# MINIMIZING CRYOPRESERVATION-INDUCED LOSS OF DISC CELL ACTIVITY FOR STORAGE OF WHOLE INTERVERTEBRAL DISCS

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## Abstract

Severe intervertebral disc (IVD) degeneration often requires disc excision and spinal fusion, which leads to loss of spinal segment mobility. Implantation of an allograft disc or tissue engineered disc construct emerges as an alternative to artificial disc replacement for preserving the motion of the degenerated level. Establishment of a bank of cadaveric or engineered cryopreserved discs enables size matching, and facilitates clinical management. However, there is a lack of understanding of the behaviour of disc cells during cryopreservation, as well as how to maximize their survival, such that disc graft properties can be preserved. Here, we report on the effect of alterations in cooling rates, cryoprotective agents (CPAs), and duration of pre-cryopreservation incubation in CPA on cellular activity in whole porcine lumbar discs. Our results indicated that cooling rates of -0.3°C/min and -0.5°C /min resulted in the least loss of metabolic activity in *nucleus pulposus* (NP) and *annulus fibrosus* (AF) respectively, while metabolic activity is best maintained by using a combination of 10% dimethylsulphoxide (DMSO) and 10% propylene-glycol (PG) as CPA. By the use of such parameters, metabolic activity of the NP and the AF cells could be maintained at 70% and 45%, respectively, of that of the fresh tissue. Mechanical testing and histological evaluation showed no significant differences in mechanical properties or alterations in disc structure compared to fresh discs. Despite the limitations of the animal model, our findings provide a framework for establishing an applicable cryopreservation protocol for human disc allografts or tissue-engineered disc constructs.

**Key words:** Intervertebral disc, nucleus pulposus, *annulus fibrosus*, dimethylsulphoxide, propylene glycol, cryopreservation, metabolic activity, transplantation.

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## Introduction

Back pain is one of the most common healthcare problems globally with a reported prevalence in the USA of 31%. It is associated with degenerative disc disease (DDD) (Strine and Hootman, 2007). Conventional surgical methods for treating severe back pain associated with DDD involve a fusion of the segment and therefore result in loss of segmental motion (Lee and Langrana, 2004). Approaches to preserve motion of the spine include total disc replacement with an artificial disc, allogeneic disc transplantation, or replacement with engineered disc tissues. While the success rate of artificial disc replacement in the short term is promising, it may eventually fail leading to reoperation (Freeman and Davenport, 2006).

Biological intervertebral disc (IVD) transplantation has an advantage over artificial disc replacement owing to its capability of self-integration through natural remodelling. This may be achieved in the future through tissue engineered solutions (Kandel *et al.*, 2008), or by allogeneic human disc transplantation (Luk *et al.* 1997). The latter has been shown in animal studies and pilot clinical trials to be feasible (Frick *et al.*, 1994; Katsuura and Hukuda, 1994; Luk *et al.*, 1997; Luk *et al.*, 2003; Luk and Ruan, 2008; Matsuzaki *et al.*, 1996; Olson *et al.*, 1991; Ruan *et al.*, 2007). Five years follow-up results in the human clinical trial of whole IVD replacement using the cryopreserved disc allograft has shown encouraging potential for the preservation of spinal stability and segmental motion (Luk and Ruan, 2008; Ruan *et al.*, 2007). However, the transplanted disc progressively degenerates, and this is thought to be associated with suboptimal cell activity from cryo-damage, hence compromising the ability to maintain fully functional integrity of the IVD in the long term (Katsuura and Hukuda, 1994; Matsuzaki *et al.*, 1996; Luk *et al.*, 1997; Luk *et al.*, 2003).

Effective clinical application of disc grafts involves cryopreservation of the IVD and the establishment of an IVD bank. This would resolve issues of organ donation, preservation, disc size matching and immunocompatibility (Luk *et al.*, 2003). The method of cryopreservation must retain both mechanical properties and cellular activity for effective downstream applications. Freezing of the IVD has been shown to alter mechanical properties (Bass *et al.*, 1997; Dhillon *et al.*, 2001), but the effect of cryopreservation on disc mechanical properties itself has not been reported. Previous studies of canine IVD cryopreservation using step-wise freezing with dimethyl

sulphoxide (DMSO) as the cryopreservative agent (CPA) reported inconsistent loss of disc cell metabolic activity (Katsuura and Hukuda, 1994; Matsuzaki *et al.*, 1996). It is therefore necessary to understand how cryopreservation alters both mechanical properties and cellular activity and survival of the disc to advance the science of long-term storage.

Tissue and organ cryopreservation are more complicated than cell suspension; it is determined by the inherent tissue properties and affected by the diffusion rate of solutes, as well as the rate of ice propagation within cells and tissue. Different tissue types would require different cryopreservation methodologies or conditions for optimal cell survival due to the differences in matrix and cell composition (Meryman, 2007). In this study, we hypothesize that the degree of loss of cell activity in the cryopreserved whole IVD is dependent on the conditions of cryopreservation and that by exploring these relationships we may establish an enhanced protocol for tissue banking of IVD allografts. We optimized the survival of the disc cells by modulating the cooling rates, CPA concentration and incubation time using whole porcine lumbar disc as a model and evaluated the effect of cryopreservation on mechanical properties and tissue structure.

## Materials and Methods

### Porcine disc harvesting

Fifty-two porcine lumbar IVD ($L_{2-3}$ to $L_{4-5}$) were obtained from 22 pigs (weight between 37-42 kg) within 2 hours of sacrifice using a pentobarbital injection. This allowed a minimum of 5 IVDs per test group. Thus, 15 discs were used for testing 3 different rates of cooling, 15 discs for testing 3 combinations of CPAs, 10 discs for testing 2 different incubation times in CPA, 15 discs for mechanical testing, and 6 discs for histology. IVD harvesting and preparation techniques were carried out as previously described (Chan *et al.*, 2010). In brief, discs with endplates were harvested using a handsaw and trimmed by blade. The endplate surfaces were lavaged with saline solution using the Pulsavac™ wound debridement irrigation system (Zimmer, Münsingen, Switzerland) to remove cutting debris and blood clots. The discs had a mean length, width and height of 30.05 ± 4.33 mm, 23.26 ± 3.86 mm, and 7.33 ± 0.74 mm, respectively.

