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Rapid Thawing Increases the Fragility of the Cryopreserved Arterial Wall

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Objective: *to extend present knowledge of the biomechanical and structural changes which occur in the cryopreserved, rapidly thawed arterial wall.*

Materials and methods: *minipig iliac arterial segments were cryopreserved at −196 °C in either minimum essential medium or Wisconsin solution. Fresh segments served as the control group. After 1 month, the specimens were rapidly thawed (37 °C) and processed for biomechanical, ultrastructural, morphological and immunohistochemical (MMP-1, MMP-2, MMP-3 and MMP-9) analysis. Visualisation of apoptotic cells was performed by TUNEL method. For the mechanical distension analysis, an* in vitro *circuit was designed.*

Results: *the cryopreserved segments showed a 42% incidence of spontaneous fracture and the appearance of microfractures which affected the endoluminal third of the vessel. An accumulation of liquid in the subelastica was observed. An increased expression of wall-degradative enzymes (mainly MMP-2) was also observed following cryopreservation. No significant differences were detected in the proportional elasticity module or tensile strength of the specimen groups. No differences in mechanical distension were observed between groups after the vessel segments were subjected to the pulsatile circuit flow for 72 h. Cell damage was most intense in the specimens cryopreserved in Wisconsin solution.*

Conclusions: *cryopreservation in both the solutions employed, followed by rapid thawing, induce changes in the permeability which increase the fragility of the cryopreserved arterial wall. Both increased expression of wall-degradative enzymes and accumulation of liquid may contribute to graft failure after implantation.*

Key Words: Rapid thawing; Cryopreservation; Endothelial damage; Metalloproteinases; Apoptotic cell.

Introduction

Small or medium calibre prosthetic bypass is associated with a high incidence of failure[1–3] and in patients without autogenous material alternative bypass conduits are required.

Human vessels subjected to cryopreservation currently provide such an alternative.[4–6] However, vascular cryopreservation may induce changes in the structural integrity of the vessel which lead to dilatation and/or rupture after implantation.[7,8] The freezing effects depend on numerous variables including the solutions used for preservation, storage temperature, thawing procedure and vascular source (veins vs. arteries).

Previous findings with regard to the media and solutions used for cryopreservation are inconsistent.[9–13]

It is generally accepted that the freezing process should be slow. The storage of vessels at a temperature of −80 °C seems to lead to better results than temperatures as low as −160 °C/−190 °C.[10,12,14] Thawing is generally effected rapidly in a 37 °C water bath;[15] although some authors report improved preservation of the endothelium after thawing at 10 °C for 10 min.[4] The time of cryopreservation has not been standardised and ranges from short[14,16] to undefined[17] periods.

Veins are the most commonly used source of vessel and the general consensus is that arteries are more antigenic.[17,18] The use of arterial allografts[2,3,16] has been related to leukocyte infiltrates in the adventitial and medial layers, nerve damage and intimal hyperplasia. Damage to the muscular and elastic components has been infrequently described. The physical characteristics of the arterial wall (resistance, elasticity and flexibility) are generally well preserved, although there is some controversy related to these properties.[17–19]

The ideal size of the vessel to be cryopreserved has not been addressed. Although experimental studies

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involve the use of small segments, previous experience[11] suggests that the fragility of specimens may warrant the establishment of a critical length of specimen.

This study was designed to evaluate the effects of cryopreservation in minimal essential medium (MEM) and University of Wisconsin (UW) solution and rapid thawing on the structural and biomechanical properties of vessels which could condition future viability.

Materials and Methods

Experimental animals and specimens

Thirty-two minipigs weighing 20–30 kg were used. Animal care and experimental protocol were in compliance with guidelines of the European Community Standards on the Care and Use of Laboratory Animals (N. 28871-22A9).

The animals were subjected to general anaesthesia involving the administration of thiopental (2 mg/kg), endotracheal intubation and the inhalation of a mixture of oxygen and nitrous oxide. Anaesthesia was maintained by the intravenous administration of sodium thiopental, pancuronium bromide and phentanilum chloride. Following midline laparotomy, both the right and left common iliac arteries (4–6 mm internal diameter) were dissected to obtain segments 3–4 cm in length. The arteries were harvested by the *no touch* method. The segments were preserved at 4 °C in MEM or UW until cryopreservation.

