

Cryopreservation of Complex Systems: The Missing Link in the Regenerative Medicine Supply Chain

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ABSTRACT

Transplantation can be regarded as one form of “antiaging medicine” that is widely accepted as being effective in extending human life. The current number of organ transplants in the United States is on the order of 20,000 per year, but the need may be closer to 900,000 per year. Cadaveric and living-related donor sources are unlikely to be able to provide all of the transplants required, but the gap between supply and demand can be eliminated in principle by the field of regenerative medicine, including the present field of tissue engineering through which cell, tissue, and even organ replacements are being created in the laboratory. If so, it could allow over 30% of all deaths in the United States to be substantially postponed, raising the probability of living to the age of 80 by a factor of two and the odds of living to 90 by more than a factor of 10. This promise, however, depends on the ability to physically distribute the products of regenerative medicine to patients in need and to produce these products in a way that allows for adequate inventory control and quality assurance. For this purpose, the ability to cryogenically preserve (cryopreserve) cells, tissues, and even whole laboratory-produced organs may be indispensable. Until recently, the cryopreservation of organs has seemed a remote prospect to most observers, but developments over the past few years are rapidly changing the scientific basis for preserving even the most difficult and delicate organs for unlimited periods of time. Animal intestines and ovaries have been frozen, thawed, and shown to function after transplantation, but the preservation of vital organs will most likely require vitrification. With vitrification, all ice formation is prevented and the organ is preserved in the glassy state below the glass transition temperature (T_g). Vitrification has been successful for many tissues such as veins, arteries, cartilage, and heart valves, and success has even been claimed for whole ovaries. For vital organs, a significant recent milestone for vitrification has been the ability to routinely recover rabbit kidneys after cooling to a mean intrarenal temperature of about -45°C , as verified by life support function after transplantation. This temperature is not low enough for long-term banking, but research continues on preservation below -45°C , and some encouraging preliminary evidence has been obtained indicating that kidneys can support life after vitrification. Full development of tissue engineering and organ generation from stem cells, when combined with the ability to bank these laboratory-produced products, in theory could dramatically increase median life expectancy even in the absence of any improvements in mitigating aging processes on a fundamental level.

INTRODUCTION

CRYOBIOLOGY, THE SCIENCE OF LIFE at low temperatures, is a broad and dynamic area of basic and applied research that is becoming increasingly important for the practice of medicine. Sperm banking, frozen blood, and frozen human embryos are longstanding and well-known medical contributions of the field of cryobiology outside the treatment of aging. More recently, the principles of cryobiology have begun to be applied to the products of regenerative medicine and thereby to the problems of aging. Of particular note is the fact that human embryonic stem cells have been cryopreserved successfully¹ and are now available for attempts at rejuvenation.² More broadly, successes in freezing human ova³ and ovarian tissue⁴ promise to allow women to prolong their fertile years and survive cancer with their fertility intact, and the practice of freezing cord blood stem cells for uses that may well involve rejuvenatory medicine in the future also has become well known. Other proposed applications of cryopreservation (preservation at cryogenic temperatures) to the treatment of aging are feasible in principle but have yet to be realized in practice. Many years ago, Makinodan and colleagues⁵ proposed that healthy T cells be collected in youth, frozen, and given back to the donor in old age to correct immunodeficiency. As immunologic tolerance induction becomes more successful, cryopreservation of young donor organs to allow time for donor-specific tolerance to be developed in the planned older recipient^{6,7} could have a major impact on the present field of transplantation. The focus of the present paper is on the relevance of cryopreservation to the next few steps in regenerative medicine, in which increasingly complex new products of tissue engineering and stem cell biology are successfully applied to the correction of age-related deficits. This prospect brings with it both greatly improved scope for major amelioration of aging and greatly increased requirements for advanced cryopreservation technology.

TRANSPLANTATION AS A BRANCH OF REJUVENATORY MEDICINE

Aging entails both the loss of cells and the loss of the functional capacity of organs. There-

fore, reversal of aging symptoms may be achievable, at least in part, by replacing cells that have been lost and organs whose function is no longer adequate. On this basis, transplantation can be seen as a form of piecemeal rejuvenation or "brute force rejuvenation" that is in fact clearly successful at extending the human lifespan even with the current limitations of immunosuppressive therapy.

Unfortunately, human cadaver sources of replacement tissues and organs have been notoriously inadequate for many years, and xenografts remain problematic. Accordingly, as of 2001, there were 73 US companies devoted to tissue engineering and regenerative medicine and at least 16 counterparts in Europe and Australia, all devoted to closing the gap between the supply and demand for cells, tissues, organs, and structural elements.⁸ The US annual expenditure for these efforts was over \$600 million, and the estimated total value of these companies was as much as \$7 billion.⁸ More recently, the obstacles to making the transition from the laboratory to the clinic have winnowed the field considerably,⁹ but both the total investment made to date and the persistence of substantial ongoing efforts testifies to the basic and persistent need for transplantable replacements.

