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G. Pasquinelli
University of Bologna

G. L. Faggioli
State University of New York

P. Preda
University of Bologna

M. Vici
University of Bologna

G. N. Martinelli
University of Bologna

See next page for additional authors

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Are Long Term Cryopreservation and Patency of Vein Allograft Truly Achievable?

Authors

G. Pasquinelli, G. L. Faggioli, P. Preda, M. Vici, G. N. Martinelli, R. Giardino, M. D'Addato, and J. J. Ricotta

ARE LONG TERM CRYOPRESERVATION AND PATENCY OF VEIN ALLOGRAFT TRULY ACHIEVABLE?

G. Pasquinelli*, G.L. Faggioli³, P. Preda, M. Vici, G.N. Martinelli, R. Giardino¹, M. D'Addato², J.J. Ricotta³

Inst. of Clinical Electron Microscopy, ¹Inst. of Experimental Surgery, ²Dept. of Vascular Surgery, University of Bologna, Bologna, Italy; and

³Division of Vascular Surgery, Dept. of Surgery, State University of New York at Buffalo, NY, USA

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Abstract

Despite extensive experimental work, neither the effect of long term cryopreservation on vein graft architecture nor the failure of alloveins due to graft rejection have yet been investigated. Herein, we investigated ultrastructurally: **a)** the integrity of rabbit jugular veins following 1, 2 and 3 months of cryopreservation; **b)** the outcome of the three-month cryopreserved vein auto- and allografts after 1 month of implantation in the rabbit carotid artery; and **c)** the immunologic response to cryopreserved vein allografts with and without seeded autologous endothelium. Prior to implantation, the cryopreserved rabbit veins were well-maintained except for endothelial cell damage. Following implantation, the cryopreserved vein autografts were comparable to fresh veins with a complete endothelial lining. Conversely, only one of the allograft was still patent with features of acute rejection. After seeding with autologous endothelium, these explants failed shortly after surgery. We found absence of endothelium and necrosis of the media components with neutrophil infiltration. Although three months of cryopreservation does not affect vein graft architecture significantly, endothelial cells are damaged irrespective of the time of cryopreservation. Vein autografts promptly healed after one month of implantation at which time a viable endothelial cell lining was restored from the host artery. Conversely, vein allografts, with and without seeded autologous endothelium, failed due to graft rejection. This study highlights that current methods of cryopreservation do not reduce antigenicity of venous allografts significantly.

Key Words: Vein allograft, vein autograft, graft rejection, vein cryopreservation, scanning electron microscopy, transmission electron microscopy.

*Address for correspondence:

G. Pasquinelli,
Inst. of Clinical Electron Microscopy
Ospedale S. Orsola, via Massarenti 9,
40138, Bologna, ITALY

Phone No.: 0039-51-302874
FAX No.: 0039-51-306861

Introduction

As compared with earlier days (Kunlin, 1949), the improvements in cryo-techniques have resulted in significant tissue preservation following freezing and thawing of human saphenous vein allografts (Ligush *et al.*, 1991). Preliminary clinical trials (Fujitani *et al.*, 1992; Harris *et al.*, 1993; Shah *et al.*, 1993; Walker *et al.*, 1993) have indicated the possibility of successfully using the cryopreserved venous allografts for lower extremity arterial reconstructions with the infected field and the unavailability of fresh autologous tissue being the major targets for their use. Consequently, tissue banking of venous allografts is turning into a major task for people involved in current surgical practice. Despite extensive experimental work in this field (for a review see, Faggioli *et al.*, 1990; Greisler, 1991), no result is yet available on the possibility of cryopreserving vein grafts efficiently for longer than 1 month.

On the other hand, animal studies (Street *et al.*, 1988; Showalter *et al.*, 1989) suggest that a short time of cryopreservation does not drastically affect the integrity of endothelial vein graft coverage. Functional results are contradictory. Findings suggesting a subtle endothelial cell and smooth muscle cell dysfunction, i.e., decreased prostacyclin synthesis (Showalter *et al.*, 1989) and lack of response to serotonin (Brockbank *et al.*, 1990), are at variance with those of a recent study which documents biochemical and metabolic characteristics of human venous allografts to be similar to those of fresh veins (Ligush, *et al.*, 1991). Finally, the pathological evaluation of explanted failed allografts have indicated the importance of residual vein antigenicity and graft atherosclerosis as additional problems (Greisler, 1991). In the following experiment, we investigated morphologically: **a)** the effect of long term cryopreservation (up to three months) on rabbit venous graft architecture and endothelial cell residual integrity; **b)** the outcome of venous auto- and allografts following 1 month of implantation into rabbit carotid arteries; **c)** the potential host immunologic response to venous allograft implantation.

Materials and Methods

Vein harvest and cryopreservation

A total of 12 New Zealand white rabbits were used to harvest fresh veins. The animals were anesthetized by intravenous sodium pentobarbital injection (30 mg/kg) and maintained under halothane and oxygen by automatic ventilation. Jugular veins were excised with no-touch dissection and gently flushed with warm (37°C) Medium 199 (Gibco, Grand Island, NY, USA). Veins were then ligated at both extremities, cannulated and gently distended with freezing medium containing NCTC 135 (Gibco), 20% fetal bovine serum (FBS; Gibco), 17.5% dimethylsulfoxide (DMSO; Gibco) and 1% non-essential aminoacids (NEAA; Gibco). Each of the distended veins was placed into a cryogenic vial and frozen to -196°C at the controlled-rate of -5°C/sec. All steps were performed under sterile conditions. The specimens were then kept frozen at -196°C for 1, 2 and 3 months, respectively.

At the end of the designated period, thawing was accomplished by fast rewarming of the cryogenic vial to 37°C. After gentle flushing with Medium 199, the veins were processed for ultrastructural investigation.