### Disc cryopreservation

All samples were cryopreserved with single changes to the standard protocol for disc cryopreservation (Luk *et al.*, 2003) at a time. Briefly, the standard protocol entails: harvested discs were incubated for 2 hours at 4°C with 10% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) in Corneal-Potassium TES 2 (CPTES2). They were then cooled to -80°C by placing in containers inside a -80°C freezer overnight. The discs were then stored at -196°C in liquid nitrogen for one week and quickly thawed at 37°C in a water bath and washed with 4°C Hanks' balanced salt solution (HBSS) before analysis. The CPTES2 solution was prepared according to Pegg's protocol (Pegg *et al.*, 2006). It is a potassium-rich balanced salt solution containing an impermeant anionic pH buffer (TES) originally used for cornea preservation (Taylor and Hunt, 1985).

### Control of cooling rate

To simulate different cooling rates, whole IVD samples were immersed in cryopreservation solution in a 50-mL Falcon tube and then placed in three various types of containers: (A) a pre-cooled glass container filled with -80°C isopropanol, (B) a pre-cooled glass container filled with 4°C isopropanol, or (C) a 16 x 11.5 x 21cm polystyrene box of 1.6cm thickness. The samples were kept in a -80°C freezer overnight and then stored in liquid nitrogen. At the initial stage of cooling, the temperature change of the cryopreservation solution was monitored every 5 min using a thermocouple wire (Lutron TM-914C, Taiwan). The cooling rate (°C /min), corrected to 1 decimal place, was calculated as the slope of the linear line drawn from the starting and the ending temperature.

### Incubation with different CPAs

The effect of the modified cryopreservation solution formulas was tested against the traditional formula (using 10% DMSO as CPA). Three formulas were tested: [1] contained 10% DMSO, [2] contained 20% CPA (10% DMSO + 10% propylene glycol (PG) (Sigma-Aldrich), and [3] contained10% DMSO + 0.1% Supercool X-1000 (21 Century Medicine, Fontana, CA, USA). All IVDs were cooled to -80°C by overnight freezing at the determined optimal cooling rate and then stored in liquid nitrogen.

### Modifying pre-freeze incubation time

The length of incubation time prior to cooling influences the penetration of the CPA into the tissue and hence the protection offered by the CPAs during cryopreservation. The effect of pre-cooling incubation of the samples in CPA was tested by altering the incubation time between 2 and 4 hours in cryopreservation solution composed of the determined optimal CPA formula. The IVDs were then cooled to -80°C by overnight freezing at the optimal cooling rate and stored in liquid nitrogen.

### Evaluation of cell metabolic activity

The metabolic activity of the cryopreserved discs was evaluated by the AlamarBlue™ assay (Biosource™, Camarillo, CA, USA). This is a fluorometric indicator based on detection of metabolic activity of cells (Ahmed *et al.*, 1994). The cryopreserved disc samples were thawed by incubation in a 37°C water bath. After thawing and washing, the endplates were removed, and the *nucleus pulposus* (NP) and *annulus fibrosus* (AF) were isolated from the samples. Three pieces of tissue specimen from each region were obtained using a 4 mm biopsy punch. The specimens were incubated in 1ml of *Dulbecco's Modified Eagle Medium (DMEM)* (Sigma-Aldrich, USA) with 10% AlamarBlue under standard cell culture conditions for 24 hours (Jonsson *et al.*, 1997). Control experiments (fresh control) were performed using specimens from freshly harvested disc samples instead of

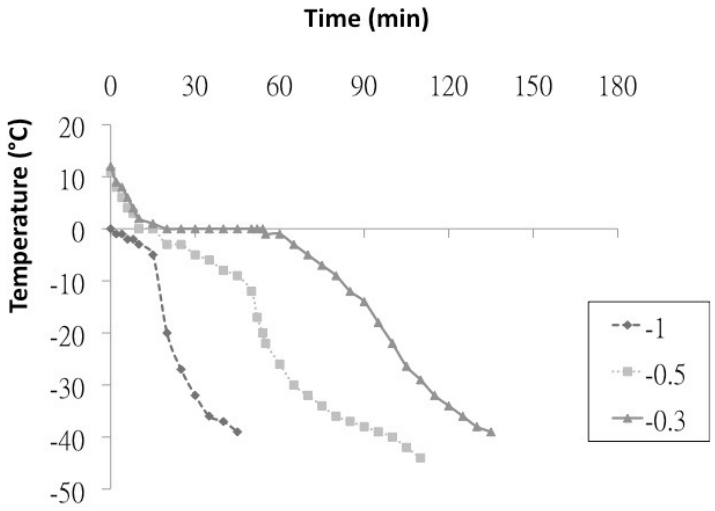


**Fig. 1.** Cooling profile of the CPTES2 solution in different containers. **(◆)** in -80°C isopropanol, **(■)** in 4°C isopropanol, **(▲)** in polystyrene box.

cryopreserved tissues. The amount of fluorescence was measured in duplicates by the multimode microplate reader (Beckman Coulter DTX 880, Brea, CA, USA) at an excitation wavelength of 535 nm and an emission wavelength of 595 nm. The specimens were dried using a lyophilizer and the dry weight was measured. For each experimental group, cell metabolic activity was expressed as relative fluorescence units (RFU) per mg dry weight. Results from the test specimens were compared with those of the fresh control to determine the percentage recovery of normal activity.

