Vitreous cryopreservation maintains the function of vascular grafts

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Avoidance of ice formation during cooling can be achieved by vitrification, which is defined as solidification in an amorphous glassy state that obviates ice nucleation and growth. We show that a vitrification approach to storing vascular tissue results in markedly improved tissue function compared with a standard method involving freezing. The maximum contractions achieved in vitrified vessels were >80% of fresh matched controls with similar drug sensitivities, whereas frozen vessels exhibited maximal contractions below 30% of controls and concomitant decreases in drug sensitivity. In vivo studies of vitrified vessel segments in an autologous transplant model showed no adverse effects of vitreous cryopreservation compared with fresh tissue grafts.

Keywords: cryopreservation; vitrification; vascular graft; smooth muscle; tissue preservation.

Conventional approaches to cryopreservation cannot be applied to more complex natural, or engineered, multicellular tissues due to the destructive effect of extracellular ice formation[1–4]. Tissues are much more than simple aggregates of different cell types; they have a highly organized structure that influences their response to freezing and thawing. The formation of extracellular ice is known to be a hazard to structured tissues and organs[2,4–7]. Restricting the amount and size of ice crystal formation during cryopreservation can be achieved by using sufficiently high concentrations of cryoprotectants to promote amorphous solidification (vitrification) rather than crystallization[8–10]. Vitrification is a relatively well-understood physical process, but its application to the preservation of biological systems is difficult since the high concentrations of cryoprotective agents (CPAs) used are potentially toxic. Limiting toxic effects depends on using the least toxic CPAs at the lowest concentration that still permit glass formation[8,11].

Earlier attempts at ice-free cryopreservation of tissues focused on an "equilibrium approach" in which the tissue was exposed to increasing concentrations of CPA during cooling so that the system remained above the equilibrium freezing point [12,13]. In principle, freezing could be avoided completely, irrespective of cooling rate, provided the tissue was fully equilibrated with CPA at each stage. This approach, which was tested using smooth muscle cooled and kept unfrozen at –79°C, failed to provide adequate contractile function until adequate steps were also taken to optimize the ionic composition of the CPA medium[14,15]. Nevertheless, this equilibrium approach suffers from several problems. First, it requires lengthy periods of exposure to toxic solutes. Second, studies have shown that it may not be possible to achieve adequate exchange of tissue water with CPAs at subzero temperatures, either on a practical time scale or without exceeding the tolerable limits of solute toxicity in the tissue[13,15].

It is estimated that as many as 30% of patients who require arterial bypass will have no saphenous veins suitable for use in vascular reconstruction[16]. Tissue engineered, or allogeneic blood vessels provide alternative conduits for these patients, and the demand for these grafts is growing. Between 1985 and 1992, approximately 3,000 cryopreserved allogeneic vein segments were used for arterial bypass[17]. Nevertheless, the conventional cryopreservation methods used in these clinical procedures produced less than satisfactory results, demonstrating the need for better methods of cryopreservation[18,19]. In vitro studies employing 1 M [ref. 20] or 2 M [ref. 21] dimethyl sulfoxide (DMSO) yield less than 50% recovery of smooth muscle function. Cryoinjury was clearly associated with freezing and thawing because vascular smooth muscle can tolerate exposure to 3 M DMSO in the absence of freezing[22]. Available commercial techniques used clinically employ 1 M DMSO with slow cooling[20,23]. The present study was designed to evaluate a vitrification approach to storing a vascular tissue model (rabbit jugular vein) of human blood vessels or living-tissue-engineered vascular grafts.

Results and discussion

In vitro function of preserved vein rings. In vitro function of fresh control tissue from each rabbit, or preserved rings, was assessed using a physiological organ-bath technique[21,22]. Isometric contractile tensions were measured in response to a variety of agonists and antagonists by recording the changes in developed tension relative to baseline values. Drugs used in this study included histamine, bradykinin, angiotensin II, norepinephrine, sodium nitroprusside, and acetylcholine. Because baseline responses can vary between different freshly isolated veins, the experimental design included paired controls for each preserved vein by testing the contractile responses of fresh untreated sample rings from each jugular vein collected for the preservation studies.

