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RESEARCH ARTICLE

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Cryopreserving the intact intervertebral disc without compromising viability

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Abstract

Background: Tissue cryopreservation requires saturation of the structure with cryoprotectants (CPAs) that are also toxic to cells within a short timeframe unless frozen. The race between CPA delivery and cell death is the main barrier to realizing transplantation banks that can indefinitely preserve tissues and organs. Unrealistic cost and urgency leaves less life-threatening ailments unable to capitalize on traditional organ transplantation systems that immediately match and transport unfrozen organs. For instance, human intervertebral discs (IVD) could be transplanted to treat back pain or used as ex vivo models for studying regenerative therapies, but both face logistical hurdles in organ acquisition and transport. Here we aimed to overcome those challenges by cryopreserving intact IVDs using compressive loading and swelling to accelerate CPA delivery.

Methods: CPAs were tested on bovine nucleus pulposus cells to determine the least cytotoxic solution. Capitalizing on our CPAs Computed Tomography (CT) contrast enhancement, we imaged and quantified saturation time in intact bovine IVDs under different conditions in a bioreactor. Finally, the entire protocol was tested, including 1 week of frozen storage, to confirm tissue viability in multiple IVD regions after thawing.

Results: Results showed cryopreserving medium containing dimethyl sulfoxide and ethylene glycol gave over 7.5 h before cytotoxicity. While non-loaded IVDs required over 3 days to fully saturate, a dynamic loading protocol followed by CPA addition and free-swelling decreased saturation time to <5 h. After cryopreserving IVDs for 1 week with the optimized CPA and permeation method, all IVD regions had 85% cell viability, not significantly different from fresh unfrozen controls.

Conclusions: This study created a novel solution to a roadblock in IVD research and development. Using post-compression swelling CPA can be delivered to an intact IVD over 20× more quickly than previous methods, enabling cryopreservation of the IVD with no detectable loss in cell viability.

KEYWORDS
cryopreservation, disc transplant, intervertebral disc, transport phenomena

1 | INTRODUCTION

Back pain is a nearly universal issue with substantial effects on patients' lives including disability, loss of income, loss of independence, depression, and loss of social support. In the US alone over 500 000 people annually undergo invasive surgery, such as spinal fusion, discectomies, and laminectomies to alleviate symptoms caused by IVD degeneration.[1] However, none of these procedures treat degeneration, and can lead to further health complications from limiting the spine's range of motion and patient's mobility.[2] For instance, fusions are known to cause adjacent segment issues that could require additional surgery at those adjacent levels. Artificial IVDs are an attractive alternative to fusion because they maintain a more natural range of motion,[2,3] and reduce risk of adjacent segment degradation. But there are concerns with artificial IVDs as well, including lifespan limitations, heterotrophic ossification, and facet issues to name a few.[4] Moreover, artificial IVDs add additional complexity to revision operations. Problems associated with current surgeries have driven the clinical and scientific research community to seek biological solutions for IVD structural failure.

1.1 | Biological treatment strategies for IVD pathologies

Mild to moderately degenerated discs are an attractive target for biological therapies such as cell therapy, biologics, or even tissue engineered nucleus pulposus (NP) replacement. One major complexity to this strategy is the intricate crosstalk between the exogenous cells, endogenous cells, mechanical loading, inflammation, and potential nutrient limitation that exist in degenerated human IVDs.[5] To optimize therapy, experimental systems are needed that account for this crosstalk, but it is difficult to recapitulate all these aspects of degenerated human IVDs in animal and cell culture models.[6] Whole IVD bioreactor culture models can represent more of these factors and help researchers determine method feasibility more quickly.

Severely degenerated IVDs are poor candidates for regeneration as there is often not enough tissue left to stimulate a full recovery. Whole IVD biological replacements, engineered or natural, are a potential strategy in this case, as they have the hypothetical ability to reduce complications associated with artificial IVD replacement such as limited lifespan. For this reason, cervical IVD allograft transplantations have been performed in human patients.[7] However, this practice has not gained widespread popularity or undergone clinical trials with ample sample sizes due to the necessity to size match the donor and recipient.[8] The reliance on size matching creates additional logistical obstacles to an already challenging protocol.

In effect, both research utilizing whole IVD culture and clinical research on IVD allograft transplantation suffer from similar logistical roadblocks.

