Compendium of Organ & Tissue Banking Concepts (2015)

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Abstract

The preservation and banking of organs and tissues after donor harvest is a longstanding medical goal that has until recently seen only incremental progress. However, in the same way that we currently bank stem cells, sperm, embryos, some tissues and organ slices, it is possible to control biological time for whole organs and other large tissue systems through methods like vitrification and controlled hypothermia. An ever increasing shortage of organs and tissues leads to millions of premature deaths and results in massive costs to society. The ability to bank organs and tissues would have an immediate impact on transplant medicine, surgical cancer treatment, the treatment of combat trauma and industrial accidents, and on the ability to prepare for mass casualty events. Based on progress over the last 10 years it now looks like the goal of organ and tissue banking may be within reach for the first time.

In addition to its relevance for Department of Defense health goals, several other US Government agencies have a vested interest in such a capability, including the Department of Health and Human Services, the National Institutes of Health, the Veteran's Administration, the Biomedical Advanced Research and Development Authority (BARDA), the Office of the Assistant Secretary of Preparedness and Response, and the multi-billion dollar US Project Bioshield as well as NASA's Technology Roadmap for Human Health, Life Support and Habitation Systems.

For example, outside of the military health goals, an estimated 35% of all annual U.S. deaths could be prevented or substantially delayed by organ transplantation. And two million civilians are living with limb loss in the US with an additional 185,000 amputations conducted each year.

The publication of three SBIR grant topics on organ and tissue banking during the 2015.1 cycle represents the largest opportunity for funding of innovative organ and tissue banking efforts so far.

Keywords: Organ, tissue, banking, preservation, health, regenerative, medicine, defense, combat, injury, disease, trauma

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Table of Contents

Introduction

The feasibility of many transplant and trauma procedures is limited not by the availability of donor tissue but by the time required to deliver that tissue to the recipient.[1,2] In the case of solid organ transplant there is further suffering, death, and cost due to required immune suppression because of non-ideal organ matching which results from these time constraints for organ delivery. In cases of trauma, the ability to preserve tissue and severed limbs until advanced surgery is possible would mean far fewer amputations and vastly superior outcomes. This has tremendous implications for military trauma medicine.

The development of methods to extend the viability of tissues beyond several hours post-harvest would transform the practice of transplantation and reconstructive medicine by making tissue available to many more recipients than is currently possible – the health of millions could be restored and the life of further millions could be saved. No methods presently exist to reliably preserve the biological viability of most solid organs beyond 3-12 hours or composite tissues beyond 2 hours.[1–3] **Extending this time by even only 2-fold would be transformational.** Stem cells, sperm, and embryos have been cryopreserved for decades, and progress in cryobiology has made it possible to bank arteries, heart valves, corneas, organ slices and more. We have also seen some progress with rat hearts, pig livers, sheep ovaries, pig uteri, rodent limbs, and the cryopreservation and successful transplantation of a rabbit kidney.

Building on this to make organ and tissue banking routine will require a concerted effort to address six key engineering challenges:

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

Importantly, due to the body's own regenerative abilities, each sub-problem need not be perfectly solved, only sufficiently controlled.[4]

Organ and Tissue Banking SBIR Topic Highlights

A unique convergence of expertise and tools in other rapidly advancing domains is beginning to empower cryobiologists and can be leveraged now to make breakthroughs in each of the six sub-challenges. To catalyze the formation of such highly interdisciplinary efforts in this space the DoD has published three Small Business Innovation Research (SBIR) topics. Two of these topics appear in the *Defense Health Program (DHP)* SBIR program and the third topic appears in the *Army SBIR program*. The topic numbers, titles and objectives for each topic are:

1. **Topic DHP15-013:** Optimization of Cryoprotectants, Cryotherapeutics, and Protocols for Cryopreservation of Large Tissue Systems

   Objective: Development of novel cryoprotectants, cryotherapeutics, and cryo-preservation protocols that will permit clinically effective banking of large complex vascularized composite tissues such as vital organs and limbs.

2. **Topic DHP15-014**: Optimal Rewarming Solutions for Cryopreserved Tissue Systems

   Objective: A capability is sought to solve one of the remaining barriers towards true banking of organs and vascularized composite tissues – optimal rewarming methods of large cryopreserved tissues.

   Both DHP15 topics can be found at: www.dodsbir.net/solicitation/sbir151/dhp151.htm; search for "cryo" to find the two topics

3. **Topic A15-059**: Cryopreservation for Regenerative Medical Applications

   Objective: A capability is sought to develop cryopreservation methods that can place organs and vascularized composite tissues into metabolic stasis for at least 24 hours with full functional recovery post-cryopreservation. The ability to cryopreserve complex biological tissues in this manner will enable a wide range of medical interventions to treat trauma and disease.

   This Army topic can be found at: www.dodsbir.net/solicitation/sbir151/army151.htm; search for "cryo" to find the topic

The three SBIR topics were written to address the six engineering challenges in a broad but integrated manner that allows for teams with expertise across various domains to make an immediate impact. The three SBIR Topics are included in slightly modified form from their published versions in the Appendix.

Further information on the needs and opportunities in this space can be found in the press-release "*World-leading Surgeons, Scientists, and the Organ Preservation Alliance Applaud Defense Department-Initiated First Ever Government Grant Programs Targeting Cryobanking for Transplants*": www.organpreservationalliance.org/s/Press-Release.pdf.