The maximal contraction of fresh, frozen, and vitrified vein rings in response to the panel of agonists is shown in Figure 1. The maximum contractions achieved by the vitrified blood vessel rings in response to all four agonists were greater than 80% of fresh matched controls. The maximum contraction index for frozen rings was less than 30% of fresh matched controls. Analysis of variance confirmed that the maximum physiological response of frozen jugular vein segments to each of the agonists was significantly lower than fresh controls or vitrified samples (Table 1). The responses of vitrified samples were not significantly different from those of controls for three of the four agonists. Smooth muscle relaxation tests using sodium nitroprusside (endothelium-independent) showed that vitrified veins produced maximum relaxation of the precontraction. This response was similar to that of fresh control veins (Fig. 2A). In con-

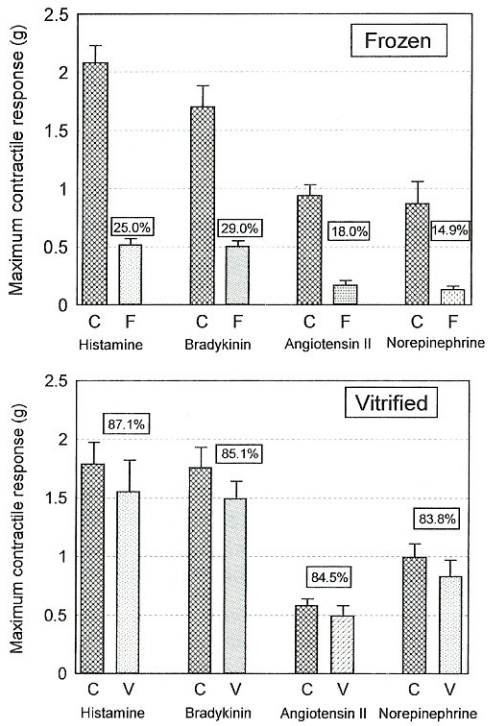

Figure 1. Contractile responses for frozen or vitrified vein rings in response to a panel of agonists. Mean responses for frozen (F), and vitrified (V) are shown with the corresponding matched control (C) values. Normalized responses as a percentage of the matched controls are indicated. For the experiments involving freezing, n = 12–16 for the controls, and n = 28–32 for the experimental groups. For the vitrification experiments, n = 11–15 for the controls, and n = 26 for the vitrified groups. Responses of frozen samples and their matched controls was statistically significant by student's t-test ($P < 0.001$), and there was no significant difference between the responses of vitrified samples compared with controls. ANOVA of these data (Table 1) leads to the same conclusion.

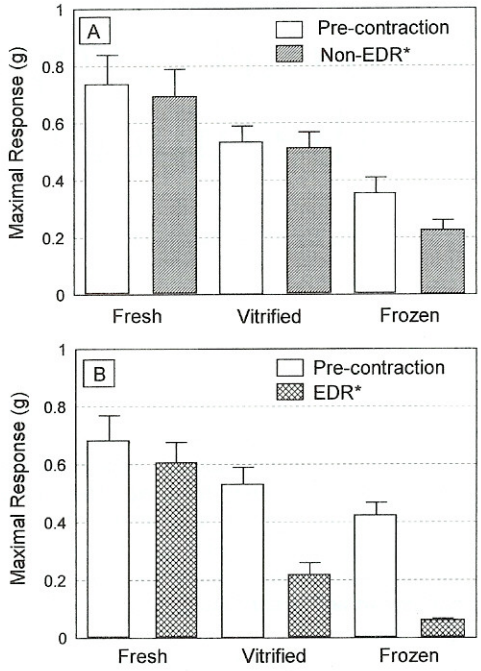

Figure 2. Maximal smooth muscle precontraction and relaxation in fresh, vitrified, and frozen veins. The maximal physiological responses to 10^{-5} M norepinephrine (precontraction), 10^{-4} M sodium nitroprusside (A, non-endothelium-dependent relaxation, Non-EDR) and 10^{-4} M acetylcholine (B, endothelium-dependent relaxation, EDR) are expressed in grams of force increased or decreased. Values are means ± s.e.m. Fresh (n = 23–35); vitrified (n = 50); frozen (n = 12–16).