An idea of the potential need for laboratory created substitutes for vital organs alone can be gleaned from an analysis of mortality data compiled in the National Vital Statistics Report.¹⁰ Table 1 shows the annual death rates caused by organ failure that could in principle be corrected by transplantation. Of the four organs whose failure is responsible for the most deaths each year, the heart makes the greatest contribution by far. Together, the preventable deaths listed in Table 1 account for nearly 900,000 deaths per annum, or a staggering 36% of the total US death rate from all causes. By comparison, the number of human organ transplants carried out annually in the United States today is only on the order of 20,000, or about 2% of the total number needed.

Figure 1 presents a reconstruction of the present human mortality curve from available statistics on age-specific death rates from all causes and compares it to the expected mortality curve if the deaths described in Table 1 could be prevented by organ transplantation.

TABLE 1. CONTRIBUTION OF DEATHS PREVENTABLE BY TRANSPLANTATION TO THE TOTAL DEATH RATE IN THE UNITED STATES

Organ	<i>Annual US deaths caused by organ failure that could be treated by transplantation</i>
Heart	710,760
Lung	122,009
Kidney	37,251
Liver	16,214
All of the above	886,234
All of the above, per day	2,428
All of the above, as a percentage of total US mortality ^a	36.2%

^aBased on total 2005 US annual mortality.

From: Table 1. Natl Vital Stat Rept 2002;50(16):15–48.

Each year, all newly surviving transplant recipients are assumed to be subject to all other nonexcluded causes of death with the same probabilities as prevail for the rest of the population. The results of intervention as estimated in this way are highly significant. According to this analysis, comprehensive replacement of the most critical four organs as needed would double the likelihood of reaching the age of 80 and would increase the probability of living to the age of 90 by nearly 20-fold. Although much of this benefit will undoubtedly be achieved by other means, such as lifestyle changes and statins to reduce cardiovascular risk factors, there will undoubtedly remain a major need for organ replacement for many years to come.

THE NEED FOR CRYOPRESERVATION

What has been overlooked generally in the global effort in regenerative medicine is the significance of the gap in time that must exist normally between the moment a given replacement is created and the occasion of its final transplantation. In some cases, particularly in a scenario in which replacements are made that are genetically identical to the recipient and plenty of time is available to produce and transplant the constructs, this gap may be short and insignificant. However, this scenario has limitations of economic viability, flexibility of scheduling, inability to supply replacements in case of acute injury or illness, and inability to produce replacements long in advance of need

unless cryopreservation is available (Table 2). Greater economic viability could be achieved in a scenario of mass-produced allografts enjoying the major economies of scale associated with such a production mode. In this scenario, life-saving constructs would be produced and distributed with the benefit of donor-specific tolerance induction, the feasibility of which has already been strongly supported,^{11–16a} and the gap between production and use of replacements generally would be much longer than can be appropriately managed by continued maintenance at 37°C or refrigeration (see

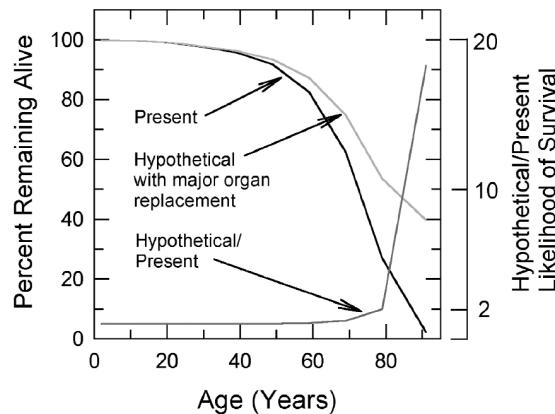


FIG. 1. Projected potential effect of heart, lung, kidney, and liver replacement on the human mortality curve, assuming no bottlenecks in organ supply, no surgical deaths, and no deaths from immunosuppression. All other causes of death in the general population are assumed to occur with the same prevalence in transplanted and nontransplanted individuals of the same ages before and after transplantation. The control curve (black) was constructed as follows. First, 100% (the survival rate at age zero) was multiplied by the infant mortality rate and the result was subtracted from 100% to yield the starting survival percentage for the same cohort over the next age interval. Second, this process was repeated, age by age, to obtain the cumulative effect of age-specific mortality until the age of 80. After the age of 80, age-specific mortality data were sparse and were estimated from the overall mortality rates at ages 85 and 85+. The effect of transplantation (upper curve) was estimated using the same method but, at each age, subtracting the fraction of deaths that would be likely to be preventable by transplantation from the total mortality rate for that age. The final curve is the ratio of the modified mortality curve to the preintervention curve and represents the age-specific increase in the probability of survival expected as a result of comprehensive and fully successful major organ transplantation. Interestingly, the effect is most pronounced at more advanced ages because the death rates at younger ages result more from cancer than organ failure, whereas at more advanced ages organ failure outstrips cancer as the leading cause of death. Results for all races and both sexes.

