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Advances in Machine Perfusion, Organ Preservation, and Cryobiology: Potential Impact on VCA

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Abstract

Purpose of review: In this review, we discuss novel strategies that allow for extended preservation of vascularized composite allografts and their potential future clinical implications for the field of vascularized composite allotransplantation (VCA).

Recent findings: The current gold standard in tissue preservation – static cold preservation on ice – is insufficient to preserve VCA grafts for more than a few hours. Advancements in the field of VCA regarding matching and allocation, desensitization, and potential tolerance induction are all within reasonable reach to achieve; these are, however, constrained by limited preservation time of VCA grafts. While machine perfusion holds many advantages over static cold preservation, it does not significantly elongate the preservation time. More extreme preservation techniques, such as cryopreservation approaches, are, however, specifically difficult to apply to composite tissues as the susceptibility to ischemia and cryoprotectant agents varies greatly by tissue type.

Summary: In the current scope of extended preservation protocols, high subzero approaches of VCA grafts will be particularly critical enabling technologies for the implementation of tolerance protocols clinically. Ultimately, advances in both preservation techniques and tolerance induction have the potential to transform the field of VCA and eventually lead to broad application of reconstructive transplantation.

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Conflict of interest statement: Dr. Uygun is inventor on pending patents relevant to this study (WO/2011/002926; WO/2011/35223) and Drs. Uygun and Tessier are inventors on several provisional patents on the topic of cryopreservation of cells, tissues, and organs, including high subzero preservation. Dr. Uygun has a financial interest in Organ Solutions, a company focused on developing organ preservation technology. Dr. Uygun's interests are managed by the MGH and Partners HealthCare in accordance with their conflict of interest policies. Dr. Brandacher is a medical advisor to X-Therma Biomimetic Nanotech and a Scientific Advisory Board Member of the Organ Preservation Alliance.

Keywords

vascularized composite allotransplantation; organ preservation; machine perfusion; cryobiology

INTRODUCTION

Vascularized composite allotransplantation (VCA) has increasingly become a viable clinical reconstructive option for the treatment of patients with amputations or devastating craniofacial tissue defects (1,2). To date, more than 200 patients worldwide have benefited from VCA, the majority receiving hand/upper extremity or face transplants. However, the potential impact of VCA is exponentially larger: currently, there are about 2 million people living with limb loss in the U.S. 185,000 amputations are performed annually with 83,000 amputations due to trauma alone that could potentially benefit from VCA (3–5). In addition, there are 3 million facial injuries in the U.S. per year with about 0.5% (15,000) cases considered catastrophic without any conventional reconstructive option. In particular, reconstruction of functional subunits such as eyelids, ears, lips, or the nose is extremely challenging and in many instances after multiple surgeries the outcomes are poor (6,7). On top of these civilian numbers, there have been approximately 1600 wounded warriors that sustained amputations and 4000 with craniofacial injuries in the recent conflicts in Afghanistan and Iraq. The total economic cost of these conditions is estimated at about $3 billion (8).

In 2017, 5.8 million reconstructive procedures were performed in the United States alone according to the American Society of Plastic Surgeons (9). In order to achieve the goal of optimal aesthetic and functional outcome, reconstructive surgeons operate via the principle of restoring *“like with like”*. Complex tissue injuries involving multiple tissue types, e.g. craniofacial and upper limb injuries, are, however, rarely sufficiently reconstructed by autologous tissue transfers and are ideally reconstructed by composite tissue allografts. VCA combines expertise of reconstructive surgery and organ transplantation, filling in the gap for treatment of complex tissue injury.

However, VCA currently faces two main hurdles that have limited its widespread application, including i) optimization and minimization of immunosuppression protocols as well as ii) logistical and matching problems. With respect to immunosuppression, an advanced understanding of alloimmunity related to solid organ transplantation has paved the way for VCA. Initial opinion that skin-containing VCA would require alarming high doses of immunosuppression have been proven obsolete (10–12). Nevertheless, the unfavorable risk-benefit ratio of life-long immunosuppression for a life-enhancing rather than life-saving treatment drives research in the development of immune tolerance induction specifically via mixed-chimerism protocols (13–18).