1.2 | IVD organ procurement logistical challenges

Scientists and clinicians must overcome the following administrative and logistical challenges to work with whole IVDs:

1. They must have or develop a new relationship with an organ procurement organization (OPO). OPOs are incredibly important as the coordinating body for harvesting organs from human donors while tissues are still viable and transporting the tissues within hours to the matching recipient's location.
2. Some OPOs will not harvest spine tissue due to the risk of transmissible spongiform encephalopathy contamination and the need for extensive aesthetic reconstruction of the donor body when not being cremated.
3. Legal agreements, typically materials transfer agreements, must be made between the investigator and OPO.
4. To perform an allographic transplantation, size matching between donor and recipient, shipping of the IVD, and implantation into the recipient needs to occur in a short time window. This expedience is not practical for an elective procedure, and there is no current system to coordinate a transplantation of this nature.
5. Acquisition of a whole spine by researchers (23 IVDs) is most respectful of the donor gift, economical, and efficient. Still, it requires immediate culture initiation, with incubator or bioreactor capacity for all levels and reagents available to start experiments. Whole spine acquisition necessitates flexibility concerning gender, age, IVD size, and IVD degeneration grade, which might not be supported by the research question.

Biobanking of cryopreserved IVDs would allow scientists and clinicians to obtain IVDs with desired specifications at the optimal time for their incubation space, funding, reagent availability, experimental demand, staff time, or patient needs.

1.3 | Cryopreservation

Cryopreservation provides a solution to the long-term storage of cells and tissues by lowering the specimen's temperature to below −80°C, where cellular functions are limited and preservation can occur.[9,10] This process has revolutionized allograft clinical research in other medical fields by overcoming the supply–demand imbalance for viable specimens.[11,12]

There are two competing challenges for cryopreservation of any whole organ:

1. Cryoprotectants (CPAs) are required to prevent cell damage during freezing,[10] and they must permeate the entire organ.
2. Most CPAs are cytotoxic to mammalian cells and cause cell death or damage within a matter of hours unless frozen before that time.[13]

These challenges are notable in the transport limited IVD structure.[14] Studies of IVD nutrition show that transport of small molecules

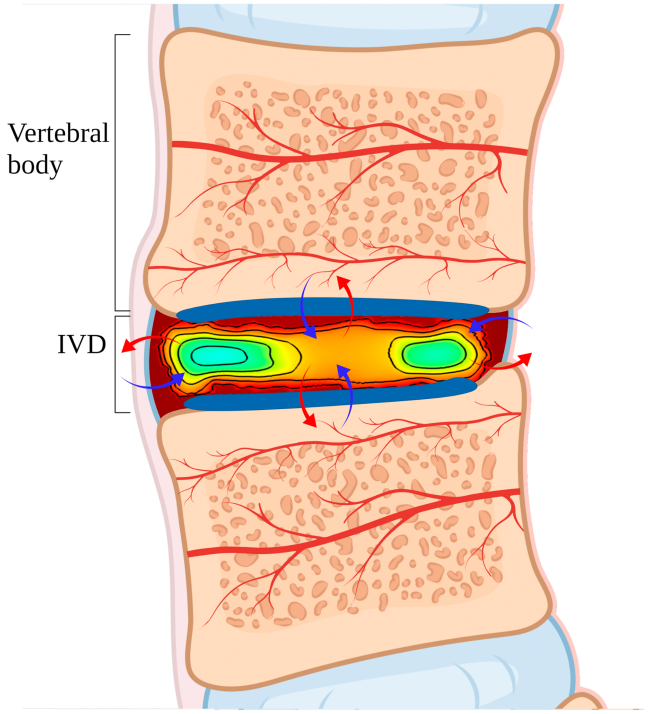

FIGURE 1 Schematic of small molecule distribution, such as glucose, in the IVD. Red are high concentrations of solutes and blue are low concentrations of solutes.

occurs through the cartilaginous endplates and radially through the annulus fibrosus, primarily reliant on a slow diffusion mechanism.[14,15] This results in the metabolite distribution shown in Figure 1. Assuming similar mechanisms for CPA transport in the IVD, this would result in the cells in the cartilaginous endplate (CEP) and annulus fibrosus (AF) being in contact with cytotoxic CPA immediately and needing to remain so until the outer NP/inner AF is permeated by the CPA. Several cryopreservation protocols for the IVD have been developed using various CPAs and incubation times ranging from 2 to 20 h.[16–19] Several CPA formulations have been shown to maintain NP cell viability and multipotency potential following cryopreservation.[20,21] Unfortunately, cell viability has not yet proven sustained across the intact entire structure.

