THE ROLE OF CRYOPRESERVATION IN THE BIOMECHANICAL PROPERTIES OF THE INTERVERTEBRAL DISC

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Abstract

Implantation of intervertebral disc (IVD) allograft or tissue engineered disc constructs in the spine has emerged as an alternative to artificial disc replacement for the treatment of severe degenerative disc disease (DDD). Establishment of a bank of cryopreserved IVD allografts enables size matching and facilitates logistics for effective clinical management. However, the biomechanical properties of cryopreserved IVDs have not been previously reported. This study aimed to assess if cryopreservation with different concentrations of cryopreservant agents (CPA) would affect the dynamic viscoelastic properties of the IVD. Whole porcine lumbar IVDs ($n = 40$) were harvested and processed using various concentrations of CPA, 0 % CPA, 10 % CPA and 20 % CPA. The discs were cryopreserved using a stepwise freezing protocol and stored in liquid nitrogen. After four weeks of storage, the cryopreserved IVDs were quickly thawed at 37 °C for dynamic viscoelastic testing. The apparent modulus, elastic modulus (G'), viscous modulus (G") and loss modulus (G"/G') were calculated and compared to a fresh control group. Cryopreserved IVD without cryopreservants was significantly stiffer than the control. In the dynamic viscoelastic testing, cryopreservation with the use of CPA was able to preserve both G' and G" of an IVD. No significant differences were found between fresh IVD and IVD cryopreserved with 10 % CPA or 20 % CPA. This study demonstrated that CPAs at an optimal concentration could preserve the mechanical properties of the IVD allograft and can provide further credence for the application of long-term storage of IVD allografts for disc transplantation or tissue engineered construct applications.

Keywords: Intervertebral disc, allograft, transplantation, cryopreservation, biomechanics.

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Introduction

Low back pain is a debilitating condition with tremendous socioeconomic and health-care expenditure (Hart *et al.*, 1995; Andersson, 1999; Deyo *et al.*, 2006; Gray *et al.*, 2006; Katz, 2006; Freburger *et al.*, 2009). Degenerative disc disease (DDD) is an etiological factor associated with lower back pain (Luoma *et al.*, 2000; Bendix *et al.*, 2008; de Schepper *et al.*, 2010). Currently, the most common treatment for DDD is spinal fusion (Fritzell *et al.*, 2001; Brox *et al.*, 2003). However, fusion of the vertebral segments restricts spinal motion, which may increase stress and motion at the adjacent segments; thereby, contributing to the degenerative process at adjacent levels that may become symptomatic and necessitate surgical intervention (Ghiselli *et al.*, 2004; Hilibrand and Robbins, 2004; Park *et al.*, 2004; Hoogendoorn *et al.*, 2008).

To counter the effects of spinal fusion and preserve motion, the uses of intervertebral disc (IVD) artificial implant devices have been advocated. However, the success of an artificial disc implant relies heavily on the design and positioning of the implant in the interbody space to achieve the required biomechanics that would recreate the natural properties of the human disc (Huang *et al.*, 2003; Lee and Langrana, 2004). Studies of artificial disc implants have suggested that the total disc replacement causes permanent changes in the kinematics of the spine and cannot fully restore normal mobility (Dooris *et al.*, 2001; Tournier *et al.*, 2007; Rohlmann *et al.*, 2009). Such permanent changes in the kinematics of the spine can cause instability and increase stress forces at the adjacent segments that may also contribute to the degenerative process at those levels (Lee and Goel, 2004; Lee and Langrana, 2004).

As an alternative to artificial disc replacement, intervertebral disc allograft transplantation has been developed (Olson *et al.*, 1991; Frick *et al.*, 1994; Matsuzaki *et al.*, 1996; Luk *et al.*, 2003) with noted satisfactory clinical outcomes in humans at five-year follow-up (Ruan *et al.*, 2007; Luk and Ruan, 2008). However, appropriate size-matching of the allograft to the recipient site is one of the key factors in ensuring transplantation success. As such, preserving and storing allograft IVD of different sizes in an IVD bank is essential before the procedure can be popularised. Cryopreservation is one method that can enable tissue storage. It is used widely for preserving sperm, ovum, and even embryo. However, some studies have reported that freezing the IVD can cause permanent changes to its mechanical properties (Flynn *et al.*, 1990; Bass *et al.*, 1997; Gleizes *et al.*, 1998). Other researchers have shown that sheep IVD