Described first in the 1950’s (19), transplant tolerance refers to a state of donor-specific immune hypo- or unresponsiveness. In kidney transplantation, tolerance induction has been successfully achieved clinically through the induction of mixed chimerism subsequent to a nonmyeloablative preoperative regimen combined with donor bone marrow transplantation. These protocols allow for long-term allograft survival without the need for maintenance

immunosuppression (20). As tolerance and decreased need for immunosuppressive medication would allow for mitigation of drug-related side effects, the potential to expand prospective VCA recipients is substantial. However, this requires time preoperatively to apply a conditioning regimen prior the transplant, which would necessitate longer preservation of the tissue for transplant. This cannot be achieved with the current static cold preservation technology in a cadaveric donor setting.

The matching of scarce VCA donor grafts to recipients is also complicated by small geographic allocation windows while working with strict biological and anatomical matching criteria. Close matching is critical for both graft acceptance and immunomodulation; however, with current time and distance constraints, many viable organs are unable to be allocated to the best-matched recipient in a timely manner and are thus either unused or transplanted into a less-ideal candidate (21,22). Beyond this, VCA grafts suffer damage from tissue necrosis during the preservation period and increased ischemia-reperfusion injury following transplantation (23–26). Another big roadblock in VCA allocation is the significant amount of presensitization present in potential recipients secondary to prior blood transfusions and reconstructive efforts (27,28). For these patients, a desensitization regimen would decrease morbidity associated with transplant and make these patients candidates for VCA. However, currently available desensitization strategies are confined to a living donor setting.

Optimal matching and allocation, preconditioning desensitization, and potential tolerance induction are all within reasonable reach to achieve, barred, however, by the time constraints associated with VCA. Thus, despite the tremendous need and potential of reconstructive transplantation, the transformational potential of VCA remains severely limited by short preservation times (29). In this review, we discuss novel strategies with the potential to allow for longer preservation of vascularized composite allografts and their potential future clinical implications for the field of VCA (Table 1).

PRESERVATION

In all fields of transplantation, organ viability is inextricably linked to transplant success. Since the first successful organ transplantation performed in 1954 by Dr. Joseph E. Murray, cooling organs has been the key element in maintaining organ viability during the period of organ recovery until transplantation. Organs are rapidly flushed with a cold preservation solution (4–10 degrees Celsius) and transported in an ice-filled box until transplantation, referred to as static cold storage (SCS). During cold ischemia, organs are deprived of oxygen, resulting in ischemic cell injury of nearly all cells triggered by adenosine triphosphate (ATP) depletion, impairment of mitochondrial respiratory function, and acidosis from glycolysis (30). Moreover, upon restoration of blood flow, cell injury and damaging pathways are further aggravated. This biphasic phenomenon is referred to as ischemia-reperfusion injury (IRI). In solid organ transplantation, ATP depletion prior to transplantation strongly correlates with delayed graft function and impaired post-transplant outcome (31). Though cold ischemia itself is harmful to the organ, hypothermia still has a central role in preservation; a decrease of 10 degrees Celsius slows down metabolism by factor 1.5 to 3 (32), thereby lowering the demand for oxygen and slowing down ATP

depletion. This provides a time window wherein organs are kept viable *ex situ*, i.e. a maximum of 4 hours in hearts and 12 hours in livers, while cold ischemia time for kidneys can be extended up to 24–36 hours (33–36). VCA grafts are, however, composed of biologically heterogeneous tissues (skin, vessels, nerves, muscles, bone and even bone marrow) originating from different embryological germ layers (37) with varying degree of susceptibility to ischemia. Hand and limb grafts are mainly composed of muscle tissue, the cells of which are known to have an increased susceptibility to cold ischemia due to high metabolic activity. Experimental limb allograft models have shown that the degree of muscle damage correlates with cold ischemia times and that irreversible myocyte damage occurs between 3 to 6 hours (38,39). Another cell type that is very easily and severely affected by cold ischemia are the endothelial cells lining the vasculature. Loss of endothelial cells disrupts the first vascular barrier which elects a site of pro-coagulation and inflammation (40). As a consequence of endothelial dysfunction, production of nitric oxide (a potent vasodilator) decreases resulting in poor tissue perfusion and hypoxia (41).

