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In Vivo Evaluation of the Effects of a New Ice-Free Cryopreservation Process on Autologous Vascular Grafts

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ABSTRACT Conventionally cryopreserved vascular grafts have performed poorly as arterial grafts. One possible mechanism that causes the poor function is the extracellular ice damage in tissue. We used a novel new ice-free cryopreservation (namely, vitrification) method for prevention of ice formation in cryopreserved venous grafts. This study was designed to evaluate the in vivo effects of the vitrification process on autologous vascular grafts using a short-term transplantation model and to examine the morphology and patency of vitrified grafts in correlation with control grafts. New Zealand White rabbits underwent a right common carotid interposition bypass graft. Fresh and vitrified reversed ipsilateral external jugular veins were used as autologous grafts. Animals were sacrificed at either 2 or 4 weeks after implantation, and fresh and vitrified vein grafts were harvested for histology studies. The results, comparing the patency of fresh and vitrified grafts, demonstrated similar short-term patency rates (~90%). There were no signs of media disruption, aneurysm, or graft stenosis in vitrified vein grafts. Vitrification had not altered the pathophysiological cascade of events that occur when a vein graft is inserted into the arterial system. The vitrification process had no adverse effects locally or systemically in vivo. In addition, vitrification has preserved endothelial cell and smooth muscle cell integrity posttransplantation. In conclusion, this study, using an autologous animal model, clearly demonstrated a significant benefit of vitrification for preservation of graft function, and vitrification may be an acceptable approach for preservation of blood vessels or engineered tissue constructs.

KEYWORDS autologous grafts, cryopreservation, vascular grafts, vein, vitrification

It has been estimated that as many as 20% of the patients who require arterial bypass procedures will have no saphenous veins suitable for use in vascular reconstruction, and in 10% of cases no autogenous vein from any source (i.e., lesser saphenous vein, brachial veins) is usable [1]. Many of these patients do not have autologous veins suitable for grafts due to the absence of long graft length, poor quality of distal runoff, or extensive prior lower extremity or coronary revascularization. Synthetic grafts such as polytetrafluoroethylene (e-PTFE) and human umbilical veins have disappointing patency rates [2]. The demand for cryopreserved allogeneic veins is growing despite the well-documented immune response to these grafts and the low clinical patency rates. Between 1985 and 1992, approximately 3000 cryopreserved allogeneic vein segments were used for arterial bypass [3]; however, the allograft veins cryopreserved using conventional cryopreservation methods produced less satisfactory results. Walker et al. reported that the cumulative survival rate was 14% and the cumulative secondary patency rate was 37% at 18 months [4]. The grafts demonstrated reduced endothelial cell functions and impaired smooth muscle contractility after cryopreservation in vitro and poor long-term patency rates in vivo [5–11]. One possible factor that may cause the poor vein graft functions is freezing damage associated with ice formation during cooling and warming. The rationale that we have adopted in our studies is that prevention of ice formation in blood vessels by an alternative cryopreservation approach, namely, vitrification, might optimize cell functions and minimize extracellular matrix damage resulting in more effective, durable grafts. Vitrification, an amorphous solidification of a supercooled liquid, can be achieved by adjusting the solute composition and the cooling rate such that formation of ice nuclei and growth of ice crystals is essentially prevented. Our approach attempts to develop methods for ice-free cryopreservation of small caliber blood vessels for clinical implantation. This study was to use a short-term autologous transplantation model to evaluate the in vivo effects of the vitrification process on small-diameter vascular grafts.

MATERIAL AND METHODS

New Zealand White rabbits (male, average weight 2.0 to 2.5 kg) underwent a right common carotid interposition bypass graft. The vitrified reversed ipsilateral external jugular veins were implanted into carotid artery, as autologous grafts. Fresh autologous vein grafts were used as controls. The rabbits were sacrificed at 2 or 4 weeks postimplantation, and the patency and morphology of the vitrified vein grafts were examined in correlation with control grafts. Animal care was carried out in compliance with the "Guide for the Care and Use of Laboratory Animals" issued by the National Institutes of Health (U.S. Department of Health and Human Services, revised 1996).