Experimental design

The following study groups were established:

Group I (Control, *n*=20): fresh arterial segments transported and preserved (4 °C) in MEM (*n*=10) or UW (*n*=10) until processing;

Group II (*n*=22): arterial segments cryopreserved in MEM plus 10% DMSO (−196 °C);

Group III (*n*=22): arterial segments cryopreserved in UW plus 10% DMSO (−196 °C).

Cryopreservation

The arterial segments were submerged in MEM or UW plus dimethylsulphoxide (DMSO) at a proportion of 9:1. The DMSO was added gradually over the 24 h following extraction. The controlled, automated cryopreservation process was performed in a biological freezer (CM25 P.115 Carburos Metalicos S.A., Madrid, Spain) and involved a 1 °C/min reduction in temperature to approximately −100 °C (Fig. 1). Following an overnight temperature stabilisation process at −80 °C, the segments were stored in liquid nitrogen at −196 °C for a period of 30 days. After the storage period, the specimens were subjected to rapid thawing in a water bath at 37 °C.

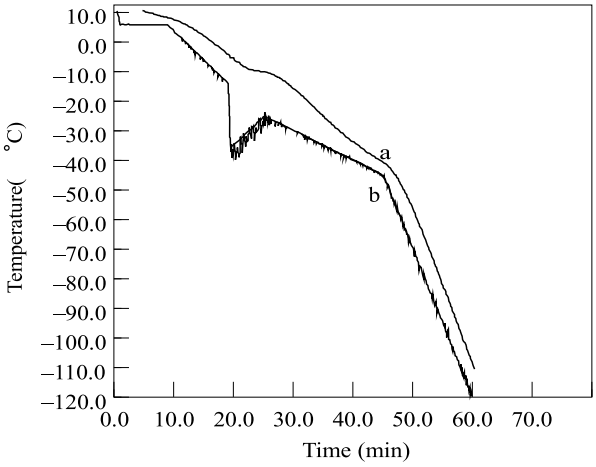

Fig. 1. Freezing process: (a) probe inside the cryotube records the temperature of the sample. (b) Data from a probe placed inside the freezing chamber.

Morphological analysis

Fresh/control and cryopreserved specimens were processed for light microscopy and transmission and scanning electron microscopy (TEM, SEM). Specimens for light microscopy were fixed by immersion in 10% formaldehyde and embedded in paraffin to obtain 5-mm sections. The sections were deparaffinised, hydrated and stained using haematoxylin–eosin, orcein and Masson's trichrome stains.

Specimens for ultrastructural analysis were fragmented, fixed for 1 h in 3% glutaraldehyde, placed in Millonig buffer (pH 7.3) and postfixed in 2% osmium tetroxide. Once dehydrated in a graded series of acetone, they were embedded in Araldite to obtain thin cuts. The cuts were contrasted with lead citrate and observed under a Zeiss 109 transmission electron microscope. The specimens for scanning electron microscopy were opened longitudinally and immersed for 1 h in 3% glutaraldehyde. They were subsequently transferred to Millonig buffer (pH 7.3) for 1 h and dehydrated in a graded series of acetone reaching a critical point in an E3000 Polaron with CO_2. Once metallised with gold-palladium, they were observed

variable was expressed as the elasticity proportional

under a Zeiss 950 DSM scanning electron microscope.

Immunohistochemical analysis

Specimens for immunohistochemical analysis were fixed by immersion in 10% formaldehyde and were embedded in paraffin to obtain 5-mm cuts. They were then deparaffinised, hydrated and equilibrated in TBS buffer.

Four monoclonal antibodies to metalloproteinases were used as primary antibodies: MMP-1 (interstitial collagenase; Biogenesis, Sandown, U.S.A.), MMP-2 (gelatinase A; Binding Site Ltd, Birmingham, U.K.), MMP-3 (stromelysin 1; Binding Site Ltd, Birmingham, U.K.) and MMP-9 (gelatinase B; Biogenesis, Sandown, U.S.A.). To detect the antigen–antibody reaction, the peroxidase–antiperoxidase (PAP) technique using a rabbit/mouse staining kit (Sigma Chemical, St Louis, MO, U.S.A.) was employed. The chromogenic substrate used in this kit was 3-amino-9-ethyl carbazole in N,N-dimethylformamide. The labelled specimens were examined under a light microscope (Zeiss, Jena, Germany).

