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Cryopreservation procedure does not modify human carotid homografts mechanical properties: an isobaric and dynamic analysis

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Abstract

The viscoelastic and inertial properties of the arterial wall are responsible for the arterial functional role in the cardiovascular system. Cryopreservation is widely used to preserve blood vessels for vascular reconstruction but it is controversially suspected to affect the dynamic behaviour of these allografts. The aim of this work was to assess the cryopreservation's effects on human arteries mechanical properties. Common carotid artery (CCA) segments harvested from donors were divided into two groups: Fresh ($n = 18$), tested for 24–48 h after harvesting, and Cryopreserved ($n = 18$) for an average time of 30 days in gas-nitrogen phase, and finally defrosted. Each segment was tested in a circulation mock, and its pressure and diameter were registered at similar pump frequency, pulse and mean pressure levels, including those of normotensive and hipertensive conditions. A compliance transfer function (diameter/pressure) derived from a mathematical adaptive modelling was designed for the on line assessment of the arterial wall dynamics and its frequency response. Assessment of arterial wall dynamics was made by measuring its viscous (η), inertial (M) and elastic (E) properties, and creep and stress relaxation time constant (τ_C and τ_{SR}, respectively). The frequency response characterization allowed to evaluate the arterial wall filter or buffer function. Results showed that non-significant differences exist between wall dynamics and buffer function of fresh and cryopreserved segments of human CCA. In conclusion, our cryopreservation method maintains arterial wall functional properties, close to their fresh values.

Abbreviations: BF – arterial buffering function; CF – arterial conduit function; ARX – autoregressive with exogenous input model; CCA – common carotid arteries; τ_C – creep time constant; f_C – cut-off frequency; E – elastic index; ECMM – esternal cleido mastoid muscle; M – inertial index; τ_{SR} – stress relaxation time constant; η – viscous index

Introduction

It is known that large arteries have two distinct but interrelated major functions: (a) to be low resistance blood distribution conduits to deliver an adequate supply of blood to the peripheral organs, named conduit function (CF), and (b) to smooth out the pressure and flow pulsatility due to intermittent ventricular ejection, in order to transform it into an almost continuous arteriolar-capillary flow and pressure, named buffering function (BF) (Bia et al. 2003, 2005). Additionally, the BF exerts a protective arterial wall action (Pontrelli and Rossoni 2003). The efficiency of CF and BF depends on viscoelastic properties of arterial wall tissues (i.e. elastin and collagen fibres, smooth muscle cells) and the geometric (i.e. diameter, length) characteristics of the arterial segments.

At date, it is accepted that, when an arterial segment is replaced, the ideal arterial graft used for arterial bypass and/or reconstruction, must share identical geometric and viscoelastic properties with the host artery (Seifalian et al. 2002). In order to reduce the geometrical and mechanical-mismatch between prosthetic graft and native artery, and thereby to improve the long-term graft patency rates, different alternatives have been developed. In this context, cryopreserved vessels are an interesting option as arterial substitutes for vascular reconstruction (Buján et al. 2001; Pascual et al. 2002), and the use of vascular allograft tissues from banks is increasing (Buján et al. 2001). Cryopreservation has the advantage of long-term storage of arteries, but the consequences of cryopreservation (regarding the acceptance and performance of these arteries when they are used as allografts), is unclear (Vischjager et al. 1996).

Arterial wall dynamic and mechanical properties, can be assessed by analysing the pressure-diameter loop relationship (Bia et al. 2003, 2004, 2005). In a pressure-diameter analysis, the arterial wall shows (a) a non-linear behaviour that can be associated with a pressure-volume exponential response and (b) viscous and inertial mechanical properties (Armentano et al. 1995a; Bia et al. 2004, 2005). Therefore, in order to perform an appropriate mechanical comparison between vascular and/or graft conduits, it is necessary an isobaric, isofrequency and dynamic study, where physiological pressure signals are applied to arterial or graft segments (Armentano et al. 1995a; Bia et al. 2003, 2004). To our knowledge, an isobaric, isofrequency and dynamic mechanical comparison between cryopreserved and fresh human carotid arteries has not been reported.

The aim of this work was to analyse the capacity of the cryopreservation method used in our tissue bank, to preserve the vascular viscoelasticity and inertia in arteries that will be transplanted as vascular allografts.

The dynamics of fresh and cryopreserved/defrosted arteries (analysed in an *in vitro* system) were compared, at identical pressure and frequency levels. The comparison includes a frequency domain analysis and the assessment of the biomechanical parameters commonly used in the arterial function characterization studies.

Material and methods

In vitro pressure and diameter registration of fresh and cryopreserved/defrosted common carotid artery (CCA) segments obtained from multiorgan donors were employed:

Bullet Group I ($n = 18$): fresh segments.
Bullet Group II ($n = 18$): cryopreserved/defrosted segments.