Venous autograft and allograft implants

After 3 months of cryopreservation, both vein autografts ($n = 3$) and allografts ($n = 3$) were implanted in a group of six animals. The vein grafts were sutured with two end to side anastomoses in the rabbit carotid artery using 9-0 nylon sutures under microscopical control. The carotid artery between the two anastomoses was ligated at the end of procedure but not cut. Four weeks after surgery, each animal was anesthetized, the graft dissected free and the rabbit euthanized. The animal care followed the "Principles of Laboratory Animal Care" and the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No 80-23, revised 1985).

Controls were performed by implanting non-cryopreserved autologous veins for four weeks.

Seeding with autologous endothelium

Twelve animals were used to perform the following experiment. To enzymatically harvest rabbit vein endothelial cells (RVECs), freshly excised jugular veins ($n = 6$) were incubated twice (20 minutes each step) with a 0.2% collagenase dispase (Boehringer Mannheim Co., Indianapolis, IN, USA) solution. Following incubation, the solution containing the RVECs was centrifuged at 300 g for 10 minutes at 4°C, the supernatant discharged and the pellet suspended in 4 ml of endothelial cell complete medium (ECCM) [Medium 199 containing 20% FBS, 100 IU/ml heparin (Organon, Inc.), 10 µg/ml endothelial cell derived growth factor (Boehringer Mannheim Co.), 1% vitamins (Gibco), 1% NEAA, 1% sodium pyruvate (Gibco), 0.1% PSN (penicillin, streptomycin, neomycin; Gibco), and 0.05% gentamicin (Gibco)]. The RVECs were then plated into T25 flask and incubated at 37°C with 5% CO₂. After 24 hours,

the cells were washed three times with PBS and refed with fresh ECCM. The medium was changed three times a week. When 90% confluent, the cultures were trypsinized and replated at a 1:6 ratio. Cells at the first or second passage were used to seed the vein grafts. RVECs identity was confirmed by the typical cobblestone appearance, with positive immunofluorescent staining for Factor VIII RA and negative actin staining.

Six animals were used as vein allograft donors. Following excision, the jugular veins were de-endothelialized with a collagenase dispase solution and cryopreserved for three months. At the time of the seeding experiment, the vein allografts were thawed, vigorously and repeatedly rinsed with warm Medium 199 and seeded with approximately 2×10^6 autologous endothelial cells per cm² surface area. Each vein was then placed horizontally into a sterile tube with warm media, and incubated at 37°C with continuous rotation (1 rpm). After 2 hours, the veins were removed from the sterile tube and implanted into the carotid artery of the rabbit which was previously used as source of endothelium. As a result, six rabbits in this group received a venous allograft seeded with autologous endothelial cells. Explants were performed between 4 days and 2 weeks.

Controls were included to verify that the autologous RVECs effectively adhered and spread onto the venous allografts.

Transmission and scanning electron microscopy

Representative samples from each experimental step were processed for transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Each of the explanted venous grafts was cannulated, perfused under physiologic pressure (100 mm Hg) with warmed (37°C) Krebs-Henzleit saline solution (Sigma, St. Louis, MO, USA) and fixed with cacodylate-buffered glutaraldehyde *in-situ*. Subsequently, the specimens were fixed with glutaraldehyde for 2 hours, opened along their ventral surface, and cut into serial strips. After 1 hour of 1% osmium tetroxide post-fixation, the samples were repeatedly washed in distilled water and dehydrated in a series of graded alcohols. Samples for TEM were embedded in epoxy resin. Thin sections were counterstained with uranyl acetate and lead citrate and observed in a Philips 400T transmission electron microscope. Samples for SEM were critical point dried, sputter-coated with 10 nm of gold and viewed in a Philips 505 scanning electron microscope.

Results

Ultrastructure of cryopreserved vein grafts

Following 1, 2 and 3 months of cryopreservation, no significant ultrastructural difference was found among rabbit vein grafts.

By SEM, the vein graft surface was corrugated and partially covered by endothelial cells (Fig. 1). Foci of endothelial cell shedding associated with surface blebbing and loss of intercellular contacts were seen.

By TEM, most of the endothelial cells showed pyknotic nuclei and cytoplasmic vacuoles (Fig. 2). The basal lamina was present. The elastic lamina showed a slight dissociation of elastotubules from the elastic moieties. Smooth muscle cells were generally unremarkable except for variable lysis of myofilaments and cytoplasmic vacuolization (Fig. 3).

Ultrastructure of autologous venous explants

After 1 month of implantation, the 3-month cryopreserved vein autografts as well as the non cryopreserved autografts (control) were patent and showed identical ultrastructural findings.

By SEM, the cryopreserved autografts were continuously lined with flat endothelium (Fig. 4). The endothelial cells were orderly aligned along the direction of the blood flow. Neither platelets nor fibrin were observed. Leukocyte adherence was unremarkable.

TEM of endothelium showed nuclei with finely dispersed chromatin and an occasional nucleolus (Fig. 5). The cytoplasm contained cisternae of rough endoplasmic reticulum, mitochondria and abundant intermediate filaments. Stress fibers were found in endothelial cells lining the perianastomotic regions of the graft. Tight junctions were present. Scant leukocytes were observed in the subendothelial space. The elastic lamina was continuous. In the media, the elastic fibers increased throughout and focally condensed into small laminae. Many of the smooth muscle cells had dilated cisternae of rough endoplasmic reticulum containing moderately-dense synthesized material (Fig. 6).

Ultrastructure of vein allograft explants

After 1 month of implantation, only one of the 3 months cryopreserved vein allografts was still patent (patency rate 12.6%). The other explants were thrombosed and transformed into fibrotic conduits.