In situ *apoptotic cell labelling*

Visualisation of the apoptotic cell fraction was performed by modification of the TUNEL method.[15] Specimens were subjected to microwave irradiation (SANYO EM-704T) for 5 min (350 W) in 0.01 M citrate buffer (pH 6). The detection of DNA fragmentation was performed using a kit (Calbiochem, CN Biosciences Inc., U.S.A.). The specimens were examined and labelled cells counted under a light microscope. The Mann–Whitney *U*-test was used to compare the apoptotic cell fractions in each study group.

Biomechanical analysis

Four-centimetre arterial segments were used for the biomechanical tests. The segments (13 group I, eight group II and six group III) were placed in an INSTRON tensiometer (A7411). This instrument has controlled temperature (−75 °C–200 °C), a force of 19.6-98 000 N and a cross-head speed of 50 cm/min to 0.005 cm/min. Both the tensile strength (i.e. the break-load or maximum force the artery will withstand before breaking) and viscoelasticity were determined. This latter module calculated from the break-load tracing. The biomechanical variables obtained for each group were compared using the Mann–Whitney *U*-test.

Mechanical distension analysis

For the mechanical distension analysis, an *in vitro* circuit was designed to incorporate control arterial segments and those subjected to cryopreservation and thawing.

Arterial segments were cannulated at each end and placed in a conical tube closed at both ends by screw caps that were perforated in the centre. The vessel segments could thus be positioned in the central part of the tube, with the free ends of the cannulae protruding through the holes in the caps. Both cannulae were connected to a silicon tube using three-way valves which was, in turn, connected to a peristaltic pump of adjustable velocity MASTERFLEX L/S model 7518-50 (COLE-PARMER, Illinois, U.S.A.). In this way, a closed circuit loop was obtained which was filled with M-199 complete medium via the three-way valves. To avoid the drying out of the specimens, the tube housing the vessel segments was filled with the same medium (M-199) so that the specimen was completely submerged. The flow velocity was set at 100 ml/min for a circuit time of 72 h.

Results

After thawing, fractures sufficient to render the vessels non-viable were observed in 42% of the cryopreserved specimens. The lines of fracture emerged from within the arterial segments and extended towards the outside generally leaving the tunica adventitia intact (Fig. 2a). In addition to these fractures which affected the endothelium and the tunica media, microfractures damaging the intima and superficial layers of the media were also observed (Fig. 2b).

Morphological findings

No changes in the normal structure of the arterial wall were observed in the group I (control) fresh arteries preserved in MEM or UW until fixation and processing. In the group II specimens, the endothelial cells of the intima were observed by SEM to be slightly swollen and turgid although the endothelium was