Procurement and Preparation of Vein Segments

To prevent endothelial cell damage, a "no-touch" technique was applied to isolate the external jugular vein. After the small branches of the jugular vein had been ligated, a double suture was made around the outside of the maxillary vein just above the bifurcation, and both maxillary veins and linguo-facial veins were ligated. The external jugular vein was cannulated with a silicone tube (OD 1.96 mm, ID 1.47 mm), and the cannula was tied firmly to prevent detachment from the vein during the procedure. The external jugular vein was then perfused with HEPES-buffered Dulbecco's modified Eagle's medium (DMEM). Perfusion was at physiologic pressure. The reservoirs were adjusted to give a hydrostatic pressure of 80–100 mm Hg at the cannula [12]. After blood was cleared from the lumen, the external jugular vein was ligated at the proximal end and freed from surrounding tissues. The vein was stored in HEPES-buffered DMEM and transported to the laboratory on ice. The warm ischemia time was less than 1 min and cold ischemia time was 60 to 120 min.

Surgical Procedures

Anesthesia was induced with an injection of a mixture of ketamine hydrochloride (60 mg/kg) and

xylazine (6 mg/kg), and maintained using isoflurane delivered in oxygen. A single-dose antibiotic prophylaxis in the form of enrofloxacin at 5 mg/kg was given intramuscularly at the time of induction. The operation was performed with an operating microscope (Zeiss Opmi 6S, Carl Zeiss, Inc., Germany) under sterile conditions. After exposure through a right longitudinal neck incision, the right external jugular vein was identified, its branches were cauterized, and the vessel was then removed, and fresh veins were immediately implanted into the carotid artery. Vitrified veins were rewarmed in the laboratory and transported to the operating room in DMEM on ice. At the time of implantation of both fresh and vitrified vessels, the right common carotid artery was identified and dissected. Heparin (200 IU/kg) was administered intravenously. A proximal longitudinal arteriotomy was made, and one end of the reversed jugular vein was anastomosed to the artery end-to-side with continuous 8-0 microvascular nylon suture. The distal anastomosis was performed similarly. Throughout the procedure, care was taken to avoid unnecessary manipulation of the vein graft. The right common carotid artery between the two anastomoses was ligated with 4-0 silk ligatures. Hemostasis was achieved, and the wound was subsequently closed in layers. Upon recovery, analgesic (buprenorphine, 0.05 mg/kg, sc) was provided as necessary. Animals were observed daily for signs of infection, illness, injury, or abnormal behavior. Sick or injured animals were referred immediately for veterinary care or euthanized.

Vitrification

The vitrification solution employed in this study consists of three components, 2.2 M (16.8% w/v) propylene glycol, 3.1 M (14.0% w/v) formamide, and 3.1 M (24.2% w/v) dimethyl sulfoxide (DMSO) [13]. The total concentration of these glass-inducing solutes is 55% (w/v), hence the acronym VS55. A vitrification method was developed for the rabbit external jugular vein segments (40–50 mm long, internal diameter 3–5 mm). Perfusion was performed for addition and removal of vitrification solution in the isolated vein segments (Figure 1). Vitrification solution was added in 6 steps for 15 min each. After the addition of the vitrification solution, the vein segment together with the silicone tube, was placed in a glass scintillation vial (diameter × height, 25 × 60 mm) containing 1 ml of the same precooled (4°C) vitrification solution. The top of the vitrification solution was covered at 4°C with 0.7 ml of 2-methylbutane (isopentane, freezing point −160°C, density 0.62) to prevent direct contact with air. Samples were cooled rapidly (43 ± 2°C/min) to −100°C followed by slow cooling (3 ± 0.2°C/min) to −135°C and finally stored at −135°C freezer for at least 24 h. Each vitrified vein was rewarmed in two stages, slow warming to −100°C (30 ± 2°C/min) and rapid warming to about −70°C (225 ± 15°C/min). After the vein was rewarmed, the vitrification solution was removed by perfusion in a stepwise manner.