SEM of the patent allograft showed an endothelialized graft surface with marked leukocyte adherence (Fig. 7). The surface inflammatory infiltrate, starting a few mm from the anastomotic sites, was homogeneously distributed along the length of the graft. Many leukocytes were observed while migrating through evident interendothelial gaps, into the subendothelial space. Retraction of the endothelium was noticed especially in the mid-portion of the graft (Fig. 8).

By TEM, endothelial cells had convoluted nuclei with finely dispersed chromatin and abundant cytoplasm. Apart from the usual assortment of organelles, the cytoplasm contained a number of ribosomes, polyribosomes, and small profiles of rough endoplasmic reticulum. Weibel-Palade bodies were also found. The subendothelial space was occupied by small and large, 'blast-like', lymphocytes, monocyte/macrophages as well as migrated smooth muscle cells (Fig. 9). These latter cells had a few peripheral contractile filaments and numerous cisternae of rough endoplasmic reticulum. The elastic laminae were fragmented; in the media, lymphocytes were associated with smooth muscle cells having a synthetic appearance, as well as fibroblasts (Fig. 10). The

adventitia contained a marked perivascular infiltrate of lymphocytes, plasma cells, monocyte/macrophages and neutrophils.

Ultrastructure of seeded vein allograft explants

Prior to implantation, controls showed a discontinuous crowding of rounded endothelial cells on the surface of the vein allograft (Fig. 11). Some endothelial cells spread over the graft surface (Fig. 12); filopodial contacts were observed.

After implantation, five of the six vein grafts thrombosed within a short time (four days to two weeks). In three cases, the thrombus was organized within a fibrotic vein. Technical defects were ruled out. One vein graft was patent with no gross evidence of thrombus formation (patency rate 12.6%).

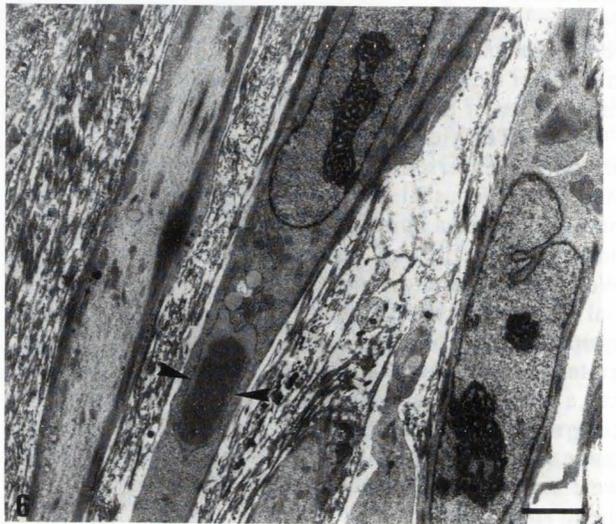
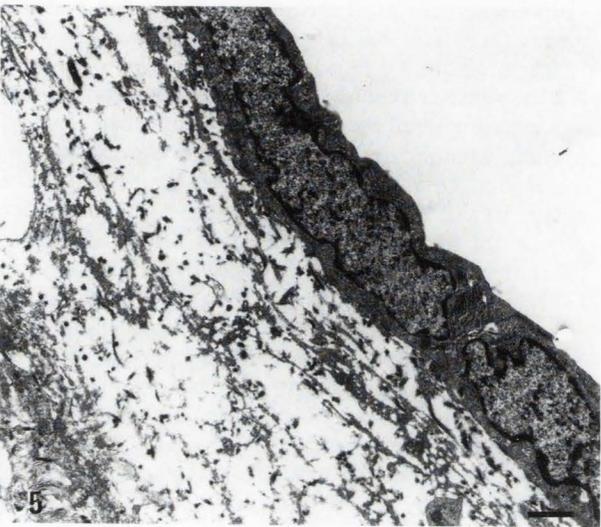
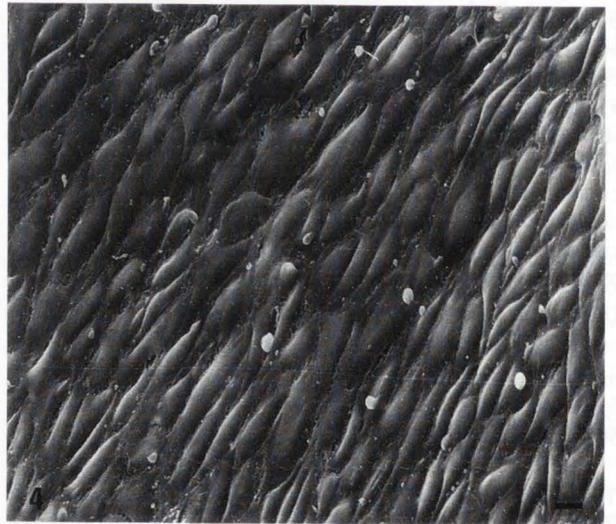
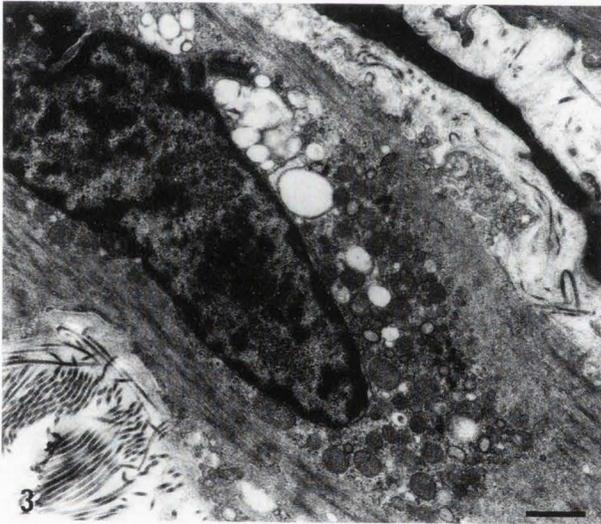
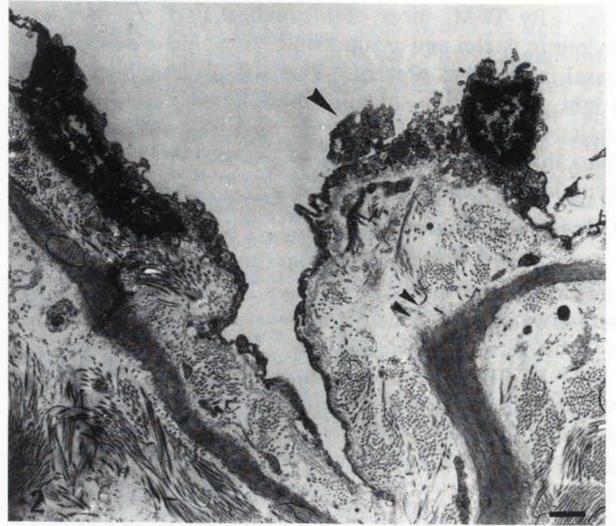
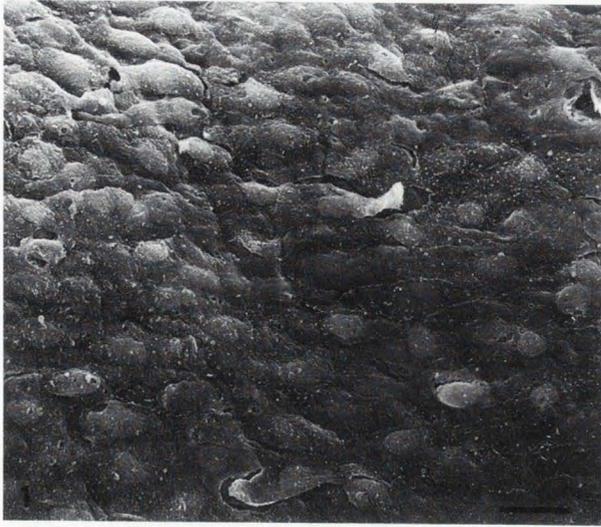
By SEM, the patent allograft was de-endothelialized. The surface showed a fibrin-mesh with activated platelets and erythrocytes (Fig. 13). Microthrombi were also present.