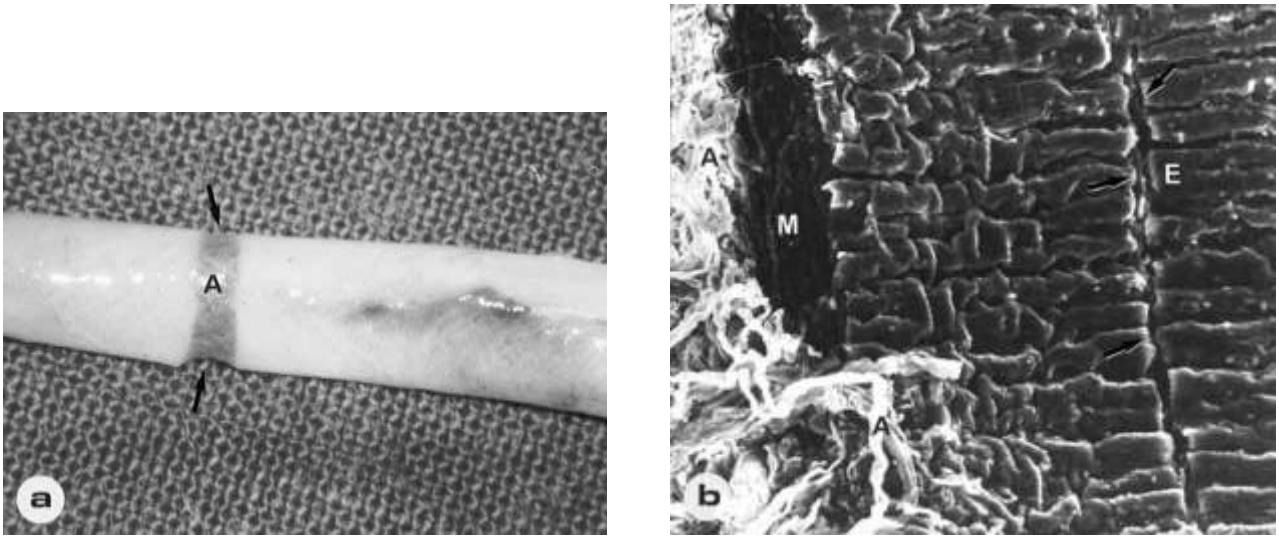

Fig. 2. (a) A cryopreserved/rapidly thawed arterial segment (MEM). Note how the spontaneous fracture affects the entire wall (arrows) except the tunica adventitia (a). ×25. (b) SEM of the fracture above, showing clean cutting of the muscle layer (M) and disorganised fibres in the adventitia (a). Also, on the luminal surface, a microfracture (arrows) may be seen to only involve the endothelium (E) and the internal elastic lamina. ×200.

well preserved with no loss in continuity. Endothelial detachment was most evident in the group III specimens, where the endothelial cells could be seen to become unstuck from the subendothelial bed and acquire increasingly globular, fusiform shapes, eventually escaping into the vessel lumen. In some of the group III specimens, folding of the endothelium and the internal elastic lamina resulted in the formation of large spaces between these layers and the media with the accumulation of fluid. Under the light microscope, longitudinal sections showed oedematous areas of different size, mainly in the cleavages of the internal elastic lamina (Fig. 3a,b).

Ultrastructurally, the group II endothelial cells showed a different morphology to control cells including oedematous cytoplasms and mitochondrial swelling and rupture of crests (Fig. 4a), while the endothelial cells of group III specimens showed damage to the nucleus and the cytoplasm confirming the previous histological observations (Fig. 4b).

Immunohistochemical observations (MMPs)

This analysis was aimed at detecting possible changes in the expression of the MMPs 1, 2, 3 and 9 after the cryopreservation process. Changes in MMP labelling patterns were observed, although the most significant changes were related to MMP-2 expression. It is proposed that this metalloproteinase is an important marker of tissue remodelling after implant. In the control specimens, MMP-1 expression was only observed in the endothelium. MMP-2 expression was discretely seen both in the endothelium and in the media while MMP-3 and 9 were not detected. The group II specimens showed MMP-1 expression in the endothelium, a high degree of MMP-2 expression in the endothelial and medial layers and, to a lesser extent in the adventitia, and MMP-9 expression in the intimal and adventitial layers. These specimens showed no MMP-3 activity. The findings of all study groups were shown in Table 1.

Apoptosis

Following cryopreservation, cell viability is reduced and reflected by an increase in the number of apoptotic cells. No labelled cells were observed in the control specimens. The group II specimens showed some labelled cells in the endothelium, although the medial layer was well preserved. However, in the group III specimens practically all the endothelial cells and some of the cells of the tunica media were TUNEL positive
(Fig. 5). Accordingly, total cell viability was lower in specimens which had been subjected to cryopreservation in UW, compared to MEM (18.7±1.8 vs. 36.3±4.1% apoptotic cells).

Biomechanical observations

The cryopreserved vessels showed similar biomechanical behaviour to the control, fresh arterial