trast, frozen, cryopreserved veins reached only 66% relaxation ($P < 0.01$). Endothelium-dependent smooth muscle relaxation (EDR) was tested using acetylcholine. Although the maximum response in vitrified samples was compromised when compared with fresh controls (41% vs. 80%), the mean response was superior to the frozen vessels, which achieved only 13% relaxation (Fig. 2B). Although this difference did not reach statistical significance, improved endothelial function in the vitrified group suggests that tissue integrity might be further improved by optimization of the vitrification procedure. Moreover, the dose/response curves (Fig. 3) showed that the vitrified vessels demonstrated similar drug sensitivities compared with untreated controls, whereas frozen vein rings exhibited decreases in drug sensitivity (illustrated for two agonists in Fig. 3). Comparison of agonist potencies in frozen, vitrified, and control tissues was performed for evaluation of tissue sensitivity. The isometric responses of the vessel rings were converted to percent maximal response and plotted against the negative logarithm of the agonist dose to produce dose/response curves (as shown in Fig. 3). The EC50 value, the concentration for the half-maximal response, for each agonist and each ring was calculated by logistic analysis[30] and is expressed as the pD2, which is defined as –log10[EC50]. The sensitivity data is summarized in Table 2. It can be seen that vitrified veins did not show significantly different sensitivities to any of the agonists compared with controls, whereas frozen veins were significantly less sensitive to histamine compared to matched controls and were significantly less sensitive to three of the agonists compared with the response of vitrified samples.

Table 1. Analysis of variance for maximum physiological responses of jugular veins[a]

	Control (g)	Frozen (g)	%	Vitrified (g)	%
Histamine	1.94 ± 0.12	0.52 ± 0.05[b]	27	1.55 ± 0.17	80
Bradykinin	1.72 ± 0.12	0.50 ± 0.05[b]	29	1.49 ± 0.15	87
Angiotensin II	0.76 ± 0.07	0.17 ± 0.04[c]	22	0.49 ± 0.09[d]	65
Norepinephrine	0.93 ± 0.11	0.13 ± 0.03[b]	14	0.83 ± 0.14	89

[a]Statistical p-values were calculated by one-way ANOVA (Kruskal–Wallis with Dunnett's Multiple Comparison Test). A p-value less than 0.05 was considered as significant. Data are expressed in grams of maximal tension generated. Values are means ± s.e.m. %, Percentage of fresh controls. Frozen, Vein rings cryopreserved with 1.0 M DMSO (n = 28–32).Vitrified, Vein rings vitrified with vitrification solution (n = 26). Control, Fresh vein rings. Control for cryopreserved vein rings (n = 12–16); control for vitrified vein rings (n = 11–15).
[b]$P < 0.001$ vs. fresh control or vitrified group.
[c]$P < 0.001$ vs. fresh control and $P < 0.05$ vs. vitrified group.
[d]$P < 0.05$ vs. fresh control.

Vitrification of vein segments for transplantation. We next evaluated a similar technique for vitrification of longer vein segments to

Table 2. Sensitivities to various contractile agonists[a]

	Control	Frozen	Vitrified
Histamine	6.22 ±0.07	5.78 ± 0.09[b,c]	6.36 ± 0.06
Bradykinin	7.62 ± 0.07	7.42 ± 0.12[d]	7.82 ± 0.11
Angiotensin II	6.82 ± 0.27	6.99 ± 0.29	7.06 ± 0.22
Norepinephrine	6.60 ± 0.19	6.05 ± 0.24[e]	6.84 ± 0.10

[a]Statistical p-values calculated by one-way ANOVA. A p-value less than 0.05 was considered significant. Control, Fresh vein rings (n = 31 for histamine and bradykinin, n = 23 for angiotensin II and norepinephrine). Frozen, Vein rings cryopreserved with 1.0 M DMSO (n = 32 for histamine and bradykinin, n = 28 for angiotensin II and norepinephrine). Vitrified, Vein rings vitrified with vitrification solution VS55 (n = 26).
[b]$P < 0.01$ compared with control group.
[c]$P < 0.001$ compared with vitrified group.
[d]$P < 0.05$ compared with vitrified group.
[e]$P < 0.01$ compared with vitrified group.