In this study, we demonstrate a method to cryopreserve intact IVDs with no loss of cell viability compared to fresh unfrozen control samples, which has major implications for the following two applications:

1. Creating a biobank of fresh frozen human IVDs for potential total disc replacement allografts.
2. Increasing access for 3D whole IVD bioreactor research, thereby accelerating development of regenerative therapies through more realistic models.

Here we outline our approach and results to: (1) increasing the time before CPA cell damage occurs, and (2) decreasing the time to fully penetrate the intact IVD with CPA. Finally, we demonstrate the protocol fully and measure cell viability throughout the structure.

2 | MATERIALS AND METHODS

To conserve resources and avoid unnecessarily repeating prior work, this study was performed in the following 3 stages:

1. Examine previously published protocols using methods that decouple the CPA cellular toxicity and tissue transport limitations.
2. Optimize the toxicity and transport conditions seperately by studying multiple CPA mixtures' cellular toxicities, evaluating CPA tissue saturation time using several protocols, and evaluating CPA expulsion.
3. Confirm the full protocol, utilizing the optimized CPA mixture and transport conditions, maintains IVD viability through the cryopreservation, thawing, CPA expulsion, and subsequent short culture period. Ensure all areas of the IVD equally viable after cryopreservaion.

2.1 | Examine previous protocols

2.1.1 | Intervertebral disc harvest

NP cells were isolated from a protocol adapted from Wiseman et al.[22] Two freshly skinned skeletally mature bovine tails were obtained from a local abattoir, disinfected for 5 min in a 1% Betadine solution, and dried in a sterile environment for 10 min. Muscle and fat were removed with a scalpel and rongeurs. Bone-IVD-Bone segments were collected from the proximal three caudal levels. Cuts through the vertebrae were made axially using a surgical saw, leaving approximately 2 mm of bone on either side of the IVD. IVDs were kept hydrated with sterile gauze soaked in 0.9% sodium chloride and 55 mM sodium citrate while awaiting further processing. The exposed boney endplates were scrubbed with the sterile buffer-soaked gauze to remove some of the exposed blood and bone marrow.

2.1.2 | Intervertebral disc cell isolation

NP tissue was removed from the whole IVD with a scalpel, minced, and digested for 1 h in Dulbecco Modified Eagle Medium (DMEM) (D6046, Sigma, MO, USA) with 0.2% Pronase E (97062-916, VWR International, PA, USA) in a humidified incubator at 37°C and 5% CO_2. A second digestion followed in DMEM with 0.025% Collagenase (103701-190, VWR International, PA, USA) for 18 h. Digested NP was filtered using a 70-µm cell strainer and washed twice with 1× PBS prior to culture in media containing 89% DMEM, 10% FBS (F0926, Sigma, MO, USA), and 1% antibiotic/antimycotic solution (A5955, Sigma, MO, USA). Cells were passaged thrice prior to performing viability assays.

2.1.3 | Effect of standard cryopreservation medium on NP cells in monolayer

NP cells were cultured in monolayer in 6 well-plates to elucidate the cytotoxicity of a standard cryopreservation medium[16] composed of 80% DMEM, 10% dimethyl sulfoxide (DMSO) (IC0219605590, VWR International, PA, USA), and 10% propylene glycol (PG) (P4347, Sigma, MO, USA). The wells were assigned randomly to incubation times of 2, 6, 12, 18, or 24 h ($n = 3$) at 4°C. Cells were stained with a fluorescent LIVE/DEAD™ assay (L3224, ThermoFisher, MA, USA) and scanned using Keyence BZ-X700 fluorescence microscope (Keyence, IL, USA). The captured scans were processed on ImageJ 1.53i to estimate cell viability.[23] Due to the propensity of dead cells in monolayer to detach and wash away during staining, live cell counts were normalized to the seeding density.

2.1.4 | CPA transport in the IVD

We assessed the transport of DMSO in unloaded bovine IVDs ($n = 3$) incubated in 10% DMSO for 3 days. DMSO was visible using Computed Tomography (CT) scans taken at 0, 24, 56, and 72 h with a Toshiba Aquilion 64-slice CT. Acquisition parameters were 120 kVp, 240 FoV, 0.6 pitch, and 250 mA.