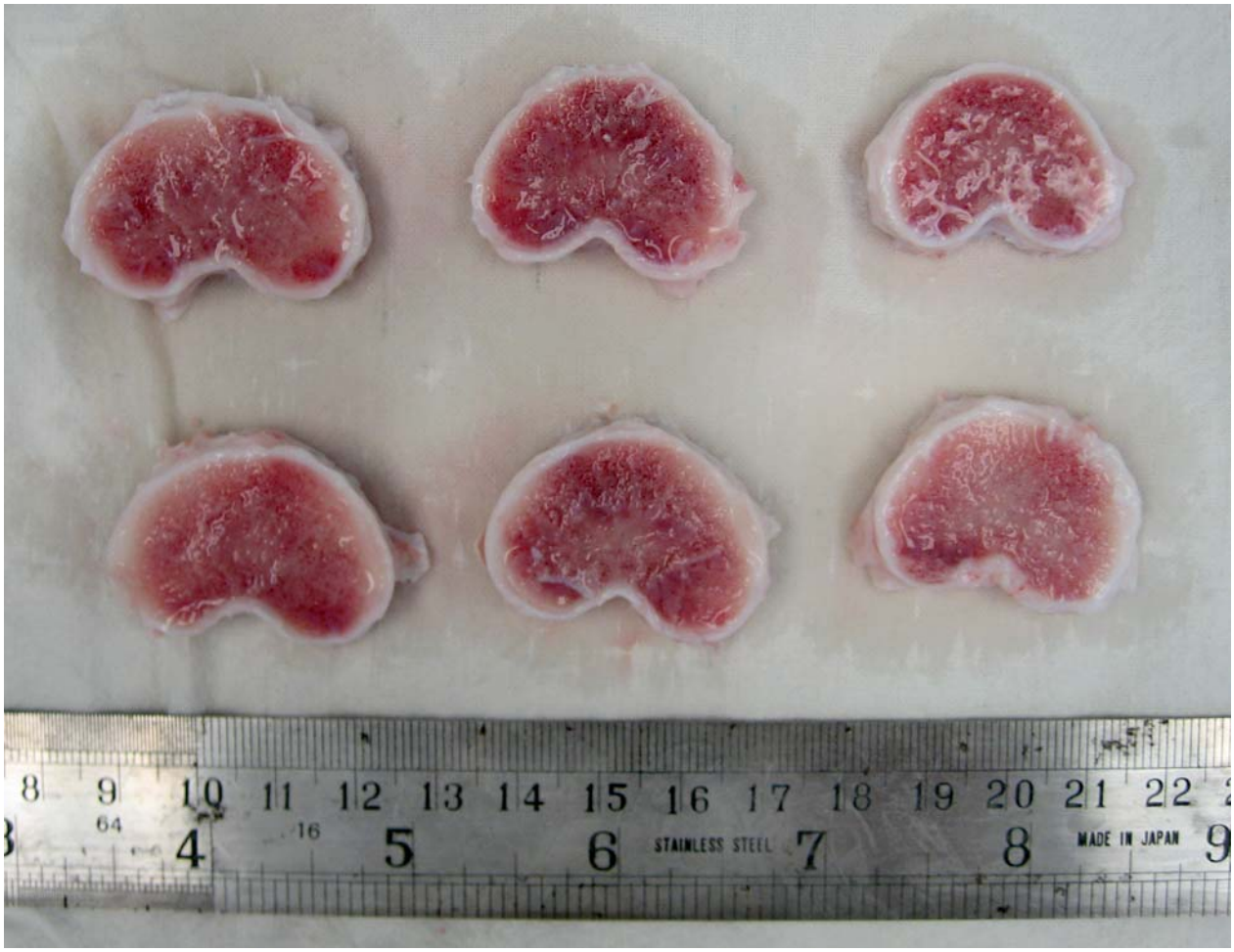

Fig. 1. Porcine lumbar intervertebral disc allografts.

frozen without cryopreservants for a period of three months resulted in irreversible deterioration of the biomechanical properties of the IVD (Gleizes *et al*., 1998). Likewise, some authors have contended that frozen storage permanently alters the permeability and creep behaviour of the porcine IVD, while studies on the human IVD revealed that a typical freezing protocol did not significantly alter matters (Dhillon *et al.*, 2001). Furthermore, freeze-drying the IVD may compromise the stiffness of the spinal segments to a significantly greater extent than deep-freezing because the water content within the matrix of the disc is removed through the lyophilisation process of freeze-drying (Flynn *et al*., 1990).

Long-term storage of IVD allografts of different sizes in an IVD bank is an essential component for IVD allograft transplantation. The use of cryopreservant agent (CPA) is vital to the safe preservation of the IVD (Matsuzaki *et al*., 1996; Bass *et al*., 1997). There is no consensus in the literature on a cryopreservation protocol that can best retain the whole IVD allograft. The effect of different strengths of CPA on the physical properties of the composite is also not known. It is important that the mechanical properties of the IVD allograft implant following cryopreservation are characterised to see if it can retain similar properties as a normal IVD because it has been established that the mismatch of mechanical properties between the implant and the host tissue can be detrimental to the performance of the implant (Bao *et al.*, 1996). Moreover, the difference in mechanical properties may change the pattern of loading through the spine and can cause changes to the kinematics as well as degeneration of the facet joints (Dooris *et al*., 2001). Therefore, the objective of this study was to investigate the effects of CPA concentration on the biomechanical properties of the IVD allograft.

Materials and Methods

Porcine disc harvest

Porcine lumbar discs (L1 to L6) (n = 40) were harvested from 9 pigs (weight range = 37 to 42 kg) that were freshly sacrificed with an injection of an overdose of pentobarbitone (200 mg/kg). After removal of the surrounding muscles and posterior elements, an osteotomy was made at the endplates approximately 10 mm above and below the disc. The endplate-annulus fibrosus-nucleus pulposus composite was removed *en-bloc*. A high-speed burr (Stryker, Mahwah, NJ, USA) was then utilised to further trim down the bony endplate to a thickness of 1-2 mm in a manner avoiding the development of fractures. The whole discs were kept in Hank's buffered saline solution (HBSS) with 50 mmol sodium citrate (Sigma-Aldrich, St. Louis, MO, USA), 1 % Penicillin/Streptomycin, and 0.4 %