TABLE 2. SUPPLY CHAIN MANAGEMENT ISSUES IN REGENERATIVE MEDICINE

For allografts (assuming tolerance induction; manufactured or natural organs)
1. It must be possible to ship inventory to any desired location to allow wide geographic distribution without product deterioration en route.
2. After shipment, it must be possible for inventory to be stored in local hospitals until needed.
3. Inventory reserves must be available to meet sudden unforeseen increases in demand (natural disasters, accidents, terrorist attacks, wars).
4. It is necessary to retain flexibility of production (to allow retooling for process modifications, emergency shutdowns for repairs, decontamination, or inspections) without interruptions in supply.
5. Quality control issues include the desirability of: lot archiving for subsequent quality analysis; lot quarantine to meet regulatory requirements; large lot sizes to ensure product consistency over time; lot protection from contamination and genetic drift.
6. Natural organ allografts may require weeks or months of banking to permit donor-specific tolerance induction before transplantation.
For autografts (either natural or laboratory produced and genetically identical to the recipient)
1. Some organs may be removed during chemotherapy and/or radiation therapy for cancer, banked, and replaced after cancer eradication.
2. Organs for slowly developing diseases such as end-stage renal failure may be produced well in advance of the unpredictable time of need so as to be available without long delays when the need arises.
3. In some cases, like spare tires, individual-specific organs may be banked in local hospitals in case of sudden need (accidents, disease, attempted homicide, occupational hazards).
4. In some cases, organs produced for elective use may be banked if something interferes with their scheduled transplantation.

Table 2). According to former officials of the now-defunct Advanced Tissue Sciences, Inc., a minimum storage period of 3 to 6 months is necessary for the adequate management of the regenerative medicine supply chain even for a relatively simple engineered tissue such as skin (Applegate, personal communication). Even if it were technically feasible to maintain manufactured tissues and organs indefinitely in culture at 37°C, the cost would become prohibitive over 3 to 6 months or longer,¹⁷ and there would be a constant danger of bacterial or viral contamination and genetic drift. By comparison, maintenance in liquid nitrogen is very inexpensive, and cryopreservation is the only preservation approach that can allow any desired storage time to be selected as required by changing circumstances. Because cooling to below T_G arrests biologic changes over time, cryopreservation would make tissue archiving possible for quality assurance purposes, and large inventories could be prepared and stored to ensure product uniformity over time¹⁸ without concern over outdated. Other critical applications of cryopreservation as listed in Table 2 are self-explanatory.

In principle, the use of stem cells to repair organs *in situ* in the living patient would obviate the need for exogenously produced transplants and would be a less traumatic and much

less costly remedy for the current organ failure problems.^{19,20} In practice, the ability to control stem cell targeting and differentiation *in vivo*, although quite promising, is still in its infancy, and involves many unanswered questions about safety, efficacy, and adequacy.²¹ An organ already ravaged with disease and burdened by extensive fibrosis may not always be reparable by the mere presence of stem cells in the vicinity of diseased cells. Such an organ may require in addition the targeted death of the diseased cells to make way for new cell replacements and/or a method for removing scar tissue to allow the invasion of new cells.^{19,22} The arts of selectively clearing diseased but not dead cells and scar tissue from amidst healthy tissue are far from established at the present time. In addition, concerns over tumorigenicity of undifferentiated stem cells remain (e.g., see Ref. 22a). Questions also exist concerning the ability of stem cell treatments to reverse damage to nonliving extracellular structures important for organ function, such as the glomerular basement membrane of the kidney. The simple excision of unwanted tissues or organs and their replacement with more youthful tissues and organs that have been fully, stably, and reliably differentiated *in vitro* is a more foolproof scenario for the time being. It is also the scenario in which the greatest investments

of time, money, and personnel have been made to date. Ultimately, *in vivo* stem cell therapy¹⁹ or endogenous stem cell creation or activation²⁰ and endogenous regeneration²² will surely be the future of medicine. However, in the interim the more traditional approach of transplantation may provide more immediate help to patients who cannot wait for more sophisticated methods to be developed.