Donor criteria selection

All procedures of vascular tissue procurement and processing took into account ethical and safety concerns for therapeutic use, and include consent documentation according to legal rulers N° 14005 and N° 17668 of República Oriental del Uruguay. General exclusion criteria were taken in agreement with International Atomic Energy Agency (IAEA, International Standards for Tissue Banks). Particular exclusion criteria for vascular tissue procurement was taken in consonant with AEBT (Estándares de la Asociación Española de Bancos de Tejidos 2nd Ed, 2002, 83–89). Donors age was 23–45 years (Mean = 29.4 years).

Tissue procurement

The CCA used in this work for investigation propose, were procured with chirurgical aseptic

technique during multiple organ and tissue harvesting, under transplant program of Banco Nacional de Organos y Tejidos (BNOT) of República Oriental del Uruguay (Alvarez et al. 2003), from 18 donors in brain death condition.

Aseptic cleaning on carotidal region with 7% povidone-iodine solution was made after aortic clamp and cardiac arrest. Sterile drapes were used to establish chirurgical field. Skin incision was made following anterior border of esternal cleido mastoid muscle (ECMM). Carotid was carefully cleared from the surrounding tissues after removing laterally the ECMM with forceps. Measurement was taken in anatomical position, from proximal situation of skin incision behind ECMM, until distal bifurcation of CCA and then a 5 cm length segment of the artery was cut and removed. In each donor after harvesting, both CCA (right and left) were washed with saline solution and stored at 4 °C. The warm ischemia was 55–66 min (Mean = 60.2 min). The cold ischemia was 24–48 h (Mean: 36 h). Arteries to be used as fresh-controls were sent to Vascular Physiology Laboratory to biomechanical test.

Experimental design, cryopreservation, and thawing

Carotids used as cyopreserved/defrosted probands (Group II) were immersed during 30 min in a final volume of 85 cc cryopreservant solution at 20 °C, with Culture Medium (RPMI 1640): 85%; Human Albumin Solution (20%): 5%; and Dimethilsulphoxide: 10%, in a cryo resistant bag (Joisten and Kettenbaum, D51429, Bereisch Gladbach, Mod.011342) sealed with thermal machine, in a laminar flow cabinet (Microflow, Laminar Flow Work Station, MDH Ltd, Wal Worth Road Andover Hants England SP.10.5.AA) (Fahy et al. 1987, 1990). Programmed cryopreservation was carried out in a Controlled Rate Freezing System (Model 9000, Gordinier Electronics, Inc. 29975 Parkway, Roseville, Michigan 48066 USA).

Related to the cooling rate we chose a modified protocol from Pegg et al., which was made on rabbit's CCA (Pegg et al. 1997). Our protocol consisted in two operative time steps. First, a slow programmed cooling rate with a mean value of 1 °C/min until −90 °C. Second, a rapid cooling rate obtained by the immediate transference of the bag to the gas phase of liquid nitrogen compartment (−142 °C). The frozen arterial specimens were stored for 30 days at −142 °C (Mark III, Temperature and Liquid Level Controller, Taylor, Wharton, Theodore, Alabama USA).

After the storage period the vessels were defrosted. In our standard warming time protocol a two stage processing model was made; also taking into account Pegg et al. work, but modifying their protocol (Pegg et al. 1997). The warming referential protocol highlights the importance of the thawing rate, in order to avoid the fractures (Hunt et al. 1994; Wassenaar et al. 1995; Pegg et al. 1997). The first step in our thawing protocol was a slow warming process, achieved by transferring the bag from the nitrogen gas phase to the room temperature (20 °C) during 30 min. Then the bag was removed and rapid warming by transferring it to a 40 °C water bath until completely defrost. To prevent cellular damage, once the segments had thawed, the cryoprotectant was gradually removed in four 10 min-steps by immersion in tapered concentrations (10, 5, 2.5, and 0% of DMSO), at +20 °C. The total time of the removal of the cryoprotectant was 40 min (Fahy et al. 1987). Finally, the cryopreserved/defrosted arteries were sent in RPMI 1640 bath to the Vascular Physiology Laboratory to biomechanical test.

In vitro mechanical tests

In order to perform the mechanical tests, each segment (fresh or cryopreserved/defrosted) was non-traumatically mounted on specifically designed cannulas of the flow circuit loop (in vitro system; see Figure 1) and immersed and perfused with a thermally regulated (37 °C) and oxygenated Tyrode's solution, with pH = 7.4 (Fischer et al. 2002). The *in vivo* arterial length was preserved during *in vitro* tests. The perfusion line consisted in polyethylene tubing and a Windkessel chamber powered by a pneumatic pump (Jarvik Model 5, Kolff Medical Inc., Salt Lake City, Utah, USA). The pneumatic device was regulated by an air supply machine that allowed fine adjustments of the pump rate, pressure values and waveforms (Fischer et al. 2002).

