Original Article

Changes in Mechanical, Structural Integrity and Microbiological Properties Following Cryopreservation of Human Cadaveric Iliac Arteries

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Abstract

Introduction: The study seeks to investigate how the duration of storage of cryopreserved human cadaveric iliac arteries impacts their mechanical, structural and microbiological properties as compared to their fresh sample. Materials and Methods: Iliac arteries were harvested from 12 human cadavers and divided into 2 groups. One group underwent mechanical stress-strain assessment immediately and another was cryopreserved for a pre-determined time-period (range, 29 to 364 days). Mechanical functionality was assessed with a customised clamping mechanism. The arteries' microbiological properties were studied pre- and post-cryopreservation. The post-thawed arteries were also assessed histologically for structural integrity. Results: Of the 12 pairs, only 7 (58, 119, 150, 252, 300, 332 and 364 days) iliac arteries were included in the final analysis. The other 5 pairs (29, 90, 188, 205 and 270 days) had abundant local calcification and their stress-strain curves could not be characterised. From the curves, pre- and post-cryopreserved arteries had the most similar mechanical properties when stored for 119 days. A trend of increasing relative stiffness with increased duration of storage was noted. The post-thawed arteries demonstrated minimal fragmentation except in atherosclerotic areas. Majority of the arteries were not contaminated by bacterial or fungal infection pre- and post-cryopreservation. Also, 2 arteries (364 and 332 days) which had initial bacterial colonisation showed no bacterial growth on their post-thawed sample. Conclusion: Mechanically, non-atherosclerotic cryopreserved arteries can be a good substitute to their corresponding fresh arterial graft. However, the length of cryopreservation has an effect on the relative stiffness of the pre- and post-cryopreserved arteries. Histological and microbiological findings suggest that cryopreservation have little impact on an artery structural integrity and may possibly have a role in maintaining sterility and sterilising the arteries.

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Introduction

Advancement in cryopreservation techniques have led to mounting interest in the use of arterial allograft for vascular reconstructive procedures and for treating arterial infections.[1,2] Cryopreservation of arterial allografts at deep sub-zero temperature offers the benefit of ensuring a ready supply of vascular tissues for emergency use or when electively needed. At deep sub-zero temperature, all biochemical reactions that lead to cell death are effectively stopped. Theoretically, cryopreservation would thus offer indefinite longevity to cells and possibly indefinite storage.

Current cryopreservation techniques involve the use of cryoprotectants agents (CPA) and an automated gradual freezing and rewarming process to minimise cryoinjuries and improve tissue preservation.[3-5] However, the effects of storage time on vascular allograft have yet to be extensively studied.

The long-term safety of the use of cryopreserved arterial grafts is influenced by its post-thawed haemodynamic properties, arterial tone and susceptibility to infections.[2,6,7] These factors can be measured via changes in mechanical properties, extent of disruption of the arterial structural integrity and microbiology results of the pre- and post-cryopreserved arteries. Deficiencies in these aspects can lead to the development of secondary vascular disease and eventual failure of vascular grafts.[8,9]

The aim of this study is to analyse and evaluate the effects

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of variable storage time on (i) the mechanical properties, (ii) structural integrity, and (iii) microbiological properties of pre- and post-cryopreserved human cadaveric iliac arteries.

Materials and Methods

Iliac arteries were harvested from 12 human cadavers aged between 45 and 71 years. Two iliac arteries were obtained from each cadaver, where they were excised from the end of the abdominal aorta. Arteries were removed immediately after death and transported to the laboratory in an ice box with histidine-tryptophan-ketoglutarate (HTK) solution before the experiments. The arteries were kept in HTK solution prior to cryopreservation or mechanical stress-strain testing for not more than 1 hour at room temperature. HTK solution is used to preserve the arteries during its hypothermic transport to the laboratory.

The iliac arteries from each donor were divided into 2 groups (Group A and Group B) and each artery was cut further into 2 pieces, one measuring 40 mm and the other measuring 10 mm. The 40 mm artery was used for mechanical stress-strain assessment while the 10 mm piece was used for pre-microbiological properties assessment. Group A underwent mechanical stress-strain assessment within 1 hour of its arrival to the laboratory as well as microbiology evaluation within 24 hours from harvesting. Group B underwent cryopreservation via a standardised protocol and each artery had its mechanical, histological and microbiological properties assessed only after a pre-determined period of time between 29 days and 364 days.