Materialize Mimics® 21.0 was used to segment the IVD's soft tissue (AF and NP). A code was developed in MATLAB® 9.9 to perform a Gaussian curve-fitting analysis of the segmented scans, estimating the peak signal intensity (Figure 2). To relate mean signal intensity at the various time points (IVD_n) to saturation percentage we used Equation (1):

$$\text{DMSO saturation} = \frac{IVD_n - IVD_0}{IVD_f - IVD_0} \quad (1)$$

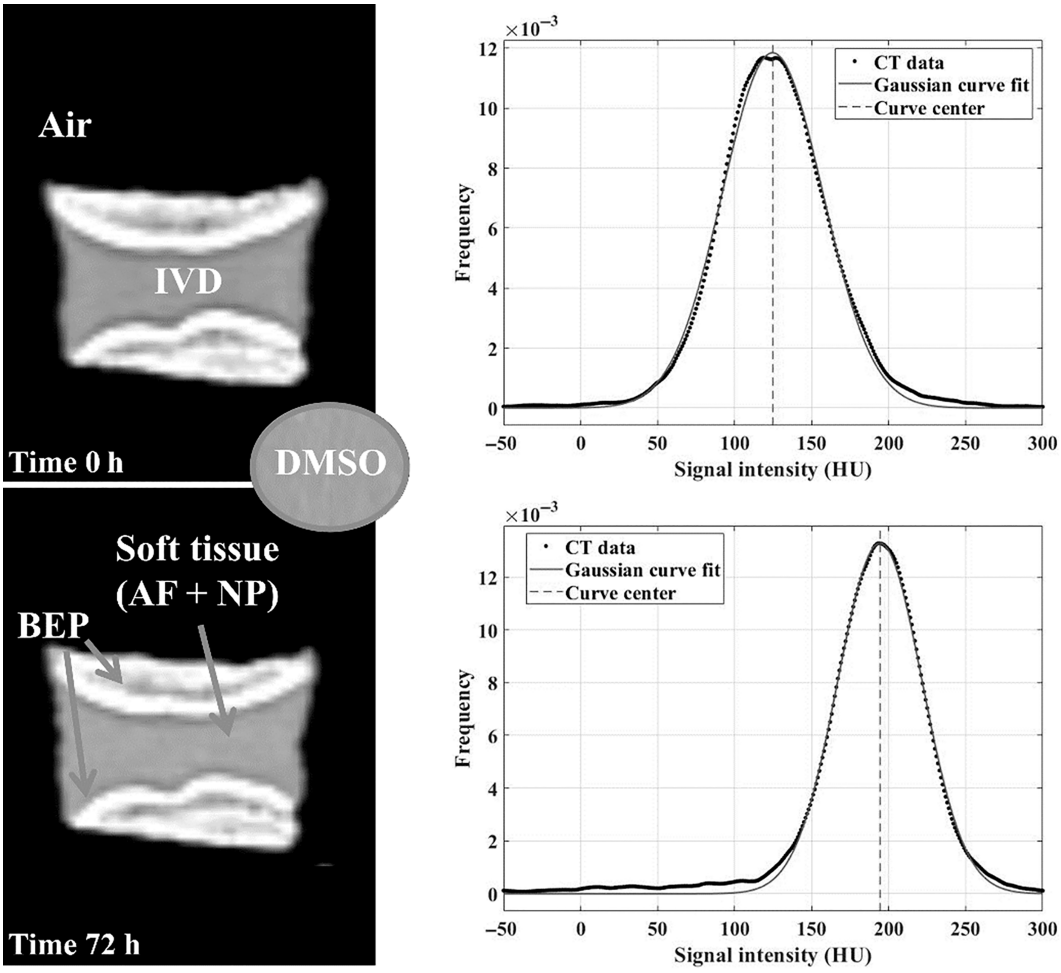

FIGURE 2 The mean signal intensity of the intervertebral disc's (IVD) soft tissue (Annulus Fibrosus, AF, Nucleus Pulposus, NP) increased due to the penetration of dimethyl sulfoxide (DMSO), though is not apparent to the naked eye. To quantify the intensity change several image processing steps were required. CT scans of IVDs were segmented in Materialize Mimics® 21.0 (left) with the boney endplate (BEP) excluded. Histograms of the segmented regions were created in MATLAB® 9.9 and the mean signal intensity (Hounsfield units, HU) was estimated by fitting with a Gaussian curve (right). The images on top show the CT scan and histogram before the addition of DMSO, while the bottom show the scan and histogram 72 h after DMSO addition and no IVD compression.

where IVD_0 and IVD_f are the mean signal intensities before exposure to DMSO and after complete saturation with DMSO, respectively. IVDs were assumed fully saturated after 1 week, at which point the mean signal intensity had reached an asymptote.