Machine perfusion

Over the last decade, machine perfusion has gained a lot of attention as an alternative method of organ preservation (or perhaps may become an essential counterpart to static preservation methods). While SCS is sufficient to preserve good quality donor organs, it is less suitable to preserve or recover suboptimal donor grafts that are being used more aggressively in order to meet the worldwide donor shortage (42,43). During machine perfusion, organs are mechanically perfused via the vasculature with a perfusion solution (either oxygenated or non-oxygenated, and cellular or acellular). As compared to SCS, machine perfusion has the opportunity to provide essential nutrients, “wash out” toxins, resuscitate the organ, and assess its viability prior to transplantation. In the literature, the different methods of machine perfusion are mainly classified by the temperature at which it is used: hypothermic (0–12°C), mid-thermic (13–24°C), subnormothermic (25–34°C), or normothermic machine perfusion (35–38°C)(44). In 2010, Guarrera et al. published the first clinical series of hypothermic machine preservation (HMP) in human liver transplantation (45) showing feasibility and safety of the technique. Whether oxygenated HMP is superior to SCS in reducing ischemic cholangiopathy after transplantation is currently being investigated in a clinical trial (ClinicalTrials.gov NCT02995252). Subnormothermic machine perfusion of both rat and human livers prior to transplantation has shown to reverse ischemia-induced damage to the liver (46–48). Recently, normothermic machine perfusion (NMP) of human donor livers was compared to conventional SCS in a randomized controlled trial (49). Friend and colleagues found that NMP resulted in 50% lower levels of graft injury, a 50% lower rate of organ discard, and a 54% longer mean preservation time.

In 2011, Constantinescu et al. applied the technique of extracorporeal blood perfusion to a VCA graft by preserving amputated extremities for 6 hours in a porcine model (50). They found that muscle stimulation was possible throughout the entire perfusion, whereas a complete loss of response was noted in static cold preserved controls. In 2016, Ozer et al. published perfusion of a swine limb with autologous blood at 27–32 degrees Celsius up to 24 hours (51). While neuromuscular stimulation remained intact until the end of preservation in the machine perfusion group, a neuromuscular response was absent in

control limbs (preserved at 4 degrees for 6 hours). The same research group was the first to report the use machine perfusion to preserve a human VCA graft. In a report of 5 human limbs maintained for 24 hours with *ex situ* perfusion at 30–33 degrees Celsius (52), the neuromuscular electrical stimulation continually displayed contraction until the end of perfusion, and the histology showed no myocyte injury. In this study, cold ischemia time was limited to an average of 76 minutes, ranging between 40 to 100 minutes. Machine perfusion, therefore, can be considered promising to extend the current preservation time up to several hours.

Cryopreservation

Cryopreservation is an important enabling technology for the clinical utilization of blood components/transfusions, bone marrow transplantation, artificial insemination, and *in vitro* fertilization (53,54) and will play an essential role in overcoming the many barriers facing organ transplantation (55). Cryopreservation is the use of very low temperatures (typically ≤ −80°C) to dramatically reduce enzymatic and chemical activity thereby slowing down cellular functions while maintaining three dimensional and cellular structures of living cells and tissues (56). At low enough temperatures, such as that of liquid nitrogen (−196°C), all biological and chemical processes are suspended and can be, at least theoretically, kept indefinitely (53). However, conventional cryopreservation is fatal to most biologics for several reasons. First, during freezing, water is trapped as ice, thereby changing the concentration of solutes in the extracellular milieu, and cell membrane properties are altered as cells/tissues are exposed to non-physiological conditions (57). Further, intracellular ice formation can rupture cells, and extracellular ice can result in severe mechanical stress. Thus, classical cryopreservation methods can cause damage to cells and tissues by mechanical factors, solute, and chilling effects, with the formation of intracellular ice an ever-present risk.

A critical consideration for any cryopreservation protocol is the inclusion of cryoprotectant agents (CPA) which can mitigate many of the aforementioned stressors through alteration of the freezing behavior. In 1948, glycerol was accidently discovered to have cryoprotective abilities as it protected spermatozoa from freezing injury (58). Over the years, many agents (i.e. trehalose, sorbitol, 1,4-butanediol) have proven to be beneficial in reducing cryo-injury. The use of many CPAs (i.e. dimethyl sulfoxide [DMSO], ethylene glycerol, 2,3-butanediol) is, however, limited due to the chemical toxicity at high concentrations or at certain temperatures. Moreover, even the process of loading/unloading of the most effective CPAs can be challenging; osmotic changes occur as the agents enter the cell more slowly than water which can lead to cell injury and death. The work of Pegg et al. thoroughly demonstrates the importance of CPA use during cryopreservation (56).

