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Impact of freezing/thawing procedures on the post-thaw viability of cryopreserved human saphenous vein conduits ☆

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Abstract

Background: Cryopreserved human blood vessels are important tools in reconstructive surgery. However, patency of frozen/thawed conduits depends largely on the freezing/thawing procedures employed.

Methods: Changes in tone were recorded on rings from human saphenous vein (SV) and used to quantify the degree of cryoinjury after different periods of exposure at room temperature to the cryomedium (Krebs–Henseleit solution containing 1.8 M dimethyl sulfoxide and 0.1 M sucrose) and after different cooling speeds and thawing rates following storage at $-196\,°C$.

Results: Without freezing, exposure of SV to the cryomedium for up to 240 min did not modify contractile responses to noradrenaline (NA). Pre-freezing exposure to the cryomedium for 10–120 min attenuated significantly post-thaw maximal contractile responses to NA, endothelin-1 (ET-1) and potassium chloride (KCl) by 30–44%. Exposure for 240 min attenuated post-thaw contractile responses to all tested agents markedly by 62–67%. Optimal post-thaw contractile activity was obtained with SV frozen at about $-1.2\,°C/min$ and thawed slowly at about $15\,°C/min$. In these SV maximal contractile responses to NA, ET-1 and KCl amounted to 66%, 70% and 60% of that produced by unfrozen controls. Following cryostorage of veins for up to 10 years the responsiveness of vascular smooth muscle to NA was well maintained.

Conclusion: Cryopreservation allows long-term banking of viable human SV with only minor loss in contractility.
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Keywords: Cryopreservation; Human saphenous vein; Contractile activity; Long-term storage; Dimethyl sulfoxide

While prosthetic material can be used for bypass of larger diameter vessels, its patency is poor when used as conduit to replace small diameter vessels. In these cases and if autologous tissue graft is not available, banked human saphenous veins (SV)[1] especially in combination with tissue engineering [10] are promising alternatives. The key advantage of cryopreservation as a technique for tissue preservation is the fact that storage at deep subzero temperatures allows long-term banking of blood vessels for elective use in reconstructive and vascular surgery [37]. However, reported clinical results for cryopreserved vein allografts are conflicting and graft patency after bypass surgery is poor. In coronary bypass surgery cryopreserved human SV have been found to serve as effective conduits with excellent initial

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[1] *Abbreviations used*: ANOVA, one-way analysis of variance; Me$_2$SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; E_{max}, maximal response; ET-1, endothelin-1; FCS, fetal calf serum; SV, saphenous vein; KCl, potassium chloride; NA, noradrenaline; pD$_2$, negative logarithm of the molar concentration of an agonist producing 50% of E_{max}; RPMI, RPMI 1640 culture medium.

success [30] but turned out to provide poor long-term patency rates [13]. Similar conflicting results have been reported when cryopreserved human SV were used as infrainguinal grafts [1,9,11]. Various factors, such as post-operative anticoagulation therapy [4,28] and immunosupression to prevent rejection [6] are important determinants of allograft patency. Furthermore, the surgical preparation of human SV including distension and storage may provide severe damage to both the endothelial and medial cell layers and may be responsible for certain failures [7]. In addition, damage to the tissue during the freezing/thawing procedure is an important factor vital to both viability and patency of cryopreserved grafts. Recently it has been documented that cryopreserved human SV retain their viscoelastic properties [2]. The aim of the present study was to investigate and evaluated the impact of various steps of the cryopreservation procedure on the post-thaw functional activity of the smooth muscle in human SV.

Materials and methods

Tissue preparation

Samples of human saphenous veins (SV) were taken from 26 patients undergoing bypass surgery (age 51–85 years) and from 3 multiple organ donors (34, 39 and 46 years old) after obtaining permission from the local ethical committee. The tissues were placed in cold Krebs–Henseleit (KH) solution (mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.25, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11) and transported to the laboratory within 6 h after removal. The veins were cleaned of loose connective tissue and cut into segments of about 20 mm or into rings of about 2–2.5 mm length to be investigated either unfrozen or after cryopreservation in organ bath studies.

