000 atm. They found that an increase in pressure substantially reduces the concentration of chemical additives required to induce vitrification [126].

More recently, Rubinsky et al. have developed the fundamental thermodynamics of isochoric (constant-volume) systems for cryopreservation [20, 159, 170]. Particularly significant is the theoretical work by Szobota and Rubinsky [158], who showed that the probability for random ice nucleation decreases in an isochoric system; therefore, they predicted that the probability for vitrification will be enhanced in an isochoric system. Additionally, Ukpai et al. [171] reported the thermodynamic profiles of solutions of Me_2SO in pure water during cooling

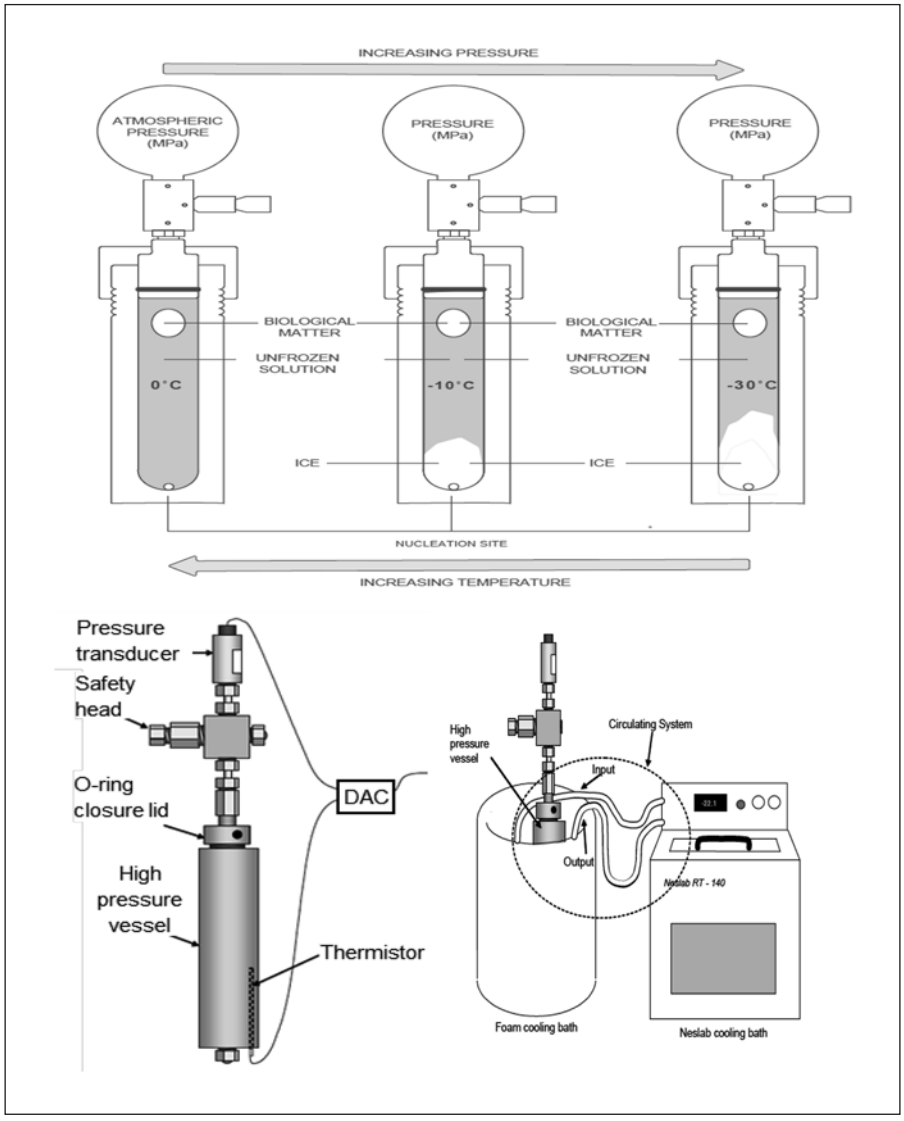


Fig. 5. Top panel: schematic of an isochoric (constant-volume) system, in which the biologic in solution is cooled; the temperature is progressively reduced from left to right to control ice nucleation separated from the biological sample in a thermodynamically equilibrated way. Isochoric preservation apparatus. Lower left panel: isochoric preservation vessel, pressure and temperature measurement, and DAC-computer connection assembly. Lower right panel: example of a system for cooling the isochoric chambers. The cooling bath (NESLAB RT-140) with hoses running out of the cooling bath and into the foam bath. One hose outputs the cooling fluid from the cooling bath to the foam bath. Another hose carries fluid from the foam bath to the cooling bath. The fluid used is a 50% by volume ethylene glycol/water mixture. (Drawings courtesy of P.A. Perez.)