Regenerative medicine has had many successes, and many promising designs are in development.²³ For engineered cells and simple engineered tissue, traditional methods of cryopreservation may well be adequate.²⁴ Attempts to reconstruct large whole organs are relatively rare, but major successes already have been achieved. The bioartificial liver is a particularly prominent example of a device created entirely *in vitro*,²⁵ and important strides also are being made in the development of bioartificial cardiac assist devices.^{25a} Perhaps the most impressive achievement in the field to date is the creation of whole, functioning proto-kidneys from bovine embryonic stem cells made by somatic cell nuclear transfer and their long-term survival after transplantation into the nuclear donor.²⁶ However, spectacular success also has been achieved in the creation of artificial vaginas, uteruses, bladders, urethras, and other constructs that are presently functioning in real human patients.^{26a} Therefore, although mass supplies of US Food and Drug Administration-approved extracorporeal or permanently implantable organ substitutes are undoubtedly still years away, it appears likely that they are indeed on the way. When they arrive, methods for their long-term preservation will be necessary, and conventional freezing methods will not be equal to the task.²⁷⁻²⁹

Given that the development of methods that will be able to meet the challenges of preserving complex vascularized extended systems also will be complex and will require time, it is fortunate that research on the preservation of whole organs at cryogenic temperatures is already well underway. In the meantime, methodology being developed at the authors' laboratory for the preservation of large organs is finding very important applications for the preservation of simpler systems of great im-

portance in regenerative and rejuvenatory medicine today.³⁰

ORGAN CRYOPRESERVATION BY FREEZING

The earliest attempts to achieve the successful cryopreservation of whole mammalian organs involved freezing. In the late 1950s, Smith and Farrant were able to show the recovery of contractile responses in guinea pig uteri *in vitro* after freezing to the temperature of dry ice and thawing,³¹ but they did not attempt to transplant these organs. Hamilton, Holst, and Lehr achieved the next major breakthrough in 1973, freezing lengths of dog intestine in liquid nitrogen, thawing them, and obtaining long-term survival of a minority of these segments after transplantation.³² In 2002, considerable attention was given to a report of partial success with intact rat ovaries³³ that were frozen, thawed, and transplanted by vascular anastomosis. A minority of these ovaries survived, but one survivor was able to give rise to developing pups.³³ Unfortunately, this model does not really test the suitability of vascular preservation after whole organ freezing because rodent ovaries can survive freezing, thawing, and transplantation even without vascular anastomosis^{34,35} and can even self-assemble from enzymatic digests of ovarian tissue and form pups after natural mating.³⁶ A report of the freezing, thawing, and transplantation of sheep ovaries appeared in 2003.³⁷ Vascular patency was retained in three of 11 grafts, and follicle-stimulating hormone levels were kept to within normal limits by the three surviving grafts.³⁷ A second report claims higher patency rates using a directional freezing approach,³⁸ but damage remained extensive.³⁹ Although this might represent a step forward for the reversal of sterility after chemotherapy, it is unlikely to be an optimal approach (see the following).

It remains the case that no vital organ (particularly a heart, liver, or kidney) has ever been frozen to a temperature low enough for long-term storage and subsequently thawed, transplanted, and found to support life, let alone to do so on a consistent and reliable basis. The rea-

sons for this failure are numerous,⁴⁰ but one of the most critical problems is vascular damage.^{41–43} A great deal of vascular damage was undoubtedly induced by freezing in the canine intestine and sheep ovary, but both are able to accommodate substantial edema without developing tissue pressure sufficient to terminate further perfusion, so this injury can be healed in some cases. Unfortunately, in vital organs such as the heart, liver, and kidney, vascular damage leads to permanent vascular obstruction and tissue necrosis.

Vascular damage from freezing is apparently a consequence of mechanical effects of ice on the vascular wall^{28,42–44} and can be induced by the formation of very little ice.⁴¹ Consequently, if the reliable banking of vascularized natural or bioartificial organs is to be feasible, the formation of ice must be minimized or eliminated entirely.

PROGRESS TOWARD ORGAN CRYOPRESERVATION BY VITRIFICATION

The possibility of avoiding ice formation by depressing the freezing point to temperatures approaching the sublimation temperature of dry ice (-79°C) was first demonstrated for red blood cells by Huggins⁴⁵ and for guinea pig uteri by Farrant⁴⁶ in 1965. However, the problem of vascular integrity was not explicitly addressed in these studies, nor was the problem of delivering and removing cryoprotectants by perfusion. In addition, these studies resulted in storage in the liquid state, which is inadequate for truly long-term changeless storage. Finally, attempts to apply Farrant's method to kidneys^{47–50} and even to kidney slices^{24,51} were not successful.