2.2 | Optimize conditions

2.2.1 | Optimization of cryopreservation medium composition in 3D alginate culture

We assessed toxicity of the standard cryopreservation medium[16] and a new cryopreservation medium, this time in 3D culture to improve model accuracy. The new cryopreservation medium contained 10% ethylene glycol (EG, 324558, Sigma, MO, USA) instead of 10% PG. To generate 3D alginate cultures, passage 3 cells were suspended in 2% low-viscosity alginate (A0682, Sigma, MO, USA) in 0.15 M NaCl to a final density of 300 000 cells/mL. The alginate cell solution was slowly dropped into 0.15 M $CaCl_2$ (C4901, Sigma, MO, USA) using a 16G needle, incubated for 15 min on a rocker, washed twice with 0.15 M NaCl, and incubated in cell culture media for 24 h at 5% CO_2 at 37°C before assessing viability. The live/dead assays were performed as above using 5 cell-seeded alginate beads per well and incubated for 0, 2, 4, 8, or 48 h.

2.2.2 | Optimization of CPA penetration into IVD

We also assessed the effectiveness of PrimeGrowth™ reagents ($n = 3$) and compression ($n = 3$) in improving the transport of DMSO in intact IVDs and decreasing cell CPA exposure time compared to unloaded IVDs ($n = 3$). PrimeGrowth™ Isolation Medium is designed to clear blood clots in the CEP, improving transport through its pores and channels,[24] while axial compression could enhance the convective transport of fluids in the IVD. The two experimental groups were treated as follows:

1. IVDs treated with PrimeGrowth™ reagents were incubated in a 30 mL PrimeGrowth™ Isolation Medium for 1 h and rinsed in an equal volume of PrimeGrowth™ Neutralization Medium for 2 min, then transferred to 10% DMSO.[24]
2. IVDs treated with compression were dynamically loaded (0–0.25 MPa at 0.5 Hz) in culture medium in a custom-built bioreactor, modeled after Haglund et al.[25] The IVD height change approached its minimum asymptote after 3–4 h. Then 10% DMSO was added and the IVDs were allowed to free-swell. IVD height change was monitored using the displacement readout from the bioreactor.

DMSO penetration in IVDs was tracked via CT scans at 0, 5, 8, 24, and 36 h and compared to a control group ($n = 3$) not treated with PrimeGrowth™ Isolation Medium or compression.

2.3 | Confirm whole protocol maintains cell viability

2.3.1 | IVD cryopreservation

IVDs were cryopreserved using the optimized method comprised of compressing the IVDs, then allowing free-swelling in the new cryopreservation medium containing 10% DMSO and 10% EG. To test cell viability after the full protocol 20 bovine IVDs were assigned randomly to four groups:

1. Fresh unfrozen controls
2. No compression and no CPA
3. With compression and with DMSO+EG
4. No compression, but with DMSO+EG

The IVDs were cooled from 4 to −80°C in sterile specimen cups containing 50 mL of cryopreservation media in a polystyrene box. A freezing rate of −1°C/min was measured in previous samples with a thermocouple placed in the center of an IVD. After 1 week of storage at −80°C, the IVDs were thawed in a 37°C water bath and DMSO was removed into 1X PBS through a step-wise dilution process using 3–4 h of compression, a PBS exchange, and 3–4 h of free-swelling at 4°C. Once the DMSO was removed, all IVDs were cultured in 89% DMEM, 10% FBS, and 1% antibiotic for up to 12 h at 37°C, then dissected to estimate cell viability. A central sagittal cross section 2 mm thick was acquired for a LIVE/DEAD™ assay (L3224, ThermoFisher, MA, USA) and imaged using the Keyence BZ-X700 fluorescence microscope.[23]

Three additional IVDs were used to measure the rate at which the CPA was expelled during post-thaw compression and free-swelling cycles. The IVDs were first treated with compression to saturate them with the CPAs (10% DMSO and 10% EG), frozen and thawed as described above. Then the IVDs were compressed for 3–4 h in 100 mL of 1X PBS, the PBS was replaced and the IVD then allowed to free-swell for 3–4 h. This process was repeated for 22 h. Osmolarity in the solution was measured with a micro-osmometer (Advanced® Model 3320, Advanced Instruments, MA). A mole balance was used to calculate the percent of CPA remaining over time.