Process Summary

All three cryopreservation topics are in SBIR cycle "DoD SBIR 2015.1". The key dates for this cycle are found at: http://www.acq.osd.mil/osbp/sbir/sb/schedule.shtml

Between 15 January and 18 February proposers can ask questions through the SBIR Interactive Topic Information System (SITIS) interface and any responses to those questions will be published to the SITIS page for the respective topic. The link to the SITIS page is: http://www.dodsbir.net/sitis/

SBIR Solicitation Opens: 15 January 2015
Final Proposals Due: 18 February 2015 (6am EST)

Huge Need and the Value for Transplant, Trauma, and Regenerative Medicine

Globally the vast and growing shortage of organs leads to premature death for millions and costs to society are immense. There is further suffering, death, and cost due to needed immune suppression and non-ideal organ matching. In the U.S., the official need for organ transplants severely outweighs the supply – by a factor of 5 to 1, and the gap continues to grow. In 2012, there were 120,873 *official* waiting list candidates, but only 28,052 transplants from 14,013 donors. In the same year, more than *10,000* people were removed from the waiting list because they died or became too sick to receive a transplant. The *official* organ waiting list, however, represents only one part of a bigger problem. **The true need is almost 10X larger in the U.S. An estimated 35% of all annual U.S. deaths could be prevented or substantially delayed by organ transplantation** and more through broad tissue engineering.[5–7] In addition, beyond the U.S., **the W.H.O estimates that organ transplants are currently meeting *less than 10%* of the worldwide need**.[i]

In addition to the lack of vital organs there is a huge lack of vascularized composite tissues for transplant, trauma, reconstructive and regenerative medicine. Approximately 1,600 wounded service members sustained amputations from the wars in Iraq and Afghanistan; close to 500 of those suffered amputations of more than one limb and 4,000 service members sustained facial injuries.[8] While the number of service members who sustained these catastrophic combat injuries to the face and limbs is unacceptably high, in truth the number of civilians who suffer similar injuries is even greater.

For instance, **two million people are living with limb loss in the US; 185,000 amputations are conducted each year** – where approximately 100,000 of those patients have vascular disease and/or diabetes and roughly 83,000 are due to trauma (and often young individuals and even children).[9,10]

Three million facial injuries are treated in emergency rooms in the US each year.[11] Conservative estimates suggest that if even 0.5% of those are catastrophic injuries, then 15,000 patients each year suffer dramatically life-changing facial disfigurement and disability and would be potential candidates for a face transplant.

Likewise there is a **tremendous need for skin and vascular grafts to treat the two most common complications of lifestyle diseases, namely, diabetic ulcers and coronary artery bypass**. In addition, advances in skin and vascular production or banking would also address skin and vascular injury resulting from accidents, fires or combat trauma.

Furthermore it seems that banking of tissues perhaps could help meet government mandates given to the Biomedical Advanced Research and Development Authority, the Office of the Assistant Secretary of Preparedness and Response, and the multi billion dollar US Project Bioshield to **stockpile biological materials, needed in case of mass casualty** events like a nuclear power plant failure or terrorist attack. Tissue and organ banking would transform the power of both civilian and military transplantation and trauma medicine and would help with each of the enormous problems above.

Progress here would also help other areas. There is a significant opportunity to provide preservation methods of tissues, both natural and engineered, for research on diseases like cancer and heart disease as well as for drug development and chemical screening – where banked human tissue would reduce cost, increase efficiency and decrease the need of animals in research. Progress in developing methods for large volume tissues will also likely transform current cell and thin tissue banking industries by providing better protocols and solutions that improve cell viability, cell yield, and tissue function and by providing alternatives to the currently widespread use of dimethylsulfoxide (DMSO) and other toxic compounds used in virtually all cell preservation media. Also, organ and tissue preservation methods such as vitrification[ii] and controlled hypothermia are emerging as viable alternatives. These improvements would in turn help accelerate the overall field of regenerative medicine toward faster clinical translation.

The feasibility of many transplant and trauma procedures is limited by the time required to transport donor tissue to the recipient.[1,2] In cases of trauma the ability to preserve tissue and severed limbs until advanced surgery is possible would mean far **fewer amputations and less debilitating tissue loss**. Extending the viability of tissues beyond several hours post-harvest would transform transplantation and reconstructive medicine and would make vitals tissues available to many more recipients than is currently possible. Presently no methods exist to reliably preserve solid organs beyond 3-12 hours or composite tissues beyond 2 hours.[1–3] **Extending this by 2-fold would impact millions of potential recipients.**

Many harvested donor organs (especially heart, lung, and pancreas) are discarded because of logistical demands that exceed their storage lifetime. Total costs for heart transplant procedures can exceed $1M per transplant with extreme logistical demands that require transplantation within 3 hours of organ harvest. Over 70% of donor hearts are left un-harvested largely due to these logistical demands. In the case of cardio-pulmonary transplants if just half of the non-used hearts and lungs (from deceased donors from which other organs were harvested) could be used, **the U.S. waitlist for these organs would be extinguished in 2-3 years**. An organ banking capability would also eliminate the need for partial immune matched organ transplants which requires lifelong aggressive immunosuppression

resulting in 5 year mortality rates above 40%. **Organ banking would thus address organ shortage, logistical constraints of organ donation, cost from waste and procurement, and poor immune-compatibility matching that occurs due to time constraints.**

In addition, the nascent field of tissue engineering also faces a fundamental limitation in that any engineered tissue must be used immediately after manufacture. The DoD is currently funding over 15 tissue engineering companies to manufacture skin, bone, cartilage, peripheral nerve grafts, blood vessels, connective fascia, and adipose tissue (and BARDA and NIH and others also supporting similar efforts). These products have virtually no **shelf-life** because viable preservation methods do not exist. This forces a 'just in time manufacturing' model which is not compatible with urgent clinical use. **Solving the preservation problem would remove the single most important constraint on the total size of the tissue engineering market**. The ability to bank engineered tissues would transform this field and create huge new markets. It would also immediately improve the logistical utility of existing products such as engineered skin for trauma indications. **The ability to preserve complex engineered tissues for even several weeks would completely transform the industry by opening new markets and incentivizing the development of engineered tissues for new clinical indications. The DoD and other government agencies would also overcome the single biggest limitation to the use of engineered tissues to treat trauma and in other cases of urgent need.**

Additionally, tissue and organ banking should be valuable with respect to several NASA goals including NASA's Technology roadmap for Human Health, Life Support and Habitation Systems as well as Sections 4.3, Human Health Countermeasures, and Section 4.5, Space Radiation of NASA's Human Research Program (HRP). Several of those needs could be met and key hazards could be mitigated through the ability to bank organs and tissues for off-the-shelf, on-demand availability.