**Mechanical testing**

After defining the preferred cryopreservation protocol as 2 hours of incubation, a cooling rate of -0.3°C/min and using 20% CPAs, the mechanical property of the cryopreserved disc allograft was evaluated. Non-destructive uniaxial compression testing was performed using an MTS 858 Bionix Testing Machine (MTS System Inc., Minneapolis, MN, USA). The samples were placed on a porous puck inside a 37°C normal saline (0.15 mol/L sodium chloride) bath and then another porous puck, connected to the loading shaft, was loaded onto the samples. A basal compressive load of 10 N was applied so as to maintain contact between the puck and the samples. Then, preconditioning was performed by applying 20 pre-cycles of 100 N compressions and a slow compression ramp of 200 N was applied at a rate of 0.05 mm/sec. The deformation of the disc was assumed to be the change in the distance between the fixtures, measured using the test system linear variable differential transformer (LVDT). The apparent Young's modulus of each sample was calculated as the tangent of the linear portion of the stress-strain curve during the 200 N compression cycle.

**Histological evaluation**

The morphology of the IVD following our preferred cryopreservation method was investigated. IVDs from each group were taken out from the liquid nitrogen, thawed as described above and then fixed with 4% neutral buffered formaldehyde. The fixed discs were then dehydrated and embedded without decalcification in methyl-methacrylate (Ni *et al.*, 2006). After polymerization, IVD allograft specimens were cut in midsagittal sections with a high speed, water cooled diamond saw (EXAKT 300 CP Band System, Norderstedt, Germany). The sections were ground down to 100-200 μm thickness using a grinding machine (EXAKT 400 CP Band System), and the surface was polished with number 4000 garnet paper (Ni *et al.*, 2006). All sections were stained with haematoxylin and eosin (H&E) stain, and safranin O stain for histological observation under a Nikon (Tokyo, Japan) H600L microscope and image analysis system.

**Statistical analysis**

One-way ANOVA followed by the least significant difference (LSD) *post-hoc* test was used to evaluate the statistical difference among different treatment groups in cell metabolic activity measured by the AlamarBlue assay and Young's modulus obtained from MTS mechanical testing. A $p$ value $< 0.05$ was considered as significant.

## Results

**Effect of cooling rate**

We first examined whether the cooling rate of cryopreservation process affects post-cryopreservation disc cell metabolic activity. Different cooling rates were simulated by placing the whole IVD samples inside various media or containers and cooling in the -80°C freezer. Based on the cooling profiles (Fig. 1), the cooling rates were calculated as (A) -1.0°/min when cooled in -80°C isopropanol, (B) -0.5°C /min when cooled in 4°C isopropanol, and (C) -0.3°C /min when cooled inside a polystyrene box. NP metabolic activity at the cooling rate of -0.3°/min (79.2 ± 18.6% of the fresh control) was significantly higher than that at other cooling rates (~40% of the fresh control). Cooling the IVD at -0.5°C /min resulted in significantly higher metabolic activity in the AF (33.8 ± 25.9% of the fresh control) than at other cooling rates (Fig. 2). From these data, a cooling rate at -0.3°C / min was considered optimal for preserving the overall disc metabolic activity, and was adopted in all subsequent tests.

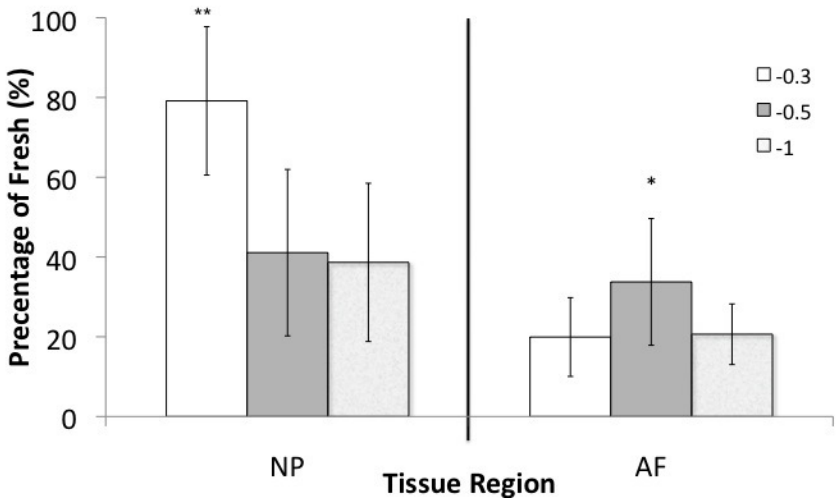


**Fig. 2**. Effect of different cooling rates on metabolic activity of the cryopreserved IVD. The discs were cryopreserved with 10% DMSO. *Post-hoc* multiple comparison: ** $p<0.01$, * $p<0.05$ (N=5, Mean ± SEM).

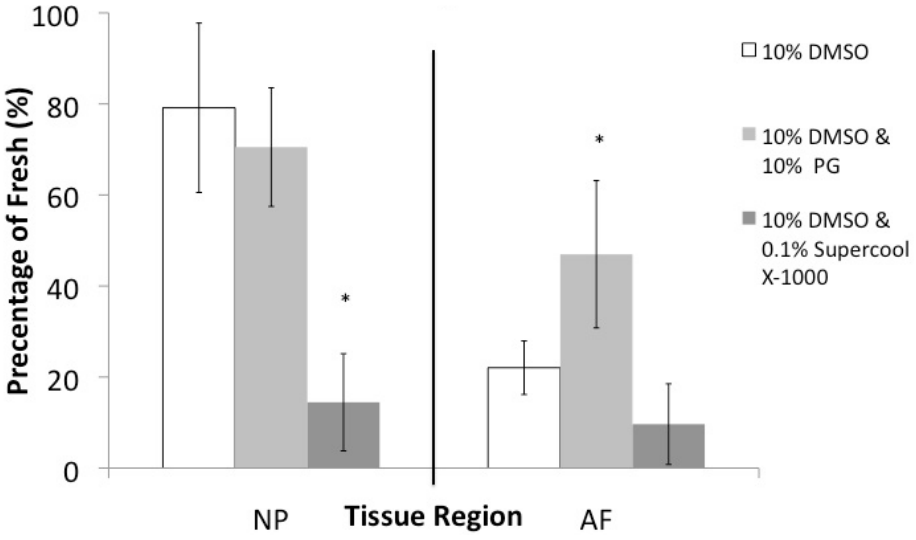


**Fig. 3.** Effect of using additional cryoprotective agents on the metabolic activity of the cryopreserved IVD. Samples were cryopreserved at -0.3°C/min in various CPAs. DMSO: Dimethyl Sulphoxide, PG: propylene glycol. * $p<0.05$, post-hoc multiple comparison, (N=5, Mean ± SEM).