To measure arterial pressure, each segment was instrumented with a pressure micro transducer

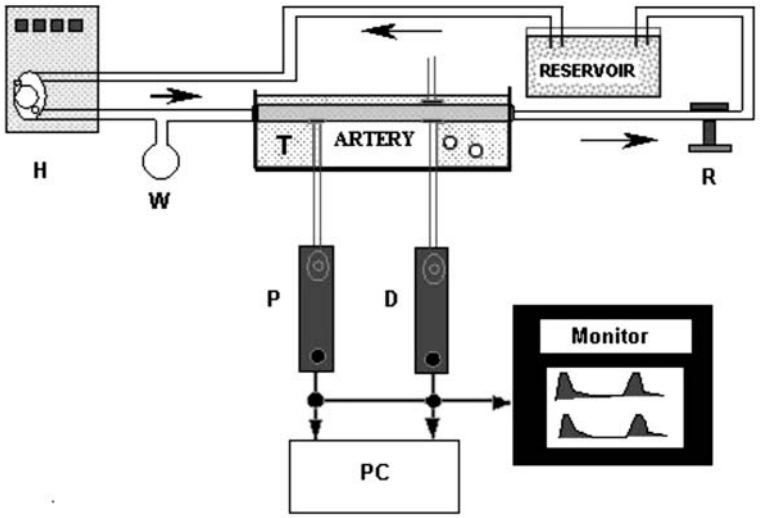

Figure 1. In vitro system. H: Pneumatic pump for the perfusion line. W: windkessel chamber. R: resistances modulator. T: thermally regulated chamber containing Tyrode's solution at 37 °C. Reservoir: contains Tyrode with oxygen bubbles at 37 °C. P: pressure signals. D: diameter signals. W, R and the air supply were used to perform necessary adjustments. Large arrows indicate flow directions.

(1200 Hz frequency response, Konigsberg Instruments, Inc., Pasadena, CA, USA) inserted in the vessel through a stab wound. To measure arterial external diameter a pair of ultrasonic crystals (5 MHz, 2 mm diameter) was sutured to the adventitia of the artery. The transit time of the ultrasonic signal (1580 m/s) was converted into distance by means of a sonomicrometer (1000 Hz frequency response, Triton Technology Inc. San Diego, CA, USA). Optimal positioning of the dimensional gauges was assessed by an oscilloscope (model 465B, Tektronix). Each pressure transducer had been previously calibrated using a mercury manometer.

Experimental protocol

Once placed in the organ chamber and instrumented, the arterial segments were allowed to equilibrate for a period of 10 min under a steady state of flow and mean pressure of 83 ± 7 mmHg. Fresh and cryopreserved/defrosted CCA's pressure and diameter signals were recorded and stored during an unique steady state, at similar mean and pulse pressure levels selected in order to cover commonly human normotensive and hypertensive pressure ranges (Armentano et al. 1995b). In all cases the pump frequency or stretching rate was fixed at similar level of human heart rate, and the pump, windkessel chamber and the tubing resistance were regulated in order to generate adequate pressure waveforms (Fischer et al. 2002).

In vitro data collection

External diameter and internal pressure were continuously measured under dynamic conditions, displayed in real time, digitised every 5 ms (12 bits) and stored for off-line analysis. In each state condition, approximately 20–30 consecutive beats were sampled and analysed.

Data analysis

A computerised procedure was used to determine the pressure-diameter loop and to calculate the mechanical parameters using an original system developed in our laboratory (Armentano et al. 1995a; Gamero et al. 2001). Pressure and diameter waveforms were shifted to correct the distance between pressure and diameter sensors. A linear autoregressive with exogenous input model (ARX) was used to fit the data. The general discrete time ARX model is given by:

$$D[k] = -\sum_{j=1}^{n_y} a_j D[k-j] + \sum_{i=0}^{n_x} b_i P[k-i] \qquad (1)$$

where D is the estimated diameter, P the arterial pressure, a_j and b_i are the coefficients to be estimated from the measured data. The value of n_x and n_y defines the model order. The ARX model was applied to the input–output (pressure-diameter) data to assess for the arterial system dynamics. In this way, the arterial diameter is estimated from the input of the model using the identified coefficients a_j and b_i. The parameters for each model fit were estimated using the least square algorithm (Ljung 1999). As a special case of the general model (1), we considered the general third order model. This model was chosen as the mean order model using the Akaike information criterion (AIC) statistic (Akaike 1969) over the whole population. Considering the estimated coefficient $\{a_j,\ j=1,\ldots,3\}$ and $\{b_i,\ i=0,\ldots,3\}$ the general transfer function in the z-transform domain is:

$$H(z) = \frac{D(z)}{P(z)} = \frac{b_0 + b_1 z^{-1} + b_2 z^{-2} + b_3 z^{-3}}{1 + a_1 z^{-1} + a_2 z^{-2} + a_3 z^{-3}} \qquad (2)$$

The inverse bilinear transformation was applied to (2) in order to obtain a continuous transfer function (from the z-plane to the s-plane):

$$H_1(s) = \frac{m_3 s^3 + m_2 s^2 + m_1 s + m_0}{n_3 s^3 + n_2 s^2 + n_1 s + n_0} \qquad (3)$$

The third order model, within the physiological range, does not present differences if the highest frequency pole and zero are eliminated (m_3 and n_3). The model reduction is then:

$$H_2(s) = \frac{m_2 s^2 + m_1 s + m_0}{n_2 s^2 + n_1 s + n_0} \qquad (4)$$

From this model, creep time constant (τ_C) was derived from the dominant polo and stress relaxation time (τ_{SR}) was calculated from the first zero. Both constant (τ_C and τ_{SR}) allows the evaluation of arterial wall viscoelasticity.