Organ banking might also provide a foundation for all the progress in the last decade in **immune tolerance induction** to finally make its way into clinical practice. Organ Transplant Nobel Prize Winner Peter Medawar has called immune tolerance induction "*the Holy Grail of Transplantation*."

Summary of Current Challenges: Cryopreservation has been used successfully for several decades to preserve individual cells, colonies of cells, and human embryos for periods exceeding several years.[12] More recently, blood vessels, cartilage, corneas, and small animal organs and limbs have been cryopreserved with some success, showing that cryopreservation is possible for complex tissues.[13,14,5,15] However, current approaches have shown only limited success with digits, limbs, whole organs, and composite tissues. **The field suffers from historical fragmentation of expertise and under-funding of new research. Important progress has occurred both within the field of cryobiology and in many fields around it that could be brought to bear on solving the remaining challenges. This presents an opportunity to seed the many new ideas that have accumulated but which remain unexplored, support pioneers, and exploit recent developments in several key fields outside of cryobiology to drive the formation of world-class teams that can make transformational advances to reach the goal of limb, tissue, and whole organ preservation**.

DoD Programs in Regenerative Medicine and Vascularized Composite Allo-transplantation

Tissue and Organ banking breakthroughs would provide enormous benefits many other key DoD programs related to tissue injury and regenerative medicine.

Until relatively recently (mid-2014) the DoD's regenerative medicine program was actually a large collection of separate efforts managed by various program offices. The **Tissue Injury and Regenerative Medicine (TIRM) program office** at Ft. Detrick, MD was established in 2014 to consolidate many of these separate efforts under the purview of a single program office. As of January 2015 the TIRM program office manages close to 200 early-stage (pre-preclinical) projects and 17 clinical trials (Phase I and II).

Organ banking would significantly enable and accelerate much of the work conducted within the Armed Forces Institute of Regenerative Medicine (AFIRM) - the single largest regenerative medicine effort managed within TIRM. AFIRM and AFIRM II cooperative agreements total over $300M in direct investment and nearly $1B when matching, in-kind, and venture capital contributions are included.

Other programs within TIRM that strongly would benefit from organ and tissue banking include the vascular composite tissue allo-transplantation (VCA) program which funds the hand and arm transplant program and the face transplant program. The DoD has been the single largest investor in the world in this area.

Numerous other efforts in regenerative medicine and that relate to tissue injury are funded through the Congressionally Directed Medical Research Program (CDMRP) are also managed within TIRM.

This diverse portfolio covers virtually every tissue injury type for which regenerative medicine can provide immediate and projected solutions. It also creates significant synergy with many solid organ efforts funded through the NIH in liver, heart, kidney, and lung. Organ and tissue banking would have an immediate impact on all these programs by establishing a fundamental technology platform that enables both researchers and physicians to store and uses tissues when and where they are needed.

In addition to the value of improvements in access to vital organs like hearts, livers and kidneys that the DoD shares with NIH, VA, and others; under TIRM the DoD's regenerative medicine portfolio has the following five focus areas:

1. Skin repair (burn and trauma)
2. Extremity repair (all extremity tissues including bone, cartilage, nerve, muscle, & vasculature)
3. Cranomaxillofacial repair
4. Genitourinary repair (restoration of continence, sexual function, and fertility)
5. Vascular composite tissue allo-transplantation (VCA)

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[i] This section borrows substantial content from a proposal to the White House Office of Science and Technology Policy (OSTP) entitled "Building a TERM Roadmap for Scientific and Technological Solutions to Organ Disease & Injury" that was coordinated by the Organ Preservation Alliance and New Organ and for which the author (Alvarez) was part of the advisory committee. It also includes input from various DoD and HHS program offices.

[ii] Vitrification is the state transition of water from a liquid to a glass (vitrified) state without the formation of ice (the solid crystal phase of water). Limiting excessive ice formation in organs and tissues as the temperature is reduced is one of the six key engineering challenges whose solution would make whole-organ and limb cryopreservation possible.

Appendix on the Organ and Tissue Banking SBIR Grant Calls

TOPIC #: DHP15-013

TITLE: **Optimization of Cryoprotectants, Cryotherapeutics, and Protocols for Cryopreservation of Large Tissue Systems**

KEY TECHNOLOGY AREA(S): Transplant Medicine / Regenerative Medicine

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OBJECTIVE: Development of novel cryoprotectants, cryotherapeutics, and cryopreservation protocols that will permit clinically effective banking of large complex vascularized composite tissues such as vital organs and limbs.

DESCRIPTION: The development of methods to cryopreserve complex tissues such as organs and limbs without loss of function would revolutionize several areas of medicine and biomanufacturing. Such a capability would alter the practice of transplant medicine, trauma treatment, fertility treatment, and the manufacturing of engineered tissues for regenerative medical applications. A key challenge in this area is the widespread use of dimethyl sulfoxide (DMSO) and similar cryoprotectant compounds. DMSO and related compounds are used in virtually all current cell preservation media and are of great concern due to their toxicity. Any methods to replace or reduce their use or mitigate their effects would transform the cell and tissue banking industries and enable the development of cryopreservation for larger tissues.

The preservation of organs and vascularized composite tissues after donor harvest is a central problem in transplant and reconstructive medicine. The feasibility of many transplant procedures (for solid organs like hearts, limb, or face) is limited not by the availability of donor tissue but by the transportation time required to deliver donor tissue to the recipient [1]. There is a vast and growing shortage of organs leading to premature death for millions. Costs to society are immense and there is further suffering, death and cost due to required immune suppression and non-ideal organ matching. Cryopreservation of organs can help solve each of these problems. It would also enable doctors to extend the benefits of fertility-related cryopreservation to women by enabling the preservation of whole ovaries. This is particularly important in women who suffer combat-related or civilian trauma or who are undergoing cancer treatment. It would also better enable a greater number of digit, hand, face and limb transplants. Moreover, organ banking will be a valuable complement to and facilitator of tissue engineering and vice versa. Each of these applications will fuel mutual demand for the other. [2] The development of methods to extend the viability of tissues beyond several hours post-harvest would transform the practice of transplantation and reconstructive medicine by making donor tissue available to many more recipients than is currently possible.