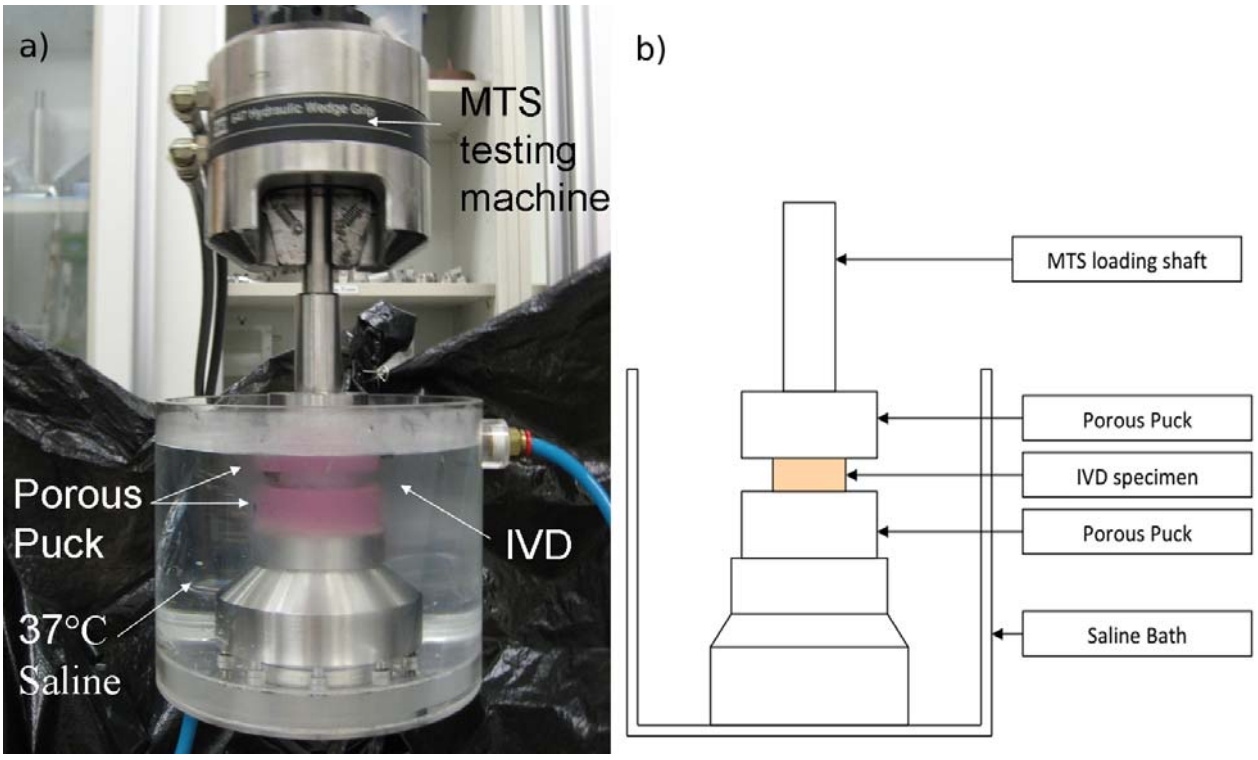

Fig. 2. Mechanical testing setup for intervertebral disc allograft.

fungizone (Gibco, Invitrogen, Carlsbad, CA, USA) during processing (Chan *et al.*, 2010). The endplates surfaces were lavaged with saline solution using the Pulsavac™ wound debridement irrigation system (Zimmer, Münsingen, Switzerland) to remove cutting debris and clotted blood (Gantenbein *et al.*, 2006). The discs had a mean length, width and height of 30.1 ±4.3 mm, 23.3 ±3.9 mm, and 7.3 ±0.7 mm, respectively (Fig. 1).

Intervertebral disc allograft cryopreservation

Corneal Potassium Tes 2 (CPTES2) solution was used as the CPA carrier solution. The CPTES2 contained the following: Na^+ 31 mmol/L, K+ 100 mmol/L, Mg^{2+} 1 mmol/L, Ca^{2+} 1 mmol/L, Cl^- 62 mmol/L, HCO_3^- 30 mmol/L, $H_2PO_4^-$ 1 mmol/L, SO_4^{2-} 1 mmol/L, glucose 5 mmol/L, TES 100 mmol/L (all chemicals from Sigma-Aldrich). The discs were randomly cryopreserved in various concentration of CPA as follows: (1) 0 % CPA (CPTES2 solution only) (n = 10); (2) 10 % CPA (10 % DMSO in CPTES2 solution) (n = 10); and (3) 20 % CPAs (10 % DMSO +10 % Propylene glycol (PPG) (Sigma-Aldrich USA) in CPTES2 solution) (n = 10). The discs were incubated with the CPA for 2 h at 4 °C, frozen stepwise to -80 °C at -0.3 °C/min overnight and then stored in liquid nitrogen for four weeks (Chan *et al.*, 2010). Before the biomechanical testing, all IVD allografts were thawed quickly at 37 °C in a saline bath until all the CPA had melted and testing were performed within 30 min after the IVD allografts were taken out of the liquid nitrogen. Fresh untreated IVD from 9 animals were used as controls (n = 10). All specimens were examined for any damages to the endplates following cryopreservation and were radiographed (Faxitron X-Ray Corporation, Wheeling, IL, USA). The x-rays were digitised using an x-ray digitiser (Vidar, Herndon, VA, USA) with a resolution of 2800 x 2507 pixels. The cross-sectional area of the IVD were measured using Image Pro Plus (Media Cybernetics, Bethesda, MD, USA), and disc cross-sectional area was computed as an ellipse using the formula π/4 x d1 x d2, where d1 and d2 were the lateral and anteroposterior widths.

Mechanical testing

Non-destructive uniaxial compression testing was performed using MTS 858 Bionix Testing Machine (MTS System Inc., Minneapolis, MN, USA). Samples were placed between two porous platens (pore size 20 µm) inside a 37 °C normal saline (0.15 mol/L NaCl) bath as shown in Fig. 2. All samples were loaded with a basal compressive load of 10 N so as to maintain contact between the puck and the samples during the testing.