In overcoming the issues observed with cryopreservation, vitrification is a promising alternative approach whereby cells/tissues are cooled to cryogenic temperatures in the absence of ice (59). In some cases, vitrification shows a clear improvement in cell viability (60) as compared to cryopreservation in the presence of ice (reviewed in (61)). However, vitrification protocols require high molarity cryoprotectant agents which further complicate the osmotic effects and increase the risk of cryoprotectant toxicity (59). Moreover,

vitrification is severely limited by the extremely high cooling/heating rates required to achieve the glassy state, to inhibit the growth of crystalline structures, and to prevent fracturing. Another form of preservation that heavily relies on the use of chemical and physical compounds is desiccation. During desiccation, all water is subtracted from tissue by addition of a hydroscopic agent (i.e. cellulose, zinc oxide) that can retract and hold the water, leaving the tissue in a state of extreme dryness. In various plants and animals (e.g. algae, seeds, certain shrimp, frogs), desiccation is used to suspend metabolism to overcome extreme environmental stressors (62). Although this method is successfully used to preserve food, currently it needs further optimization to preserve mammalian cells (63). In summary, new methods and cryoprotectant agents are constantly being investigated to overcome the various mechanisms of injury as a result of preservation.

Successful cryopreservation of composite, heterogeneous tissue is, however, much more complicated, and reports on VCA cryopreservation are scarce. Rinker et al. reported transplantation of 10 cryopreserved rodent epigastric flaps that were perfused with DMSO/trehalose, cooled in a controlled fashion to −140 degrees Celsius, and stored for 2 weeks (64). The authors reported survival of all 10 transplant recipients, ranging from 7 to 15 days with one outlier of 60 days of survival. Wang et al. reported successful cryopreservation (with DMSO stored for 2 weeks at −140 degrees Celsius) and replantation of Syme's amputated (above the ankle) rodent limbs after up to three months. However, all above-knee amputated limbs failed upon replantation as the limbs became immediately edematous upon restoration of blood flow resulting in blood vessel compression. The amount of muscle in both graft types was the discriminating factor in transplant success. Nearly a decade later, Arav et al. reported 'directional freezing' (with DMSO, ethylene glycerol and trehalose, at −80 degrees Celsius for 7 to 30 days) and replantation of 6 above-knee amputated limbs, but authors were unable to show long term survival (maximum survival 3 days) (65). The variable response of different tissue types to freezing, thawing, and CPAs complicates the quest for one generalized protocol for cryopreservation of all VCA grafts.

High Subzero Storage

Whereas cryopreservation protocols can be very damaging to diverse biologics, several specimens in nature have developed ingenious ways of halting biological activity and entering a state of "suspended animation." For example, the freeze-tolerant wood frogs can survive temperatures as low as −18 degrees Celsius for months without injury (66). One critical feature of freezing survival is the rapid mobilization of hepatic glucose, which enables cells and tissues to withstand the stress caused by freezing and thawing (67). In many ways, CPAs are used via similar principles. Thus, new methods aimed at drawing lessons from nature – such as leveraging high subzero temperatures or mimetic CPAs – hold great promise.

A novel alternative to cryopreservation is supercooling (ice-free), which stores cells, tissues, and organs at high subzero temperatures while also avoiding any phase transition. In contrast to conventional cryopreservation and vitrification, the temperature range of supercooling is just below zero (68,69). Even the slightest temperature drop below zero, −0.8 degrees Celsius, greatly improves ATP content when compared to livers stored at 4 degrees Celsius

(70). In a recent study, our group was able to demonstrate successful transplantation of rat livers after 4 days of supercooling at −6 degrees Celsius with long term survival (68). An essential component to this protocol was the inclusion of the glucose derivative 3-*O*-methyl-d-glucose (3-OMG) (71). 3-OMG is a viable mimetic alternative to the glucose used by freeze-tolerant wood frogs as it is relatively metabolically inert and thus can accumulate in the intracellular environment. Our group is currently working on a protocol to apply the technique of supercooling to VCA grafts. While promising, supercooling is an unstable equilibrium state and holds the risk of accidental ice formation. The smallest impurity or vibration could therefore initiate freezing of the whole system. Thus, other high subzero preservation techniques which aim for an equilibrium state are regaining interest (72). For example, a technique originally proposed by Farrant (73) and later termed “liquidus tracking” uses progressively higher concentrations of CPAs during cooling to depress the solution’s freezing point and eliminate the possibility of ice formation. Using this technique, subzero temperatures can be achieved without the unwanted risk factor of accidental ice formation (74).