Current formulations of cryoprotective agents (CPAs) suffer from several limitations that prevent their use in the clinically-relevant cryopreservation of large complex tissues such as organs and limbs. This topic focuses on three fundamental problems that govern the performance of CPAs in these types of

applications: 1) CPA effectiveness per unit of toxicity 2) mass exchange issues and material properties of CPAs (e.g., diffusivity and viscosity) that impact the effectiveness of ice inhibition and the ability of the CPAs to uniformly diffuse into tissues prior to cooling and to diffuse back out of tissues following rewarming, and 3) the ability to intervene therapeutically to mitigate the unwanted effects of CPAs and thermomechanical stress. Further, currently utilized CPAs and cryopreservation strategies typically limit their effects to that of physical and chemical control of the impact of the freeze-thaw process while minimally addressing the molecular-biological driven stress response of cells and tissues to the process. [3],[4]. This molecular control aspect continues to gain attention in the research community and has provided a new path in the development of CPA formulations targeting not only improved survival but function as well [5].

A key physical parameter that impacts the outcome of cryopreservation of large tissue systems is the nature of the aqueous state transition in and around cells from liquid water to either an amorphous glassy state or ice [6],[7]. The formation of ice in or between cells can be lethal and is problematic during both the cooling and warming phases of cryopreservation. Previous studies have found that ice formation in hepatocytes can be lethal at levels as low as 2-4% of the total water in the cell [8]. The extent of ice formation depends on factors such as the cooling rate, solute concentrations, and the path of state transition to either ice or an amorphous glassy state. However, a promising method to manage or avoid ice formation when cryopreserving larger tissue systems exists in so-called vitrification [9],[10],[11]. Vitrification, an "ice-free" cryopreservation method, is an effective way to preserve biological matter as it avoids the damage caused by ice growth. This method involves solidification of a liquid into a glassy state without crystallization due to high values of viscosity at low temperatures and can allow for indefinite storage without any biological change.

Water can be supercooled to the homogeneous ice nucleation temperature of 235 Kelvin (K) and can be vitrified by very rapid cooling to below the water glass transition temperature of 136 K when cooled at rates exceeding 10^5 K/s [12]. This rate of cooling has not yet been demonstrated for aqueous systems wider than a few microns. However, cooling rates necessary to achieve vitrification can be reduced greatly by adding CPAs. Successful vitrification of nucleated cells using a cooling rate of only 20 K/min has been demonstrated by using a mixture of several CPAs to minimize toxicity [13]. More recent work has shown that ~10 gram rabbit kidneys can be loaded with vitrifying CPA, cooled to -45°C, and then unloaded by vascular perfusion with consistent subsequent long-term survival [14]. One kidney was successfully cooled below the glass transition temperature with post-transplant function [15]. CPAs discovered to date tend to be toxic in varying degrees for both living cells and tissues, especially at warmer temperatures. Permeability and, as temperature is lowered, viscosity can stand in the way of obtaining uniform distributions of the CPA solutions to all parts of a tissue during the desired short loading and unloading periods. The effectiveness of CPAs often requires that they be used in very high concentrations. Natural antifreeze proteins (AFPs) found in cold-adapted organisms such as some species of fish and frogs may be less toxic but have so far been prohibitively expensive. However, successful cryopreservation in combination with DMSO using relatively inexpensive recombinant insect-derived AFPs has been reported [16].

Various cell and tissue types also exhibit varied tolerance to different types of CPAs. This may require formulations of CPAs optimized for particular tissues The identification of novel biological targets for the development of cryotherapeutics to mitigate CPA toxicity and chilling injury would open additional possibilities for advancing the field of cryopreservation. Potentially promising paths that are encouraged under this topic, include, but are not limited to:

- Specialty CPAs including disaccharides, ice blockers or modulators and/or natural antifreeze compounds that in addition or in combination are less toxic.
- Applying computational chemistry and/or high throughput testing methods to design, evaluate, and optimize more effective and less toxic cryoprotectant cocktails.
- Adding accessory compounds that mitigate effects of oxidative damage, osmotic stress, and cryoprotectant toxicity (cryotherapeutics), or augment natural healing processes.
- Using modeling, simulation, and estimation, to guide optimal loading and unloading protocols while also optimizing cool down and rewarming protocols.
- Alternative techniques for CPA loading and unloading, such as electroporation, ATP–induced pore formation in cells that carry appropriate receptors, and aquaporins to increase membrane permeability.
- Using hyperbaric protocols to improve loading times and distribution of CPA and other compounds to cells and tissues.
- Identification of critical molecular cell stress response pathways activated during cryopreservation.

Convergence of multiple domains outside of cryobiology may yield important insights that would be useful in solving key challenges. A non-exhaustive list of such domains includes:

- Metabolomics, proteomics, genomics and epigenetics (to better understand and find ways to intervene in toxic reactions to cryoprotectants, osmotic stress and chilling injury).
- Modeling and computer simulations (to better guide rational compound discovery and optimal loading and unloading procedures).
- Thermodynamics of complex biological systems.
- Preconditioning and biological stress responses (to prepare and “harden” cells ahead of potential damage and optimize healing post-insult).
- Hypothermic surgery and resuscitation medicine (to minimize ischemic injury and optimize resuscitation procedures).
- Advanced imaging / phase scanning / diffusion tensor imaging techniques (to image CPA distribution, ice distribution, and stress distribution in 3-dimensional systems).