Dynamic mechanical testing

The viscoelastic properties of the cryopreserved IVD were investigated using the method described by Miyamoto *et al*. (Miyamoto *et al.*, 2006). Preconditioning was performed by applying 10 sinusoidal strain cycles with an amplitude of 10 % at 1 Hz. After a 3 min recovery from preconditioning the IVDs were tested using 6 frequencies of uniaxial compression loading (0.05 Hz, 0.1 Hz, 0.2 Hz, 0.5 Hz, 1 Hz and 2 Hz) and compressed with 10 % strain amplitude. Each frequency test was performed using one cycle of sinusoidal strain followed by 3-minute recovery (Fig. 3a). An elastic modulus (G'), a viscous modulus (G") and a loss tangent (G"/G') were determined. G' represents the elastic behaviour with its ability to store deformational energy and G" represents viscous behaviour

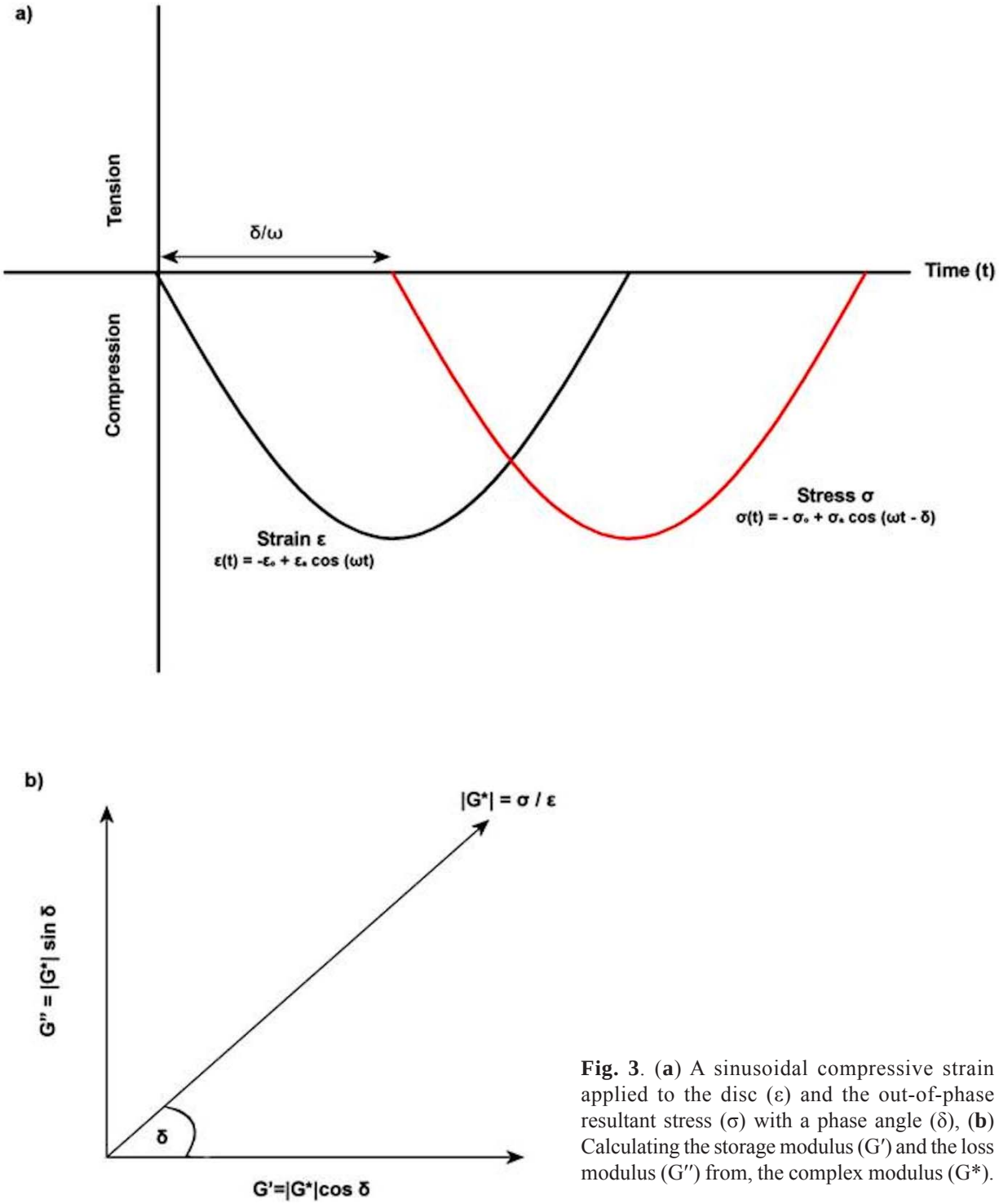

Fig. 3. (**a**) A sinusoidal compressive strain applied to the disc (ε) and the out-of-phase resultant stress (σ) with a phase angle (δ), (**b**) Calculating the storage modulus (G′) and the loss modulus (G″) from, the complex modulus (G*).

with the dissipation of energy during deformation in the IVD (Tanaka *et al.*, 2002). The complex modulus (G*) was determined by |G*| = σ/ε, where stress σ = force (F) / cross-sectional area (A), strain ε = change in disc height (ΔDH)/disc height (DH) (Miyamoto *et al*., 2006). G' was determined by G' = |G*|cos δ, G" by G" =| G*|sin δ, and G"/G' by tan δ = G"/G' (Fig. 3b).

Compression testing

The stiffness of the cryopreserved IVD was characterised. Following the dynamic compression testing, a slow compression ramp (0.5 mm/s) of maximum load 200 N of axial compression was applied (Costi *et al.*, 2002). Stiffness was calculated using linear regression for the mode of loading and was defined as the slope of the load-displacement curve over a defined range of load (Costi *et al*., 2002).

Statistical analysis

One-way ANOVA followed by LSD *post-hoc* test was used to evaluate the statistical difference in the elastic, viscous, loss tangent and stiffness between different treatment groups. A p value < 0.05 was considered to be statistically significant.