The method proposed by Huggins and Farrant involved equilibrium depression of the freezing point by the colligative action of cryoprotective agents. A more sophisticated and practical plan for eliminating ice entirely even at the temperature of liquid nitrogen was proposed by Fahy in the 1980s.^{28,29,52,53} This method involved the use of carefully balanced cocktails of cryoprotective agents in sufficiently

high concentrations to permit clear-cut supercooling into the glassy state, a process known as vitrification, rather than equilibrium freezing point depression.⁵³ A glass is a liquid whose fluidity has been lost as a result of extreme elevation in viscosity produced by cooling to or below the glass transition temperature (T_G , the temperature at which vitrification occurs).²⁹ By supercooling into the glassy state, the concentration of cryoprotectant needed for preservation was actually reduced in comparison to Huggins and Farrant's equilibrium method, and the exact concentration needed for vitrification (C_V) was shown to be predictable from phase diagram information.^{29,53} Furthermore, before Fahy's method was suggested, vitrification generally was thought to require very rapid cooling rates unattainable for objects the size of whole organs,^{54,55} and a key aspect of Fahy's proposal was the ability to transcend this limitation. The misconception that vitrification requires ultrarapid cooling has been difficult to correct, but the demonstration of applicability to whole rabbit kidneys ($\sim 11 \text{ mL}$) in 1984²⁹ and 2004²⁷ and to volumes of up to 2 L or more in 2005⁵⁶ is gradually making the broad potential utility of vitrification at low cooling rates more apparent.

Fahy's proposal, although developed to solve the problems of cryopreserving organs, rapidly became popular for the preservation of simpler systems, in part because it eliminated the need for controlled rate freezing machines and allowed prepared samples to be preserved by direct immersion in liquid nitrogen.⁵⁷ The initial proof of principle for the method, in 1985, was the successful preservation of mouse embryos by vitrification,⁵⁷ an accomplishment that continues to influence much research in reproductive cryobiology, including the cryopreservation of ova for extending the reproductive lifespan of women.^{3,30} An indication of the growth of this field is provided in Figure 2. The projected number of PubMed citations for vitrification as a method of cryopreservation was expected to reach approximately 600 by the end of 2005.

As might be expected, application of Fahy's method to large organs was less straightforward. The approach required perfusion of or-

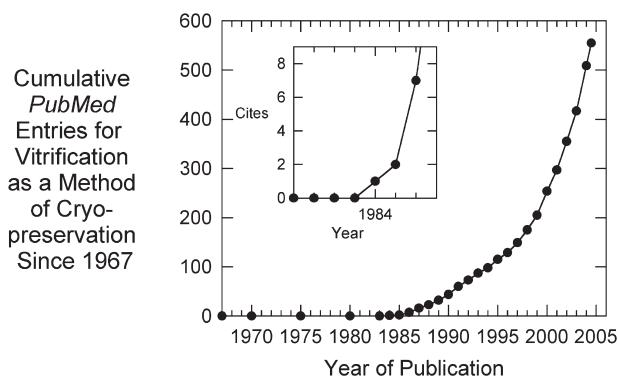


FIG. 2. Popularity of vitrification as an approach to cryopreservation, as measured by the cumulative number of citations in PubMed to the use of the method to preserve living systems at cryogenic temperatures. The onset of the curve in 1984 is from: Fahy GM, MacFarlane DR, Angell CA, Meryman HT. Vitrification as an approach to cryopreservation. *Cryobiology* 1984;21:407–426. The first proof of principle of the method in 1985 is from: Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196°C by vitrification. *Nature* 1985; 313:573–575.

gans with multimolar concentrations of cryoprotectant at relatively high temperatures. For this to be possible, new equipment, software, and protocols for automated cryoprotectant perfusion had to be created,⁵⁸ and new mixtures of cryoprotectants with less toxicity than anything available previously^{29,59–62} also had to be developed. However, once these new tools were in hand, steady progress and a series of fundamentally new advances were achieved.

The most encouraging initial development was the attainment of immediate renal function *in vitro* in about half of rabbit kidneys perfused with 7.5 M cryoprotectant.⁶³ This was followed shortly thereafter by 100% survival and retention of full histologic integrity after transplantation of kidneys perfused with 7.5 M cryoprotectant at -3°C .⁶⁴ However, this concentration, as enormous as it was, was insufficient for vitrification unless cooling was carried out under elevated hydrostatic pressure.^{59,63} Raising the total concentration to 8.4 M cryoprotectant to permit vitrification at ambient pressure was sufficient to allow the successful preservation of several simpler tissues (blood vessels, heart valves, and rabbit cartilage) by vitrification.⁶⁵ However, the survival rate of kidneys perfused

with this 8.4 M solution (known variously as VS55 or VS41A)⁵⁹ was 0% at -3°C and only about 50% when the kidneys were manually cooled to about -20°C while containing 6.7 M cryoprotectant before introducing VS41A.⁶⁶ It was apparent that further breakthroughs were needed, not only because of the damage caused by VS41A even at -20°C ⁶⁷ but also because kidneys vitrified with this cocktail could not be rewarmed without freezing on rewarming (devitrification),⁶⁵ a problem that negates the advantage of previous vitrification. Also, although many alternatives to VS41A have been used with success for the vitrification of relatively simple systems such as the cornea⁶⁸ and probably the mouse ovary⁶⁹ and ovarian tissue from several species,⁷⁰ none of these alternative solutions is likely to be less toxic than VS41A.³⁰