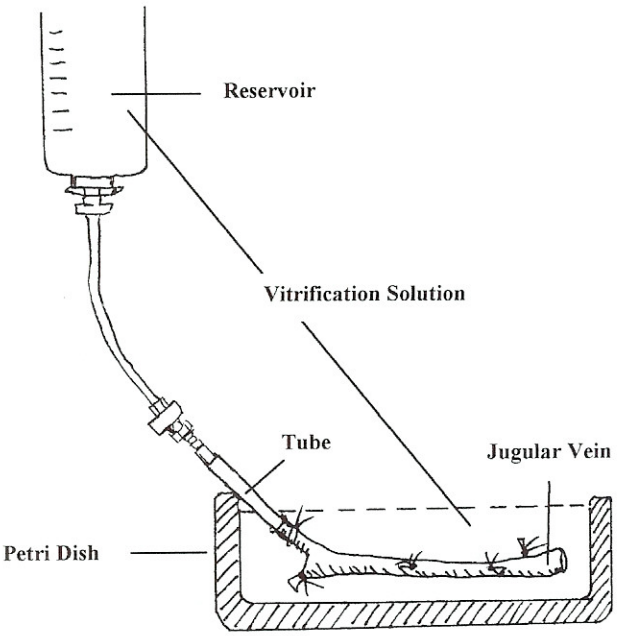

FIGURE 1 Perfusion apparatus for addition and removal of vitrification solution in vein segments. The perfusion system consisted of a reservoir (a 60-ml syringe) connected to the cannula with 2-way stopcock. The reservoir was adjusted to physiologic pressure. Each vein was placed in a petri dish (diameter × height, 50 × 15 mm) containing vitrification solution. The vitrification solution in both reservoir and petri dish was precooled (4°C), and the petri dish was placed in ice during the perfusion process.

Conventional Cryopreservation

For conventional cryopreservation involving freezing, the vein rings were processed following a commercial procedure [14, 15]. Briefly, the tissue was initially immersed for 20 min in DMEM containing 1 M DMSO, 2.5% chondroitin sulfate, and 10% fetal calf serum at 4°C. Samples were then cooled at a controlled rate of 1.0°C/min to −80°C and finally transferred to liquid nitrogen for storage. Thawing was accomplished by immersing the containers in a water bath controlled at 37°C until all ice had visibly disappeared, whereupon the containers were transferred to an ice bath for elution of the DMSO. This was achieved in sequential steps in which the tissue samples were transferred to DMEM containing 0.5 M, 0.25 M, and finally 0 M mannitol as an osmotic buffer.

Cryosubstitution

Cryosubstitution is a technique in which tissue is processed at subzero temperatures using organic solvents to dissolve ice. In this way the profile of any ice domains can be revealed in the frozen or vitrified samples. The conventionally cryopreserved and vitrified veins were stored in precooled cryosubstitution media consisting of 1% osmium tetroxide in 100% methanol at −90°C. The frozen water in these tissues was substituted by replacement with cryosubstitution medium over a period of several days. The samples were then placed in a −20°C freezer overnight followed by 4°C for 1 h and finally to room temperature. This gradual warming of the tissue and cryosubstitution media assured complete fixation of the tissue. Finally these tissues were transferred to 100% acetone and infiltrated with Araldite resin and polymerized, sectioned, and viewed by light microscopy.

Histology

At the time of graft harvest, the vein grafts were perfusion fixed in situ. Grafts were perfused with an initial infusion of DMEM followed by 2% glutaraldehyde made up in 0.1 M cacodylate buffer supplemented with 0.1 M sucrose to give an osmolality of approximately 300 mOsm. Subsequently, the specimens were further immersion fixed with glutaraldehyde for 24–48 h and divided into a proximal, middle, and distal segments. Cross sections from the central region and longitudinal sections from proximal and distal anastomosis regions were processed for either light or electron microscopy. Histology sections were stained with a Mikat elastin stain and a standard hematoxylin and eosin (H&E) stain. Electron microscopy was performed on selected