to and warming from cryogenic temperatures in an isochoric system. Subsequently, together with Rubinsky's group, we showed that pressure measurement is important for designing and control of cryopreservation protocols in constant-volume (isochoric) systems and confirmed that any ice formation is associated with an increase in pressure, and that therefore pressure can be used as a measure for the occurrence of vitrification. However, when ice is not formed, the pressure in the isochoric system does not increase during cooling, and in systems that vitrify, the absence of a pressure increase can confirm vitrification, observed pressure increases during a vitrification protocol can indicate devitrification, and temporal or static pressure measurements can be used as an objective substitute for or adjunct to more subjective visual inspection methods [28, 171, 172]. A comparison with results from the literature shows that the concentration of CPAs needed for vitrification in an isochoric chamber is substantially lower than that needed for vitrification in isobaric systems at 1 atm and in hyperbaric systems at 1,000 atm. In addition, isochoric chambers are much more effective in promoting vitrification than hyperbaric pressure chambers; in addition, they are less expensive and easier to design and implement.

Non-Newtonian and Rheomagnetic Fluids

Since this is a concept paper, we choose to outline a novel concept recently introduced by Kilbride and Morris [173, 174] – after iteration of a concept that won the Breakthrough Ideas Hackathon at the first global Organ Banking Summit at Stanford – and highly relevant to the topic under discussion but not yet investigated widely in the context of cryopreservation.

In vitrification of larger tissue systems, viscosity is a catch-22: it is required to be high during cooling/warming and low during CPA loading/unloading. This limits

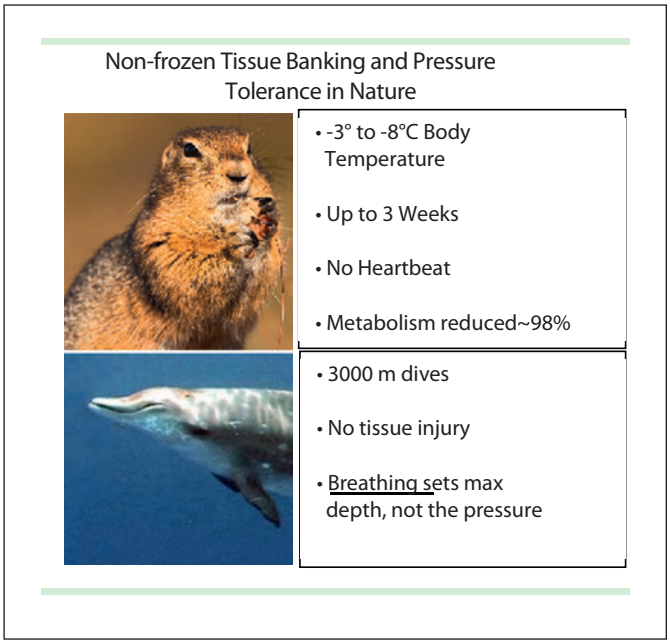


Fig. 6. At least 45 vertebrates achieve supercooling at body temperatures as low as –14 °C [74]. Mammals like the arctic ground squirrel [76] hibernate/supercool with body temperatures of –3 °C (–8 °C in the laboratory [75, 108]) for up to 3 weeks [108], with every organ "banked" without injury. Even *Caiman crocodilus*, which can grow to a weight of over 58 kg, can supercool to below –5 °C. Mammals such as the beaked whale dive to depths of 3 km [167] with no tissue injury; the lowest depths of the Mariana Trench support animals [168] at pressures of more than 110 MPa and some fish go down to almost the same depths (deeper than the height of Mt. Everest [169]).

current vitrification methods, as the viscosity of the solutions required is too high during loading, and so perfusion and permeation into tissue are slow – leading to increased exposure to toxic CPAs, which counteracts the protective benefits that the CPAs provide. After loading, viscosity needs to be much higher, but currently this is only achieved through deep cooling – a process that takes considerable time with larger tissues and increases both the exposure time to toxicity and risks of spontaneous ice formation. Yet, through bringing in cross-disciplinary learning from non-Newtonian physics and rheology, three separate approaches have been conceived that, while complementary to each other, may each individually be enough to enable vitrification of tissues and organs of any size. These three breakthrough concepts are each derived from achieving temperature-independent, on-demand, and extreme control of viscosity. This could be achieved by developing (1) non-Newtonian shear thinning and/or (2) non-Newtonian shear thickening, or (3) rheomagnetic CPA solutions. While these concepts are not currently applied to biopreservation, the use of non-Newtonian and rheomagnetic fluids is studied widely in other fields and is being translated to industrial, manufacturing, and commercial processes; moreover, these fluids also exist in nature [175–185].