In order to evaluate the arterial wall buffer or filtering function, the cut-off frequency (f_C), was computed at -3 dB point, from the 3rd order approximation of $H_1(s)$ (3).

To give physiological meanings to the coefficients, the zeros were discarded:

$$H_3(s) = \frac{m_0}{n_2 s^2 + n_1 s + n_0} \qquad (5)$$

In the continuous time domain, the model is better understood and a physical meaning can be given to the model coefficients. These coefficients are related to a linear time invariance ordinary differential equation. Assuming a second order differential equation to characterise the wall dynamics, the frequency response would be (Peterson et al. 1960):

$$H_3(s) = \frac{D(s)}{P(s)} = \frac{1}{Ms^2 + \eta s + E} \qquad (6)$$

From (5) and (6) we resume the arterial wall elastic index ($E = n_0/m_0$), viscous index ($\eta = n_1/m_0$) and inertial index ($M = n_2/m_0$). Figure 2 shows the relationship between (3), (4) and (5) that reinforces our wall transfer function approximation. In this figure, we can observe the frequency response of the ARX model given by (3) i.e. $H_1(\omega)$, frequency response of the second order system $H_2(\omega)$ and frequency response of the fresh (Group I) arterial wall $H_3(\omega)$.

Statistical analysis

All values were expressed as mean values ± standard deviation (MV ± SD). Model order selection was obtained using the Akaike information criterion (Akaike 1969). To evaluate the nature of the error of the ARX model fitting procedure, correlation and residual analysis was performed. Frequency response comparisons between fresh and criopreserved arteries, were made at each physiological frequency level, using a two tailed unpaired student t-test. The hemodynamic and mechanical data comparisons were performed using a two-tailed unpaired Student t-test. To indicate significant differences between results a $p < 0.05$ threshold was adopted.

Results

Physiological waveforms and pressure levels were simulated in the mock system, and chosen in order to cover normotensive and hypertensive pressure ranges. Table 1 shows the pressure and diameter values registered in the *in vitro* experiments. Note that both groups were tested with similar pressure

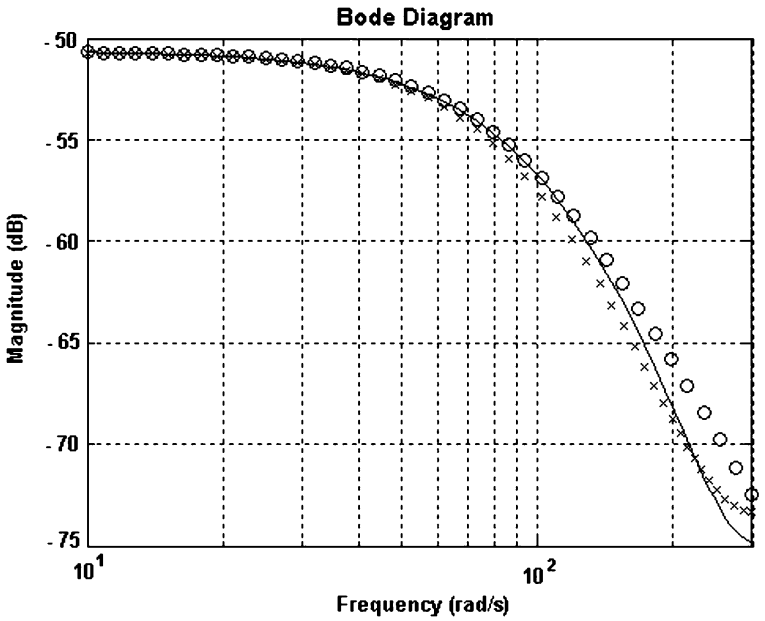

Figure 2. Solid line: absolute Bode plot of the third order approximation $H_1(\omega)$. Circle line: absolute Bode Plot of the second order approximation $H_2(\omega)$. Cross line: absolute Bode plot of the physiological model $H_3(\omega)$.

Table 1. Haemodynamic parameters.

	Group I ($n=18$)	Group II ($n=18$)
Systolic pressure (mmHg)	140.67 ± 4.60	139.20 ± 5.76
Diastolic pressure (mmHg)	69.00 ± 7.39	69.93 ± 5.77
Mean pressure (mmHg)	101.32 ± 2.66	100.83 ± 4.84
Mean diameter (mm)	9.15 ± 1.47	10.06 ± 1.54
Pump rate (Hz)	1.10 ± 0.20	1.10 ± 0.10

Mean ± SD values. Group I: fresh arteries. Group II: Cryopreserved/defrosted arteries.

and frequency levels, ensuring an isobaric and isofrequency analysis.