The arteries in Group B were preserved in 10% dimethysulphoxide (DMSO). The DMSO was added gradually in 4 x 5 minutes steps (2.5%, 5%, 7.5% and 10% dilution of DMSO). The arteries were then subjected to automated controlled freezing in a biological freezer at a temperature reduction rate of 1°C/min. The frozen iliac artery segment was stored in liquid nitrogen at -150°C for a pre-determined period of time ranging from 29 days to 364 days.

After a pre-determined storage period, the iliac artery segment underwent a staged thawing process. The artery was transferred from the storage tank and thawed at 37°C water bath within 20 minutes. Following which, the arteries were allowed to equilibrate with 5% DMSO at room temperature for 20 minutes. The cryoprotectant additives were removed gradually via dilution in a Dulbecco's Modified Eagle Medium (DMEM) at room temperature for 20 minutes.

A mechanical property testing apparatus was designed and built to access the mechanical properties of vascular tissues.[10] A customised clamping mechanism was also designed to ensure a uniformly distributed clamping force over the clamping area. In addition, a combined logarithm and polynomial strain energy equation were applied to model the elastic response of the arteries.

Tao Yang et al[10] described the apparatus as one which included a stepper motor (CTP21 DANAHER) employed to carry out basic motion such as elongation and a laser sensor (optoNCDT 1401-200, Micro-epsilon) equipped to control positioning. Also, load cells (LCM UF series) were employed to measure force imposed on the arteries and a circulator (Polyscience 8106) was employed to circulate the testing medium (Krebs Ringer solution) in the testing environment.

The stress-strain test was conducted in a water bath of circulating Krebs-Ringer solution with pH maintained at 7.3 to 7.4 and temperature at 37°C. This was done so that the experiments could be carried out in an environment that mimics body condition. Prior to testing, all visible connective tissues on the arteries were removed and all arteries had their stress-strain curve preconditioned to a steady state before data were collected. This is used to reduce variability in mechanical measurements.[7] In our experiment, each artery underwent uniaxial elongation, longitudinally with a ramping speed controlled at 2.5 mm/second.

The changes in mechanical properties (before and after cryopreservation) were studied quantitatively by matching the stress and strain curves of a cryopreserved artery and its corresponding fresh controls. Pre- and post-cryopreserved arteries that had similar trends of within 20% match when elongated at 30% strain or more were considered a good match.[11]

The arterial structural integrity was analysed via histological assessment. Samples of the artery were inked with both haematoxylin and eosin (H&E) stain and Elastic Van Gieson (EVG) to assess the extent of disruption of the internal and external elastic lamina layer after cryopreservation. The pre- and post-cryopreserved cadaveric arterial grafts were also evaluated for fungal growth and aerobic and anaerobic bacterial colonisation.

Results

Cryopreservation had been performed for a total of 12 pairs of human cadaveric arteries. The duration of storage of the cryopreserved iliac arteries ranged from 29 days to 364 days. The mechanical, histological and microbiological properties were evaluated (Tables 1 and 2).

The stress-strain curves of the cryopreserved and fresh controls of the same artery were matched and the relationship of the curves was analysed (Fig. 1). Of the 12 pairs, 7 pre- and post-cryopreserved iliac arteries (58, 119, 150, 252, 300, 332 and 364 days) were evaluated. Out of the 7 specimens, all of them showed no evidence that cryopreservation will "weaken" an artery. Also, 4 post-thawed cryopreserved

Table 1. Histological Analysis of the Structural Integrity of Post-thawed Cryopreserved Arteries