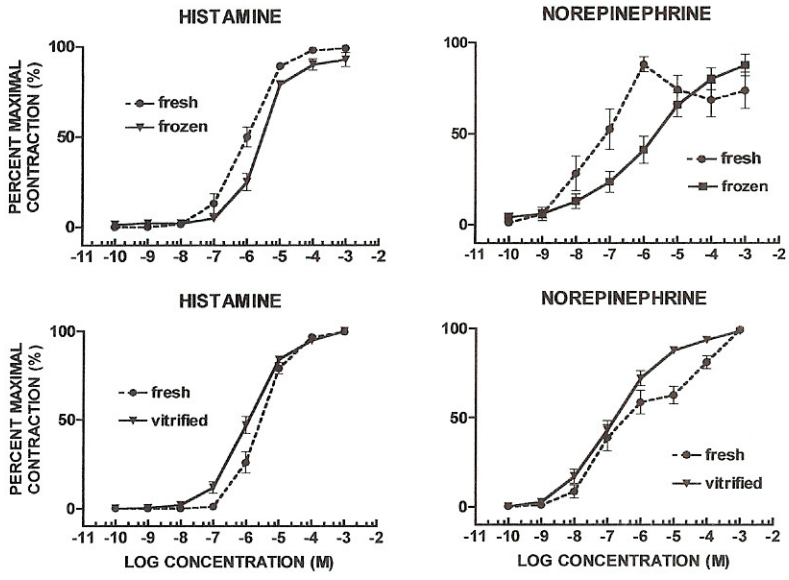

Figure 3. Contractile dose/response curves for fresh, frozen, and vitrified veins. Venous contractile function after conventional cryopreservation (upper panel) and vitrification (lower panel). The panels show the cumulative dose/response curves to histamine and norepinephrine in fresh, frozen, and vitrified external jugular veins. Values are the means (± s.e.m.) percentage of the maximal force generated. Fresh controls (n = 11–16); frozen veins (n = 26–32); vitrified veins (n = 26).

assess by both in vitro contractile function and in vivo implantation. After rewarming, the vitrified blood vessel segments were either cut into rings for in vitro contractility testing, or transplanted in rabbits for evaluation of in vivo viability. The rabbits were killed at two or four weeks postimplantation and the grafts examined for patency.

Contractility studies demonstrated that there was no significant difference in the maximum physiological responses of batches of vein rings prepared either as vitrified rings, or sectioned from vitrified vein segments. Moreover, the autologous vein implantation studies showed that the patency of fresh and vitrified rabbit jugular veins was similar after two or four weeks, with both groups exhibiting short-term patency rates of ~90%. To date, 10 fresh grafts have been collected with 9 vessels remaining patent, and a total of 12 vitrified grafts have yielded 11 patent vessels two to four weeks postoperatively. These data contrast with reports in the literature for frozen autologous grafts in which less than 67% of grafts remained patent after four weeks in the arterial circulation[24,25].

The feasibility of ice-free cryopreservation by vitrification has already been established for some biological systems, such as monocytes, ova, early embryos, and pancreatic islets[8,26]. Each of these mammalian systems is also able to be cryopreserved by conventional procedures involving freezing, but the successful preservation by vitrification of mammalian cells or tissues that cannot be cryopreserved adequately by classical methods has not previously been established to our knowledge. It is probable that in cell systems, vitrification provides equivalent—but not better—preservation because, unlike organized tissues, the ultimate viability of these systems is not greatly affected by extracellular ice formation during freezing and thawing. However, this study in a multicellular tissue demonstrates, for the first time, that vitrification can have a salutary effect on the cryopreservation of a system that sustains significant injury from freezing. In these experiments, the absence of ice during cooling and warming was verified by macroscopic visual inspection. This was regarded as sufficient in view of the extensive calorimetric evidence previously published by Mehl defining the conditions necessary to achieve vitrification in this medium during cooling, and to avoid devitrification during warming[28]. The technique used in this study safely exceeded these requirements. Microscopically, Figure 4 shows clearly the extent to which tissue architecture is distorted by ice during conventional cryopreservation involving freezing, and that this was avoided in the vitreous preservation protocol. Although the possible formation of microscopic ice nuclei cannot be unequivocally ruled out in the vitrified samples in these experiments, it is clear that in the vitreous protocol ice crystal formation was avoided, or at least the size and amount of ice was