By TEM, no endothelial cells could be recognized. The inner elastic lamina was interrupted and had a sieve-like appearance (Fig. 14). There was necrosis of the media with residual smooth muscle cells having pyknotic nuclei and degenerated cytoplasm (Fig. 15). Cell debris and neutrophilic infiltration were common findings (Fig. 16). Neutrophils were also observed fixed in a state of granule exocytosis.

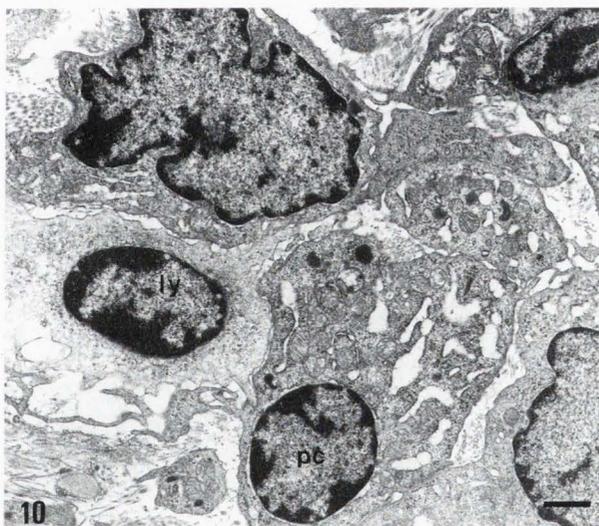
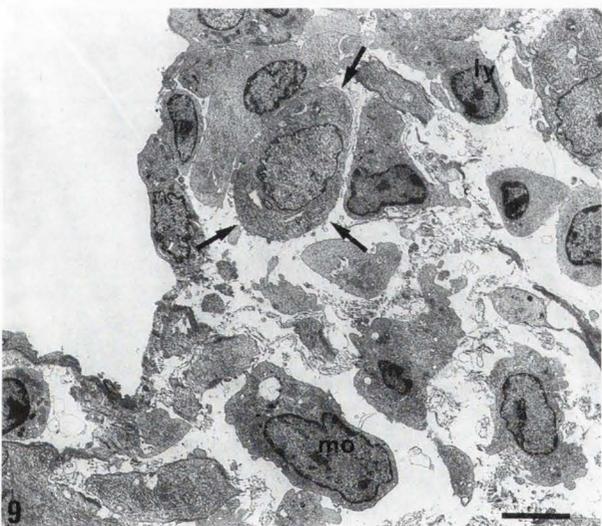
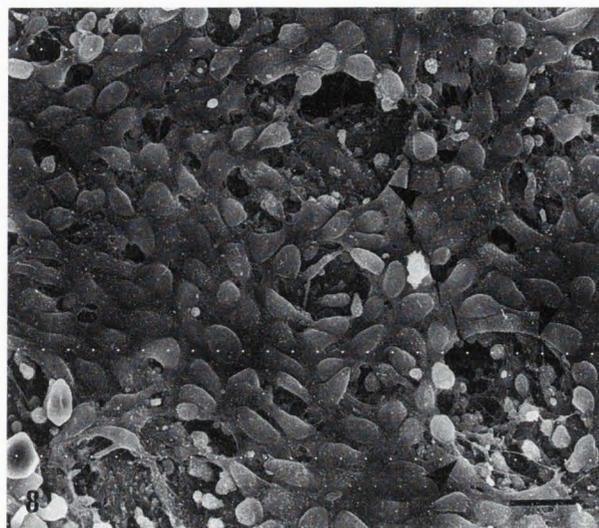
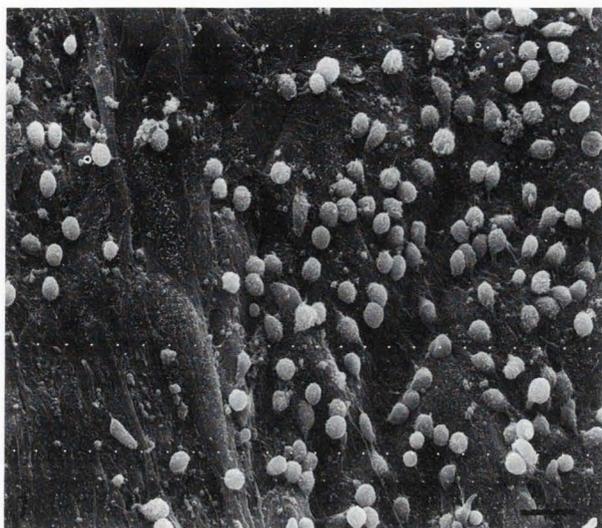
On the contrary, TEM of the thrombosed allografts showed a fibrotic media containing fibroblasts and scant inflammatory cells. Smooth muscle cells were virtually absent.