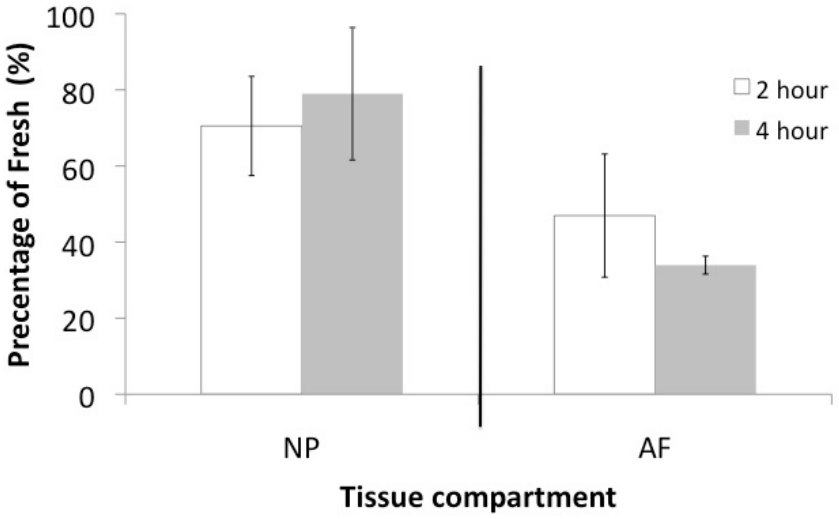


**Fig. 4.** Effect of pre-cooling incubation on metabolic activity of the cryopreserved IVD. The discs were incubated for either 2 or 4 hours in 20% CPA at 4°C before cryopreservation at -0.3°C/min (N=5, Mean ± SEM).