Experimental design

Venous rings were placed in 2 ml Liquid Nitrogen Storage Ampoules (Gibco AG, Basel, Switzerland) filled with 1.6 ml vehicle solution (KH solution, Dulbecco's modified Eagle medium or RPMI 1640 culture medium) containing 1.8 M dimethyl sulfoxide (Me_2SO) and 0.1 M sucrose as cryoprotecting agents [19]. In a first series of experiments the samples were placed into the vials at different times so that the tissues were exposed for 10, 60, 120 and 240 min to the cryomedium at room temperature before being placed into a polystyrene box ($11 \times 11 \times 22$ cm) and slowly frozen at a mean cooling rate of about 1.2 °C/min in a freezer maintained at −70 °C. After about 2 h the vials were transferred into liquid nitrogen (−196 °C) where they were stored until use in organ bath studies after thawing at 15 °C/min.

When the effect of different cooling rates was investigated, samples were equilibrated with the cryomedium at room temperature for 10 min before the vials were placed either directly into liquid nitrogen and frozen at about

−150 °C/min or samples were first placed for about 60 min into dry ice to be frozen at a mean cooling speed of −5.7 to −70 °C before being transferred into liquid nitrogen. Slow freezing rates of −1.2 and −0.6 °C/min were achieved by placing the vials into a polystyrol box with 10 and 45 mm wall thickness, respectively, in a freezer maintained at −70 °C. About 2 h later, the vials were transferred into liquid nitrogen and stored there at −196 °C until use in organ bath studies.

When the effect of different thawing rates upon the post-thaw functional activity was investigated human SV were equilibrated for 10 min with the cryomedium (KH-solution containing 1.8 M Me_2SO and 0.1 M sucrose) before being frozen at a mean speed of about −1.2 °C/min. Before use the samples were thawed either rapidly at about 100 °C/min by placing the vials for 4 min into a 40 °C water bath, or thawing in a 40 °C water bath was preceded by exposure of the vials for 6 min to room temperatures giving a mean thawing rate of approximately 30 °C/min, or samples were placed for 10 min on dry ice and then exposed for 6 min to room temperature before being thawed within 4 min in a 40 °C water bath giving a mean thawing rate of about 15 °C/min. For post-thaw removal of the cryomedium venous samples were placed for about 5 min in KH solution containing 50% cryomedium before being rinsed in a dish containing 20 ml KH solution at room temperature. In some experiments thawed venous rings were incubated for 24 h in tissue culture polystyrene plates filled with 2–6 ml Dulbecco's Eagle medium containing 100 U/ml penicillin–streptomycin at 37 °C and investigated in organ bath studies 24 h later.

In each series of experiment rings from the same venous segment were randomly allocated to control and experimental groups.

Organ bath experiments

Venous rings were mounted between two hooks of stainless steel wire (diameter 0.5 mm) and suspended in 10 ml organ baths containing KH solution at 37 °C, gassed continuously with 5% CO_2 in oxygen. Changes in tone were recorded isometrically under a resting tension of 1 g with electromechanical transducers (Statham model UC 3) and a potentiometric recorder. The preparations were allowed to equilibrate for about 3 h in the bathing medium. During this time the vascular preparations were challenged twice with noradrenaline (1 μM) and the baseline tension of the rings was readjusted to 1 g if required. Contractile–response curves to agonists were determined by cumulative additions, the concentration in the organ bath being increased when the maximum effect had been produced by the previous concentration.

Temperature measurements

Temperature courses during freezing and thawing were measured by type du3s of Ellab a-s (Ellab Instruments, Copenhagen, Denmark). In these experiments the tip of a flexible thermoprobe was inserted into a 15 mm long

segment of human saphenous vein and fixed there by a cotton thread. The preparation was then frozen and thawed as described above while temperature measurements between -60 and $+20\,°C$ were recorded digitally every 15 s.