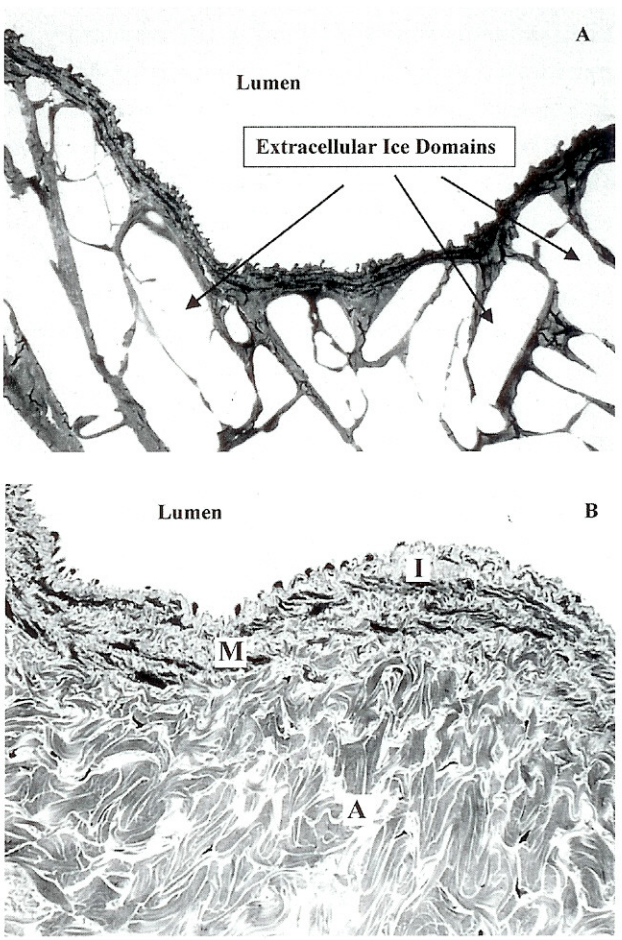

FIGURE 2 Morphology of frozen and vitrified veins cryosubstituted at −90°C. Low-power micrographs of rabbit jugular vein segments cryosubstituted with methanol at −90°C reveal ice domains within the frozen (A) or vitrified (B) tissue. The structure of the frozen sample was noticeably distorted by the prevalence of variable-sized ice crystal domains scattered throughout the extracellular matrix of the vessel wall. In marked contrast, the vitrified sample appeared to be free of ice, and the normal structural features of the tunica intima (I), the tunica media (M), and tunica adventitia (A) are clearly discernible at −90°C. In each case the lumen of the cryosubstituted vein rings is indicated. Toluidine blue staining, ×40.

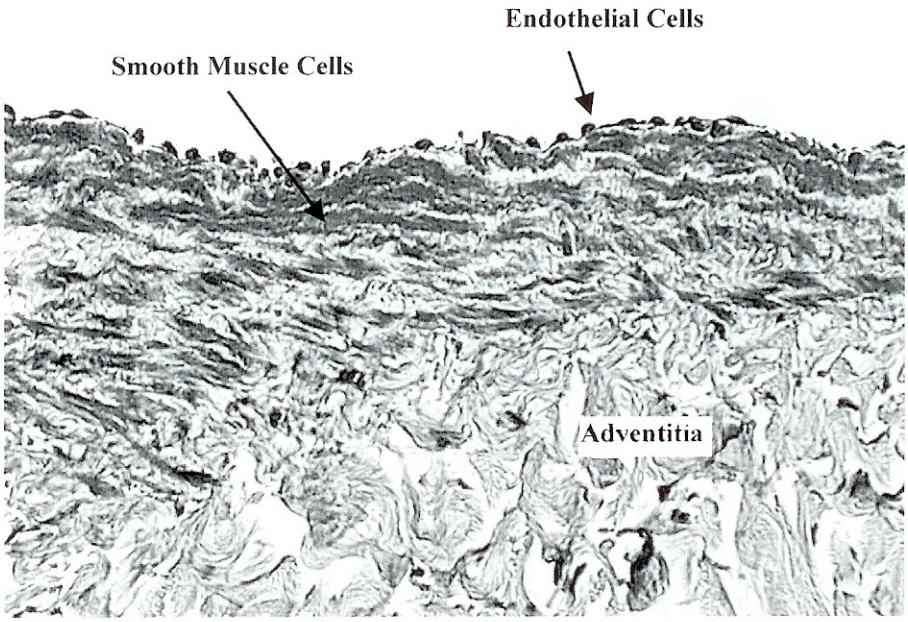

FIGURE 3 Morphology of vein segment after vitrification. Histological section of a vitrified rabbit jugular vein shows intact morphological features, including endothelial cells, smooth muscle, and the connective tissue. Mikat staining, ×40.

specimens to further characterize posttransplantation tissue changes. Veins were processed for electron microscopy by placing the tissue in a 2% cacodylate buffered glutaraldehyde solution for approximately 2 h. The samples were then rinsed in phosphate buffered saline and subsequently fixed in buffered 1% osmium tetroxide for 1 h. Dehydration was achieved using a graded series of acetone followed by infiltration of Araldite 502 resin and polymerization at 60°C for 18 h. Electron microscopic studies were performed on 80-nm-thick sections, which had been stained with lead citrate and uranyl acetate, using a Hitachi H-7000 transmission electron microscope.