Discussion

In this study, we first evaluated the ultrastructure of the rabbit vein tissue after 1, 2 and 3 months of cryopreservation, respectively. Basically, no significant difference was found within the investigated specimens. Consequently, an adverse effect of storage time (up to 3 months) and procedures (storage under liquid nitrogen at -196°C) on vein graft architecture was ruled out. This means that tissue banking of cryopreserved vein grafts is feasible within a reasonably short period of time. All the specimens showed marked endothelial cell damage including, cell shedding, nuclear pyknosis, and cytoplasmic vacuolization, along with good preservation of the medial components, i.e., smooth muscle cells and elastic tissue. Unexpectedly, the use of improved techniques for tissue cryopreservation, such as, delicate procedures of tissue handling, vein irrigation with warm (37°C) solutions, the use of DMSO as a cryoprotectant (Barner *et al.*, 1966; Boren *et al.*, 1977; L'Italien *et al.*, 1979; Sitzmann *et al.*, 1984), rapid freezing ($-5^{\circ}\text{C}/\text{sec}$), and fast thawing, did not prevent damage to the venous endothelial surface at all. This finding is at variance with previous studies (Dent *et al.*, 1974; Weber *et al.*, 1976; Ladin *et al.*, 1982; Lindanauer *et al.*, 1982; Street *et al.*, 1988; Brockbank *et al.*, 1990) which found morphologically intact endothelium after vein storage at low



Ultrastructure of vein allografts



- Figure 1.** Three month cryopreserved vein graft showing the endothelial cell lining. SEM. Bar = 10 μ m.
- Figure 2.** Three month cryopreserved vein graft. The endothelial cells show surface blebbing (large arrowhead) and pyknotic nuclei. A slight dissociation of the elastotubules is also observed (small arrowheads). TEM. Bar = 1 μ m.
- Figure 3.** Three month cryopreserved vein graft. Smooth muscle cells are unremarkable. TEM. Bar = 1 μ m.
- Figure 4.** Cryopreserved autologous vein graft. Following one month of implantation, the graft surface is completely lined with a flat endothelium. SEM. Bar = 10 μ m.
- Figure 5.** Cryopreserved autologous vein graft. One month of implantation. By TEM, the endothelial cells show oval nuclei with finely dispersed chromatin. The cytoplasm contains a few profiles of rough endoplasmic reticulum, mitochondria and intermediate filaments. TEM. Bar = 1 μ m.
- Figure 6.** Cryopreserved autologous vein graft. One month of implantation. The smooth muscle cells show abundant rough endoplasmic reticulum; some cisternae are dilated and contain moderately-dense synthesized material (arrowheads). TEM. Bar = 10 μ m.
- Figure 7.** Cryopreserved allograft. Following one month of implantation, SEM shows a continuous endothelium with a marked leukocyte adherence. SEM. Bar = 10 μ m.
- Figure 8.** Cryopreserved allograft. One month of implantation. Retraction of endothelial cells (arrowheads) as observed in the mid-portion of the graft. SEM. Bar = 10 μ m.
- Figure 9.** Cryopreserved allograft. One month of implantation. TEM of the subendothelial space shows lymphocytes (ly), monocytes (mo), and modified smooth muscle cells (arrows). TEM. Bar = 5 μ m.
- Figure 10.** Cryopreserved allograft. One month of implantation. Lymphocytes (ly), plasma cells (pc), and smooth muscle cells with a synthetic appearance are observed in the media. TEM. Bar = 1 μ m.