PHASE I: The performer will carry out early foundational work to develop an understanding of biological pathways that can inform novel candidate CPA formulations, identify targets for cryotherapeutics, and/or molecular/stress modulation, and develop cryo-protocols that would be expected to increase efficacy and minimize damage during cryopreservation of large complex tissues. The performer will propose, or adapt, existing model systems that are suitable to evaluate and clearly demonstrate efficacy and toxicity outcomes. This model or set of models will be used in Phase II. The focus of this phase is on developing the understanding required to rationally develop CPA formulations, identify targets for cryotherapeutics, develop cryo-protocols, and select biological model systems that are suitable for use in Phase II. Computational modeling and simulation of system performance during Phase I is encouraged. Preliminary evaluation and testing of novel CPAs, cryotherapeutics, and cryo-protocols in the selected biological model system is encouraged, but not required. The biological model system may be engineered tissue or tissue derived from an animal. Screening may employ cell-based assays.

PHASE II: The performer will build on the foundational work performed during Phase I to develop and evaluate novel CPA formulations, screen stress modulators and cryotherapeutics, and test cryo-

protocols to demonstrate their effectiveness using the biological model system identified in Phase I. Although screening and early evaluation may be carried out in smaller cell-based biological model systems this phase will require demonstration of performance of top CPA(s), stress modulator(s), cryotherapeutic(s), and cryo-protocol(s) in suitable tissue-based engineered constructs, human or animal explants, or an in vivo small animal model, with preference given to larger, more complex model systems. The deliverable in Phase II will be a novel CPA formulation, therapeutic modulator of cyro-injury, model system to evaluate cryo-preservation, or protocol for performing cryo-preservation.

PHASE III: Cryopreservation of complex biological tissues is an open problem with a large potential market in cell banking, organ banking, or storage of engineered tissues and with direct applicability across the full spectrum of medical treatment, diagnostics, and long-term unattended biologically based sensor platforms. Progress in developing methods for large volume tissues will also likely transform current cell and thin tissue banking industries. By providing better protocols and solutions improving cell viability, cell yield, and tissue function, and by providing alternatives to the currently widespread use of dimethyl sulfoxide (DMSO) and other toxic compounds used in virtually all cell preservation media. There is also a significant opportunity to provide preservation methods of tissues, both natural and engineered, for chemical and drug screening to reduce cost, increase efficiency and decrease the need of animals in research. The performer or a suitable partner will pursue development of the approach to permit the cryopreservation of successively larger tissues and organs. This award mechanism will bridge the gap between laboratory-scale innovation and entry into a recognized FDA regulatory pathway leading to commercialization. Specific transition to commercial viability will require clinical validation of the product using accepted trial methods. FDA approval or licensure of the product or device is the ultimate objective. Military applications include within the DoD's regenerative medicine program at USAMMDA where several companies are funded to develop engineered tissues and would benefit from cryo-preservation for long-term storage and shelf-life extension of engineered tissues.

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KEYWORDS: Cryopreservation, CPAs, extension of biological function, stasis, organ transplant preservation, tissue engineering, regenerative medicine, vitrification

This topic may have been modified from the official version published on SBIR.gov; consult the official version online at www.sbir.gov for all purposes related to submitting a proposal under the SBIR program

TOPIC #: DHP15-014

TITLE: **Optimal Rewarming Solutions for Cryopreserved Tissue Systems**

KEY TECHNOLOGY AREA(S): Transplant Medicine / Regenerative Medicine

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OBJECTIVE: A capability is sought to solve one of the remaining barriers towards true banking of organs and vascularized composite tissues – optimal rewarming methods of large cryopreserved tissues.

DESCRIPTION:
This solicitation calls for the development of optimal warming methods that can be applied to volumetrically large complex biological tissues. While examples of potentially promising methods are discussed below, this call is not limited to those approaches but seeks any and all promising approaches for the optimal re-warming of complex tissue post cryopreservation.

The preservation of organs and vascularized composite tissues after donor harvest is a central problem in transplant and reconstructive medicine. The feasibility of many transplant procedures is limited not by the availability of donor tissue but by the transportation time required to deliver donor tissue to the recipient.

The development of methods to extend the viability of tissues beyond several hours post-harvest would transform the practice of transplantation and reconstructive medicine by making donor tissue available to many more recipients than is currently possible. It is envisioned that cryopreservation methods may also be used to salvage mangled extremities following trauma by permitting their preservation until advanced surgical procedures are available to repair the limb. One aspect of cryopreservation that remains a challenge regardless of the approach is the rewarming phase. This topic focuses on the optimization of solutions and methods for rewarming of cryopreserved tissues.

Cryopreservation through vitrification holds great promise as demonstrated in cells and described in theory for tissues in the mid 1980s,[1]. Vitrification involves freezing to a “glassy” rather than crystalline phase, thereby avoiding damaging intra- and extracellular ice crystals that are known to damage cells and tissues in the frozen state. In practice vitrification relies on loading a high enough concentration of a cryoprotectant (CPA) (up to 50% w/w) and cooling rapidly enough such as to reach below the glass transition temperature (T_g) while minimizing or avoiding nucleation of ice. The full realization of this technology could potentially make hand, face, limb, vital organ, and ovary banking part of medical practice. It would also enable storage of 3D engineered tissues for regenerative medicine. [2] However, practical application in tissues has been difficult to realize due to diffusive heat and mass transfer and phase-change limitations that cause the procedure to fail. For instance, insufficient diffusive loading of the cryoprotective solution, insufficient cooling or warming rates, and thermal gradients that can impose thermal stress can all lead to vitrification failures. These are manifested as ice growth due to devitrification and stress driven fractures and cracking during rewarming. More recently some groups have shown promising approaches to address these diffusive limitations in tissues, by working with thin

veins, blood vessels [3],[4] small mammalian organs [5],[6],[7] and limbs [8]. This has continued to highlight the promise of vitrification, but also underlines the need to find a way to broaden the ability to work with thicker bulk tissue systems to fully realize the potential of the technology [9].