An adaptative procedure was developed to fit pressure and diameter data in the time domain. An initial third order model was chosen, and a low pass response was observed in the transfer function, representing the compliance transfer function response of the arterial wall in frequency domain (Figure 2).

Figure 3 shows the frequency response of the compliance transfer function $H_1(\omega)$ for fresh arteries with their confidence interval. Note that the cryopreserved/defrosted arteries rely within that range showing no significant differences.

The arterial wall can be represented by three main indexes that characterize its elastic, viscous and inertial behaviour respectively. Accordingly, a second order reduction was tested to reduce the order model, $H_2(s)$. Finally, the numerator coefficients were neglected to preserve the physiological correlation of its second order differential equation, $H_3(s)$. As can be seen in Figure 2, the frequency response of the three transfer functions at each frequency level, did not show differences in the physiological ranges ($p < 0.01$).

Table 2 shows the biomechanical parameters obtained during experimental conditions. Note that after cryopreservation, non significant differences between fresh and cryopreserved/defrosted biomechanical parameters were evidenced. Creep and relaxation time constants, τ_C and τ_{SR}, respectively showed higher deviation values than the other observed parameters.

Discussion

At date, as far as arterial transplantation is concerned, cryopreservation is an usual method to preserve allografts for a while. Besides, vessel reconstruction with cryopreserved segments has increased (Buján et al. 2001). However, some clinical observations reported cases of mechanical

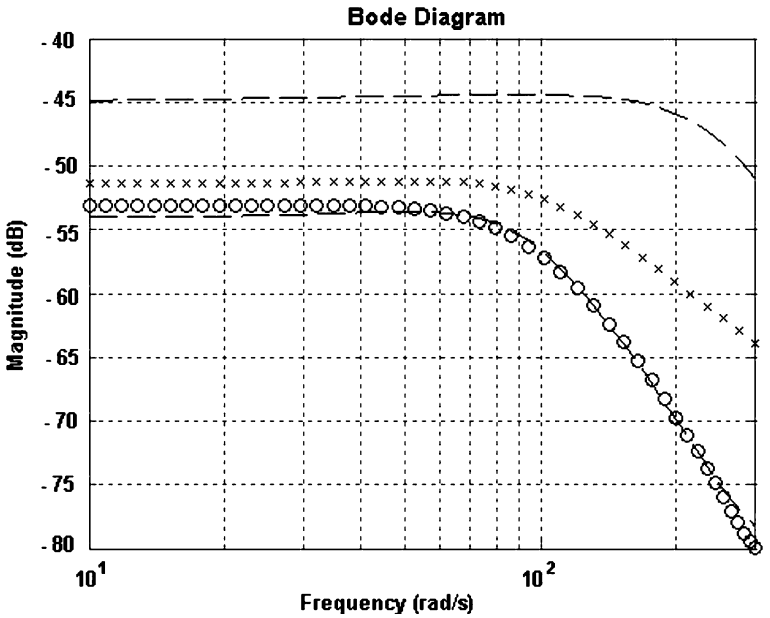

Figure 3. Cross Line: Mean third order model $H_1(\omega)$ for fresh arteries. Dot Line: confidence intervals for $H_1(\omega)$. Circle line: Mean cryopreserved third order model $H_1(\omega)$. Cryopreserved frequency response was placed inside the fresh (95%) confidence interval.

Table 2. Biomechanical parameters.

	Group I ($n=18$)	Group II ($n=18$)
E (mmHg/mm)	314.53 ± 33.24	305.18 ± 33.94
η (mmHg.s/mm)	6.12 ± 0.67	6.53 ± 1.11
M (10^{-2})(mmHg.s^2/mm)	3.37 ± 0.67	3.37 ± 0.71
$\tau_C(10^{-2})$ (s)	1.40 ± 0.24	1.52 ± 0.17
$\tau_{SR}(10^{-3})$ (s)	5.07 ± 1.14	4.95 ± 1.08
f_C(Hz)	11.10 ± 0.92	10.43 ± 0.87

Mean $\pm$ SD values. E, η and M: elastic, viscous and inertial indexes; τ_C and τ_{SR}: creep and stress relaxation time constants; f_C: cut-off frequency. Group I: fresh arteries. Group II: Cryopreserved/defrosted arteries.

modifications of allografts (i.e. fissuring, rupture, aneurysm) (Lehalle et al. 1997). Whether the cryopreserved procedures modify viscoelastic and inertial wall properties and geometrical arterial properties remains controversial (Blondel et al. 2000; Buján et al. 2001).

In this context, the purpose of this work was to characterize, to compare and to analyse, in isobaric, isofrequency and dynamic ways, the effects of our cryopreservation protocol on the human CCA wall dynamics. Assessment of arterial wall dynamics was made by measuring its viscous (η), inertial (M) and elastic (E) properties, and creep and stress relaxation time constant (τ_C and τ_{SR}, respectively). The frequency response characterization allowed to evaluate the arterial wall filter or buffer function performance. This study provides a complete description of biomechanical behaviour of CCA, with and without cryopreservation effects (Fresh vs. Cryopreserved/defrosted).