		Histological properties	
Duration of storage	**Atherosclerosis (AS)**	**Lumina elastica interna**	**Lumina elastica externa**
364 days	Atherosclerosis (2/3)	Mild fragmentation (1/3) Areas involved by AS extensive fragmentation (3/3)	Mild fragmentation (1/3) Areas involved by AS not optimally visualised
332 days	No atherosclerosis	No fragmentation	No fragmentation
300 days	Atherosclerosis (1 – 2/3)	Moderate fragmentation (1 – 2/3) especially when AS is more pronounced	Moderate fragmentation (2/3) especially when AS is more pronounced
270 days	No atherosclerosis	No fragmentation	No fragmentation
252 days	Atherosclerosis (1/3)	Mild fragmentation (1/3)	Mild fragmentation (1/3)
205 days	Atherosclerosis (2 – 3/3)	Extensive fragmentation (2 – 3/3)	Mild fragmentation (1/3)
188 days	Atherosclerosis (1/3)	Mild fragmentation (1 – 3)	Mild fragmentation (1/3)
150 days	Atherosclerosis (1/3)	Mild fragmentation (1/3)	Mild fragmentation (1/3)
119 days	Atherosclerosis (2/3)	Mild fragmentation (1/3) Areas involved by AS extensive fragmentation (3/3)	Mild fragmentation (1/3) Areas involved by AS extensive fragmentation (3/3)
90 days		Not assessed due to incomplete section	
58 days	No atherosclerosis	Mild fragmentation (1/3)	Mild fragmentation (1/3)
29 days	No atherosclerosis	Mild fragmentation (1/3)	Mild fragmentation (1/3)

Table 2. Comparison of Microbiological Properties of Pre- and Post-Cryopreserved Arteries

Duration of storage	**Bacterial culture***	**Pre-cryopreserved microbiological properties†**	**Post-thawed microbiological properties†**
364 days	Aerobic Anaerobic	*Acinetobacter* species *Delftia acidovorans* *Enterococcus faecalis*	Negative
332 days	Aerobic Anaerobic	Negative	Negative Negative
300 days	Aerobic Anaerobic	*Diphteroid bacilli* Negative	Negative Negative
270 days	Aerobic Anaerobic	Negative Negative	Negative *Propionibacterium* species
252 days	Aerobic Anaerobic	Negative	Negative
205 days	Aerobic Anaerobic	Negative	Negative
188 days	Aerobic Anaerobic	Negative Negative	Negative
150 days	Aerobic Anaerobic	Negative Negative	Negative Negative
119 days	Aerobic Anaerobic	Negative Negative	Negative
90 days	Aerobic Anaerobic	Negative	Negative
58 days	Aerobic Anaerobic	Negative	Negative
29 days	Aerobic Anaerobic	Negative *Propionibacterium* species	Negative *Propionibacterium* species

*Bacterial growth was labelled as negative after 5 days of no bacterial growth.
†No fungal growth was detected after 14 days on pre- and post-thawed arteries for all duration of storage.

arteries showed good match with its fresh control on stress-strain curve within 30% strain. The match was noted to be good as mechanically, the stress-strain relationship of the arteries had a closeness of match of within 20% (arteries stored for 119, 150, 300 and 332 days). The remaining 5 pairs (29, 90, 188, 205 and 270 days) of arteries had abundant calcification noted on visual inspection. Their stress-strain curves were characterised by premature breaks and their elasticity were compromised due to stiffening of the arteries by calcification (Fig. 2).

From the experiments it was noted that the stress (i.e. pressure) required to strain (i.e. elongate) a cryopreserved artery as compared to its fresh artery varied with the duration of cryopreservation. At 58 days, the cryopreserved artery was noted to be less stiff as compared to its fresh sample. At 119 and 150 days of storage, the post-thawed arteries were noted to match the fresh samples tightly. This trend of increasing stiffness continued and at 364 days, the cryopreserved artery was noted to be much stiffer as compared to its corresponding fresh control (Fig. 1).

The histology of the 12 cryopreserved iliac arteries was analysed and most arteries showed minimal histological changes (Table 1). For the arteries noted to have atherosclerotic plaques present, fragmentation of the internal and external lumina elastica layers were noted. In addition, increased fragmentation was noted over the area involved by atherosclerosis (arteries stored for 119, 205, 300 and 364 days). Correspondingly, arteries with no or only mild atherosclerosis have no fragmentation or only mild fragmentation of the lumina elastica layers. All these arteries had well preserved cellularity of the medial layers. The luminal surface for all the arteries appeared to be well preserved with only some randomly distributed areas noted to be lacking endothelium.