Assuming sufficiently uniform cryoprotective loading can be achieved as previously reported [1],[10], the most important issues to address relate to uniformity and speed of cooling and rewarming rates such that failures such as cracks and devitrification (i.e. crystallization) can be avoided. While cracking and fracturing can already occur within smaller tissue systems, these problems only grow as tissues scale up in volume. Thermo-mechanical fractures are created by differential contractions in the tissues; and they may be caused by differences in coefficients of expansion in different tissue types, by thermal gradients, and perhaps by other means. Vitrifiable tissues larger than a few cubic centimeters often develop these large-scale fractures [11].

With regards to devitrification or ice nucleation, several studies suggest that the successful outcome of cryopreservation is often limited by the re-warming, not the cooling step [12],[13]. More specifically, the rates of cooling necessary to achieve vitrification are often orders of magnitude less than those needed to avoid devitrification during warming [15]. This is important since approaches to cool to the vitrified state in bulk tissues such as kidneys already exist [1],[10]. Importantly, some ice growth can be compatible with viability and function post cryopreservation, but the level of ice must be held to a very low percentage within the sample [1],[4],[10]. Ice nucleation within a vitrified aqueous phase can during rewarming lead to large intracellular ice crystal growth and preservation failure. Avoiding this devitrification is directly related to the warming rates and concentration of added cryoprotectants. Encouragingly, unlike cooling, warming can be accelerated by application of penetrating radiofrequency or microwave energy [15] and other methods such as the use of hyperbaric pressure and warmed gas persufflation have been proposed [16]. The most well recognized attempt at achieving uniform heat generation in cryobiology has been with microwave rewarming [17-23]. Importantly, the study of electromagnetic warming, coupling with magnetic nanoparticles and gas persufflation are all well researched domains in other fields, but have thus far had limited applications in cryobiology. Recently, initial success has also been demonstrated with the combination magnetic nano particles and radio frequency (winning the 2013 J.K. Crister Award at the Society for Cryobiology's 50th Anniversary meeting) [9].

All the methods discussed above have the potential to enable increased speed and uniformity of warming that can help avoid cracking or fracturing and devitrification. The temperature zone where previously-nucleated ice crystals grow most rapidly typically spans tens of degrees Kelvin below the melting temperature, above which ice cannot exist, and below which ice growth is kinetically inhibited by viscosity [24],[25]. Each of the methods mentioned above has the potential to rapidly and uniformly traverse that specific temperature zone of maximum ice growth where risk of devitrification and damage from ice is greatest and increased warming rates are needed the most. Further, any method that enables faster rewarming and hence less devitrification can decrease the needed amount/concentration of cryoprotectants. Since cryoprotectant toxicity increases non-linearly with concentration, even small reductions in concentration can yield large decreases in toxicity [26]. Faster rewarming also decreases the toxic effects of the cryoprotectants as toxicity is directly dependent on the exposure time.

Approaches based on electromagnetic fields (microwave and RF) and/or magnetically susceptible nanoparticles have the advantage that in principle these bulk warming methods can be chosen to couple

with the cryoprotectant solution as a function of temperature, thereby slowing the speed of thaw for annealing close to the glass transition temperature [11], but allowing one to speed up to avoid devitrification during subsequent warming. This allows rapid passage through the ice growth temperature zone while also reducing the mechanical stress by annealing and thereby avoiding both devitrification (ice growth) and cracking or fracturing [28].

While the intent of the call is to improve vitrification in large tissue systems, there will be simultaneous benefit at all scales in cryopreservation. Development of new technology in this space would likely capture substantial commercial value in the existing cell and thin tissue banking industries. For instance, increasing the uniformity and speed of thawing will extend the abilities of almost any cryopreservation solution in the field, and therefore its use on any system large or small. Specifically, faster warming will allow the molarity of cryopreservation solutions to be reduced thereby allowing less toxic solutions to be developed and used [10]. This will be a large step forward in cryopreservation that will have lasting impact in both cellular and tissue-based regenerative medicine.

Beyond the physical challenges associated with rapid and uniform warming of frozen or vitrified tissues, another significant challenge to current organ and cell transplant procedures is the control of ischemia/reperfusion (I/R) injury that occurs as a result of re-warming, re-oxygenation, and the resultant reactivation of biochemical functions. It is known that hypothermia causes tissue damage which influences the extent of I/R injury thereby directly impacting organ/graft rejection [30]. What is less appreciated is the role I/R injury plays in cell and tissue demise following cryopreservation. Given that all cells, tissues, and organs undergoing freezing and thawing are subjected to prolonged exposure to hypothermic conditions all these biologics are impacted to a degree by I/R injury upon rewarming and implantation [31]. This is true for both hypothermically stored and cryopreserved products. The rewarming process can result in the creation of a delayed I/R injury environment resulting in continued compromise of cell retention, decreased tissue quality and function, as well as, the initiation of an immune response yielding delayed engraftment [32],[33]. As such, there is a compelling need for the development of strategies to mitigate this molecular biochemical response within tissues during the thawing and recovery process. The development of new strategies (reagents and protocols) designed to reduce the level of I/R related damage would provide for improving cell survival, function and engraftment of tissues and organs post-thaw [34]. This would not only impact organ cryopreservation but would also be beneficial for current and future cell and tissue banking industries, such as cell therapy products and vascular grafts, as well as offering the potential to improve more traditional organ transplantation protocols by providing a post-storage pre-implantation tissue recovery process.

PHASE I: The performer will demonstrate successful development of warming methods that minimize vitrification failures due to ice growth and mechanical (i.e. thermal) stresses thereby allowing for reduced molarity and different types of CPAs to be evaluated. This should then allow protocol development that reduces toxicity while maximizing viability of tissue systems post-preservation. Proposals may include devices, protocols, or reagents among others. Phase I can be used to demonstrate feasibility on suitable model systems rather than working directly with complex vascularized tissue systems. The deliverable of Phase I will be a technical report and does not need to be a product or device.