Drugs

The following compounds were used: $(-)$-noradrenaline hydrogentartrate, dimethyl sulfoxide (Me_2SO), sucrose, D$(+)$-trehalose, (Fluka, Buchs, Switzerland), human endothelin-1 (ET-1, Novabiochem AG, Läufelfingen, Switzerland), RPMI 1640 culture medium, Dulbecco's Eagle medium (Cat. No. 11880) and penicillin–streptomycin solution was purchased from Gibco BRL, Life Technologies A.G., Basel, Switzerland.

ET-1 was dissolved in 0.1% acetic acid and diluted to give a 0.1 mM solution containing 0.01% acetic acid. Samples of ET-1 (10 µM) were stored at $-20\,°C$ until use. Further dilutions were made in physiologic salt solution. Drug concentrations are given as molar concentrations throughout.

Data analysis

Concentration-response curves were analyzed by means of a curve-fitting computer program in RS/1 (BBN Software Products Corporation, Cambridge, Mass., USA) and parameters for efficacy (E_{max} = maximal effects) and drug affinity (pD_2 values = negative logarithm of the concentrations of the agonists producing 50% of E_{max}) were derived from this analysis. Where appropriate, statistical analysis of data was performed using one-way analysis of variance (ANOVA) followed by the Bonferroni-corrected t-test to assign differences to individual between-group comparisons when overall significance ($P < 0.05$) was attained. Data are given as means ± SEM.

Results

Equilibration experiments

Exposure of venous rings to the Me_2SO-containing cryomedium for 10–240 min without subsequent freezing did not significantly modify contractile responses of the venous smooth muscle (Fig. 1, left). If, however, equilibration with the cryomedium was followed by freezing, maximal post-thaw responses to noradrenaline of these veins were significantly diminished. Pre-freezing equilibration with the cryomedium for up to 120 min reduced post-thaw maximal responses to noradrenaline by 34–44% ($P < 0.05$) whereas equilibration with the cryomedium for 240 min attenuated contractile responses to noradrenaline by 67% compared to that obtained with unfrozen controls ($P < 0.001$, Fig. 1, right). Similar results were obtained when ET-1, the contractile effect of which is mediated through ET_A-receptors [14] was used as the agonist (not illustrated) and the same was true for contractile responses to depolarisation by potassium chloride. Pre-freezing equilibration with the cryomedium for 10–

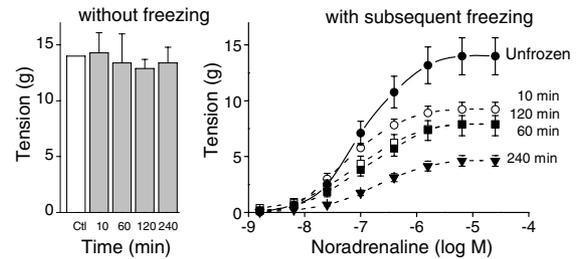Contractile responses to noradrenaline after exposure to cryomedium

Fig. 1. Left, maximal responses to noradrenaline of rings from unfrozen human saphenous veins after 10, 60, 120 and 240 min exposure to the cryomedium (KH-solution containing 1.8 M Me_2SO and 0.1 M sucrose) at room temperature. The bars represent means ±SEM from five individual experiments. Right, concentration-response curves for noradrenaline on rings from human saphenous veins from unfrozen (—) and cryopreserved (- - -) veins after pre-freezing equilibration for 10, 60, 120 and 240 min with the cryomedium at room temperature. Thawing was performed at 15 °C/min. The bars represent means ± SEM from six individual experiments.

120 min diminished responses to KCl by about 40% ($P < 0.05$), the contractile–response curves being nearly superimposable, equilibration for 240 min, however, attenuated maximal depolarisation-induced contraction by 67% of that produced by unfrozen controls ($P < 0.001$, Fig. 2).