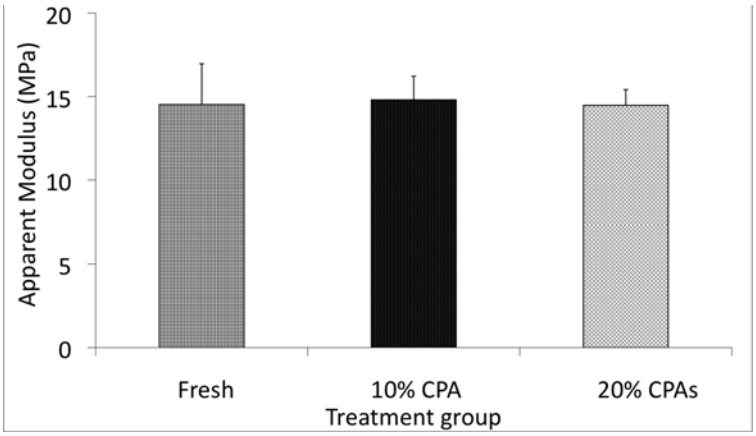


**Fig. 5.** Effects of CPA concentration on the apparent modulus of porcine IVD (Mean±SEM).

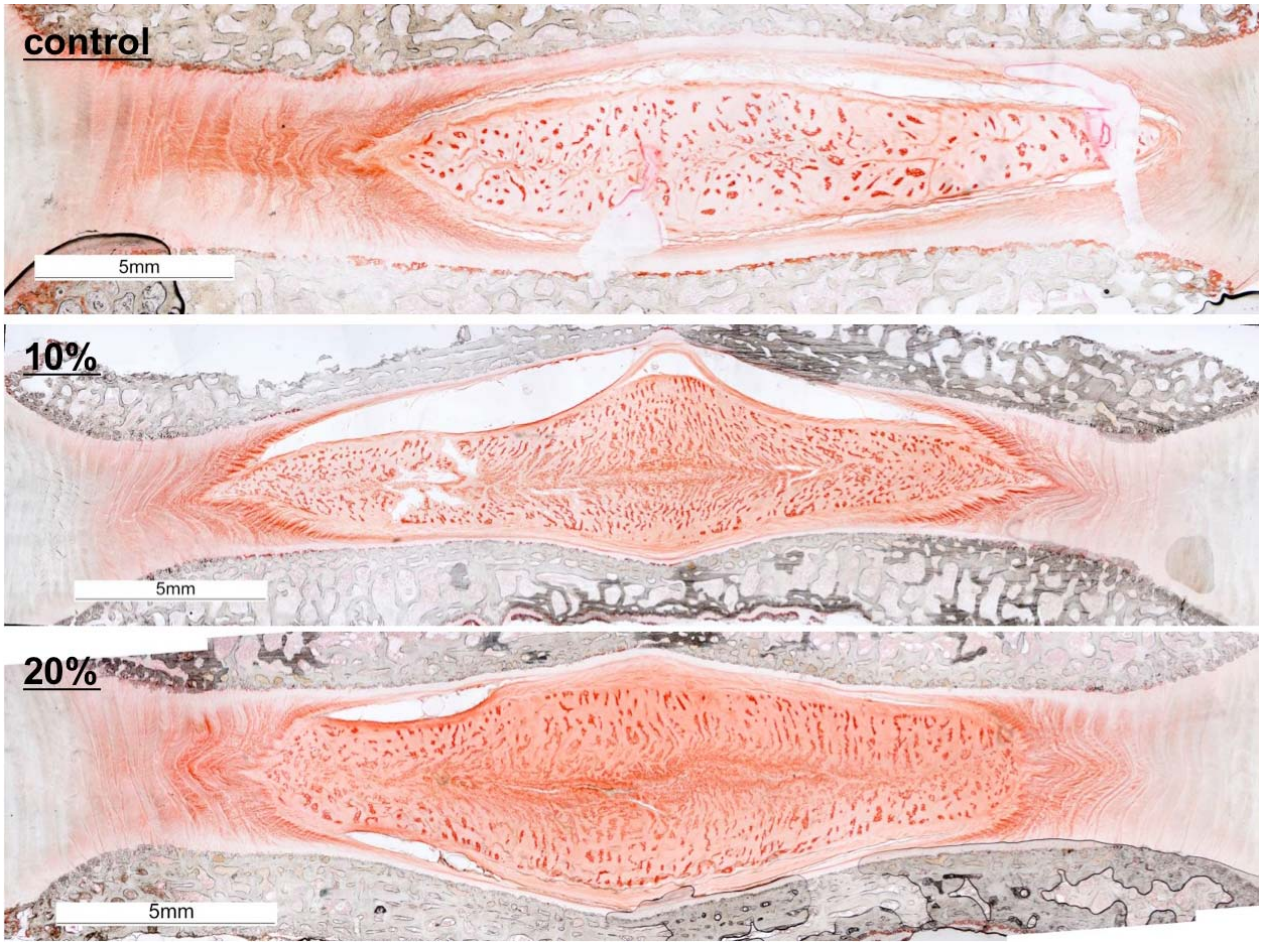


**Fig. 6.** Safranin O stained sections of the IVD allograft. Positive sulphated GAG orange staining was observed in the IVD following cryopreservation. Normal morphology, including oval NP, organised annular lamellae as well as clear boundary between AF and NP was observed. Top: fresh control, middle: cryopreserved with 10% CPA, bottom: cryopreserved with 20% CPAs. Scale bar = 5mm

**Effect of cryoprotective agents**

Using the cooling rate of -0.3°C/min deduced in the previous study, the effects of 3 different formulae of cryopreservation solutions on preserving disc metabolic activity were tested. In the NP, no significant difference was found between using the conventional agent 10% DMSO (10% CPA) or a formula of 10% DMSO +10% propylene glycol (20% CPA), with a metabolic activity between 70 to 82% of the fresh control (Fig. 3). Cryopreserving the IVD in 10% DMSO plus Supercool X-1000 resulted in significantly lower cell metabolic activity in the NP (14.5 ± 10.7%) (Fig. 3). In the AF, a significantly higher metabolic activity was found in the 20% CPA group (47.0 ± 16.2% of the control) compared with other groups (between 17- 30% of the control) (Fig. 3). The results suggest that mixing 10% DMSO and 10% PG favours maintenance of the overall disc metabolic activity during the cryopreservation process. This formula of CPA was adopted in subsequent tests.