Statistical Analysis

Differences in graft patency rates between the fresh and vitrified groups were analyzed for statistical significance by Fisher's exact test. A p value less than .05 was considered significant.

RESULTS

During rewarming of vitrified samples, a transparent glassy vitrification solution can be visualized at the early stage of warming. Cryosubstitution confirmed there is no ice formation in vitrified veins (Figure 2). However, veins cryopreserved by the traditional methods demonstrate high levels of ice cavities in the extracellular matrix. The morphology studies demonstrated that the structural integrity of vein segments was preserved following vitrification and before transplantation (Figure 3).

Graft Patency

The autologous vein implantation studies showed that the patency of fresh and vitrified rabbit jugular veins were not significantly different after two and four weeks ($p > .99$), with both groups exhibiting short-term patency rates of ~90% (Table 1). To date, 12 vitrified grafts have been harvested and 11 grafts were patent, and 13 fresh grafts have been harvested and 11 grafts were patent 2 weeks postoperatively. One rabbit was sacrificed at day 16 due to complications unrelated to grafting; however, the fresh

TABLE 1 Patency of fresh and vitrified autologous grafts

Type of graft	*2 weeks*	*4 weeks*
Fresh	11/13	8/9
Vitrified	12/13	10/11

Note. Number of grafts found patent at harvest is displayed over numbers of grafts at risk in each harvest interval.

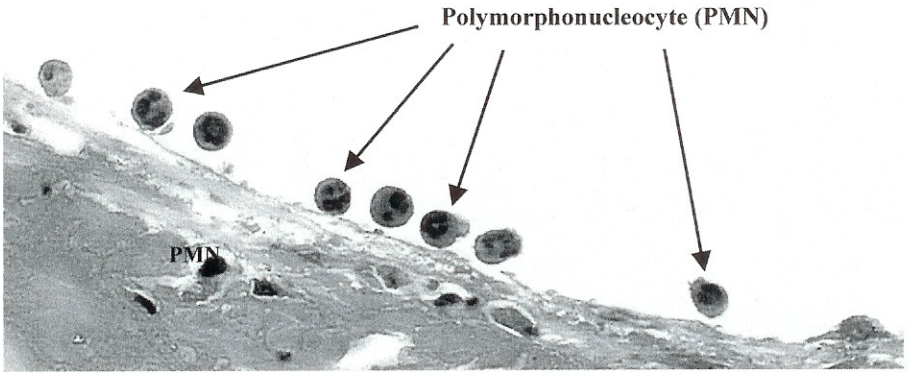

FIGURE 4 Light-microscopic micrograph of a vitrified vein graft after 2 days in the arterial circulation. Numerous polymorphonucleocytes (PMNs) are deposited on the intimal surface of a vitrified graft. Few PMNs are seen in the subendothelial layer and media. H&E staining, ×100.

graft was patent. At 4 weeks postimplantation, 10 of 11 vitrified grafts were patent. The failed graft was found at the time of harvest due to a surgical error made in the proximal anastomosis that blocked blood flow. Eight of 9 fresh grafts remained patent 4 weeks postoperatively. Infection was not observed in any of the grafts. Graft rupture, aneurysm, thrombosis, or inflammatory infiltration was not noted in