Freezing at different cooling rates

Concentration–response curves to NA revealed that optimal preservation of the contractile function was obtained when human SV were frozen at a mean cooling speed of about -0.6 to $-1.2\,°C/min$. Increasing the cooling speed attenuated progressively the post-thaw contractile function (Fig. 3).

Thawing experiments

Temperature changes, recorded inside the lumen of a human SV during three different thawing protocols giving

Contractile responses to potassium chloride after exposure to the cryomedium
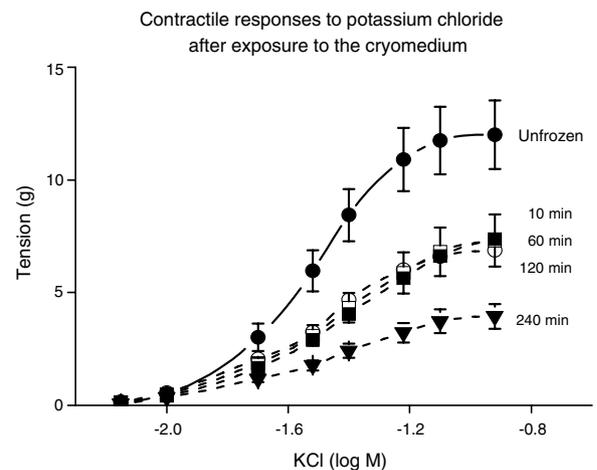

Fig. 2. Concentration-response curves for KCl on rings from human saphenous veins cryopreserved after different prefreezing equilibration times of 10, 60, 120 and 240 min with the cryomedium at room temperature. Thawing was performed slowly at 15 °C/min. The bars represent means ± SEM from six individual experiments.
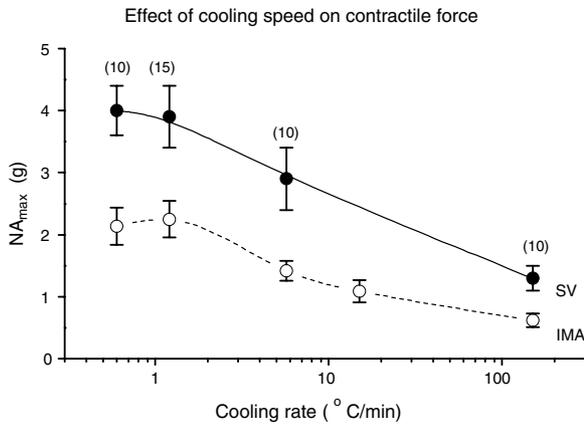
Fig. 3. Maximal responses to noradrenaline of human saphenous veins (SV, number of determinations in brackets) and internal mammary arteries (IMA, data taken from reference [20]) following cryopreservation at different cooling rates. All samples were equilibrated for 10 min with the cryomedium before starting the cooling process. The bars represent means ± SEM.

mean thawing speeds of 100, 30 and 15 °C/min, are shown in Fig. 4, left. Concentration–response curves to noradrenaline (Fig. 4, right) and endothelin-1 (not illustrated) revealed no significant changes of the contractile responses following fast or slow thawing, though post-thaw contractile force proved to be slightly higher in samples thawed slowly at a mean thawing rate of 15 °C/min.

Post-thaw removal of the cryomedium

Post-thaw removal of the cryomedium by dilution improved significantly contractile responses to noradrenaline and endothelin-1 by 26% and 20%, respectively compared to veins transferred directly from the cryomedium into KH solution. At the same time the pD_2 values were significantly reduced becoming similar to unfrozen control values (Table 1). Additional post-thaw incubation of venous rings for 24 h in Dulbecco's Eagle medium at 37 °C

revealed a small but significant improvement of the maximal contractile responses to noradrenaline by 15% ($P < 0.01$, not illustrated).