2.3.2 | Statistics

Statistica™ software (TIBCO, Hamburg, Germany) was used to perform all statistical analyses. In the first portion of the study, where a conventional protocol was examined, a one-way analysis of variance (ANOVA) was performed for the viability and DMSO saturation experiments with $p < 0.05$ considered significant. When the ANOVA showed significance, post hoc Tukey Honestly Significant Difference (HSD) tests were performed to compare time points. The same analysis was performed for the CPA removal experiment. In the second and third portion of the study, where multiple treatment groups were tested, a two-way ANOVA was performed for the viability and DMSO

saturation experiments. When the ANOVA showed significance, Post hoc Tukey HSD tests were performed. All data are reported as means ± standard deviation.

3 | RESULTS

3.1 | Examine previous protocols

Conventional protocols for cryopreservation of intact IVDs demonstrated the need for reduction of CPA toxicity and/or faster IVD saturation. Here, the percentage of cultured NP cells remaining decreased from 100 ± 56% in the control to 6 ± 3% after 6 h of incubation in the medium containing 10% DMSO and 10% PG (DMSO +PG) (Post hoc, Tukey HSD, $p = 0.004$) (Figure 3A).

To examine the rate of DMSO penetration into IVD tissue, DMSO attenuation of CT beam intensity was measured. An unloaded IVD in DMSO achieved 55 ± 4% saturation after 24 h (Post hoc, Tukey HSD, $p = 0.07$ compared to time 0) increasing to only 76 ± 4% by 72 h of incubation (Post hoc, Tukey HSD, $p = 0.02$ compared to time 0). The observed rate of DMSO saturation would lead to total cell death in the IVD before reaching 100% saturation (Figure 3B).