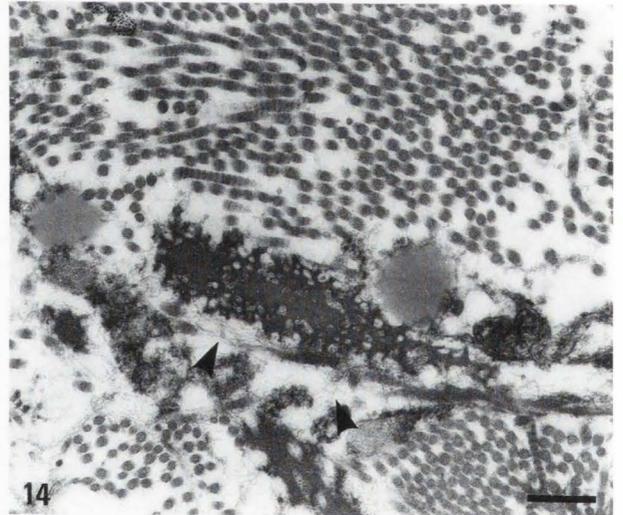
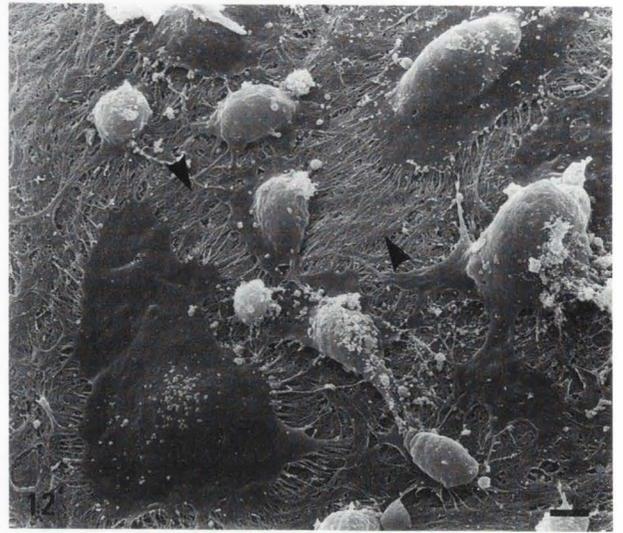
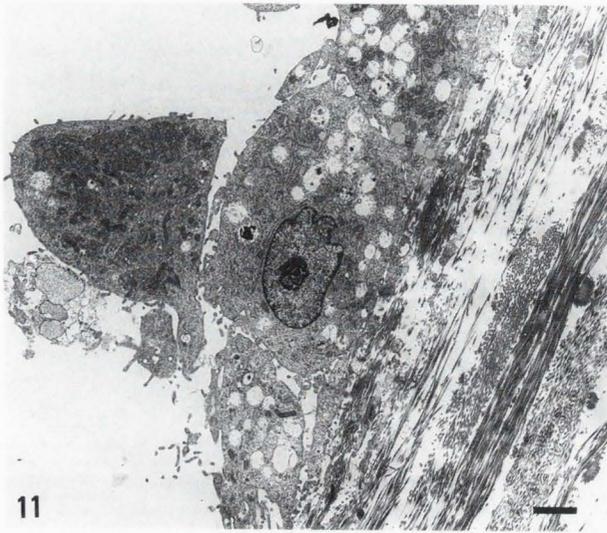


Figure 11. Allovein graft seeded with autologous endothelium. Prior to implantation, TEM shows rounded endothelial cells on the graft surface. TEM. Bar = 5 μm .

Figure 12. Allovein graft seeded with autologous endothelium. SEM shows some spread endothelial cells with numerous filopodial projections. Note that subendothelial matrix is exposed at different sites (arrowheads). SEM. Bar = 2 μm .

Figure 13. Allovein graft seeded with autologous endothelium. Following four days of implantation, the graft surface is covered with fibrin, dendritic (arrowheads) and fully spread (arrows) platelets, as well as erythrocytes. SEM. Bar = 5 μm .

Figure 14. Allovein graft seeded with autologous endothelium. The four day explant shows frayed elastic lamellae with a sieve-like appearance. Elastotubules (arrowheads) and lipid droplets are also evident. TEM. Bar = 0.5 μm .

Figure 15. Allovein graft seeded with autologous endothelium. After four days of implantation, TEM shows necrosis of smooth muscle cells (arrowheads). TEM. Bar = 1 μm .

Figure 16. Allovein graft seeded with autologous endothelium. Four days after implantation. A neutrophil is observed in correspondence with cell debris and remnants of elastic tissue (arrowheads). TEM. Bar = 1 μm .

temperatures in the presence of a cryoprotectant agent (ammonium acetate, glycerol, and DMSO). In accordance with our results, disruption of the endothelial layer was ultrastructurally demonstrated in human specimens by Ligush *et al.* (1991). We further documented this issue indirectly by evidencing a failure in culturing endothelial cells from analogous rabbit cryopreserved specimens before implantation (Faggioli *et al.*, 1993). Thus, our view is that current methods of cryostabilization do not maintain endothelial cell integrity effectively.

However, from our results, the significance of preserving an intact endothelium remains questionable for at least two reasons. First, four weeks after implantation, all the cryopreserved vein autografts behaved like fresh autografts. They were patent and completely endothelialized with early features of media arterialization. Since endothelium injured by cryopreservation is extremely susceptible to sloughing when exposed to the arterial flow (Elmore *et al.*, 1991; Faggioli *et al.*, 1993), it can be supposed that a reendothelialization takes place in cryopreserved rabbit vein autografts, the source of the neo-endothelium being the host arterial tissue. Second, unlike previous statements (Schwartz *et al.*, 1967; Tice and Zerbino, 1972; Dent *et al.*, 1974; Axthelm *et al.*, 1979; L'Italien *et al.*, 1979; Malone *et al.*, 1980; Theide *et al.*, 1981; Ladin *et al.*, 1982; Showalter *et al.*, 1989; Brockbank *et al.*, 1990; Fujitani *et al.*, 1992), we feel that cryopreservation does not significantly reduce the antigenicity of venous allografts.

Additionally, the presence of residual endothelium on vein allografts can be deleterious rather than beneficial since injured endothelium can drastically trigger immunological reactions leading to graft failure. Further, they can determine production of procoagulant factors, such as thromboplastin and factor V, although this issue has not been yet investigated in cryopreserved veins. Additionally, Elmore *et al.* (1991) showed that the endothelium of cryopreserved veins has more vasoconstrictive properties than the fresh endothelium. Accordingly, in the present study, all but one of the cryopreserved vein allografts were thrombosed and fibrosed after one month of implantation.