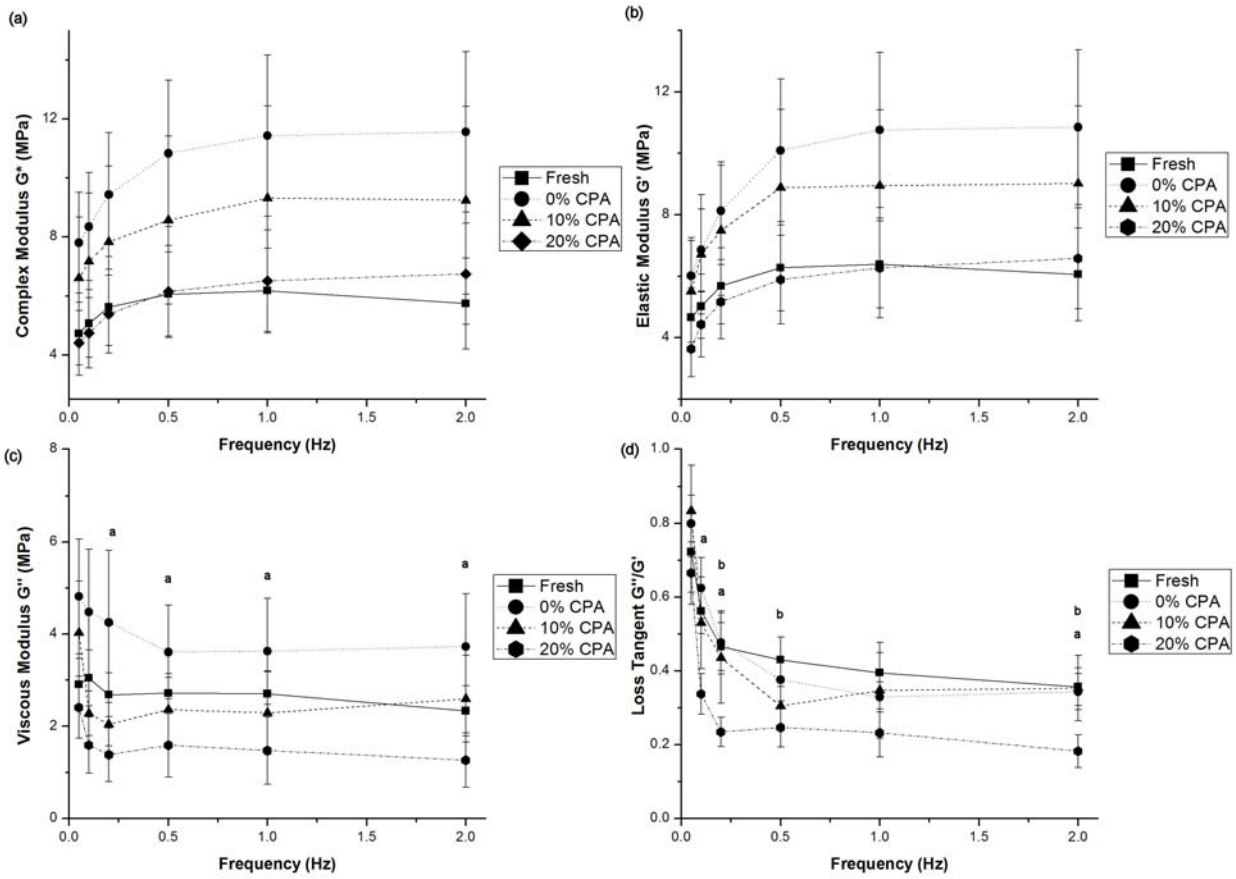

Fig. 4. Effects of cryopreservation (CPA) concentration on the apparent modulus of the porcine intervertebral disc (mean ±SEM). [a] denotes statistical significance at $p < 0.05$.

Results

Dynamic mechanical testing

The G*, G', G" and G"/G' of the fresh control and different treatment groups over the range of testing frequencies are presented in Fig. 4.

G' was observed to increase with increasing frequency rate. The range of the G' for the fresh control was 4.66 ±0.96 MPa at 0.05 Hz to 6.39 ±1.41 MPa at 1 Hz, while the G' range of the 10 % CPA group and 20 % CPA group was 5.51 ±1.65 MPa at 0.05 Hz to 9.02 ±2.52 MPa at 2 Hz and 3.64 ±0.91 MPa at 0.05 Hz to 6.58 ±1.64 MPa at 2 Hz, respectively. The 0 % CPA group recorded the highest G' properties, 6.02 ±1.24 MPa at 0.05 Hz to 10.85 ±2.52 MPa at 2 Hz. The 20 % CPA group retained the closest G' properties to the fresh IVD group, but no statistical significant differences in the G' properties were detected among the different groups across the frequencies.

The G" properties were observed to decrease with increasing frequency rate. No statistical significant differences in G" properties were detected between the fresh control group and the treatment groups. The G" range of the fresh control was 3.05 ±0.60 MPa at 0.1 Hz to 2.33 ±0.55 MPa at 2 Hz while the 10 % CPA group recorded a G" range of 4.03 ±1.12 MPa at 0.05 Hz to 2.04 ±0.47 MPa at 0.2 Hz. The G" properties of the 20 % CPA group range from 2.41 ±0.67 MPa at 0.05 Hz to 1.27 ±0.59 MPa at 2 Hz. The 0 % CPA recorded the highest G" range of 4.81 ±1.24 MPa at 0.05 Hz to 3.61 ±1.02 MPa at 0.5 Hz. A statistically significant difference was noted between 0 % CPA and 20 % CPA groups in the G" for 0.2 Hz (p = 0.026), 0.5 Hz (p = 0.04), 1 Hz (p = 0.016) and 2 Hz (p = 0.026).