For microbiological assessment, majority of both pre- and post-cryopreserved arteries did not have any bacterial (aerobic and anaerobic) or fungal growth after 5 and 14 days respectively (Table 2). Interestingly, for the artery stored for 300 and 364 days, its post-thawed bacterial culture failed to detect any bacterial growth though both aerobic and anaerobic species were detected initially before cryopreservation (Table 2). For the artery stored for 29 days, *Propionibacterium* species was grown in both pre- and post-cryopreservation. Only in 1 out of the 12 post-thawed artery, was bacterial cultured (*Propionibacterium* species) when previously no bacterial growth was noted in the pre-cryopreserved specimen (artery stored for 270 days).

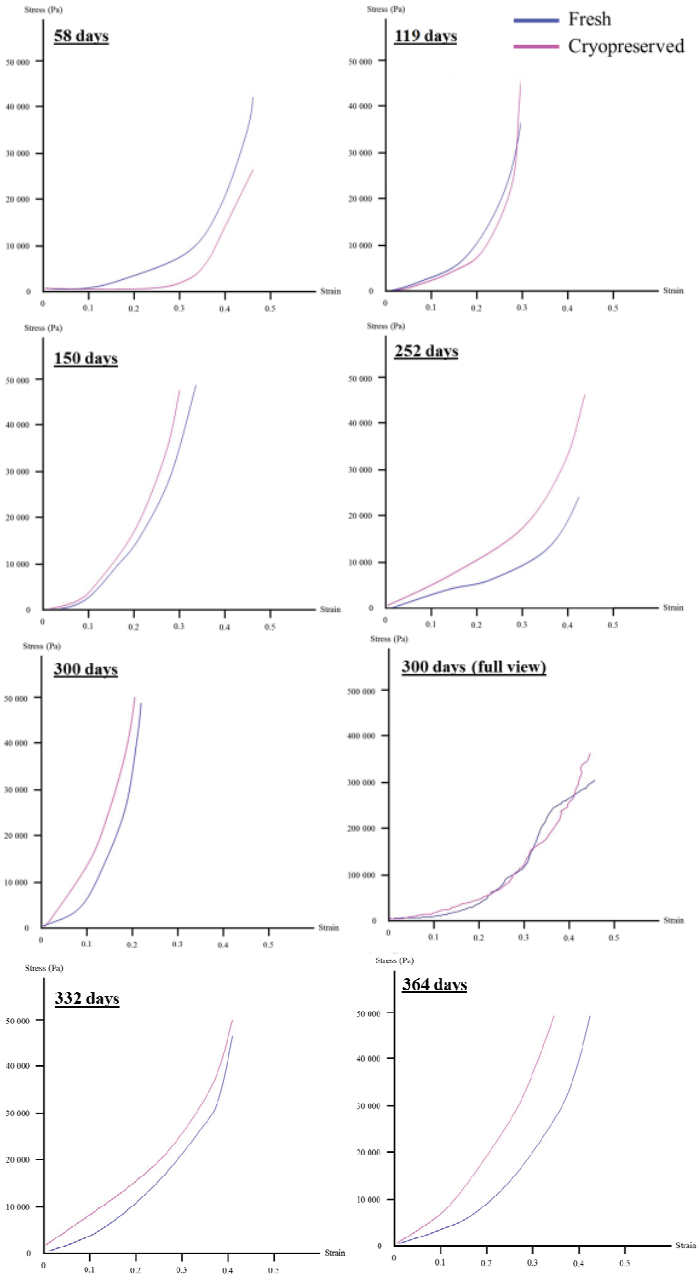

Fig. 1. Stress and strain curve of pre- and post-cryopreserved harvested human cadaveric iliac arteries (58, 119, 150, 252, 300, 332 and 364 days).