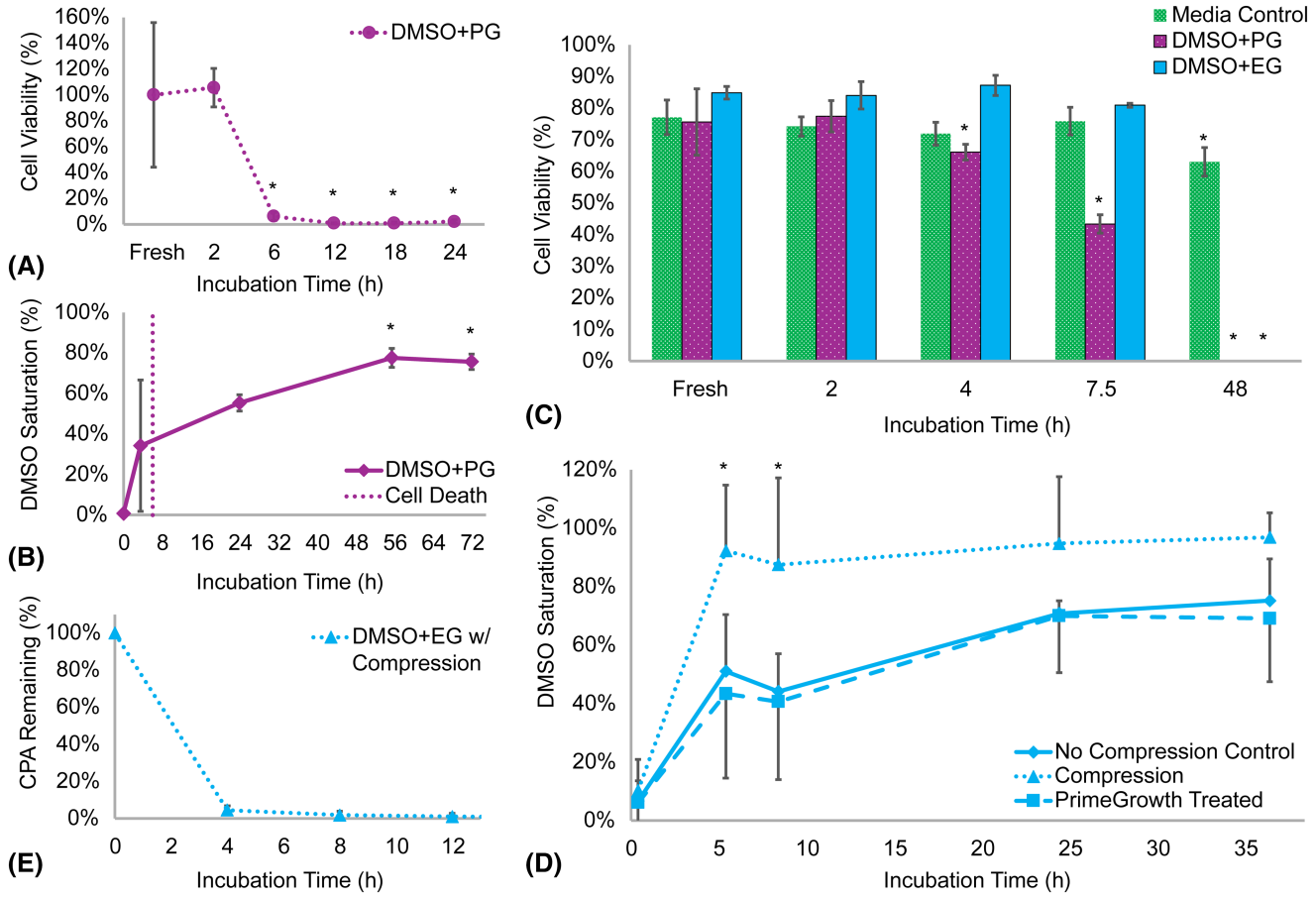

FIGURE 3 (A) Shows cell viability of Nucleus Pulposus (NP) cells in a 2D monolayer culture exposed to standard cryopreservation medium (10% DMSO, 10% PG) at 4°C for 24 h ($n = 3$). Significance was denoted by an * comparing treatment groups with the fresh control (post hoc Tukey HSD, *$p < 0.005$). (B) Average dimethyl sulfoxide (DMSO) saturation in unloaded bovine IVD organ culture explants ($n = 3$) was measured via CT at 5, 24, 56, and 72 h of incubation. Mean DMSO saturation in the IVD tissue was significantly different after 56 h of incubation compared to time 0. The data was normalized to IVDs that had soaked in 10% DMSO for 1 week. The dashed line shows the time at which significant cell death was observed in the cell culture cytotoxicity experiment (A). * indicate significance when comparing time point with $t = 0$ (post hoc Tukey HSD, *$p < 0.02$). (C) The effect of cryopreservation medium composition and incubation time on the average nucleus pulposus (NP) cell viability in 3D culture ($n = 4$). Cells exposed to dimethyl sulfoxide and propylene glycol (DMSO+PG) exhibited decreasing viability at 4 h compared to the control at time 0. Dimethyl sulfoxide and ethylene glycol (DMSO+EG) was not observed to affect cell viability at 7.5 h of incubation. At 48 h, cell viability was estimated to be 0% for both CPA groups. Significance was estimated by comparing the samples with the control at time 0 with * indicating $p < 0.05$ (post hoc Tukey HSD). (D) Optimization of DMSO saturation in bovine IVD organ culture explants ($n = 3$). Compression increased DMSO saturation to 92% at 5 h. IVDs treated with PrimeGrowth™ Isolation Medium did not observe improved transport when compared to the control at each time point. * indicates significance of a group compared to the control group at the same time point (post hoc Tukey HSD, $p < 0.05$). (E) The percent of DMSO+EG remaining in the IVD during post thaw loading was measured by tracking the osmolarity of the surrounding fluid and performing a mass balance. The percent DMSO+EG remaining did not change significantly after 4 h (one-way ANOVA, $p = 0.81$). Error bars indicate standard deviation.

3.2 | Optimize conditions

Optimization of CPA type required finding a CPA mixture with lower cytotoxicity than the standard cryopreservation medium, which was observed to be devastating for cell viability in a monolayer cell culture (Figure 3A). The new cryopreservation medium, which replaced PG with EG, decreased cytotoxicity and improved overall cell viability for at least 7.5 h (Figure 3C), extending the time available to saturate the IVD with CPA.

Next, methods to improve CPA penetration through the IVD were studied. Compression and PrimeGrowth™ were hypothesized to decrease the overall time it required DMSO to fully saturate the IVD tissue. Results demonstrated compression to be superior to PrimeGrowth™, decreasing the time it required DMSO to saturate the disc 92 ± 19% to 5 h, a significant improvement from the control which only reached 51 ± 22% in the same amount of time (post hoc Tukey HSD, $p = 0.01$) (Figure 3D). In contrast, PrimeGrowth™ showed no detectable improvement to DMSO transport from the control (post hoc Tukey HSD, $p = 0.85$).

3.3 | Confirm protocol maintains cell viability

Based on the results presented above, we hypothesized that IVDs could be effectively cryopreserved using a less toxic cryopreservation medium containing 10% DMSO and 10% EG (DMSO+EG), as well as compression and free-swelling to accelerate DMSO+EG transport in the IVD tissue.