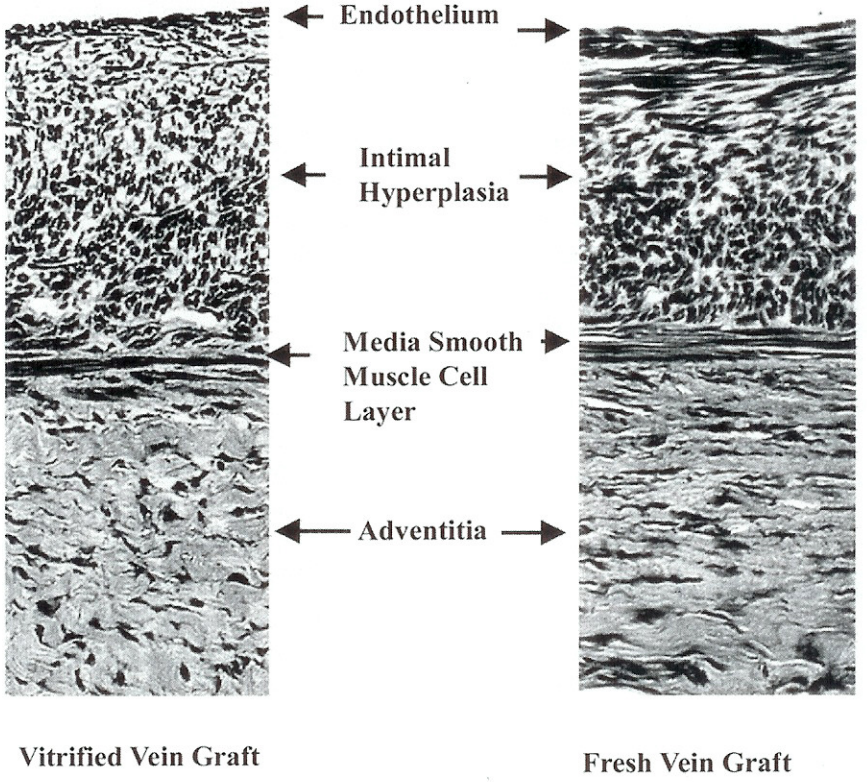

FIGURE 5 Composite micrograph of fresh and vitrified vein grafts after 4 weeks in the arterial circulation, as representative cross-section from the wall of grafts. Due to the lack of a well-defined internal elastic lamina in veins, the separation between intimal and medial layers was delineated by identification of the demarcation between the crisscross orientation of the intimal hyperplastic smooth muscle cells and circular smooth muscle cells in the media. The outer limit of the media was defined by the interface between the circular smooth muscle cells of the media and the connective tissue of the adventitia. There is little well-defined collagen in the intimal hyperplasia in both grafts. Collagen is, however, prominent in the media and in the adventitia of vitrified veins. Mikat staining, ×20.

any of the patent grafts. Five rabbits were sacrificed before 2 weeks due to severe edema within the region of the incision site, severe pulmonary edema, or lung infection. All these grafts were patent at the time of harvest.

Graft Histology

Vitrified grafts removed at 1 to 2 days postoperatively due to non-graft-related animal death showed extensive polymorphonucleocyte (PMN) adhesion on the intimal surface and some cells penetrated into the subendothelial layer (Figure 4). At 2 weeks, both the control and vitrified grafts had similar macroscopic appearances at harvest. The walls of fresh vein grafts appeared grossly thicker than those of the vitrified grafts. The smooth muscle cells in the intima were arranged in a random pattern of orientation with connective tissue found between the cells. The media of the grafts from both groups were also similar in appearance. However, the fresh vein grafts had a greater degree of intimal hyperplasia than the vitrified vein grafts. At 4 weeks, both the control and vitrified grafts had similar gross morphological appearances. The fresh and vitrified vein grafts had similar degrees of intimal hyperplasia and medial thickening (Figure 5). The morphological features of vessel wall in vitrified grafts are shown in Figure 6. The endothelium was clearly visible on top of the intimal layer. Smooth-muscle-cell morphology was well maintained after vitrification and tranplantation. The media demonstrated morphological features of hypertrophied smooth muscle cells. These cells exhibited a synthetic appearance when implanted into the arterial circulation. These smooth muscle cells were richly endowed with ribosomes as seen at the electron microscope level. Occasionally, two prominent nucleoli can be seen in these cells