CONCLUSIONS

Ultimately, when we talk about bringing transplantation and specifically VCA to new frontiers, *TIME* will be our most precious resource. Such additional time to prolong viability of organs can be bought currently by new developments and innovations in machine perfusion, cryopreservation, and organ banking.

This gained time will allow us to improve current processes in all of transplant but particularly in the field of VCA. Specifically, organ preservation would allow us to narrow the gap between supply and demand in transplantation. Having extended organ preservation and organ banking strategies in place will simplify logistics and enhance matching; this is particularly important for VCA, in which size, age, gender, and skin tone must be accounted for in addition to blood type and immunological markers to enable restoration of “*like-with-like”* tissue. In addition, we would be able to further enhance matching by exchanging VCAs and tissue grafts over a larger geographic area.

While VCA is most certainly a life altering procedure that greatly improves the quality of life of its recipients, it is not necessarily a lifesaving therapy. Commitment to lifelong immunosuppression is therefore a concession that must be carefully weighed against all the potential benefits of VCA. We support the belief that for VCA to become common practice, the clinical implementation of immunosuppression minimization or eventually successful tolerance protocols are vital. With advances in organ preservation we will be able to convert transplant practice and fundamentally change the field from an acute setting procedure to elective surgery. This would enable us to routinely apply tolerance protocols by allowing for recipient preconditioning in a cadaveric donor setting. And finally, cryopreservation and organ banking will facilitate pre-transplant desensitization strategies and allow us to overcome issues related to preformed antibodies and ABO incompatibility. Furthermore, this is probably only a small portion of possibilities that would arise – we would be able to explore entirely novel concepts of tolerance induction, such as immune engineering, off the shelf availability of donor-derived immune and stem cells for tolerance induction, or the

establishment of immune vaccination in the field of organ transplantation. With such novel tolerance strategies in place, we would be able to further expand the indications for VCA. Though further trials are still needed to prove the feasibility, safety, and superiority of machine perfusion over static cold preservation of VCA grafts, the technique can be used as part of extended storage protocols, which can be applied to extend the preservation time more profoundly. Ultimately, advances in preservation techniques and tolerance induction have the potential to transform the field of VCA and eventually lead to broad application of reconstructive transplantation.

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Abbreviations used:

ATP	adenosine triphosphate
CPAs	cryoprotective agents
DMSO	dimethyl sulfoxide
HMP	hypothermic machine perfusion
IRI	ischemia-reperfusion injury
NMP	normothermic machine perfusion
OPTN	Organ Procurement and Transplant Network
SCS	static cold storage
UNOS	United Network of Organ Sharing
VCA	vascularized composite allotransplantation

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** of outstanding interest

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KEY POINTS:

- The current gold standard in tissue preservation is insufficient to preserve VCA grafts for more than a few hours, creating a technological bottleneck in making VCA common practice.
- Cryopreservation of composite tissue is complicated by the varying degree of susceptibility to ischemia and cryoprotectant agents.
- Extended preservation protocols, particularly high subzero approaches, of VCA grafts will be critical enabling technologies for the implementation of tolerance protocols clinically.

TABLE 1:

Schematic overview of several different preservation techniques.

Abbreviations used; C: Celsius, CPAs: cryoprotectant agents, VCA: vascularized composite allotransplantation.

	Static cold storage	Machine perfusion	High subzero preservation	Vitrification
Description	*Current method of preservation in cold solution on ice*	*Continuous perfusion with solution*	*Storage below the freezing point without ice formation*	*Fast enough transformation of a substance into a glass state*
Mechanism	Cold	Cold or warm, wash out toxins, (non-)oxygenated	Cold	Cold
Temperature range	+4 °C	+37 to +10 °C	−4 to −6 °C	−120 to −196 °C
Thermodynamic state	Equilibrium	Equilibrium	Non-equilibrium	Non-equilibrium
Maximum storage time	Hours	Hours	Days to weeks	Years
Clinical implications for Vascularized Composite Allotransplantation				
Global matching options	No	No	Yes	Yes
Tolerance induction	No	Maybe	Yes	Yes
Scalability options	High	Low	High	Difficult
Difficulties for Vascularized Composite Allotransplantation				
Main problems	Short storage time	Storage time and labor intensive	Not optimized for VCA yet	Toxic CPAs, devitrification damage