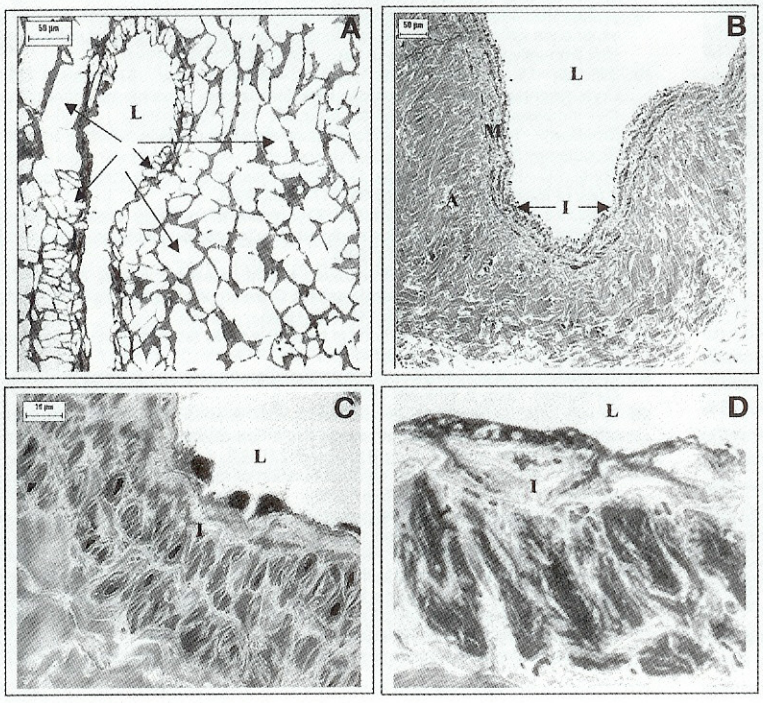

Figure 4. Micrographs of rabbit jugular vein segments cryosubstituted with methanol at –90°C from frozen (A), or vitrified (B–D) tissue. The structure of the frozen sample was noticeably distorted by the prevalence of variable sized ice crystal domains (arrows) scattered throughout the extracellular matrix of the vessel wall. The vitrified samples seemed to be free of ice, and the normal structural features of the tunica intima (I), including endothelial cells, the tunica media (M), and tunica adventitia (A) are clearly discernible at –90°C (B). Vitrified samples cryosubstituted at –90°C were also examined at higher magnification at both the light (150×) and electron (6,000×) microscopic levels. Micrographs C and D show that even at higher magnifications the samples seemed to be devoid of any apparent ice crystal domains. The endothelial cells present in the electron micrograph (D) includes some vacuolation, which can be observed in routine histology of fresh vascular endothelium and is not considered to represent ice. In each case the lumen (L) of the cryosubstituted vein rings is indicated. The method of cryosubstitution involved the transfer of frozen, or vitrified, samples to a precooled substitution medium comprising 1% osmium tetroxide in 100% methanol at –90°C. Replacement of any ice in these samples with the organic solvent was allowed to proceed isothermally for at least five days, whereupon the samples were rewarmed slowly and held at –20°C overnight before rewarming to 4°C for 1 h and then room temperature for final processing. The cryosubstituted samples were infiltrated with Araldite resin and polymerized, sectioned, and examined by light and electron microscopy.

limited to an innocuous amount sufficient to reap the practical benefits demonstrated here compared with conventional freezing. This study therefore demonstrates a strong correlation between the avoidance of freezing and improved tissue function that supports previous demonstrations of the deleterious effects of ice formation in multicellular tissues[2,4–7].