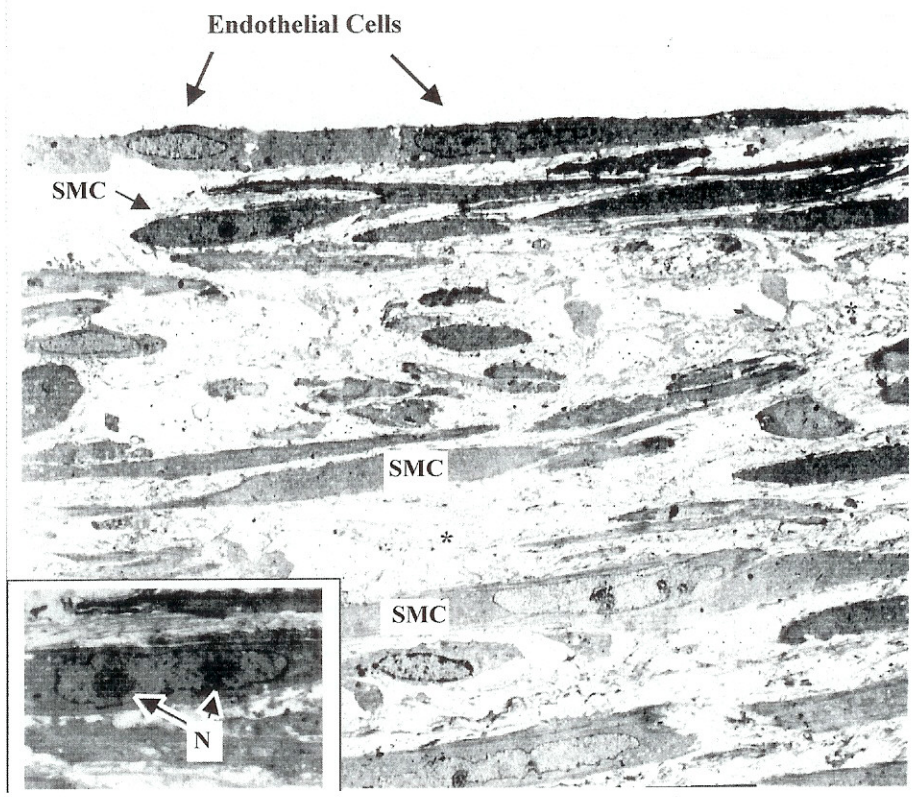

FIGURE 6 Transmission electron micrograph of a vitrified graft after 4 weeks in the arterial circulation shows a well-defined monolayer of endothelial cells. Beneath the endothelium, vitrified vein graft is composed of multiple layers of mostly obliquely sectioned smooth muscle cells embedded in a matrix of poorly developed connective tissue. Voluminous hypertrophic smooth muscle cells (SMC) can be identified. The well-organized connective tissue has been displaced by disorganized abundent collagen fibers. These fibers (asterisk) surrounded by smooth muscle cells have not yet organized into bundles. Inset: The nucleus of one of the cells in the latter stage of cell cycle displays two prominent nucleoli (N), Magnification for entire photo is ×3000.

indicating ongoing mitotic activities. Intimal hyperplastic lesions appeared pale, smooth, firm, and homogeneous; they are uniformly located between the endothelium and the media smooth muscle cell layer.

DISCUSSION

There is a growing clinical need for preservation of blood vessels for use as vascular grafts, but current techniques of cryopreservation do not produce optimum preservation. The major obstacle for functional recovery of complex multicellular structures following freezing to low temperature is the avoidance of extracellular matrix damage and retention of the normal relationships between cells and the extracellular matrix. We previously evaluated venous contractile function in vitro using the tissue organ bath technique [16, 17]. The study tested the maximal contraction of fresh, cryopreserved and vitrified vein rings in response to histamine, bradykinin, angiotensin II, and norepinephrine. A comparison of the effects of vitrification and conventional cryopreservation upon contractility showed that the maximum contractions achieved by the vitrified blood vessel rings in response to all four agonists were greater than 80% of fresh matched controls. In contrast, the maximum contractions achieved by frozen rings were less than 30% of fresh matched controls. The study demonstrated that prevention of extracellular ice formation can dramatically improve tissue function [16, 17]. Early studies on cryopreservation of vascular grafts have found that the smooth muscle function was reduced to less than 50% compared to fresh controls at the time of transplantation [12, 14, 15]. These studies employed a lower concentration of cryoprotectant (1.0 to 2.0 M DMSO) and slow cooling rates (0.5 to 1°C/min). The damage occurred during freezing and thawing, since we know that vascular smooth muscle can tolerate exposure to up to 3.0 M DMSO [18]. The reduction of smooth-muscle contractile function with respect to control after slow cooling may be caused by extracellular ice formation, since intracellular ice was most likely avoided at much lower cooling rates [19]. There are at least two known mechanisms in which extracellular ice formation causes injury in tissue. First, mechanical disruption by ice damages tissue structure and impairs the functions of grafts. Second, the solute concentration increases progressively as the water is removed due to freezing. As a result, cell membranes are damaged either by a critical reduction in size or by the high salt concentration. Therefore it would be desirable to develop techniques in which ice does not form. Ice-free cryopreservation by vitrification has previously been applied in cell systems, which resulted in equivalent but not better preservation compared with traditional cryopreservation (with ice formation) [20]. Unlike organized tissues, the ultimate viability of these cell systems is not greatly affected by extracellular ice formation [21]. Our previous study in a multicellular tissue demonstrates, for the first time, that vitrification is superior to conventional cryopreservation methods in preservation of rabbit jugular veins in vitro [16, 17].