Methodological design

In vitro test

In order to perform an appropriate mechanical comparison between fresh and cryopreserved arterial segments, several aspects should be taken into account. *First*, to reproduce the *in vivo* haemodynamic conditions, and to preserve the shape and integrity of arterial wall it might be better to analyse the arterial mechanical properties in a dynamic way using vascular segments, instead of the currently used rings or blades (Adham et al. 1996; Vischjager et al. 1996). *Second*, although most works related to cryopreservation concern on the endothelial and/or smooth muscle viability/reactivity (Vischjager et al. 1996; Rendal et al. 2004), an adequate characterization requires the evaluation of the viscous, inertial, and elastic properties of the arterial wall, which are the main determinants of CF and BF (Armentano et al. 1995a; Bia et al. 2005). Related to this, we have

proposed a characterization of the arterial mechanic behaviour on the basis of invasive or non invasive recordings of pressure and diameter waveforms (Armentano et al. 1995a, b). An improved parametric modelling-identification on line approach, taking into account the viscoelastic and inertial properties and assessing simultaneously the arterial frequency response, was further developed by our group (Gamero et al. 2001). *Third*, the non linearity of the arterial pressure-diameter relationship and the frequency-dependent properties of the arterial wall, make necessary an isobaric, isofrequency and dynamic analysis, in order to perform an appropriate mechanical comparison (Armentano et al. 1995a; Bia et al. 2003, 2004). Therefore in this work, we evaluate the viscous, inertial and elastic properties of the arterial wall, doing an isobaric, isofrequency, dynamic, and pressure-diameter test on fresh and cryopreserved/defrosted arterial segments.

Cryopreservation and thawing protocol

An optimal cryopreserved arterial conduit should have anatomic and physiologic characteristics similar to those of the fresh artery. During arterial cryopreservation procedures, several damages on the cellular (i.e. smooth muscle cells) and extra cellular matrix (i.e. elastin and collagen fibres) constitutive components of the arterial wall have been described (Karlsson and Toner 1996; Rigol et al. 2000). The injury of biological tissues by cryopreservation has been related to many factors, such as the cooling and thawing rate, the temperature range, and additionally cryoprotectant toxicity. The deleterious effects of the different factors have been ascribed to mechanic, osmotic, biochemical and thermal stresses that build up during cryopreservation (Fahy et al. 1987; Dawber 1988; Hunt et al. 1994; Wassenaar et al. 1995; Karlsson and Toner 1996; Pegg et al. 1997). These factors were considered and our cooling-thawing protocol is in agreement with those that showed the best results (Hunt et al. 1994; Wassenaar et al. 1995; Pegg et al. 1997).

Hence, between −80 and −100 °C, the minimum programmed temperature reached in cooling time, no damage is seen in vessels, our minimum temperature reached was −90 °C with a mean slow cooling rate of 1 °C/min at chamber. After that, the bag was immediately transferred at −142 °C in the gas phase of liquid nitrogen. According to the two stages rewarming process, we performed a first stage of slow warming rate by exposing the bag from −142 °C with frozen carotid to room temperature (20 °C) during 30 min, avoiding damage for thermal stress in the no thermodinamical equilibrium through glass transition temperature (T_g). In the second stage, the tissue underwent a rapid warming, in a 40 °C water bath so fast to avoid re crystallizations around (T_d) devitrification temperature post devitrification (Pegg et al. 1997). Finally, the cryostorage of arteries in liquid nitrogen accompanied by the cryoprotectant Dimethilsulphoxide, and the gradual remotion of the cryoprotectant once the artery is defrosted, has proved to be the most effective method to preserve cellular viability, structural integrity, endothelial and smooth muscle endocrine activity, and to determine the lowest immunologic response after transplantation (Vischjager et al. 1996; Pegg et al. 1997; Pascual et al. 2002). These factors were taken into account during our cryopreservation protocol design.

Arterial function characterization

The main functions of large and medium arteries are: (a) to serve as conduits for the distribution of blood to different tissues (conduit function, CF) and (b) to smooth out the pressure and flow pulsatility caused by the intermittent ventricular ejection (buffer function, BF) (Bia et al. 2003, 2005). These interrelated functions depend on the geometrical and mechanical properties of the arteries (Bia et al. 2003, 2004, 2005). The viscous, inertial and elastic mechanical properties are determined by passive (mainly elastin and collagen fibres), and active components (vascular smooth muscle cells), and are mainly set by the individual contribution of each structural constituent, their relative proportions, structural geometry and orientation, and the coupling among them (Armentano et al. 1995a; Wells et al. 1999).