Furthermore, the ultrastructure of the still patent allograft showed features of acute graft rejection. These latter allografts included a remarkable mixed inflammatory infiltrate mainly affecting the intima and adventitia. In addition to lymphocytes, which are probably responsible for initiating immune-mediated graft damage, varying numbers of monocytes, plasma cells and neutrophils were also seen. 'Activated' lymphoid cells with 'blast-like' features were also present. Interestingly, the endothelial lining, presumed to be autologous in nature, was spared; whereas, the media featured foci of inflammatory cells, modified smooth muscle cells, fibroblasts, and degenerated elastic tissue.

These findings further extend the issue of venous allograft antigenicity. Although most of previous studies have concentrated upon endothelial cell antigenicity, smooth muscle cells and extracellular matrix components, such as the elastic tissue, can play an additional role in the mechanism of vein graft rejection. Support of this hypothesis is the finding of smooth muscle cell expression of molecules (class I and II major histocompatibility complex antigens as well as vascular cell adhesion molecule-1, VCAM-1) which are known to be important in directing immune-mediated responses (Munro and Cotran, 1988; Briscoe *et al.*, 1992). On the other hand, elastic tissue can be potentially immunogenic since elastin-derived peptides were found implicated in the pathogenesis of human inflammatory abdominal aneurysms (Gargiulo *et al.*, 1993).

Further evidence of this issue comes from the observation of explants of vein allografts seeded with autologous endothelium. All but one of these grafts thrombosed within a very short time (from four days to two weeks). This early failure was believed to be a consequence of incomplete endothelial cell seeding. Ultrastructural examination of the seeded vein grafts before implantation showed many areas lacking any cell coverage with highly thrombogenic subendothelial matrix components exposed to the blood flow. Of interest, TEM of a four day patent explant showed neutrophilic infiltration and necrosis of the media. The presence of neutrophils, which are considered highly specific for the diagnosis of rejection in other tissues, is particularly important since they are suspected to augment immunological damage to target structures by delivering lytic enzymes as well as metabolic products, e.g., hydrogen

peroxide, into the matrix (Adams *et al.*, 1990). Neutrophilic recruitment followed by disruption of media components can therefore be responsible for the fibrotic evolution of the older failed explants.

The present results, which are at variance with most of the previous work on this subject, need obvious confirmation in other experimental settings, including the ongoing clinical trials as well. However, cryotechnologies for preserving vein tissue and, most importantly, the immunological characteristics of the cryopreserved vein allografts with and without endothelial cells should be more extensively investigated. In our opinion, future issues to be explored in order to resolve the immunological problems would be:

a) Improved seeding with autologous endothelium. Our preliminary results show complete endothelialization after 22 hours of cell incubation (results not shown).

b) Human leukocyte antigen (HLA) matching instead of the actually recommended ABO matching whose clinical efficacy is questionable in light of the results obtained in the clinical settings (Harris *et al.*, 1993; Shah *et al.*, 1993; Walker *et al.*, 1993).

c) Graft treatment with antibodies directed to integrins, such as, the intracellular adhesion molecule-1 (ICAM-1) and VCAM-1, which mediate adhesion of inflammatory cells to endothelial cells as well as to mesenchymal cytotypes.

d) Administration of low-dose, short term effective immunosuppressive drugs according to Augelli *et al.* (1991) who have recently found that cyclosporin A improves the patency of either fresh or cryopreserved allografts in the dog model.

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Discussion with Reviewers

K.A. Robinson: Perhaps the most interesting finding is the disparity between your results and those of previous investigators with regard to both long-term patency and immunologic rejection of cryopreserved homografts. Are methodologic differences to blame?

Authors: Early studies on this subject showed better patencies in cryopreserved allografts when compared to fresh controls. However, cryopreservation techniques in these studies were somehow "rudimental" and significant cell damage probably occurred during the freezing-thawing cycle. We believe that the cell loss may have decreased the overall antigenicity of the venous wall. In more recent studies, improved freezing techniques allowed a better preservation of all the wall components; therefore, the patencies obtained were comparable to

those of fresh allografts, which are notoriously poor. Some recent studies showed that acceptable patencies are achieved in cryopreserved venous allografts only with concomitant immunosuppressive and antiplatelet therapy [Augelli *et al.*, 1991; Miller VM, Bergman RT, Glocviczki P, Brockbank KGM. (1993). Cryopreserved venous allografts: effects of immunosuppression and antiplatelet therapy on patency and function. *J Vasc Surg* **18**: 216-226]. In this respect our study further supports these data.

K.A. Robinson: Please comment further on the rationale for autologous endothelial cell (EC) seeding of the cryopreserved allografts. Presumably one reason is to enhance compatibility in the host; yet your results indicate no difference between seeded versus unseeded cryopreserved allografts in terms of immunologic/inflammatory sequelae.

Authors: The rationale for autologous EC seeding was exactly to reduce immunoreaction by increasing compatibility. Previous studies showed that endothelium is the major immunological target in graft rejection (Greisler, 1991). Our results do not support this view. Smooth muscle cells and other components of the venous wall seem to be responsible for immunoreaction, despite the fact that some authors reported better patencies in de-endothelialized venous allografts if compared with intact allografts [Galumbeck MA, Sanfilippo FP, Hagen PO, Seaber AV, Urbaniak JR. (1987). Inhibition of vessel allograft rejection by endothelial removal. *Ann Surg* **206**: 757-764].