In 1998, another pivotal and fundamental breakthrough took place in the technology of cryoprotectant solution formulation that allowed cryoprotectant toxicity to be understood as a consequence of reduced water availability for the hydration of cellular biomolecules.³⁰ This insight allowed drastically less toxic solutions to be designed. For example, kidneys consistently experienced no postoperative creatinine peak (i.e., sustained zero damage) after perfusion at -3°C with a new solution called VMP that, like VS41A, has a total concentration of 8.4M (Fig. 3).³⁰ However, although VMP is very concentrated, it is still too dilute to escape from devitrification on warming at easily achievable warming rates for large organs; therefore, additional innovations were still required.

The next major practical step forward was the development of a working ultrastable vitrifiable solution, M22. M22 contains two novel antinucleating substances (“ice blockers”) that are dramatically effective in reducing the probability of ice formation.^{71–73} It also contains a physiologic support solution (the “carrier solution” or vehicle for M22 cryoprotectants), LM5, that is compatible with the new “ice blockers.”²⁷ M22 attains further stability by including a novel polymer that is capable of significantly slowing ice crystal growth²⁷ and has an ideal tonicity for blocking “chilling injury” (an ill-understood form of injury caused by

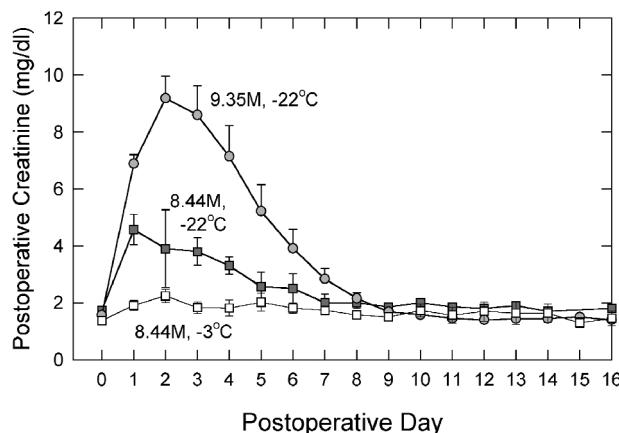


FIG. 3. Effects of perfusing the 8.44M VMP solution through rabbit kidneys at -3°C (lower curve), cooling them to -22°C while continuing perfusion with VMP (middle curve), and perfusing them with the 9.345M M22 solution at -22°C after perfusion-cooling with VMP (upper curve). All kidneys were transplanted back to their original donors after rewarming and cryoprotectant washout, and an immediate contralateral nephrectomy was performed. n values are 7, 3, and 10 for VMP at -3°C , VMP at -22°C , and M22 at -22°C , respectively. There were no nonsurvivors in any of these groups. For further discussion, see text. Data collected from: Fahy GM, Wowk B, Wu J, Phan J, Rasch C, Chang A, Zendejas E. Cryopreservation of organs by vitrification: perspectives and recent advances. *Cryobiology* 2004;48:157–178; Fahy GM, Wowk B, Wu J, Paynter S. Improved vitrification solutions based on predictability of vitrification solution toxicity. *Cryobiology* 2004; 48:22–35.

cooling per se).²⁷ Finally, M22 contains a novel low-toxicity, strongly glass-forming, and highly permeable methoxylated cryoprotectant⁷⁴ and a highly permeating novel amide.²⁷ M22 (so named because it is intended for use at -22°C), has extraordinary physical properties (Table 3); however, when used in renal cortical slice toxicity screening assays, it yielded $>90\%$ recovery of ion transport function.²⁷

Accordingly, the next step was to test M22 on whole kidneys. To do this, perfusion-cooling and -warming methods had to be worked out to allow continuous perfusion of the organ during cooling to and warming from about -22°C . Figure 3 assembles the “bottom line” of several series of optimization experiments^{27,30} relevant to the evaluation of M22. The lower curve shows the net effects of perfusing VMP at about -3°C (baseline conditions), demonstrating the remarkable lack of damage associated with this treatment. The second curve shows the effect of cooling kidneys to -22°C with VMP using an optimized protocol. Again, little damage is observed, attesting to the success of VMP at inhibiting “chilling injury” at -22°C . Finally, the upper curve shows the effect of perfusing to -22°C with VMP and then, at -22°C , switching to M22 and perfusing with

TABLE 3. PHYSICAL PROPERTIES OF M22^a

Property	Value
Total concentration, g/L	684
Total concentration, molar	9.345
Number of specific-function cryoprotectants	8
Melting point ^b	$\sim -54.9^{\circ}\text{C}$
Glass transition temperature ^c	-123.3°C
Lowest cooling rate supporting no ice in 2 liters	1°C/min
Lowest warming rate supporting no ice	4°C/min

^aThe complete formula for M22 is given in Fahy GM, Wowk B, Wu J, Phan J, Rasch C, Chang A, Zendejas E. Cryopreservation of organs by vitrification: perspectives and recent advances. *Cryobiology* 2004;48:157–178.