Comparison of various vehicle solutions

To test the effects of various modifications of the cryomedium venous rings were cryostored either in FCS-sucrose (fetal calf serum containing 1.8 M Me$_2$SO and 0.1 M sucrose), KH-trehalose medium (KH-solution containing 1.8 M Me$_2$SO and 0.1 M trehalose), in DEM-medium (Dulbecco's modified Eagle medium containing 1.8 M Me$_2$SO and 0.1 M sucrose) or in RPMI-medium (RPMI 1640 culture medium containing 1.8 M Me$_2$SO and 0.1 M sucrose). In these experiments SV rings from the same patients, cryopreserved in KH-sucrose medium, served as control preparations. After thawing responses to noradrenaline (Table 2) and endothelin-1 (Table 3) revealed that the use of RPMI-sucrose medium improved the post-thaw contractile responses to both noradrenaline and ET-1 significantly by more than 50 % ($P < 0.01$). In addition, the use of RPMI-sucrose medium improved the affinity for noradrenaline, as indicated by the pD_2 values. By contrast, contractile responses were diminished by 23% when FCS was used as the vehicle (Table 2).

Table 1
Effect of post-thaw dilution of the cryomedium

	Frozen/thawed diluted	Frozen/thawed undiluted	Unfrozen
E_{max} (g)			
Noradrenaline	8.7 ± 0.3[#, *]	6.9 ± 0.6[#]	14.0 ± 1.7
Endothelin-1	9.8 ± 0.3[#, *]	8.2 ± 0.6[#]	14.3 ± 1.3
Affinity (pD$_2$ values)			
Noradrenaline	7.09 ± 0.09[*]	7.38 ± 0.06[#]	6.99 ± 0.06
Endothelin-1	8.15 ± 0.08[**]	8.85 ± 0.13[#]	8.39 ± 0.14

Difference against values determined on unfrozen veins significant at [#]$P < 0.05$, against values determined on frozen/thawed undiluted veins significant at [*]$P < 0.05$, [**]$P < 0.001$. For each group $n = 6$ rings from the same vein of an organ donor. Data are presented as means ± SEM.

Table 2
Post-thaw parameters for noradrenaline on human SV frozen in various cryomedia

Medium	n	Test medium	Control medium (KH sucrose)	Test medium (% of control)
E_{max} (g)				
FCS-sucrose[a]	10	3.3 ± 0.6	4.3 ± 0.5	77
KH-trehalose	6	2.8 ± 0.2	3.1 ± 0.3	90
DEM-sucrose	6	3.5 ± 0.5	3.1 ± 0.6	113
RPMI-sucrose	6	6.2 ± 0.7[b]	3.7 ± 0.4	168
Potency (pD$_2$ values)				
FCS-sucrose[a]	10	6.52 ± 0.18	6.69 ± 0.14	
KH-trehalose	6	7.21 ± 0.11	7.13 ± 0.07	
DEM-sucrose	6	6.12 ± 0.12	6.31 ± 0.11	
RPMI-sucrose	6	7.35 ± 0.09[b]	7.08 ± 0.05	

[a] Data from reference [31].
[b] Difference from controls in KH-sucrose medium significant at $P < 0.01$. Data are presented as mean ± SEM.
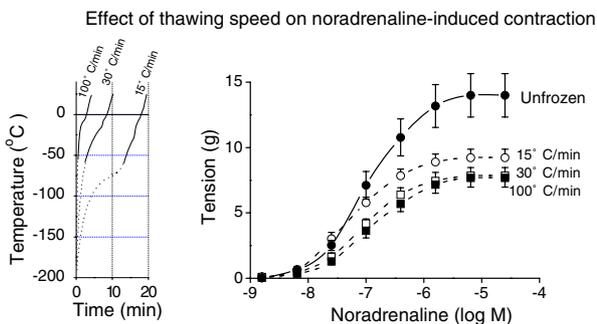

Fig. 4. Left, temperature changes recorded within the lumen of a human saphenous vein during thawing at different warming speeds. Each curve represents the mean of four recordings (single lines), extrapolated to −190 °C (dotted lines). Right, concentration–response curves for noradrenaline on rings from human saphenous veins without (—) and after cryopreservation (- - -). Venous rings were cryopreserved after 10 min incubation in KH cryomedium at room temperature and thawed at 100, 30 and 15 °C/min. The bars represent means ± SEM from six individual experiments.