In this study, we used a short-term surgical model to evaluate the in vivo effects of vitrification process on autologous vascular grafts. We demonstrated that the vitrification process had no discernible adverse effects in vivo. Examination of the morphology of vitrified vein grafts showed considerable early PMN adherence to the initimal surface, which concurs with findings in fresh autologous vein grafts [22]. Leukocyte adherence and infiltration appear to have occurred despite the initial morphologic integrity of the endothelium. PMNs can cause endothelial cell injury and detachment from the vessel wall. Within the vessel wall, PMNs can interact with the smooth muscle cells and release mitogenic factors that may contribute to the initimal hyperplastic response [23]. During this initial period, the endothelial lining appeared thinner, and was intact in the vitrified vein grafts. These morphological changes were maximal within the first 3 days after grafting [22]. There was a confluent layer of endothelial cells in grafts after this stage. At 2 to 4 weeks, a continuous layer of endothelial cells covered the intimal surface in both control and vitrified vein graft. At this time, the endothelial lining of the vitrified grafts morphologically appeared more prominent and had distinct cell borders. In contrast, the intact endothelial cells have been shown to be detached by subsequent arterial flow in conventionally cryopreserved autologous rabbit veins [24]. Similar results have been reported using cryopreserved autologous veins

in canine models [25, 26]. In these studies, the cryopreserved autologous vein grafts showed poor long-term patency and the patency rates were less than 67% 4 weeks postoperatively [24, 27]. There was universal sloughing of the endothelial layer in these grafts. Unlike allografts, extensive endothelial denudation usually does not occur in autografts [28]. The damage to endothelial cells and detachment of the endothelial monolayer may be associated with the injury caused by ice formation during the conventional cryopreservation process.

Preservation of smooth-muscle integrity is also important in cryopreservation of vein grafts. If the smooth muscle cells are damaged during freezing and thawing, late aneurysmal formation may occur, which is one of the potential causes of cryopreserved vein graft failure [26]. This study demonstrated that smooth-muscle-cell morphology and function in vitrified grafts were well maintained at the time of transplantation. During the initial 2-week grafting period, the tunica media demonstrated morphological features of hypertrophied smooth muscle cells in these vein grafts. This study also showed that the intimal hyperplastic process was delayed in vitrified vein grafts. The cause of this delay is not clear and requires further study. However, the hyperplastic process returned to normal at 4 weeks after grafting. Intimal hyperplasia is the universal response of a vein graft inserted into the arterial circulation. In general, it is a self-limiting process and usually becomes quiescent within 2 years of graft insertion [28]. It has been demonstrated that after reimplantation of grafts removed from the arterial circulation back to the venous circulation, there was a significant regression of both intimal and medial thickening in the reimplanted graft [29]. The precise initiating stimuli for intimal hyperplasia have not been fully defined, but it appears to be the response of the vascular smooth muscle cells to a combination of physical, cellular, and humoral factors accompanied by dysfunctional endothelial regulation [30, 31]. The cells appearing in the intima are considered to result from both the migration of smooth muscle cells out of the media into the intima and the proliferation of the smooth muscle cells in the intima. During this process the phenotype of smooth muscle cells has changed from contractile to synthetic [28]. The formation of intimal hyperplasia indicates the smooth muscle cells are able to proliferate and migrate; in other words, they are viable. The result that the degree of intimal hyperplasia appears comparable in control and vitrified vein grafts after 4 weeks further indicates that the process of vitrification preserved smooth muscle cell integrity.

We used a surgical model to evaluate the effects of the vitrification process on small-diameter vein grafts. In vivo autologous transplantation studies comparing the patency of fresh and vitrified rabbit jugular veins transplanted into the carotid artery demonstrated similar short-term (2–4 week) patency rates (~90%). There were no signs of media disruption, aneurysm, or graft stenosis in either fresh or vitrified vein grafts. Histological studies showed that endothelial integrity was preserved in vitrified vein grafts. Vitrification has preserved vein graft viability, which was manifest by smooth-muscle-cell proliferation in the arterial circulation. In conclusion, this study demonstrated that the autologous transplantation model was successful in evaluating vitrification as an effective cryopreservation method for preservation of blood vessels or tissue engineered constructs. Further use of this model or a large-animal model is necessary for evaluation of the intermediate and long-term function of vitrified vascular grafts.

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