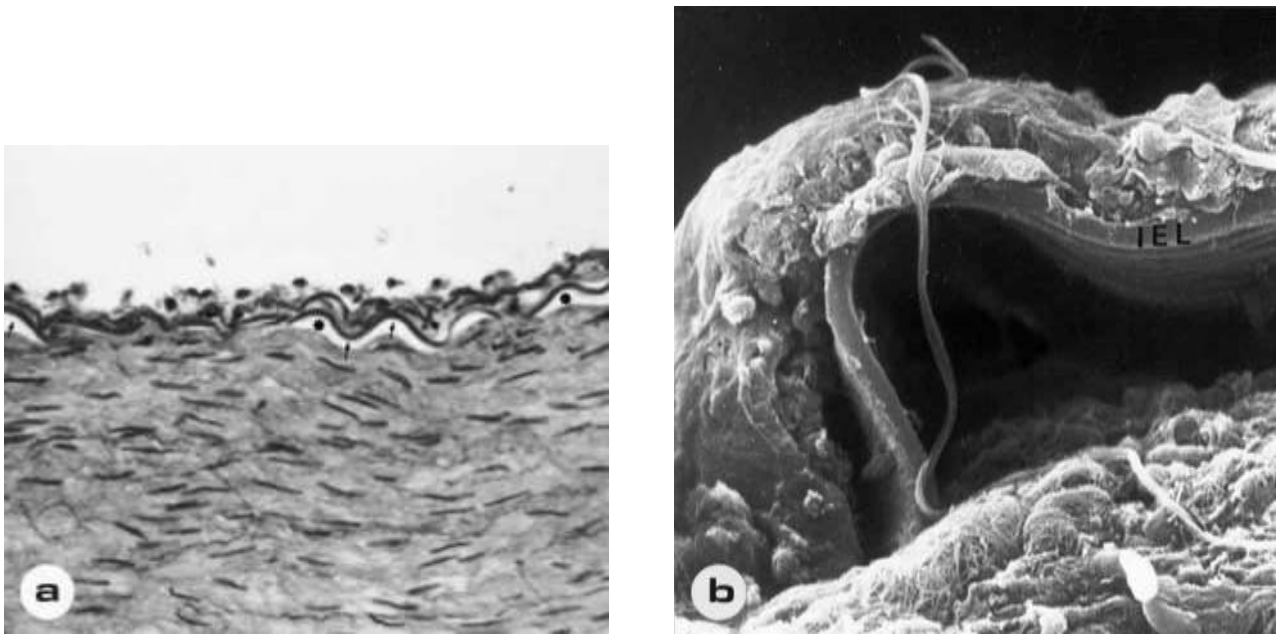

Fig. 3. (a) After rapid thawing, this artery shows detachment of the IEL (arrows) due to liquid accumulation in the general area of the subelastica (∗). ×400. (b) The same image shown by SEM. Accumulated fluid leads to wrinkling of the IEL. ×2000.

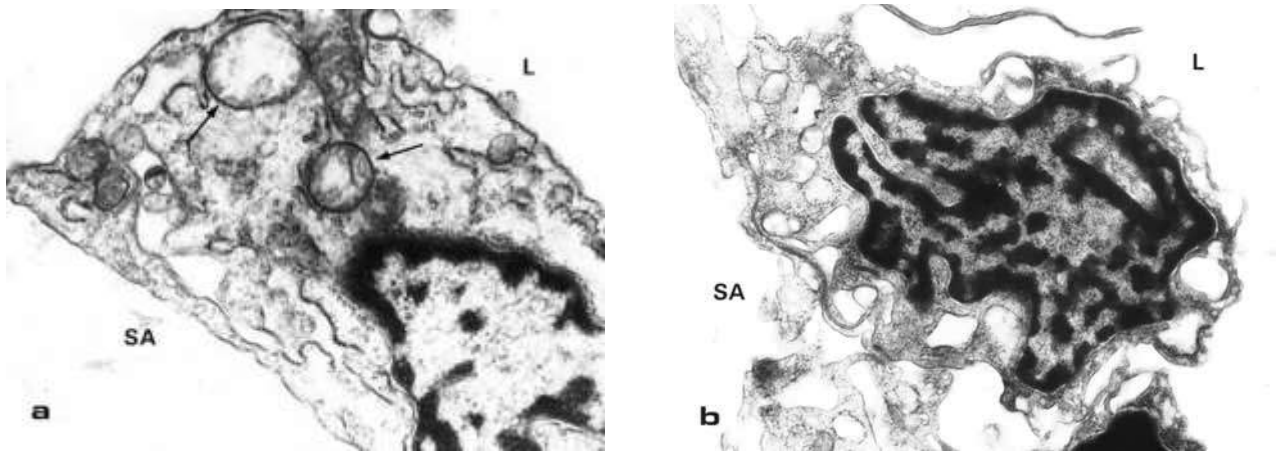

Fig. 4. (a) An endothelial cell after cryopreservation in MEM showing the swelling of some mitochondria (arrows). ×20 000. (b) An endothelial cell after cryopreservation in UW showing damage to the nucleus and cytoplasm. ×20 000. (L: arterial lumen, SA: subintimal area.)

segments. Break-load values ranged from 19.8 to 29.4 N with highest values corresponding to the group III specimens, although there were no significant differences between the groups (Fig. 6). Highest proportional elasticity module values were recorded in the group II specimens, although, once again, these differences were not significant.

All the control arterial segments were viable for use in the mechanical distension study, while some cryopreserved/thawed specimens were rejected due to spontaneous fractures affecting the entire thickness of the arterial wall. No differences in mechanical distension were observed between groups after the vessel segments were subjected to the circuit flow for 72 h. No anomalies or fractures were produced in any of the specimens.