K.A. Robinson: How does the ultrastructure (particularly of the EC) of the cryopreserved specimens compare to freshly harvested veins?

Authors: Basically, we found that cryopreservation significantly affected the endothelial cell viability. Ultrastructurally, a variable, but constant, EC loss was appreciated in the examined samples. Residual endothelium showed nuclear changes, including chromatin clumps and pyknosis, as well as features of cytoplasmic damage, such as mitochondrial swelling and vacuolization. Surface blebbing was also prominent. The EC suboptimal morphological preservation is also in agreement with the failure in culturing EC from analogous cryopreserved specimens (Faggioli *et al.*, 1993). However, it should be also mentioned that smooth muscle cells and matrix components, which showed an almost unremarkable ultrastructural morphology, were less affected by the freezing-thawing cycle.

M.F. Sigot-Luizard: You said, and I think probably rightly, that "from our results, the significance of preserving an intact endothelium remains questionable". Moreover, you supposed that the reendothelialization of the autograft vein is provided by the host arterial tissue. Did you perform some experiments to be able to confirm this hypothesis?

Authors: Yes, we did (Faggioli *et al.*, 1993). First, we studied two-hour explants of cryopreserved rabbit vein autografts and found that, at this time, the EC were completely lost from the venous surface, the surface being lined with a mesh of fibrin and fully spread platelets only. Second, we found that analogous one month explants showed a completely endothelialized graft surface. Consequently, we supposed that reendothelialization was mostly provided by the host arterial tissue.

R.M. Albrecht: The findings regarding the antigenicity of remaining EC and of the other vessel components are of considerable importance. While the non-seeded grafts were first observed at one month, the seeded grafts were observed as early as 4 days. Most of these apparently showed evidence of acute rejection. Was this simply a general inflammatory response to the damaged tissue or have the animals somehow been previously sensitized to the foreign antigen so that this very rapid response occurs? Is there any data which indicates the unseeded alloveins also shows this rejection response by the 4 day time point?

Authors: We suspect that the pathologic picture we observed in the seeded vein allografts may correspond to an early stage of the otherwise typical features of acute rejection found in the unseeded alloveins. This finding is not surprising because rabbit jugular veins from different inbred strains were employed as vein allografts. Under these circumstances, an acute graft rejection is reasonably expected within a few days or weeks depending on the number and immunogenic strength of the antigens present in the graft but absent in the recipient. Alternatively, one can speculate that an unexpected form of hyperacute graft rejection may have occurred in these specimens. As you suggest, an hyperacute rejection is generally associated with the presence of preformed circulating antibodies in the recipient directed against donor HLA or ABO antigens. However, this does not seem to be so in the present case. Further, it should be also mentioned that, at least in humans, not all patients with circulating antidonor HLA or ABO antigens develop hyperacute rejection; there are also occasional cases that demonstrate no detectable antidonor antibodies in recipient serum or allograft eluates, even if clinically and pathologically characterized as hyperacute rejection [Sanfilippo FP. (1990). Renal Transplantation. In: The Pathology of Organ Transplantation. Sale GE (ed.). Butterworths. pp. 51-101). Regarding to your last question, at the present, we have no data on the unseeded alloveins.

R.M. Albrecht: The actual rates of freezing and thawing of the cells and tissues often vary over time, i.e., are not linear, due to the latent heat of fusion, conductivity, exposed surface area, etc. Thus, even if the cooling device tools a block of metal at a certain rate it is still important to know the actual cooling curve of a biological

specimen in the cooling device. Are the actual freezing and thawing rate curves known (for example, measured by thermocouples inserted in the specimen)? Is ice crystal damage or formation of intracellular ice seen with this cryoprotectant and with these rates of cooling? Could extensive damage produced by the freezing and thawing technique effectively mask any minor damage related to the length of storage?

Authors: Thermoelectric couples were not used in our model. Although previous studies stated that DMSO, at the concentration herein employed, efficaciously prevent the formation of ice crystals intracellularly, we believe that no conclusive data exist on this issue. Strictly speaking, cryoprotection with DMSO means the addition of solutes and the reduction of intracellular free water to living cells in order to increase survival rates after freezing and thawing. Many living cells, subjected to slow freezing rates when suitably cryoprotected survive freezing and thawing as a result of the slow removal of water from cells and the growth of extracellular ice. However, survival of a living cell following the freezing-thawing cycle does not mean suitable preservation of the original fine structure of that cell. Thus, we believe that ice crystals do occur intracellularly. However, turning to the preservation of vein components and in particular of EC, we do not know how much EC damage is proportionally caused by freezing-thawing per se, or by DMSO cryoprotection, which is also potentially deleterious. In this respect, the resulting cell damage can obviously mask any additional, even though minimal, damage caused by storage conditions.

R.M. Albrecht: Were any studies performed *in vitro* or *in vivo* to see if the seeded autologous EC remained in place during pulsatile flow at arterial pressures?

Authors: We examined a couple of two-hour explants and we did not find any endothelial cell on the graft surface. However, as discussed in the paper, at that time we did not succeed in achieving a continuous and flat EC monolayer on the graft surface. Subsequently, we examined the 22-hour seeded alloveins and found that approximately 75% of the original EC were still present after 2 hours of implantation.

R.M. Albrecht: Why is bovine serum, rather than autologous rabbit serum, used in the freezing media; might this not actually increase the antigenicity of the vessels if bovine molecular species bind in the subendothelium?

Authors: This comment is very appropriate and we thank the reviewer for focusing on this subject. We chose bovine serum rather than rabbit serum because this is the technique used in most clinical studies and we elected to keep all variables as close as possible to the clinical settings. Moreover, our serum is heat-inactivated and should not interfere significantly, even if still present on the venous surface after careful washing, with immunological reactions.