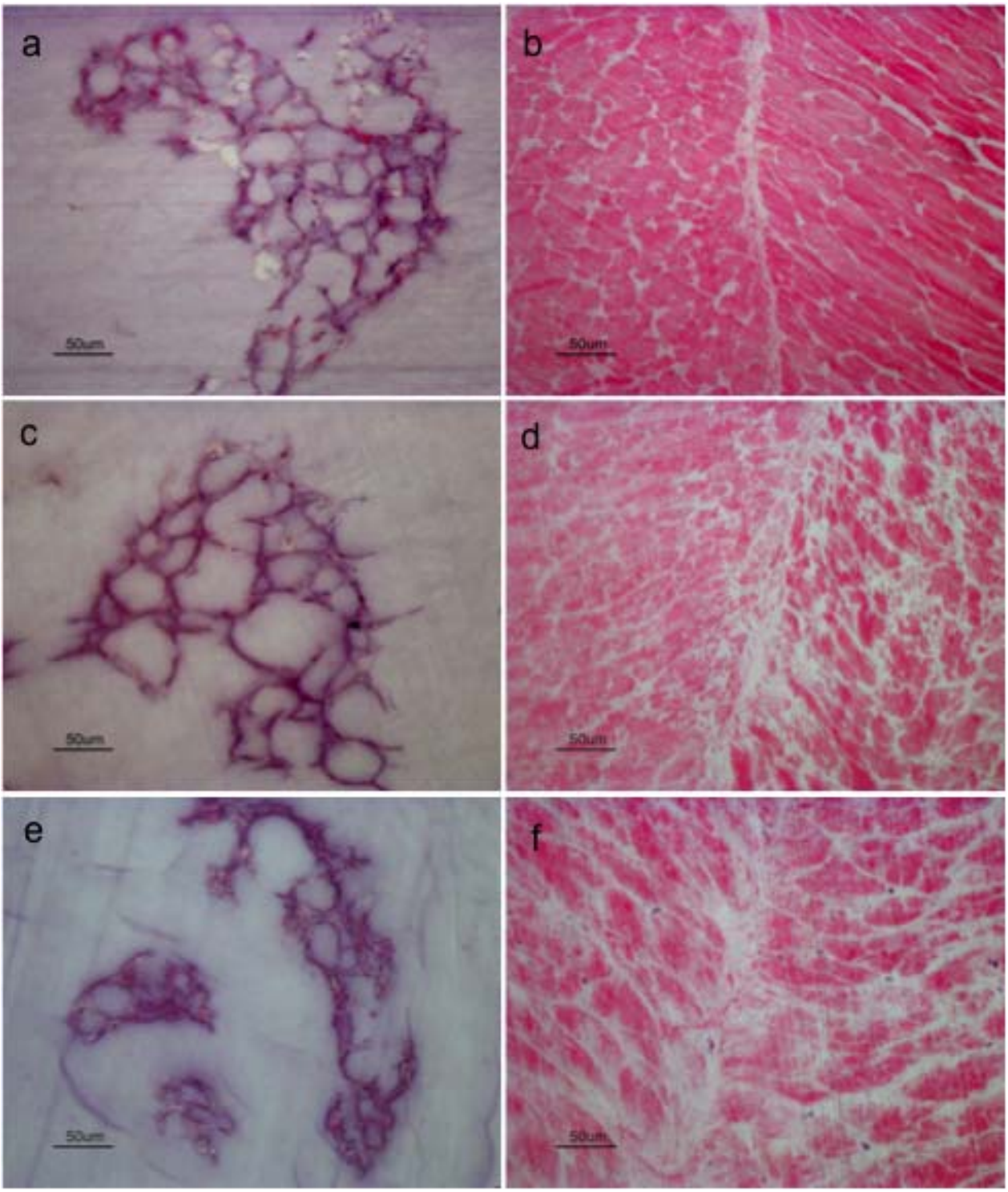


**Fig. 7**. Haematoxylin & Eosin stained sections of the IVD allograft. Top row: fresh control **(a)** NP, **(b)** AF), middle row: cryopreserved with 10% CPA **(c)** NP, **(d)** AF), bottom row: cryopreserved with 20% CPAs **(e)** NP, **(f)** AF). Large vacuolated cells stayed in cluster were found in fresh or cryopreserved samples **(a, c, e)**. NP cells in the fresh control and cryopreserved with 20% CPA appeared in round shape while those cryopreserved in 10% CPA appeared squashed. In AF, *annulus* fibres stayed in an organized manner after cryopreservation but wider white space appeared between each fibres in cryopreserved samples indicating the track of ice formation during the freeze/thaw process. Scale bar = 50μm.

**Effect of incubation time in cryopreservation solution**

Disc metabolic activity was evaluated between discs that underwent a pre-cooling incubation time of 2 and 4 hours in the cryopreservation solution consisting of 20% CPA. NP metabolic activity was 79.0 ± 17.4 % for a 4 hr incubation and 70.5 ± 13.0 % for a 2 hr incubation, while AF metabolic activity was 33.9 ± 2.4 % and 47.0 ± 16.2 % for 4 and 2 hr incubation, respectively (Fig. 4). The differences were not statistically significant.

**Effect of cryopreservation on the mechanical properties**

The apparent Young's modulus of the fresh control and different CPA concentration treatment groups are presented in Fig. 5. The apparent Young's modulus of the fresh control group was 14.5 ± 2.4 MPa, that of 10% CPA was 14.8 ± 4.4 MPa and that of 20% CPAs was 14.5 ± 0.9 MPa. *Post-hoc* tests showed no significant differences between the fresh control and the 20% CPA group ($p$ = 0.986) as well as the 10% CPA group ($p$ = 0.908). Also, no significant difference was found between the 10% CPA group and the 20% CPA group ($p$ = 0.894).

**Effect of cryopreservation on structural integrity**

The gross structure of the fresh and cryopreserved IVD was evaluated by histology. All the allografts exhibited an oval NP shape and a clear NP-AF boundary (Fig. 6). Safranin O staining showed that the cryopreservation process was able to preserve a sulphated glycosaminoglycan (GAG) content within the NP area comparable to the fresh control IVD as demonstrated by the positive orange staining in Fig. 6. Normal morphological characteristics of the IVD were observed in all cryopreserved allografts as accessed by H&E staining (Fig. 7: a,b (fresh control), c,d (10% CPA), e,f (20% CPA)). Cells were sparsely distributed in the NP, in which vacuolated cells remained in clusters (Fig. 7 a, c, e). Cells in the NP were roundish in fresh and cryopreserved with 20% CPA samples while some of the NP cells in the 10% CPA cryopreservation sample appeared squashed. An organized AF structure was also preserved (Fig. 7 b,d,f). However, *annulus* fibres appeared less densely packed in the cryopreserved disc, compared with the fresh control.

## Discussion

Regenerative medicine and tissue engineering are novel approaches for treatment of IVD degeneration. Studies have been performed on various animal models to investigate the performance of disc unit transplantation as a biological treatment for alleviating pain and preserving motion of severely degenerated discs. Feasibility of disc grafting has been studied using disc-vertebral-disc units (Olson *et al.*, 1991), IVD autografts (Frick *et al.*, 1994), fresh IVD allografts (Luk *et al.*, 1997) and recently, cryopreserved IVD allografts (Katsuura and Hukuda, 1994; Luk *et al.*, 2003; Matsuzaki *et al.*, 1996; Ruan *et al.*, 2007). Cryopreserved IVD allograft transplantation is more practical in the clinical setting than fresh allografts because of the limited allograft availability and the need for size matching. From previous studies, Luk *et al.* (2008) suggested that disc size matching and press-fit fixation is essential to achieve primary stability of the transplant. Therefore, establishing an IVD bank with various disc sizes is essential for the clinical application of IVD allograft transplantation (Luk and Ruan, 2008). Tissue allografts that underwent cryopreservation are proposed to have reduced immunogenicity (Rodrigo *et al.*, 1996). However, allograft preservation may compromise the disc cell viability as cells could be damaged due to the freezing and thawing procedures. Therefore, we aimed to improve the IVD allograft quality by investigating the effects of whole disc cryopreservation on graft integrity.

It is technically challenging to keep cells alive in large organs. Katsuura and Hukuda (1994) studied the biosynthetic activity of the cryopreserved dog lumbar disc measured by $^{35}S$ uptake and analyzed with autoradiography. The authors found an overall activity of 44% of the fresh control (Katsuura and Hukuda, 1994). Matsuzaki *et al.* (1996) compared two storage temperatures for dog lumbar disc allografts at -80°C or -196°C using conventional step-wise slow freezing, rapid thawing and 10% DMSO as freezing medium. They analyzed the $^{35}SO_4$ and $^{3}H$-proline radioactive incorporation representing proteoglycan and collagen synthesis by the disc cells respectively, which were merely 5-10% of the fresh control (Matsuzaki *et al.*, 1996). These low cell activity grafts were transplanted and showed gradual narrowing of disc space and loss of hydration beginning at 6 months. Both authors suggested that penetration of the CPA into the disc tissue, especially into the NP, is the major hurdle for successful disc graft cryopreservation. Ruan *et al.* used a similar approach for cryopreserving the human cervical disc allograft in the recent clinical trial, and the allografts were transplanted into 5 patients with cervical disc herniation (Ruan *et al.*, 2007). A five-year clinical follow-up was performed with serial MRI and static and dynamic radiographs to monitor the stability and mobility of the graft segment. The results were encouraging as the spinal stability and motion of the spine were preserved and no significant immunoreaction was observed. Nevertheless, one major issue of allograft disc transplantation is the progressive degeneration of the graft, which is believed to be partly caused by decreased cell viability and/or cell activity of the cryopreserved allograft (Katsuura and Hukuda, 1994; Matsuzaki *et al.*, 1996; Luk *et al.*, 1997; Luk *et al.*, 2003).