In the human body, the shear-thinning properties of blood are essential for life. In normal flow, blood is too viscous to flow through thin capillaries; but since blood is a shear-thinning fluid, as vessels get smaller, the shear force caused by the interaction between moving blood and stationary vessel walls increases, allowing the now more fluid blood to flow [176–178, 185]. Many fluids display both characteristics depending on the shear force used. The forces used can be mild, such as those that already occur in every organ with blood flow, and may be experienced in everyday life, such as when using a laboratory vortex mixer. This shows that every organ in the body is adapted to shear forces essential for life. Tantalizingly – or serendipitously – the base fluid used in shear-thickening body armor (PEG) is a known and extensively studied CPA [182, 186, 187]. A huge scientific opportunity exists in applying this knowledge to harnessing on-demand viscosity control, and non-Newtonian and rheomagnetic solutions could be the foundation of a highly significant advance, helping to solve the remaining sub-problems in organ cryobanking (Fig. 7).

Non-Newtonian Shear Thinning: Non-Newtonian CPA Loading/Unloading

The viscosity of non-Newtonian fluids can change by many orders of magnitude with shear stress (e.g., vibration) from a high-viscosity to a low-viscosity liquid, or from a low-viscosity liquid to a solid-like state, instantly depending on the force used [188–190]. As the viscosity of vitrification solutions is extremely high, their application to biologics larger than small-cell clusters is curtailed due to the long exposure required for the solutions to diffuse acceptably into the biologic. CPAs are less toxic at lower temperatures; at the same time, CPA loading temperatures are constrained because CPAs become more viscous at lower temperatures [121, 187]. By inducing shear thinning, where the CPA becomes more fluid on application of a shear force [188, 191], the diffusion time and temperature during CPA loading can both be significantly lowered, both resulting in considerably improved post-preservation outcomes. Analogously, on rewarming, such a decrease in viscosity can be used to accelerate unloading of the CPA and/or to start unloading at lower, less toxic temperatures, further reducing toxicity (Fig. 7).

Non-Newtonian Shear Thickening: Non-Newtonian Pseudo-Vitrification

A second independent set of advantages is shear-thickening effects (CPA viscosity increases with shear force application) [188] and/or reverse shear thinning (increasing viscosity of a shear-thinning suspension by removing