Table 3
Post-thaw parameters for endothelin-1 on human SV frozen in various cryomedia

Medium	Test medium	Control medium (KH sucrose)	Test medium (% of control)
E_{max} (g)			
KH-trehalose	4.4 ± 0.3	4.9 ± 0.4	90
DEM-sucrose	4.6 ± 0.4	4.4 ± 0.6	105
RPMI-sucrose	7.9 ± 0.7[a]	5.1 ± 0.5	155
Potency (pD$_2$ values)			
KH-trehalose	8.51 ± 0.05	8.53 ± 0.03	
DEM-sucrose	8.06 ± 0.08	8.05 ± 0.16	
RPMI-sucrose	8.59 ± 0.04	8.44 ± 0.08	

[a] Difference from controls in KH-sucrose medium significant at $P < 0.05$. Data are presented as mean ± SEM, for each group $n = 6$.

Long-term storage in liquid nitrogen

In this series of experiments samples from human SV of the same organ donor were cryostored after 10 min equilibration with the cryomedium (KH-solution containing 1.8 M Me$_2$SO and 0.1 M sucrose) and thawed at 15 °C/min before being investigated in vitro after storage for up to 10 years. Data revealed that the contractile function remained well preserved being nearly unchanged during this observation period (Fig. 5).

Discussion

The present findings on human saphenous veins (SV) are in line with the observation that cryopreservation usually attenuates post-thaw functional activity of vascular grafts. Significant factors contributing to this attenuation might be (1) the toxicity of the Me$_2$SO-containing cryomedium and (2) the rate of freezing and thawing vascular samples. Due to the formation of interstitial ice crystals, freezing of living mammalian cells without any cryoprotective additive induces severe cell injury and only few cells survive [16]. Previous experiments on canine [17] and human SV [24]
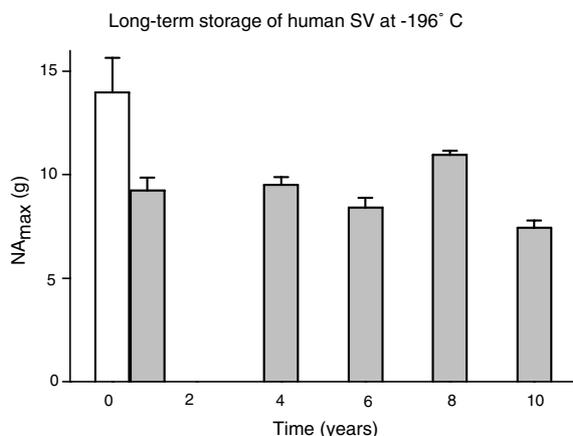

Fig. 5. Maximal responses to noradrenaline expressed in g of rings from human saphenous veins after different periods of cryostorage. Each bar indicates mean values of six rings from the same organ donor.

have shown that freezing in a medium containing 1.5–2.5 M of the permeating cryoprotectant Me$_2$SO [26] provides optimal preservation of the post-thaw contractile function. Further experiments on various animal blood vessels revealed that addition of the nonpermeating cryoprotectant sucrose to the Me$_2$SO-containing cryomedium improves the preservation of both contractile and relaxant activity of frozen/thawed vascular smooth muscle [19]. It has also been shown that for optimal post-thaw functional activity of human tissues such as bronchi [21], mesenteric and coronary arteries [22] and human SV [31] fetal calf serum (FCS), not even as additive to the cryomedium is required. Therefore, the experiments of the present study were performed in cryomedia containing no FCS. Comparing the protective effect of various solutions, optimal post-thaw contractile function of human SV was obtained when RPMI 1640 culture medium was used as vehicle for the cryomedium. This finding is supported by the observation that cell damage in cryostored human SV as assessed by morphological and immunochemical analysis is less intense when RPMI is used instead of M199 medium as vehicle solution for the cryomedium [15].