A decreasing trend was observed in the G"/G' factor with increasing frequency in all the groups. The G"/G' factor range in the fresh control was 0.94 ±0.13 at 0.05 Hz to 0.30 ±0.09 at 0.5 Hz while the G"/G' factor in the 10 % CPA group and 20 % CPA group was 0.83 ±0.12 at 0.05 Hz to 0.30 ±0.05 at 0.5 Hz and 0.66 ±0.08 at 0.05 Hz to 0.18 ±0.04 at 2 Hz, respectively. While the 0 % CPA group had a G"/G' factor range of 0.79 ±0.07 at 0.05 Hz to 0.33 ±0.04 at 1 Hz. Statistically significant differences were observed in the loss modulus where there were differences between 0 % CPA and 20 % CPA at 0.1 Hz (p = 0.009), 0.2 Hz (p = 0.024) and 2 Hz (p = 0.032). Furthermore, statistically significant differences in the loss modulus were observed between fresh control and 20 % CPA at 0.2 Hz (p = 0.049), 0.5 Hz (p = 0.02), and 2 Hz (p = 0.035).

Compression testing

The stiffness of the fresh control and different CPA concentration treatment groups are presented in Fig. 5. The mean stiffness of the fresh control group was 229.14 N/mm (±SEM = 34.49 N/mm), 0 % CPA group was 296.35 N/

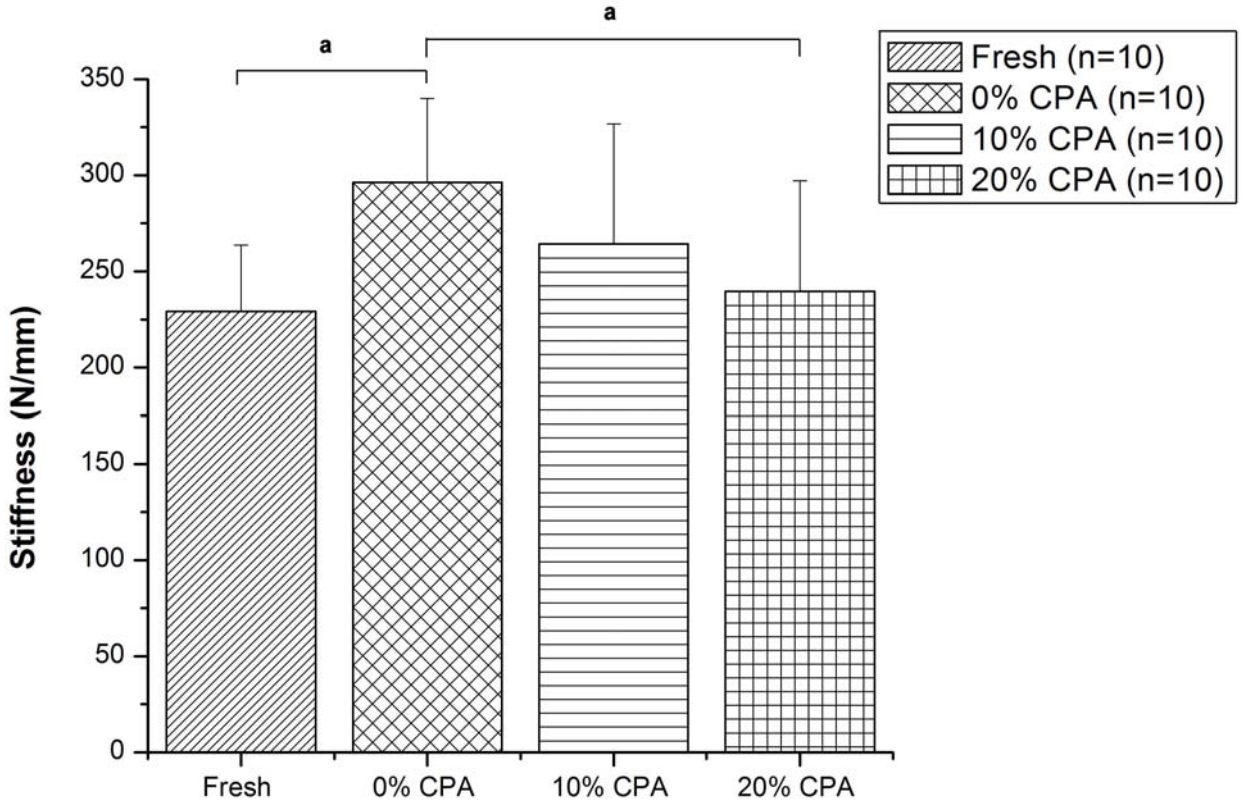

Fig. 5. Effects of cryopreservation (CPA) concentration on the (**a**) complex modulus (G*), (**b**) elastic modulus (G'), (**c**) viscous modulus (G"), and the (**d**) loss tangent (G"/G') of the porcine intervertebral disc (mean ±SEM). [a] = $p < 0.05$, 0 % CPA *vs*. 20 % CPA. [b] = $p < 0.05$, Fresh *vs*. 20 % CPA.

mm (±SEM = 43.70 N/mm), 10 % CPA was 264.17 N/mm (±SEM = 62.60 N/mm) and 20 % CPA was 239.63 N/mm (±SEM = 57.50 N/mm). *Post-hoc* test showed statistical significant differences in the apparent modulus between the fresh control and the 0 % CPA group ($p = 0.007$). Statistically significant differences were also detected between the 20 % CPA group and the 0 % CPA group ($p = 0.021$). No significant differences were found between the fresh control and the 20 % CPA group ($p = 0.659$) as well as the 10 % CPA group ($p = 0.145$). Also, no statistical significant difference was noted between the 10 % CPA group and the 20 % CPA group ($p = 0.304$).