The properties of M22 have been described in more detail in Fahy GM, Wowk B, Wu J, Phan J, Rasch C, Chang A, Zendejas E. Cryopreservation of organs by vitrification: perspectives and recent advances. *Cryobiology* 2004;48:157–178; Wowk B, Fahy GM, Toward large organ vitrification: extremely low critical cooling and warming rates of M22 vitrification solution. *Cryobiology* 2005;51:362.

^bThe melting point was estimated from an extrapolation of the melting point curve of dilutions of M22 because M22 itself could not be frozen. See: Fahy GM, Wowk B, Wu J, Phan J, Rasch C, Chang A, Zendejas E. Cryopreservation of organs by vitrification: perspectives and recent advances. *Cryobiology* 2004;48: 157–178.

^cThe glass transition temperature was measured at a warming rate of 2°C/min.

M22 for up to 25 minutes, followed by optimized rewarming to -3°C and washout with VMP. The introduction of M22 produced more damage than cooling to -22°C alone, but the damage was limited and entirely reversible within a clinically normal period of time and was less than is typically accepted in clinical human kidney transplantation today.

The next step was to test the significance of chilling injury at temperatures below -22°C . For these experiments, the authors removed the kidneys from our perfusion machine and cooled them in a rapidly moving cold atmosphere set to a temperature just below -50°C . When all parts of the kidney were between -40°C and -50°C (except for the outer cortex, which was presumably below -50°C), the organs were placed back into the perfusion machine and were rewarmed back to -22°C by external lavage with M22, then further warmed and washed free of M22 as usual. This procedure produced a substantial increase in injury compared to exposure to -22°C alone (Fig. 4), but was survived by all kidneys in the series ($n = 8$), and final creatinine levels were all in the normal range. This was the first time the routine survival of any mammalian organ from a temperature approaching -50°C (mean intrarenal temperature, about -45°C) had ever been achieved.²⁷

The exact origin of this extra injury remained to be determined. Alternatives to chilling injury included the extra total exposure time to the cryoprotectant required during the added cooling and the rewarming phase back to -22°C and injury related to the extra handling of the kidney and processes related to it. Very preliminary evidence ($n = 2$) suggests that the extra injury seen in the initial -45°C experiments is not inevitable, and can be reduced or eliminated by improvements in technique (see Fig. 4). The question of whether this suppression of injury at -45°C can be reproduced and extended to temperatures below -45°C remains open, but will soon be the subject of intensive investigation. Information obtained with renal slices suggests chilling injury will continue to increase down to about -80°C , but nevertheless can be almost completely suppressed under optimum conditions.²⁷

Although much work remains to be done, this is clearly a new era and most of what has to be done in order to achieve successful banking of complex, spatially extended living systems has been accomplished. In addition, recently the authors were able to attain the first direct proof-of-principle for organ vitrification by showing that a single rabbit kidney, after cooling to below T_g , rewarming, and reimplantation, was able to provide stable life support function until sacrificed on postoperative day 48 for histologic examination.⁷⁵ This kidney sustained major histologic damage from inner medullary ice formation in some peripelvic columns, but no such damage in others. This suggests that the problem of more uniformly distributing M22 to the inner medulla will be vital to routine recovery from below T_g , and