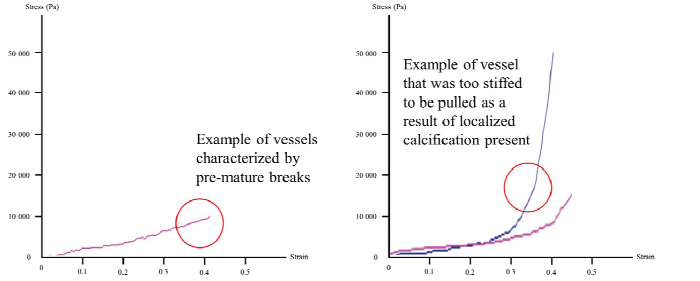

Fig. 2. Example of stress and strain curve of pre- and post-cryopreserved arteries with presence of abundant calcifications (for vessels stored for 29, 90, 188, 205 and 270 days).

Discussion

Cryopreservation is an important asset for the storage of arterial grafts. With an increasing interest in the

establishment of vascular allograft tissue banks, assessing the long-term viability of arterial grafts has important implications. However, the effects of long-term storage have yet to be extensively studied. This experimental study is the first to compare the mechanical and microbiological properties of pre- and post-cryopreserved stored for up to 1 year.

Arterial allograft cryopreserved for short periods showed promising results in terms of excellence in haemodynamic graft patency, anticoagulant need and resistance to infection.[12,13] From our study, uniaxial elongation of the arteries in a custom-designed apparatus[10] helped determine the stress-strain mechanical properties of the arteries. The elasticity of the cryopreserved and fresh arteries matched (within 20%) with the arteries stretched at 1.3 times the original length (30% strain, 1.3 stretch ratio). This was so for 4 out of the 12 harvested iliac arteries. In addition, the experimental data for all 7 out of the 12 arteries showed no evidence that cryopreservation will "weaken" an artery (i.e. making it easier to break a low strain). As such, theoretically, cryopreserved and fresh iliac arteries are a suitable substitute for each other when based on their mechanical properties. This study parallels an observation made by Jorge et al[14] who showed that mechanical properties of arteries were not significantly changed after cryopreservation for up to 2 months.

It was also observed that the duration of cryopreservation had an effect on the relative stiffness of the cryopreserved artery. In this study, the post-thawed cryopreserved artery was initially less stiff than its corresponding fresh control (58 days). This implies that less force was required to elongate the cryopreserved artery compared to its fresh control. We do not exactly understand the mechanism behind this finding; however, we postulate that it may be due to the variability of the arteries despite it being taken from the same donor. Then again, what was more apparent was that of a trend of increasing relative stiffness between pre- and post-cryopreserved arteries. The arteries appeared to be stiffer when the number of days of cryopreservation increased. After storage duration of 119 and 150 days, the stiffness of both pre- and post- cryopreserved arteries appears to match each other closely. Thereafter, the trend was that the cryopreserved arteries would require more force to elongate compared to its corresponding fresh arteries (252, 300, 332 and 364 days). This suggests that in the establishment of a vascular allograft tissue bank, vascular tissues cannot be stored indefinitely and perhaps arteries stored for up to 4 months would best mechanically substitute its fresh sample.

For the 5 arteries whose mechanical properties could not be characterised by the stress-strain curve, they were found to have abundant and localised calcifications on visual inspection. Localised calcification made it difficult to achieve uniform tensile force and the artery were inclined to break near the regions of calcification. Moreover, the calcification caused stiffening of the arteries and the elasticity was compromised. As such, their experimental data had to be discarded.

This study also demonstrated that storage for up to 1 year in liquid nitrogen (–150°C) did not negatively affect the structural integrity of the artery. Minimal histological changes were noted for most of the specimen (Table 1). This finding is similar to that described by Rigol et al[15] who noted that cryopreservation of arteries in liquid nitrogen could preserve endothelial function independent of storage time for up to 6 months. This was concluded as no interaction between the post-thawed arterial endothelium and platelet were detected on light microscopy.