Discussion

The IVD is a viscoelastic tissue consisting of a proteoglycan-rich nucleus pulposus surrounded by a collagenous annulus fibrosus (Palmer and Lotz, 2004), and it retains its mechanical nature due to its ability to attract and retain water (Bass *et al*., 1997; Maclean *et al.*, 2004; Miyamoto *et al*., 2006). The proteoglycans within the nucleus pulposus act to absorb water and osmotically exert a swelling pressure that enables it to resist the compressive loads (Farfan *et al.*, 1972; Urban and Maroudas, 1981; Urban and McMullin, 1985; Urban and McMullin, 1988; Roberts *et al.*, 1996; Urban *et al.*, 1998). Some authors have reported that frozen IVD has significantly less swelling pressure than fresh IVDs (Keller *et al.*, 1990; Johnstone *et al.*, 1992; Bass *et al*., 1997). Given that the swelling pressure directly affects the mechanical behaviour of the disc, it was believed that cryopreserving the IVD without CPA may result in the IVD having less capacity to absorb water and thus less swelling pressure; thereby, causing the frozen IVD to be more rigid and more viscous (Johnstone *et al*., 1992; Buckwalter *et al.*, 1993; Bass *et al*., 1997). In the current study, the authors tested the IVD allograft when it was cryopreserved under different concentrations of 0 % CPA, 10 % CPA and 20 % CPA. Increased viscous modulus was observed in the 0 % CPA group, which was consistent with results in the literature looking at the effects of freezing the IVD (Keller *et al.*, 1990; Bass *et al*., 1997). This increase in viscous modulus in IVDs without CPA was very similar in characteristic to degenerated or injured IVD and is believed to be related to the dehydration of the nucleus, which then was unable to bind water and generate swelling pressure (Farfan *et al*., 1972; Holm *et al.*, 1981; Keller *et al*., 1990; Bass *et al*., 1997). Also, it has been reported that the dehydration of tissue during the freezing process can cause the increase in cross-linking of collagen fibrils leading to increased stiffness of tissues (Yannas and Tobolsky, 1967; Weadock *et al.*, 1984; Dahl *et al.*, 2006). Frozen IVDs may become dehydrated when water travels from the collagen fibrils to sites of ice crystal nucleation, thereby increasing cross-linking between collagen fibrils. This increase in cross-linking from dehydration may

contribute to the higher stiffness of IVDs cryopreserved with 0 % CPA than fresh IVDs (Dahl *et al*., 2006). IVDs preserved with 10 % and 20 % CPA showed viscous characteristics similar to that of the fresh control discs, suggesting that the CPA had the ability to deter dehydration of the nucleus through substitution of water in the cells with the CPA and minimise damage caused by the ice formation during the freezing process as well as retaining its ability to bind and absorb water during the thawing process following cryopreservation (Brockbank *et al.*, 2000; Song *et al.*, 2004; Pegg, 2007).

Moreover, cryopreserved IVDs which can retain similar mechanical properties and functions to attenuating shocks as well as distributing loads as of fresh IVDs can avert any issues relating to the mismatch of mechanical properties between the implant and the host tissue as observed in artificial disc implants (Bao *et al*., 1996). Studies have shown that the difference in mechanical properties between implants and the host tissue may change the pattern of loading through the spine and can cause changes to the kinematics as well as degeneration of the facet joints. (Dooris *et al*., 2001; Rundell *et al.*, 2008; Rohlmann *et al*., 2009). Results demonstrate that the cryopreserved IVD allografts with CPA retained similar stiffness to the fresh IVD suggesting that there may not be a mismatch of mechanical properties following transplantation. Therefore, the cryopreserved IVD allografts should be able to attenuate shocks and distribute loads as of normal IVD following transplantation.

It has also been suggested that freezing can induce changes to the characteristics of the proteoglycans within the IVD and these changes were similar to those observed in aged IVDs, which have limited capacity to swell (Keller *et al*., 1990; Buckwalter *et al*., 1993; Bass *et al*., 1997). While in previous studies of IVD cryopreservation, Chan *et al*. (2010) observed that IVDs cryopreserved with CPA showed positive safranin O orange staining demonstrating that the cryopreservation process was able to preserve proteoglycan content within the NP area comparable to the fresh control IVD, suggesting that the cryopreserved IVDs maintained the capacity to bind water. Furthermore, Matsuzaki *et al*. (1996) concluded that storage duration had little impact on cell viability in the intervertebral disc and matrix synthesis, however, thermal stresses that developed during the cooling and thawing procedure may cause fractures in the tissue structure (Pegg *et al.*, 1997). The authors believe that the precise and delicate cooling, and thawing protocol applied in this study can prevent the occurrence of these fractures as histological studies of cryopreserved IVD from previous studies confirmed that the overall matrix organisation was maintained following cryopreservation with the same cryopreservation protocol. Sections of the cryopreserved IVD showed that the AF structure stayed in an organised manner when cryopreserved with CPA, however, Chan *et al*. (2010) did noticed the formation fissures between AF fibres indicating traces of ice formation during the freeze/thaw process (Chan *et al*., 2010). The combination of CPA with step-wise freezing protocol can prevent cell death associated with freezing and reduce ice crystal formation, which might potentially reduce the disruption of the IVD structure post-cryopreservation (Bass *et al*., 1997; Dhillon *et al*., 2001).