		MMP-1	MMP-2	MMP-3	MMP-9
Group I (Control)	E	+	±	–	–

Discussion

The present authors have previously reported irreversible damage to the endothelium and the extracellular matrix of the cryopreserved arterial wall which could lead to failure of the vessel after implant.[11,15] The high incidence of spontaneous fracture observed after

	M	–	±	–	–
	A	–	–	–	–
Group II (MEM)	E	++	+++	–	±
	M	+	+++	–	–
	A	+	+	–	+
Group III (UW)	E	–	++	+	+
	M	–	++	–	–
	A	–	+	+	+

Table 1. MMPs immunostaining in porcine iliac arteries.

Results are expressed as follows: (–) undetectable staining; (±) variably detectable; (+) detectable staining; (++) moderate staining; (+++) strong staining. Note that these data reflect the overall relative staining intensity and do not necessarily reflect actual quantitative differences between different MMPs: MMP-1 (interstitial collagenase), MMP-2 (gelatinase A), MMP-3 (stromelysin) and MMP-9 (gelatinase B). (E: endothelium, M: medial layer, A:
adventitial layer.)

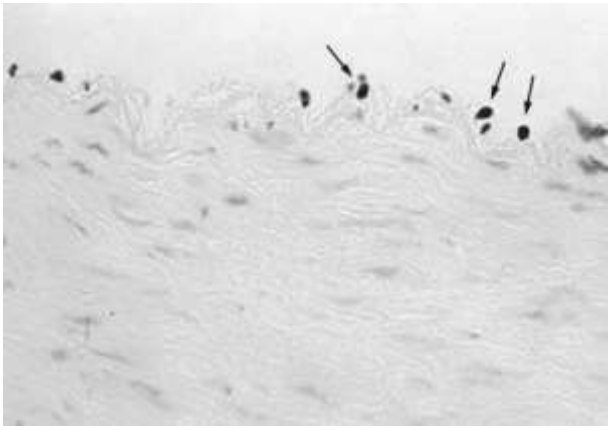

Fig. 5. Longitudinal section of the arterial wall where apoptotic – TUNEL positive – cells (arrows) may be seen in the endothelium. ×630.

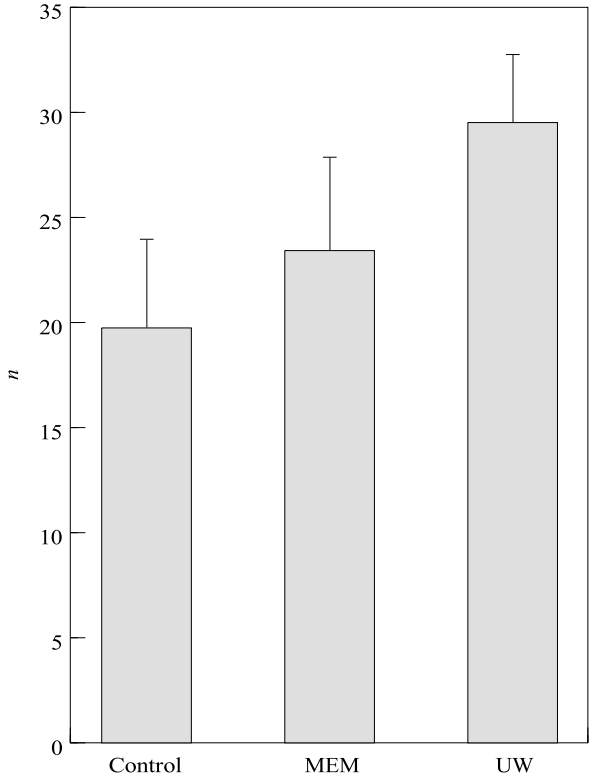

Fig. 6. Relative tensile strength of the three groups of arterial segments. No significant difference between the groups was observed (Mann–Whitney *U*-test, $p>0.001$).

intact. In the pig iliac artery, this layer is characteristically loosely reticular and predominantly elastic.

thawing has also been described by Pegg *et al.*[7,20] who attributed the phenomenon to thermal stress created by rapid warming of the vitreous material produced by freeze-concentration of the aqueous phase.