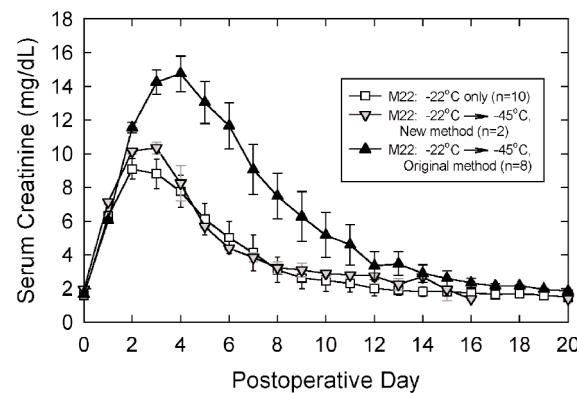


FIG. 4. Effects of cooling rabbit kidneys to a mean intrarenal temperature of -45°C as measured using invasive needle thermocouple probes in parallel experiments (modified from: Fahy GM, Wowk B, Wu J, Phan J, Rasch C, Chang A, Zendejas E. Cryopreservation of organs by vitrification: perspectives and recent advances. *Cryobiology* 2004;48:157–178). In the first method (upper curve), injury was increased by this treatment and mimicked the effects of holding kidneys at -22°C for a period similar to the time required for cooling from -22°C to -45°C and rewarming from -45°C back to -22°C (data not shown; see Fahy GM, Wowk B, Wu J, Phan J, Rasch C, Chang A, Zendejas E. Cryopreservation of organs by vitrification: perspectives and recent advances. *Cryobiology* 2004;48:157–178). In the second method, in which exposure time was reduced, all of the injury observed at -45°C was avoided. In method 2, Supercool X-1000 (Wowk B, Leitl E, Rasch CM, Mesbah-Karimi N, Harris SB, Fahy GM. Vitrification enhancement by synthetic ice blocking agents. *Cryobiology* 2000;40:228–236; Wowk B. Anomalous high activity of a subfraction of polyvinyl alcohol ice blocker. *Cryobiology* 2005;50:325–331) was omitted from M22 for reasons unrelated to this protocol, but this is not believed to have influenced the results.

the authors are presently focused on solving this problem. In addition, continued improvement of cryoprotectant technology also is ongoing in their laboratory.

Finally, direct experiments on an artificial organ model, the Cordis-Dow Artificial Kidney (CDAK), have shown that whereas both the external housing and the internal parallel hollow fibers (blood path) of the CDAK are ruptured by ice expansion during freezing in the presence of 10% dimethyl sulfoxide and 0.9% NaCl, neither the housing nor the fibers appear to be damaged by vitrification or the hazard of devitrification on warming (Fig. 5).⁷⁶ Thus, it appears that vitrification is likely to be compatible with both the living and nonliving components of future bioartificial organs.

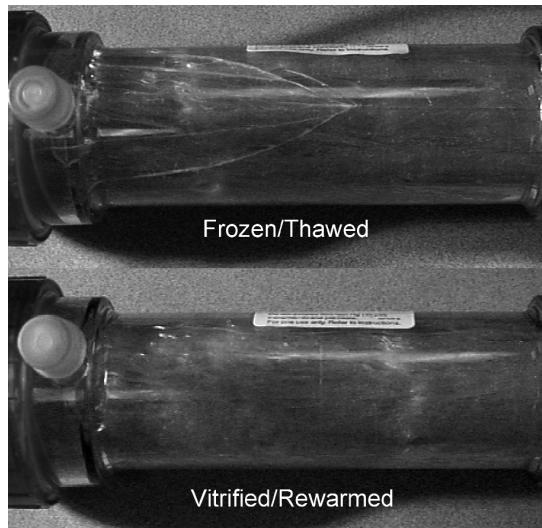


FIG. 5. Effects of freezing and thawing (*top*) and of vitrification and rewarming (*bottom*) on Cordis-Dow Artificial Kidneys (CDAKs). See: Fahy GM. The practicality of vitrification for cryopreservation of engineered tissues. *Cryobiology* 2001;43:349–350. The upper panel shows cracks formed in the housing of the CDAKs despite the use of 10% v/v dimethyl sulfoxide and 0.9% w/v NaCl to limit ice formation as in a typical cell cryopreservation procedure. These cracks, shown after thawing and closure of the cracks, were several millimeters wide when fully open prior to thawing. The CDAKs are stained blue by the use of blue dextran (2×10^6 daltons) as a marker of blood path permeability. Although blue dextran stained the hollow fibers in all kidneys, control CDAKs and vitrified-rewarmed CDAKs did not leak blue dextran from the blood path to the dialysate path, whereas the frozen-thawed CDAKs had dialysate path concentrations of blue dextran that were equivalent to those in the blood path, indicating complete breaching of the semipermeability of the hollow fibers. Note the lack of any fractures in the outer housing in the vitrified-rewarmed CDAK.

CONCLUSION

Cryopreservation currently plays an important role in the development of treatments for aging through such pathways as the preservation of stem cells and human ova and is likely to be an increasingly key factor in the success of regenerative medicine as this field progresses. Future cell-based therapeutics will have to be stored before use, and organized tissues and organ-like devices will probably not escape from this requirement. Fortunately, the science of cryopreservation is rapidly advancing and may be equal to the task of preserving even complex bioartificial implants when the time comes. In the meantime, there is evidence that low-toxicity vitrification solutions for whole organs may have applications for many simpler tissue replacements now, and even single cells subjected to freezing and thawing may benefit from the new technical advances in organ banking. Freezing can concentrate ordinary cryoprotectant solutions to toxic levels in the frozen state even when single cells are frozen, and this effect is known to be suppressed by using cryoprotectants that are less toxic even when greatly freeze-concentrated.⁷⁷ Therefore, until the long-sought goal of perpetual youth can be achieved by purely pharmaceutical and/or genetic adjustments, cryopreservation is likely to continue to be an important factor for the advanced treatment of human aging.

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