One major difference of disc cryopreservation methodology of the current study compared with previous studies is the choice of the serum free vehicle solution. Previous studies have used various cell culture media (M-199 (Katsuura and Hukuda, 1994), RPMI-1640 (Ruan *et al.*, 2007)) with 10% foetal bovine serum and DMSO for allograft cryopreservation. However, the use of bovine serum has the risk of transmitting disease such as bovine viral diarrhoea virus (BVDV) (Zabal *et al.*, 2000) and bovine spongiform encephalopathy virus (Bradley, 2004).We suggest that a serum free vehicle solution would be more appropriate for future clinical use.

Factors such as cooling rates, concentration of CPAs and pre-cooling incubation time can also contribute to the outcome of cryopreservation process (Mazur, 2004). By combining all the optimal parameters we were able to preserve porcine IVD with a post-cryopreservation metabolic activity of approximately 70% of the fresh control in the *nucleus pulposus* and 45% of the fresh control in the *annulus fibrosus* (Fig. 4). These rates are almost 2-fold higher than that of using the conventional method (Fig. 2, cooling rate at -1°C/min with 10% DMSO). Our results also indicated that different compartments within the IVD responded differently to specific parameters of the process.

Due to the differences in matrix properties and cell types, specific organ or tissue engineered constructs need unique cooling rates to minimize the damage caused by ice formation and the solution effects during cryopreservation (Pegg, 2003). In the present study, a significantly higher metabolic activity was found when the NP was cooled at a calculated rate of -0.3°C /min, while a cooling rate of -0.5°C/min was more favourable to the AF. The difference in the effect of the cooling rates on different tissue compartments is not surprising, as they differ in water content, matrix and cell composition. We understand that preserving an intact tissue structure and the amount of live cells are equally important in AF and NP for successful allograft transplantation, but we were unable to identify a cooling rate which works the best for both regions. Loss of NP cell activity was believed to be the major cause of decrease in disc height in previous studies (Katsuura and Hukuda, 1994; Matsuzaki *et al.*, 1996), therefore we aimed to preserve as many native NP cells as possible. We adopted the lower cooling rate (-0.3 °C/min) for further study since NP cell activity was dramatically preserved at the cooling rate of -0.3 °C/min compared with other cooling rates.

Cryoprotective agents function to protect the cells during the freezing process and maintain the structure of the tissue by various mechanisms (Mazur, 1984). Five to ten percent DMSO has been reported to be the optimal concentration for freezing different types of cells while concentrations higher than 12% have been described as toxic (Vos and Kaalen, 1965). Combinations of two or more CPAs may result in additive or synergistic enhancement of cell survival (Brockbank and Smith, 1993). We found synergistic enhancement in cell metabolic activity by using a combination of 10% DMSO and 10% PG as CPA compared to using 10% DMSO alone, where higher metabolic activity of the AF could be achieved. The effect of additional CPA on cellular activity in NP was, however, not significant. It is postulated that the NP itself may naturally possess anti-freeze properties due to its high proteoglycan content. Proteoglycan attracts water due to its high affinity for hydrogen bonds and may therefore act as an oncotic agent in the NP (Taylor, 1986). The natural polymer chondroitin sulphate has also been identified as very effective in maintaining stromal hydration during hypothermic storage (Taylor and Hunt, 1989). A variety of oncotic agents, such as polyvinylpyrrolidone (PVP) and dextran, has been used to restrict stromal swelling during hypothermic preservation (Taylor, 1986). Supercool X-1000, a synthetic polyvinylalcohol copolymer, is designed to suppress ice formation in tissue cryopreservation and applied at a concentration of 0.1 - 1% for tissue vitrification (Wowk *et al.*, 2000). Ice-blocking agents are usually used in tissue vitrification which employs a vitrification solution containing a combination of high concentration of CPAs and low volume of ice-blocking agents, as well as cooling (~ 30 °C/ min) and rewarming (~ 225 °C/min) at high rates (Fahy *et al.*, 2004b). We attempted to vitrify the IVD using a vitrification solution along with high cooling and re-warming rates in our pilot study, but the results were disappointing. This is very likely due to the non-homogenous change of temperature during cooling and re-warming, since the cooling and re-warming rates across the tissue samples vary depending on the geometry and the heat conductivity of the organ (Arav and Natan, 2009). At present, it is technically difficult to attain a homogenous temperature change in large organs composed of heterogenous cell types and with a high matrix content, at such high rates. Therefore, we tested a simple cryopreservation protocol with the addition of 0.1% of Supercool X-1000 with 10% DMSO at low cooling rate, but did not find any improvement in cell activity. To test the possibility of vitreous preservation of the IVD, the issue of attaining a homogenous high rate of cooling, re-warming of the organ and the complete penetration of the vitrification solution has to be resolved before further investigation can take place. Further research on directional freezing (Arav *et al.*, 2009), dielectric heating by electromagnetic re-warming systems (Wusteman *et al.*, 2004) and the use of other vitrification solution such as VS41A and M22 (Fahy *et al.*, 2004a) may help in further advancing IVD cryopreservation.