The observation of most interest was that the preservation solutions employed altered the permeability of the vessels such that fluid entering the vessel wall tended to accumulate in the cleavages of the internal elastic lamina. These sacs of fluid encapsulated in the arterial wall may correspond to the areas subjected to maximum thermal stress during thawing and induce breakage of the weaker tissue close to the vessel lumen. The microfractures observed here by SEM were also described by Pegg *et al.*[7,20] It may be speculated that

Other authors[21–24] have examined the integrity of the vessel wall following cryopreservation and ascribe the changes produced to the compliancy or rigidity of the elastic component of the vessel wall during freezing and not to the cryopreservation process. In our evaluation of mechanical properties after cryopreservation, no significant differences in the elasticity proportional module or tensile strength were detected.

Temperature and final storage in liquid nitrogen are factors known to affect endothelial preservation. According to Muller-Schweinitzer *et al.*,[25] these factors induce at least 50% endothelial denudation in the cryopreservation of 2-mm arterial rings. This finding, however, may be artefactual and, in our experience, scanning the luminal surface of 10-mm vessel segments permits the observation of irregular areas of denuded endothelium with two types of behaviour. Denudation either takes place in layers which would correspond to the entirely denuded ring or involves individual

when the fracture becomes continuous it may provoke the complete rupture of the wall. Indeed, in some of the specimens we observed how the entire wall was fractured and only the tunica adventitia remained

cells corresponding to vessel segments which appear to be endothelialised. This may account for the disparity in results regarding the viability of the cryopreserved endothelium.

Many investigations only focus on the pharmacological response of the endothelium and have also generated conflicting results. According to some authors[26] there is a good response to pharmacological stimuli after thawing in veins, while results reported for arteries are less satisfactory.[16,27] The factors which induce endothelial damage during freezing are numerous. An evident cause seems to be the loss of intraluminal pressure, which may lead to the collapse of the vessel. In addition to this, the shortening induced by contraction of the artery may lead to wrinkling of the endothelial monolayer.[28,29] Changes occurring in the smooth-muscle cells of the medial layer have been scarcely described. Such alterations have either not been observed[27] or have been detected as variations in the expression of anti-actin and perinuclear areas of vacuolisation after cryopreservation.[28] We report similar findings in the present model where evaluation of cell damage was performed by the estimation of programmed cell death. The proportion of apoptotic cells detected in arterial specimens cryopreserved in UW was approximately 36%, while specimens subjected to cryopreservation in MEM showed half this value. In both groups, the endothelial layer was most susceptible to cell damage.

The state of the components of the extracellular matrix is another factor which requires consideration. Changes in the behaviour of the fibrillar component have been previously related to the elasticity of the vessel. Accordingly, Rosset *et al.*[24] found minimum changes including breakage or dispersion of elastic fibres in a human artery model. Lehalle,[8] in an extensive experimental study, reported that changes in the elastic properties of cryopreserved vessels could constitute one of the major causes of immediate failure or of the long-term degeneration of the vessel wall.

Finally, metalloproteinases are enzymes which have been implicated in the degradative process which the extracellular matrix undergoes and, in particular, affect the collagenous, elastic fibrillar component of the vascular wall. In the present investigation, an increase in MMP-2 expression was recorded after cryopreservation. Similar changes in the expression pattern of this enzyme have been described in degenerative processes of the aneurysmal arterial wall.[30] Here, cryopreservation and thawing led to the activation of MMP-2 which could contribute to the degradation of extracellular matrix proteins which occurs in cryopreserved vessel implant failure. The lack of MMP1 expression might be attributed to the endothelial damage observed[15] which would also be in line with the fact that greatest expression of this enzyme following cryopreservation was recorded in group II, in which the endothelium of specimens was best preserved.

The present findings suggest that cryopreservation in MEM or UW followed by rapid thawing of arterial specimens induces changes in permeability and leads to the accumulation of fluid, especially in the subelastica, which may in turn lead to spontaneous fracturing of the arterial wall. The endothelial damage provoked leads to a loss in continuity of the monolayer favouring this process. After cryopreservation, there is an increase in the expression of MMP-2 in the extracellular matrix which may be implicated in the progressive degeneration of the arterial wall. These findings are felt to contribute to the current high incidence of cryopreserved vessel failure before and after implant. Future investigations should perhaps centre on the search for alternative methods of freezing/thawing aimed at reducing changes in arterial permeability and preserving tissue structures.

The findings indicate that these solutions (MEM and UW) do not affect the biomechanical strength of the vessel but do, however, modify permeability and induce changes at the cellular level.

Acknowledgements

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