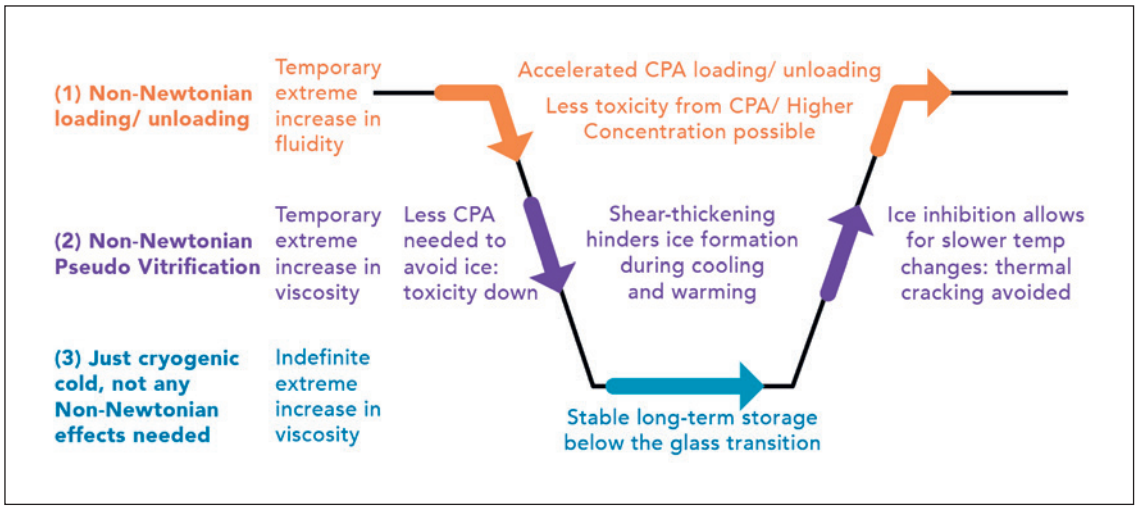


Fig. 7. Scheme illustrating three new technological approaches which could be used during both CPA loading/cooling and warming/CPA unloading. (1) On-demand non-Newtonian decrease in viscosity allows for accelerated CPA loading of tissues at lower temperatures, in turn leading to less toxicity and/or permitting the use of increased CPA concentrations. (2) On-demand non-Newtonian increase in viscosity enables ice avoidance and reduced toxicity due to lower molecular diffusion. The first effect allows decreased CPA concentration needs and the second effect allows increased CPA concentrations. All of these effects can be leveraged during both cooling and warming. Each of the on-demand increases in viscosity could also be achieved via a rheomagnetic approach. (3) Once the system is at temperatures below –60/–70 °C, the risk of ice growth during cooling or of devitrification during rewarming is no longer a problem even with current protocols, and preservation can proceed without any active viscosity control. At the same time, because any or all of these steps potentially allow the use of higher CPA concentrations, new cooling and warming protocols that proceed at slower speeds could now be used. The ability (1) to go slower and (2) thereby also to gain more degrees of freedom for annealing protocols should also permit decreased risks of fracturing or cracking. CPA, cryoprotective agent.

shear stress). With either approach, after the loading steps have been completed, the new shear force can be varied in such a way as to substantially increase the viscosity of the solution-infused tissue so that it enters a shear force-induced pseudo-"vitrified" state during cool down. This increase in viscosity (1) reduces toxicity due to lower molecular diffusion, (2) inhibits ice formation and damage, and (3) allows a decrease in the CPA concentration required. All these benefits can be leveraged again upon rewarming. Crucially, these effects only need to be applied in temperature zones down to –60/–70 °C [69], since at lower temperatures, the risk of ice growth during cooling or of devitrification during rewarming is no longer a problem even with current CPAs and cooling/warming protocols. This means that no shear forces at all would be applied in temperature regions approaching the glass transition temperature (T_g), at which tissues become brittle [69].

Rheomagnetic Pseudo-Vitrification

A third independent approach to extreme viscosity control is to utilize rheomagnetic fluids. These are fluids containing superparamagnetic (magnetic in the presence of a magnetic field) or ferromagnetic (permanently magnetic) particles whose viscosity can be controlled on demand with the use of a magnetic field. The mechanism is based on magnetic fields that can be used to induce reversible particle aggregation [181], and particle aggregation drastically increases fluid viscosity [192–194]. This can induce changes in viscosity of many orders of magnitude; hence, fluids can be loaded as low-viscosity suspensions initially, and upon application of the magnetic field, viscosity can be drastically increased after loading. Furthermore, shear stress can be applied to samples in addition to magnetic fields in order to fine-tune viscosity. The fact that many rheomagnetic fluids are also shear thinning [191, 195, 196] provides further freedom in matching the best characteristics for biological systems.

Upon relaxation of force (shear stress or magnetic), the material reverts back to its natural state, rendering each of the three processes completely reversible [188–190, 197]. While the three approaches can be combined, it is important to stress that just one of the approaches could be enough to make viable, reversible vitrification of large tissues and organ systems work.

Non-Newtonian fluids have been studied in great detail and have found a huge diversity of applications, of which those in body armor are especially impressive [175, 176, 178–183]. At the same time, while not widely known, many fluids have non-Newtonian characteristics, and the range of different shear forces that can be applied provides a large set of options. The likelihood of success is especially enhanced by the fact that by adding nanoparticles to solutions, almost all fluids adopt non-Newtonian

features [195] and can be calibrated based on the form and size of the nanoparticles – natural phenomena that could be exploited for improved methods of cryopreservation [196].

Acknowledgements

We gratefully acknowledge the input from and resources of our active collaborators in the various areas of research outlined in this article. These collaborators include: Prof. Mehmet Toner, Prof. Korkut Uygun, and Dr. Shannon Tessier (Harvard and Massachusetts General Hospital); Prof. John Bischof and Dr. Erik Finger (University of Minnesota); Prof. Boris Rubinsky (University of California – Berkeley); Dr. John Morris and Dr. Peter Kilbride (Asymptote-GE Healthcare, Cambridge, UK); Dr. Chris Hogan (University of Minnesota); Dr. Charles Lee (University of North Carolina-Charlotte); and Dr. Kelvin Brockbank (Tissue Testing Technologies). We also gratefully acknowledge the input from Jedd Lewis and Alyssa Ward at the Organ Preservation Alliance regarding the road mapping and Harvard Organ Banking Summit. The research reported was supported, in part, by grants from the National Institutes of Health (1R43HD089832, 2R44AI124835, and 1R43DK113537) and Department of Defense (contract: W81XWH-15-C-0190).

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors are all employed by Sylvatica Biotech, Inc.

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