Cell death during the freezing process may also affect the mechanical behaviour indirectly because of the degradative enzymes that are released from the dying cells in the extracellular matrix. Bass *et al*. (1997) suggested that these degradative enzymes may have deleterious effects on the tissue proteoglycans; thus, affecting the disc's osmotic pressure and its capacity to swell. Gleizes *et al*. (1998) concluded that freezing the IVD produces a dysfunction of cell metabolism, which includes modification of intra-cellular pH and release of proteolytic enzymes, which may lead to the formation of extracellular ice and the development of cytolysis. This combinational effect of cytolysis and catalytic enzymes in the extracellular environment leads to a decrease in proteoglycans, which induces a fall in osmotic pressure within the disc. (Gleizes *et al*., 1998) Since the value of the osmotic pressure directly governs the stiffness of the disc, the biomechanical properties also alter accordingly. In a previous study concerning the cryopreservation of the IVD performed by the authors, it was concluded that the use of 20 % CPA provided the most ideal condition in contributing to cell viability following cryopreservation (Chan *et al*., 2010). Both the metabolic activity analysis and the safranin O histological evaluation suggests that the CPA have penetrated through the disc into both the AF and NP areas and preserved proteoglycan levels comparable to the fresh IVDs. We propose that the use of CPA may retain the mechanical properties of the IVD following cryopreservation through preserving disc cell viability, thereby maintaining the integrity of extracellular matrix and hence the capacity of IVD to absorb water to sustain intradiscal osmotic pressure.

The loss tangent shows that the elastic modulus is more dominant over the viscous modulus suggesting that the IVD allograft behaved similar to the normal intact disc where it exhibited more elastic behaviour during compression. A significant difference in the loss tangent between the 20 % CPA and the fresh control suggests that the 20 % CPA group is more elastic in behaviour during compression. When the IVD is compressed, the pressure in the hydrated NP expands out radially and places the collagen fibres in the AF under tension and resists further nuclear expansion providing equilibrium. For all the discs, the decreasing trend observed in the loss modulus with increasing frequency indicates that the viscous behaviour of the cryopreserved IVD allograft is most prominent at the lower frequency suggesting that the NP acts more to resist compressions at lower frequency while at higher frequencies the AF is stretched and plays a more dominant role in resisting compression. Results also show that the elastic moduli as well as the viscous modulus are parameters that are highly dependent on frequency. (Kim *et al.*, 1995; Miyamoto *et al*., 2006) Preliminary tests showed that results at higher frequencies beyond 1 Hz have plateaued and it is believed that the data for 1.5 Hz will not be significantly different. Therefore, this study only tested the IVD up to a frequency of 2 Hz. The authors believe that the dynamic viscoelastic testing used in this study can effectively provide insights

into the biomechanical properties of the IVD because of its sensitivity and ability to adapt a broad range of load frequencies (Tanaka *et al*., 2002), and have used dynamic viscoelastic testing to simulate physiological loading of the IVD and demonstrated the nonlinear behaviour of the IVD tissue as well as its dependency on frequency of load (Beek *et al.*, 2001).

In this study, porcine lumbar discs were used as a model because their sizes are comparable to that of the human cervical discs (Lu *et al.*, 1999). In a study comparing the mechanical properties of glycosaminoglycans and water contents of seven different mammalian species, it was suggested that the disc tissue properties were mostly similar across animal species including pig and human (Beckstein *et al.*, 2008). Furthermore, the porcine lumbar disc model has been used in previous studies looking at disc mechanics and its properties were comparable to the human IVD in terms of axial mechanics, glycosaminoglycans and water content (Bass *et al*., 1997; Reno *et al.*, 1997). However, adolescent pig discs have notochordal cells (Zhao *et al.*, 2006). Considering that notochordal cells produce matrices of different composition to that of the chondrocyte-like cells (Horner *et al.*, 2002; Kim *et al.*, 2003; Roughley, 2004), the cryopreservation of human IVDs will be different. Therefore, whether our findings can apply to human discs necessitates future assessment. Nevertheless, this study verified the effects of different cryopreservant conditions and forms the basis for future cryopreservation and IVD tissue banking protocols assessments.

Conclusions

To the authors's knowledge, this is the first study to assess the effects of CPA on the mechanical properties of the IVD following cryopreservation. We show that in disc cryopreservation, preservation of the mechanical properties of IVD may be achieved through the use of CPAs. Our findings provide further credence for the application of long-term storage and establishment of tissue banking protocols in support of IVD allografts for disc transplantation or tissue engineered constructs for the treatment of severe DDD.

Acknowledgments

The project was supported by The University of Hong Kong: CRCG grant and Tam Sai Kit Endowment Fund. The authors wish to thank Dr. Dino Samartzis for his assistance with the preparation of this manuscript and Mr Stephen Chan for his help in the mechanical testing.

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Discussion with Reviewers

Reviewer I: Dhillon *et al*. (2001, text reference) compared the effect of freezing before and after freezing within the same disc (within level comparison) and of two adjacent discs (between level comparison, to exclude potential artifacts. What was the reason that you did not test within one level?

Authors: All the samples were randomly pooled in a bank of IVDs for storage. We did not test within the one level before and after freezing because we followed the cryopreservation protocol established in Chan *et al*. (2010, text reference), where the IVDs are cryopreserved within 2 hours death of the animal to ensure that maximum cell viability was preserved. Testing the IVD before cryopreservation will compromise the results as we believed that many of the cells within the IVD will not have survived and the mechanical testing results will not reflect the effects of the cryopreservation.

Reviewer III: A figure illustrating the penetration depth of the CPAs would greatly improve the paper and many of the points being made in the discussion. Much of the discussion hinges on the understanding that the CPAs fully penetrated the discs. Is there proof of this? How different is the concentration within the disc for 10 % *versus* 20 %? Has this been measured?

Authors: Chan *et al*. (2010, text reference) showed that the preservation of nucleus pulposus cell viability was achieved by using various concentrations of cryopreservants and histological studies suggesting that the cryopreservants had penetrated into the centre of the disc. We do not know the difference in the penetration between the 10 % and 20 % CPA and future studies directly looking at the penetration of the cryopreservant into the disc will be need to address this question.