PHASE II: The performer will demonstrate the utility of the approach from Phase I by successfully rewarming a large complex vitrified tissue system. The approach described in the technical report from Phase I will be scaled up to system sizes that allows for bulk tissues. The tissues of interest include

complex and vascularized tissue systems such as muscle and/or blood vessels, although larger systems are also of interest (i.e. animal limbs, digits, or organs). Demonstration that the large tissue achieved vitrification by some quantitative form of imaging (EM – freeze substitution, computed tomography, or other) is highly encouraged. Thermal analysis to demonstrate the avoidance of crystallization and cracking during thawing within the sample is encouraged (gross morphology, histology, EM, or other). In Phase II biological assessments that demonstrate viability and function of the tissue post-vitrification are required. Demonstration of the ability to reduce CPA toxicity by selecting less toxic CPAs and/or reducing CPA molarity as a result of the methods developed while still achieving vitrification will be a key feature of successful projects. It is expected that device(s) and methods will be developed under Phase II funding. This will form the basis for further translation and commercialization in Phase III.

PHASE III: Cryopreservation of complex biological tissues is an open problem with a large potential market and with direct applicability across the full spectrum of medical treatment, diagnostics, and long-term unattended biologically based sensor platforms. The effort should address the commercialization of the underlying technology. Potential paths to commercialization may benefit from potential future funding under programs administered through USAMRMC such as USAMMDA or CDMRP. It must describe one or more specific Phase III military applications and/or supported S&T or acquisition programs as well as the most likely path for transition of the SBIR from research to operational capability. For example, the proposal might relate the use of cryopreservation solutions, protocols or equipment to the potential use in the treatment of particular diseases or conditions of military interest. Specific commercial applications might include cell banking, organ preservation, research tools, diagnostics, or other applications where extended cell viability would be useful. Specific defense applications include preservation of engineered tissues to support long-term storage and shelf-life extension. The performer or a suitable partner will pursue development of the approach to permit the cryopreservation of successively larger tissues and organs. This award mechanism will bridge the gap between laboratory-scale innovation and entry into a recognized FDA regulatory pathway leading to commercialization.

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KEYWORDS: Cryopreservation, rewarming, ischemic, injury, extension of biological function, stasis, organ transplant preservation, tissue engineering, regenerative medicine, vitrification

ARMY SBIR TOPIC

TOPIC #: A15-059

TITLE: Cryopreservation for Regenerative Medical Applications

KEY TECHNOLOGY AREA(S): Transplant Medicine / Regenerative Medicine

OBJECTIVE: A capability is sought to develop cryopreservation methods that can place organs and vascularized composite tissues into metabolic stasis for at least 24 hours with full functional recovery post-cryopreservation. The ability to cryopreserve complex biological tissues in this manner will enable a wide range of medical interventions to treat trauma and disease.

DESCRIPTION: The preservation of organs and vascularized composite tissues after donor harvest is a central problem in transplant and reconstructive medicine. The feasibility of many transplant procedures is limited not by the availability of donor tissue but by the transportation time required to deliver donor tissue to the recipient [1].

There is also vast and growing shortage of organs leading to premature death for millions. Costs to society are immense and there is further suffering, death and cost due to needed immune suppression and non-ideal organ matching. Cryopreservation of organs can help solve each of these problems. It would also enable doctors to protect the ovaries of young women who become wounded or have curable cancer and enable more digit, hand, face and potentially even limb transplants for maimed soldiers, firefighters and civilians. Moreover, organ banking will be a valuable complement to and facilitator of tissue engineering, and tissue engineering will likewise increase the demand for organ banking. [2]

The development of methods to extend the viability of tissues beyond several hours post-harvest would transform the practice of transplantation and reconstructive medicine by making donor tissue available to many more recipients than is currently possible.

No methods presently exist to preserve the biological viability of most solid organs or vascularized composite tissues beyond 6-12 hours. Current practice relies on above freezing-point cooling which can only be used for very short durations. In contrast, cryopreservation has been used to successfully preserve individual cells, colonies of cells, and early human embryos for periods exceeding several years [3]. More recently, blood vessels, cartilage, corneas, and some small animal organs and limbs have been cryopreserved with some success, showing that cryopreservation is possible for tissues as well as cells [4],[5]. This solicitation calls for the development of cryopreservation methods that can be applied to volumetrically large complex biological tissues. At the same time, advances in above-zero preservation that may facilitate the use of cryopreservation by providing more time for cryoprotectant introduction and washout or even by preserving tissues or severed extremities long enough permit longer evacuation times to advanced medical facilities are also solicited.

A key physical parameter that impacts the outcome of cryopreservation is the nature of aqueous phase transition in and around cells from liquid water to either an amorphous glass state or ice [6], [7]. The formation of ice in cells can be lethal and is problematic during both the cooling and warming phases of cryopreservation. Previous studies have found that ice formation in hepatocytes can be lethal at levels as low as 2-4% of the total water in the cell [8]. The extent of ice formation depends on various factors such as the cooling and warming rates, solute concentrations, and the path of phase transition to either an ice or amorphous glass state. Two common methods to manage or avoid ice formation are slow equilibrium freezing and vitrification. Slow equilibrium freezing relies on the

equilibration of water activity across the cell membrane during extracellular ice formation, thus limiting intracellular ice. In contrast, vitrification relies on ultra-rapid cooling or high intracellular concentrations of vitrifying agents (such as DMSO) to trap water in an amorphous glass state that prevents ice formation. Neither of these methods as currently practiced has shown robust success with volumetrically large complex biological tissues.

Water can be super-cooled to the ice nucleation temperature of 235 Kelvin (K) and can be vitrified by very rapid cooling below the water glass transition temperature of 136 K when cooled at rates exceeding 10^5 K/s [9]. However, this rate of cooling has not yet been demonstrated for aqueous systems wider than a few microns. Two significant barriers to rapid volumetric cooling are the low thermal conductivity of biological tissues (k~0.5 W/m-K) and the emergence of the Leidenfrost effect in coolants. For example, a 0.3 m_3 cubic volume of tissue at 273 K that is hyperquenched in liquid nitrogen at 77 K would exhibit a very high Biot number (~60) and therefore experience significant volumetric temperature gradients leading to relatively slow cooling in much of the volume. This leads to significant ice formation.