The *elastic* behaviour of the arterial segments mainly depends on the elastin fibres, at low strain, and on the collagen fibres at high strain, while the vascular smooth muscle contribution to elasticity has been proposed to be exerted at intermediate strains (Armentano et al. 1995a). The arterial wall

viscous behaviour is explained by two theories. The *passive* one proposes that viscosity is a property of the arterial wall constituents, and it is generally assumed that the vascular smooth muscle is mainly responsible for this behaviour (Armentano et al. 1995a). The *active* theory, explains the viscous behaviour, taking into account the force-generating mechanism of the smooth muscle as well as the myogenic response to stretching. Both theories are not mutually excluded. Related to this, our group found that wall viscosity is higher in muscular arteries than in the elastic ones, and that it increases when the vascular smooth muscle is activated (Bia et al. 2003, 2005; Wells et al. 1999). The location of viscous elements in the infrastructure of the muscle cell has not been identified. Among several possibilities, it has been suggested that viscosity could be ascribed to (1) the protoplasm itself, (2) the viscous resistance of the cell matrix encountered by the sliding of actin and myosin filaments, and finally (3) the cell membrane that could act as a restraint presenting a viscoelastic behaviour (Armentano et al. 1995a). Finally, and rigorously speaking, the *inertial* modulus is a proportionality constant between force and the acceleration developed by a given material, and quantifies the resistance to acceleration presented by the body. In systemic arterial wall dynamics, inertial forces might develop at the beginning of systole associated to the fast increase in diameter (Armentano et al. 1995a), and the main determinant of the arterial wall inertia modulus is the wall mass (Armentano et al. 1995a).

During arterial cryopreservation procedures, damages on the smooth muscle cells, and elastin and collagen fibres, have been described (Karlsson and Toner 1996; Rigol et al. 2000). In addition, several works showed that after cryopreservation, VSM tone and reactivity are strongly affected (Vischjager et al. 1996), and that the endothelial integrity and function are impaired. These changes could be related to biomechanical impairment and arterial dysfunction and/or failure after the implant. However, the effects of the cryopreservation on the arterial elastic, viscous and inertial properties remain unclear. The following discussion focuses on the central conclusions of this study: our cryopreservation protocol does not modify arterial wall mechanical properties, and consequently the arterial CF and BF.

Our data show that the elastic, viscous and inertial properties remain unchanged between fresh and cryopreserved/defrosted arteries (Table 2). Taking into account that the viscoelastic and inertial properties are mainly determined by elastin, collagen and smooth muscle cells, our results could indicate that during our cryopreservation/defrost procedures there were not changes neither in the structure, nor in the fibrilar organization, that impaired the arterial wall mechanical behaviour.

Other reports analysing the *elastic* behaviour or the human arterial wall components of fresh and cryopreserved arteries lead to similar results. Those studies performed a partially evaluation of the mechanical behaviour of the arterial wall and among them gave complementary information. In Langerak's work (Langerak et al. 2001), the cryopreservation method did not significantly affect Young's modulus of elastin, collagen recruitment pressure, distensibility, nor the breaking stress of the human thoracic descending aorta. Pukacki et al. assessed the effects of cryopreservation on the elasticity and compliance of human iliofemoral arterial and veins segments and concluded that there were no differences between the elastic modulus and compliance of the control and the cryopreserved segments (Pukacki et al. 2000). Blondel et al. (2000), performing *in vitro* inflation static tests on segments of human fresh and cryopreserved arteries, found non significant differences between the incremental elastic and pressure-strain modulus of fresh and cryopreserved segments. Finally, Adham et al. (1996) and Gournier et al. (1993) comparing Young's module, stresses and strains in radial and circumferential directions of cryopreserved and fresh strips of human descending thoracic aortas concluded that there are not statistically significant differences between them.

Despite of the differences in the cryopreservation procedures, the arteries analysed (muscular vs. elastic) and the biomechanical test designs (static vs. dynamic; strips vs. segments), the studies' results are complementary. Related to this, in summary, the studies showed that the mechanical properties of the elastin and collagen fibres, the collagen recruitment point, as well as the breaking stress, remained unchanged after cryopreservation.

Studies that evaluate the effects of cryopreservation on the structural arterial components could

help to understand these and our mechanical findings. Related to this Gournier et al. performed histological studies before and after arterial cryopreservation, using standard and electron microscopy, and showed that arteries had normal structure after cryopreservation and that programmed cryopreservation with cryoprotectant does not alter the molecular or geometric configuration of collagen or elastic fibres (Gournier et al. 1993). Furthermore, around 20 years ago, Shepherd (Shepherd and Dawber 1982, 1984) and Dawber (Dawber 1988) confirmed in cryosurgery that collagen is particularly resistant to damage, revealing no fractures or distortion of collagen fibrils at any stage after the freeze-thaw schedules used, neither there was any alteration in the periodicity of fibrillar cross banding.

However, to our knowledge, there are not works that evaluate the cryopreservation's effects on the velocity and acceleration dependent vascular properties, that is to say, viscosity and inertia, respectively of the arterial wall. Consequently, our work presents original as well as complementary information.