Thorough penetration of CPA into the IVD is required for effective protection of the tissue from cryodamage (Ohlendorf *et al.*, 1996), an issue of high importance (Katsuura and Hukuda, 1994; Matsuzaki *et al.*, 1996). Slow permeation of CPA in cornea and cartilage has been reported, possibly due to the presence of highly charged GAG (Klossner *et al., 2008*) and collagenous network (Maroudas, 1976; Walcerz *et al.*, 1995). The IVD being a GAG- and collagen-rich tissue (Roberts and Caterson, 2004), might behave similarly with slow CPA permeation. Solutes diffuse into the disc through both the outer *annulus* and the endplates pores, but endplates pores can be frequently blocked by blood clots during tissue harvest. In this study, we used a modified IVD allograft preparation method for clearing blood clots on the endplate pores. This method has been previously verified to improve diffusion of small molecular weight solutes into the inner *annulus* and the *nucleus* (Chan *et al.*, 2010). We believe this graft preparation method has contributed to the appealing outcome for the NP. The length of incubation time in the cryopreservative solution may also determine the extent of penetration of CPA into the IVD. However, prolonged incubation of the tissue in the CPA may expose the cells to CPA toxicity (Tomford *et al.*, 1984). Kuranobu (1993) found the best result with an incubation time of 90 minutes for cryopreservation of the rabbit vertebral body-disc (Kuranobu, 1993). We intended to extend the incubation time from 2 to 4 hours for better CPA penetration but no

significant difference was found, indicating that extending the incubation time might not give additional benefit in disc cryopreservation. Other factors, such as the rate of heat and mass transfer in such a big organ, might possibly affect the result of cryopreservation and will need to be further investigated.

While metabolic activity is one of the important aspects for successful allograft disc transplantation, stability and motion of the spine will not be properly maintained if the disc's native mechanical properties are not preserved. It has been shown that frozen storage of the IVD will permanently alter the mechanical behaviour (Bass *et al.*, 1997) and caused degeneration of the biomechanical properties of the IVD (Gleizes *et al.*, 1998). Cryopreserving tissue, such as ovine cardiac heart valves, with CPA, can maintain the viability of the tissue and excellent microscopic structure with unchanged mechanical properties (Aidulis *et al.*, 2002). The thermal stresses that develop during the cooling and thawing procedure could cause fractures in the tissue structure (Pegg *et al.*, 1997), but our data suggest that the cooling and thawing protocol applied in this study can avoid the occurrence of these fractures. The mechanical property of the cryopreserved discs is similar to that of the fresh discs, suggesting that the cryopreserved IVD allograft might retain the ability to attenuate shocks and the transfer of load in the spine after being transplanted. Without the elasticity of a normal disc, a stiffer IVD cannot transmit the loading through the spine and may even change the kinematics of the spine. It is postulated that the preservation of mechanical properties of the cryopreserved IVD is related to the preservation of IVD structure (Bass *et al.*, 1997). Our study compared the disc structure between the fresh controls and those cryopreserved with 10% DMSO or 20% CPA. No significant alteration of the AF or the NP structure following cryopreservation was found. While the bulk mechanical property of the disc was not affected by cryopreservation, some of the NP cells in the disc cryopreserved with 10% CPA showed altered cell morphology compared with the fresh control, indicating that cells were stressed during cryopreservation. The relatively less densely packed *annulus* fibres also suggested the formation of ice during cryopreservation, which could account for the relatively low cell activity in the AF region.

When the animal model for the cryopreservation study is considered, the size and the geometry including surface area, volume and depth of the tissue, are of equally importance as the matrix and cell composition of the allograft (Mazur, 2004). The size of the porcine lumbar IVD used in this study is comparable to the human adult cervical IVD previously used in allograft transplantation (Lu *et al.*, 1999). Moreover, Beckstein *et al.* have demonstrated that the pig lumbar disc is comparable to the human lumbar disc in terms of disc axial mechanics, glycosaminoglycan (GAG) and water composition, and geometry (Beckstein *et al.*, 2008). However, NP cell composition is different between the human and the pig. Notochordal cells are present in the NP throughout the life of the pig but not in the human after the teenage years (Pazzaglia *et al.*, 1989). With this limitation, we believe the findings in this study may provide a guideline useful for future IVD cryopreservation studies. Verification of the protocol with human disc samples is recommended before clinical use. Since the maintenance of cell metabolic activity does not necessarily imply that the cells behave normally and retain the ability to maintain a healthy extracellular matrix. It is also not clear how well these "better-preserved" disc allografts perform when transplanted *in-vivo* in both short and long term. Thus verification of their gene expression and protein content will be important in future studies.

One additional limitation, when translating this protocol to human discs, is that disc cells can be lost during harvesting and before cryopreservation. Trout *et al.* reported that 50% of the cells in the freshly harvested adult human disc are necrotic cells (analyzed by scanning electron microscopy) (Trout *et al.*, 1982), while Jünger *et al.* reported around 80% - 90% cell viability (analyzed by Live/Dead stain and confocal microscopy) in the freshly harvested sheep disc (Junger *et al.*, 2009). Thus the starting number of viable cells may vary and even in the best-case scenario, will not be equivalent to a normal disc. However, even if cell numbers are grossly reduced, a degenerated disc does not mean a symptomatic disc, while it still maintains its mechanical and biological functions (Luk *et al.*, 2008). If cell numbers are critically reduced, possible strategies to augment this would include injection of growth factors or transplantation of nucleus or stem cells. Our previous work suggests that introduction of bone-marrow derived stem cells into a cryopreserved disc is feasible (Chan *et al.*, 2010).

In conclusion, we have investigated the response of cells in the different compartments of the IVD to different conditions of whole disc cryopreservation. The incorporation of a slow cooling rate (-0.3°C/min), a combination of cryoprotective agents (10% DMSO and 10% PG), and a limited CPA incubation time (2 hours) can favour the overall metabolic activity of disc cells. Using this preferred protocol, the overall disc cell metabolic activity was more than 60% of the fresh control, and the mechanical property and overall matrix organization were also maintained. However, there are a number of limitations in this animal model, such as the variation in cell and matrix composition, and the size difference between human and porcine discs. The performance of these cryopreserved discs in the long term is also unclear; therefore, caution should be taken when translating this work to humans. Advances in cryopreservation methodology by investigating the possibility of using a vitrification method can facilitate the development of cryopreserved IVD allografts and tissue engineered constructs for future clinical applications.

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