Cooling rates necessary to achieve vitrification can be reduced greatly by adding cryoprotectants. By using a mixture of several cryoprotectants to minimize toxicity, the first paper demonstrating successful vitrification of nucleated cells used a cooling rate of only 20 K/s [10]. More recent work has shown that ~10 gram rabbit kidneys can be loaded and then unloaded with vitrification solution by vascular perfusion with subsequent long-term survival [11]. The treatment reproducibly protected the kidneys from temperatures of -45°C, but permitted survival of actual vitrification at -130°C in only one published instance [12]. Obtaining uniform distribution of the vitrification solution to all parts of the organ was a significant problem.

Another challenge occurs during re-warming from a vitrified state. Ice nucleation within a vitrified aqueous phase can occur as the temperature rises above 150 K. This can lead to large intracellular ice crystal formation. Several studies suggest that the outcome of cryopreservation is as dependent on ice formation during warming as it is during cooling [8],[13],[14]. Critical warming rates required for ice-free warming of vitrified aqueous solutions depend on the concentration of added cryoprotectants, but are always greater than the critical cooling rate necessary to initially achieve vitrification. Encouragingly, the most advanced cryoprotectant solutions for vitrification now have critical warming rates under 5 K/min [11]. Unlike cooling, warming can also be accelerated by application of penetrating radiofrequency or microwave energy [15].

Achieving an amorphous glass state in a large-volume complex biological tissue remains a challenge at the edge of current technology. Methods to accomplish such vitrification may rely on a combination of techniques that include, but are not limited to, externally applied electromagnetic fields, acoustic, optical, or mechanical inputs to increase or decrease energy content, or to monitor phase states [16], [17]. The use of one or more vitrifying agents will likely be necessary to permit the formation of a glassy state under the thermal transport constraints mentioned previously. Accessory cryoprotectants that mitigate other effects such as oxidative damage or osmotic shock may also be required (e.g. glycerol, hydrogen sulfide, nitric oxide, or trehalose). It is also known that the cold storage solution used to carry the cryoprotectant into and out of the organ or tissue can have a powerful effect on the apparent toxicity of the cryoprotectant, so optimization of the "carrier" solution may be important in some cases as well.

PHASE I: The performer will demonstrate the ability to spatiotemporally control the phase transition of a minimum forty cubic centimeter (40 cm^3) volume of solution, of maximum linear dimension 12 cm, containing ten micrograms of linearized DNA (minimum length of 10,000 base pairs) such that the entire volume can be forced to transition from a liquid to an amorphous glass state and back to a liquid never containing more than 1% ice. Phase transition control using externally applied EM fields or other

energetic inputs that can deeply penetrate large physiological systems is both permitted and encouraged. The solution used must be compatible with survival of living cells exposed to it, preferably documented by previous study. The performer will demonstrate the ability to control the segregation of solutes that might impose gradients (osmotic or pH) detrimental to the recovery of biological function post-cryopreservation. Quantification of DNA shearing is required. Verification of less than 1% ice content by calorimetry, imaging, or other means is required. Modeling and simulation of system performance to support follow-on in vitro or in vivo study design(s) is highly encouraged. Actual demonstration of the approach using cells or tissues is not required for Phase I, but approaches that go directly to cell and tissue constructs are welcome and can be conducted in lieu of working on the volume solution & linearized DNA test. Exploration of the possibility of stable storage at temperatures close to but above the glass transition temperature is also within the scope of studies of potential interest.

PHASE II: The performer will demonstrate the utility of the approach from Phase I in a biological setting using intact vascularized tissue of minimum 4 g mass. This phase requires the performer to conduct detailed characterization of the approach in a suitable biological model and to demonstrate the ability to regain biological function post-cryopreservation. The model selected should clearly demonstrate the ability to place the biological test item under cryopreservation for 24 hours or, ideally, significantly longer and to return the test item to a fully functional biological state post-cryopreservation. Measures of functionality should be appropriate to the model selected. Deliverables will include a detailed technical profile of the approach that fully describes the methods, results, and a proposed commercialization path.

PHASE III: Cryopreservation of complex biological tissues is an open problem with a large potential market and with direct applicability across the full spectrum of medical treatment, diagnostics, and long-term unattended biologically based sensor platforms. The proposal must include a description of plans for the commercialization of the underlying technology. It must describe one or more specific Phase III military applications and/or supported S&T or acquisition programs as well as the most likely path for transition of the SBIR from research to operational capability. For example, the proposal might relate the use of cryopreservation solutions, protocols or equipment to the potential use in the treatment of particular diseases or conditions of military interest. Additionally, the Phase III section must include (a) one or more potential commercial applications OR (b) one or more commercial technologies that could be potentially inserted into defense systems as a result of this particular SBIR project. It is envisioned that the performer or a suitable partner will pursue development of the approach to permit the cryopreservation of successively larger tissues and organs. This award mechanism will bridge the gap between laboratory-scale innovation and entry into a recognized FDA regulatory pathway leading to commercialization.

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Appendix on Other Organ and Tissue Banking Resources

Organ Preservation Alliance (Non-profit foundation)
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Society for Cryobiology (Non-profit foundation)
President, Erik Woods, Ph.D.
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Society for Low Temperature Biology (Non-profit foundation)
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New Organ (Non-profit foundation)
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Further information on the needs and opportunities in this space can be found in the press-release "*World-leading Surgeons, Scientists, and the Organ Preservation Alliance Applaud Defense Department-Initiated First Ever Government Grant Programs Targeting Cryobanking for Transplants*": www.organpreservationalliance.org/s/Press-Release.pdf.