Exposure of an isolated blood vessel to a solution containing up to 15% Me$_2$SO without freezing produces no changes in contractile function neither in animal arteries, e.g., arteries from rabbits [34] and minipigs [25] nor in human internal mammary arteries [23] or human SV as demonstrated in the present study. Significant attenuation of the contractile function, however, occurred when exposure to the cryomedium was followed by exposure to sub-zero temperatures. Similar observations have been made with human internal mammary arteries [23]. In both human SV and internal mammary arteries pre-freezing exposure to the cryomedium for up to 120 min produced nearly the same reduction in contractility suggesting that this loss in contractile force was induced by the freezing and/or thawing process rather than by Me$_2$SO toxicity. Further experiments, however, are required to elucidate the mechanism causing the additional reduction of contractile force observed after prefreezing exposure for 240 min to the cryomedium.

Previous studies have demonstrated that prefreezing incubation of canine femoral arteries for up to 360 min in a medium containing 1.8 M Me$_2$SO and 0.1 M sucrose causes progressive reduction with time of exposure of both contractile and relaxant activities of the frozen/thawed arteries [18]. In those experiments optimal recovery of the functional activity was obtained when arteries had been equilibrated for only 10 min with the cryomedium before starting the cooling process while extension of the prefreezing incubation period attenuated progressively both contractile and relaxant activity. By contrast, the present study revealed that prefreezing exposure of human SV to the Me$_2$SO-containing cryomedium for up to 120 min produced nearly the same reduction in contractile responses. These data suggest, therefore, that human vascular tissues appear to be less susceptible to Me$_2$SO toxicity than animal tissues are.

Additional determinants of cell damage are the rates of cooling and thawing. Using detection of fragmented DNA as measure of smooth muscle damage Ruddle et al. [29] demonstrated that freezing of human SV in the presence of Me_2SO at $-1\,°C/min$ produces the same degree of cell damage as observed with fast freezing at $-6\,°C/min$. By contrast, the present study revealed that the post-thaw contractility of SV is reduced by about 25% when the cooling rate is increased from -1.2 to $-5.7\,°C/min$. Similarly, fast cooling at about $-5.7\,°C/min$ attenuates contractile responses of human internal mammary arteries by about 35% of that produced by arteries frozen at about $-1.2\,°C/min$ [20].

The main tissue damaging mechanism during slow freezing and rewarming is believed to be the formation and recrystallization of extracellular ice. To overcome these problems a new ice-free cryopreservation method, namely vitrification has been shown to provide significant benefit in functional studies with rabbit jugular veins [3,32,33] and even whole organs such as rabbit kidney [8]. In addition to several technical problems, however, successful vitrification of large pieces of tissue and organs requires high concentrations of cryoprotectants the tolerable limits of which will most likely be insufficient to produce the required glassy state and prevent the development of ice nuclei during cooling [8,36].

Clinical observations [35] and experiments with animal vessels [5,12,27] revealed that rapid thawing of frozen tissues can induce fractures in the vascular wall. While this phenomenon may be of less relevance if tissues are used for pharmacological experiments it can provide serious problems to the surgeon. However, in the present study different thawing protocols ranging over 100, 30 and $15\,°C/min$ on human SV, revealed very little modification of the post-thaw contractile recovery. These findings are supported by the observation that the amount of fragmented DNA in venous smooth muscle cells is not influenced by the rate of warming when samples of human SV are frozen in 10% Me_2SO [29].

In summary, the present data revealed that human venous tissues appear to be less susceptible to exposure to a Me_2SO-containing medium than animal tissues are. Optimal functional recovery of frozen/thawed human SV was obtained with venous grafts that had been immersed for 10 min in RPMI solution containing 1.8 M Me_2SO and 0.1 M sucrose, slowly frozen at a mean cooling rate of about $-1\,°C/min$ and thawed slowly at a mean warming rate of $15\,°C/min$. Under these conditions storage of living grafts for more than 10 years was possible.

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