In order to evaluate the arterial wall viscoelasticity using more classic indexes, we calculate the creep and stress-relaxation time constants. About this, during many years the arterial wall has been studied assessing such phenomena as stress relaxation and creep (Westerhof and Noordergraf 1970). In experiments dealing with stress relaxation the restoring force is recorded in strips, rings or blades, as a function of time after a step change in length has been applied. In studies on creep the length of a strip of material is observed as a function of time after the applied force has been changed, usually in a stepwise function. To improve and update this methodology to human arterial segments (and non invasive studies) we use a complete model that include creep and stress relaxation from a 3×3 adaptive parametric approach derived from the concept of the frequency dependent of the reciprocal of the Young modulus (Complex Modulus) (Westerhof and Noordergraf 1970), named Compliance Transfer Function. Our results showed that the viscoelastic behaviour, evaluated by means of the stress-relaxation and creep constant remain unchanged between fresh and cryopreserved/defrosted arteries (Table 2).

Finally, a quantitative measure of arterial wall BF can be obtained by using the cut-off frequency (f_C). Our results showed a similar f_C between fresh and cryopreserved/defrosted arteries. This can be confirmed in Figure 3 where the cryopreserved artery frequency response is included in the confidence interval of the fresh artery. For this reason, again it could be said that the cryopreservation/defrost procedures did not change the arterial wall buffering or filtering function.

Functional and clinical implications

At date it is accepted that the arterial wall CF and BF are determined not only by arterial elastic and geometrical properties, but also by the viscous properties of the arterial wall (and to a lesser extent by the inertia) (Bia et al. 2003, 2004). Hence, a proper mechanical characterization of the arterial CF and BF requires the analysis of both, wall elasticity and viscosity. One of the most important functions of the elastic properties of the arterial wall is to store part of the mechanical energy generated by the heart during systole, and to restore it in diastole, optimising the heart-vessel coupling and ensuring a continuous flow toward the tissues (Bia et al. 2004). Whereas the elastic behaviour might describe the arterial wall storage and pressure smoothing abilities, the viscous properties of the arterial wall are usually associated to an energy dissipation term (Armentano et al. 1995a; Pontrelli and Rossoni 2003; Bia et al. 2005).

Taking into account that mentioned above, arteries do not merely convey blood from one part of the circulation to another, but their viscoelastic structure and morphology, allows energy-efficient transmission of pulsatile blood flow, the simultaneous damping of excessive pressure fluctuations and the matching of the impedance characteristics of the proximal arterial tree to distal branches (Tai et al. 2000). Our data suggest that the functional behaviour of the human carotid arteries is preserved after our cryopreservation/defrost procedure.

In order to increase the vascular grafts patency rates, at date is accepted that the ideal vascular graft must have identical viscoelastic properties to that of the host artery (Seifalian et al. 2002). Commercially available prosthetic vascular grafts do not reproduce these favourable characteristics. Indeed, implantation of an incompliant graft into the arterial tree – analogous to the introduction of

an impedance into an oscillating electrical circuit – diminishes damping and perfusion efficiency, and in low-flow situations may lead to further flow stagnation and graft thrombosis (Tai et al. 2000). Disparity of viscoelastic properties and/or diameters at the junction of graft and artery, has been postulated to cause neointimal hyperplasia by stimulation of perianastomotic smooth muscle cells, that are exposed to anomalous cyclical strain forces and to mitogens released by activated platelets trapped by anastomotic flow disturbance (Tai et al. 2000). Additionally, mismatch in viscoelastic and diameter properties at the end-to end anastomosis of the native artery and the biological or synthetic graft, cause shear rate disturbances that may induce intimal hyperplasia and ultimately graft failure. The principal strategy being developed to prevent graft failure is based on the design and fabrication of compliant synthetic or innovative tissue-engineered grafts with viscoelastic properties that match those of the human artery (Tai et al. 1999).

In this way, our cryopreservation methodology could be considered a good alternative, because cryopreserved/defrosted arteries have an elastic, viscous, and inertial properties similar to control tissue. Indeed, the arterial wall buffer function (Armentano et al. 2003) and constant time parameters, were also similar to that of control arteries. Vascular tissue can thus be harvested from multiple organ donors and is likely to be cryopreserved without apparent alteration of its mechanical properties.

Further studies will be necessary to assess the effect of different tissue harvesting, processing and cryopreservation times in the arterial wall dynamics. In addition, in future studies, it will be necessary to establish the effects of our cryopreservation/defrost protocol on other vascular segments, taking into account that an identical cryopreservation procedure applied to different types of arteries could result in different responses after cryopreservation. Finally, it remains to be determined if after grafting these *in vitro observed mechanical properties will persist*.

Conclusion

In conclusion, this study suggests that, no statistical differences exist between the mechanical behaviour of the cryopreserved/defrosted arteries compared to the fresh control group. It could be concluded, therefore, that the present techniques of cryopreservation and measurement do not significantly affect the mechanical behaviour of CCA in any detrimental way.

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