In this study, the greatest amount of fragmentation was noted in areas of the arteries that had extensive atherosclerosis. Clinically, the presence of irreversible damage to the cryopreserved arterial wall could lead to failure of the arterial graft after implant.[16] As such, for vascular tissue banks to obtain and store vascular grafts, the presence of atherosclerosis in donated arteries must be minimised. Suggested stringent exclusion criteria including a cap placed on donor's age and excluding patients with known atherosclerosis or known comorbidity such as hypertension, hypercholesterolemia and diabetes mellitus should be implemented. This is suggested as patients with the above-mentioned comorbidity is predisposed to accelerated atherosclerosis.[2]

In addition, this study suggests that cryopreservation may have a role in the sterilisation of vascular allograft. This is evident from the 2 vascular grafts (artery stored for 364 and 300 days) where though they had initial bacterial colonisation, no bacterial growth was detected in the post-thawed artery. For the other specimens, majority were negative for bacterial and fungal colonisation pre- and post-cryopreservation. One vascular graft (artery stored for 270 days) stood out as an outlier whereby bacterial growth was noted on its cryopreserved artery despite negative cultures initially. This could either be due to cross-contamination or the presence of a true bacterial growth. However, on the basis that sterility was maintained or improved in the other 11 vascular grafts, we suggest that from a microbiological point of view, cryopreserved arteries are safe for use as arterial allograft.

This study is limited as it provides limited insight into the degree of function that the tissue is capable of in vivo. Even with our newly developed mechanical testing apparatus, it can never fully replicate the physiological functioning of the vascular grafts. However, what we have used is a surrogate measure that has predicted a consistent mechanical

property between pre- and post-cryopreserved arteries. In addition, we were limited by the supply of human cadaveric arteries, hence were unable to assess the arteries stress-strain properties in a uniaxial circumferential direction or biaxial loading conditions. While we have only tested mechanical properties in a uniaxial longitudinal direction, this model of testing is regarded as an acceptable estimation when assessing arterial tissue.[10] Through our study, we were thus able to evaluate the basic mechanical properties of the iliac arteries involved.

Also, as we were limited by the number of cadaveric iliac arteries, we had to settle on having only 1 sample at each time point. However, as this is an experimental and preliminary study, where we seek to investigate the effects of variable storage time for cryopreserved vessel, we decided to evaluate each vessel 30 days apart. Our study was thus better positioned to evaluate the trend of relative stiffness between pre- and post-cryopreserved vessel over a period of 1 year.

In addition, even though we set out to study the effects of the duration of cryopreservation on 12 arteries, we eventually had to exclude 5 arteries from our final analysis as these were arteries that had abundant calcification and could not undergo reliable mechanical testing. This is because, with localised calcifications, the tensile force distribution is not uniform. For example, some of the arteries were too stiff to be pulled and had hit the upper limit of our force sensor at low levels of strain. Similarly, due to calcification, some were characterised by premature breaks. Also, for the histological analysis, one sample (artery stored at 90 days) could not be assessed as it had incomplete section owing to tears present in the tissue. In addition, as the arteries did not undergo histological evaluation pre-cryopreservation, hence, for the arteries with extensive fragmentation, it cannot be concluded with certainty if its presence was due to the cryopreservation process. However, what is certain is that arteries with greater atherosclerosis have associated extensive fragmentation of the lumina elastica layers.

Long-term safety of cryopreserved arteries is determined by a multitude of factors such as haemodynamic properties, resistance to infection, anti-thrombotic properties, maintenance of vascular tone, immunologic properties and surgical technique.[12] This study does not extensively evaluate all these components. Further studies would need to be conducted to confirm the current hypothesis that cryopreserved arteries up to a year is a safe option for use as an arterial allograft.

Conclusion

In conclusion, histological and microbiological evaluation suggests that cryopreservation has little impact on an artery structural integrity and may have a role in maintaining sterility and sterilising arteries from initial bacterial colonisation. Also, this preliminary study shows that mechanically non-atherosclerotic cryopreserved arteries can be a good substitute to its corresponding fresh arterial graft. However, the duration of storage does have an effect on their relative stiffness. A larger well designed experimental study that involves the collection of only non-atherosclerotic arteries and the mechanical assessment of pre- and post- cryopreserved arteries in uniaxial longitudinal, circumferential and biaxial loading conditions would be ideal in better characterising the mechanical properties of cryopreserved arteries.

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