Experimental protocol

For conventional cryopreservation, the vein rings were processed according to Brockbank et al.[20,23]. The tissue was immersed for 20 min in HEPES-buffered Dulbecco's modified Eagle medium (DMEM) containing 1 M DMSO, 2.5% chondroitin sulfate, and 10% fetal calf serum at 4°C, and were then cooled at a controlled rate of 1.0°C min^{-1} to –80°C and transferred to liquid nitrogen for storage. Thawing was accomplished by immersing the containers in a waterbath at 37°C until all ice had disappeared, whereupon the containers were transferred sequentially to an icebath for elution of the CPA. Tissue samples were transferred to DMEM containing 0.5 M, 0.25 M, and finally 0 M mannitol as an osmotic buffer.

For vitrification of vein rings and segments, a method was developed in which a baseline vitrification medium (designated VS55 to reflect that it comprises 55% (w/v) total cryoprotective solutes, but previously designated as VS41A by its originators[27]) was used to replace at least 50% of the tissue water with a combination of CPAs. The VS55 vitrification solution consisted of 3.1 M DMSO, 3.1 M formamide, and 2.2 M 1,2-propanediol in EuroCollins solution[9,27], and the full-strength mixture was added and removed in a stepwise manner.

In the vitrification experiments with vein rings, the tissue was immersed in 1 ml vitrification solutions in glass vials (25 mm diameter × 60 mm height) at each step. Experiments with vein segments were carried out using a perfusion technique: The external jugular vein was perfused in situ to remove blood from the lumen. A 4–5 cm length of vein was cannulated in situ at its distal end and perfusion was performed for addition and removal of vitrification solution in these isolated veins. The perfusion system consisted of a reservoir (a 60 cc syringe) connected to the cannula with a three-way stopcock. The reservoir was adjusted to provide physiological pressure by adjusting its height to provide a hydrostatic pressure of 80–100 mm Hg at the cannula. The vein was placed in a Petri dish containing vitrification solution precooled to 4°C, and the Petri dish was placed on ice during the perfusion process.

Vitrification was achieved by cooling the samples rapidly (43 ± 2°C min^{-1}) to –100°C, followed by slow cooling (3 ± 0.2°C min^{-1}) to –135°C, whereupon they were transferred to a –135°C freezer for at least 24 h. Rewarming was accomplished in two stages: initially samples were warmed slowly to –100°C (30 ± 2°C min^{-1}) and then warmed rapidly (225 ± 15°C min^{-1}) to melting, whereupon the vitrification solution was eluted in a stepwise manner. The absence of ice crystallization during cooling and warming was verified by visual inspection. This was sufficient based upon Mehl's calorimetric measurements of the VS55 solution[27] in which he showed that the critical cooling rate to vitrify was >3°C min^{-1} and the critical warming rate to avoid devitrification was 40–50°C min^{-1} in bulk solution. We are confident therefore, that the samples vitrified during cooling at >40°C min^{-1} and did not devitrify during warming at >200°C min^{-1} in the tissue samples having the following dimensions: diameter = 4–6 mm; length = 5–50 mm; vessel wall thickness = 0.5 mm. Finally, all preserved samples were returned to physiological DMEM medium in preparation for viability testing, or transplantation.

In vitro function of fresh control tissue from each rabbit, or preserved rings was assessed using a physiological organ-bath technique[21,22]. Each vein ring segment was mounted between two stainless-steel wire hooks suspended in a custom organ bath (Radnoti, Monrovia, CA) containing 5 ml of Krebs'-Henseleit (KH) solution, which was gassed continuously with 95% O_2/5% CO_2 at 37°C. One hook was fixed to the base of the organ chamber and the other was connected to an isometric force transducer (Radnoti). Isometric contractile tensions were measured by adding a variety of agonists and antagonists to the tissue in the organ baths and recording the changes in developed tension relative to baseline values. In vivo viability was evaluated by carrying out vein graft implantation experiments in which recipient rabbits received either fresh, untreated control veins, or vitrified veins as reversed, autologous interposition grafts (end-to-side anastomoses) into the carotid artery[30]. The rabbits were killed two or four weeks postimplantation and the grafts examined for patency.

Statistical analysis. All data are expressed as mean ± s.e.m. and statistical differences between groups were tested using both matched paired Student's *t*-test and analysis of variance (ANOVA)[31].

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