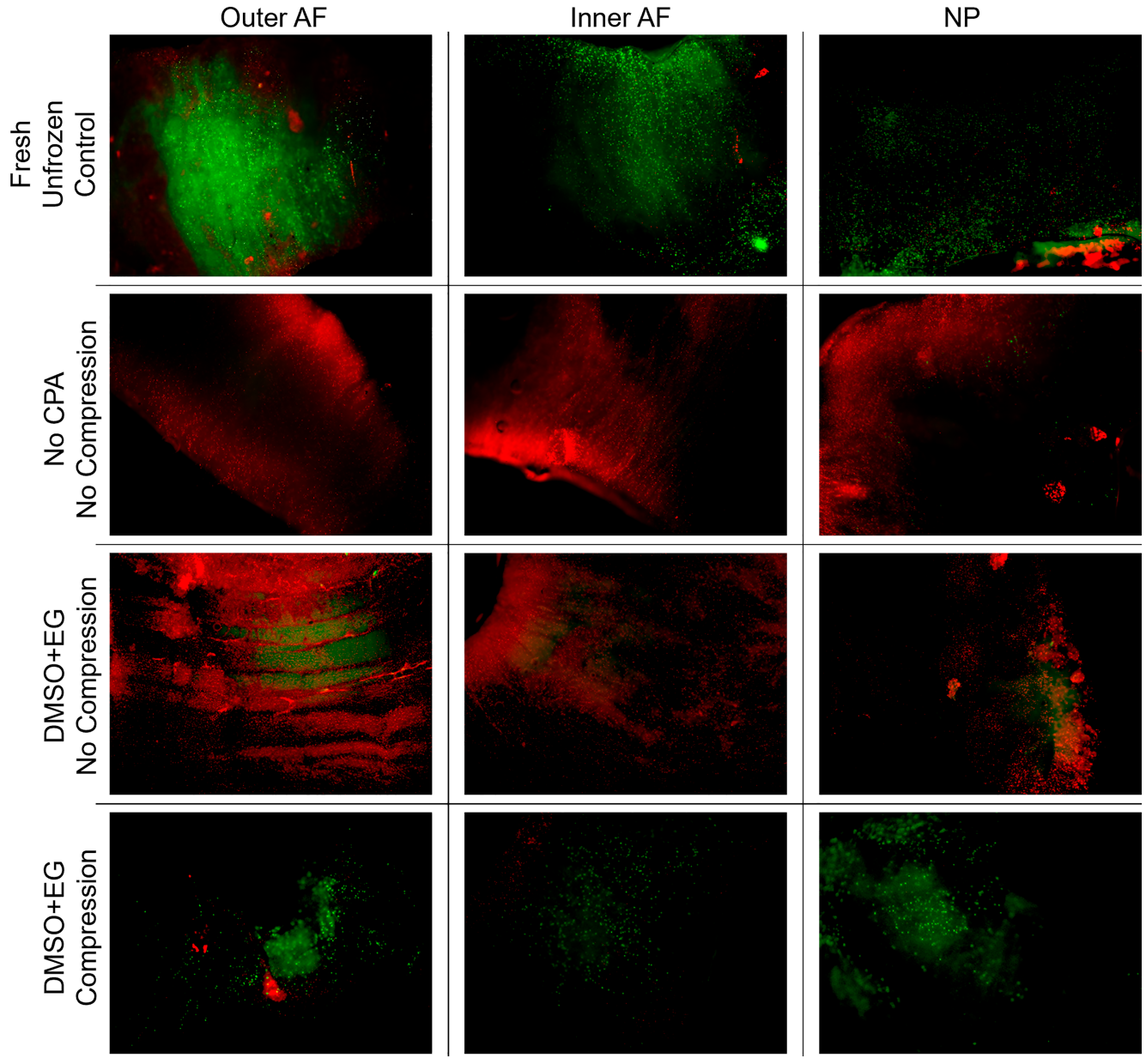

FIGURE 4 A representative sample of cell viability fluorescent images. Red is ethidium homodimer-1 staining of dead cells. Green is calcein-AM staining of live cells.

To test how quickly DMSO+EG could be removed, three IVDs treated with compression and DMSO+EG were thawed quickly, placed in fresh 1× PBS, then compressed for 3–4 h, the fluid was replaced, and the IVD allowed to free-swell for 3–4 h. The cycle was repeated several times. Figure 3E shows DMSO+EG decreasing to 4 ± 2% of the initial value after only 4 h and not significantly changing over the remaining 18 h (one-way ANOVA, $p = 0.81$). This makes the overall CPA concentrations within the IVDs 0.4% DMSO and 0.4% EG within 4 h and no longer cytotoxic.

IVDs from all groups were thawed and treated with 3–4 h of compression in 1× PBS, followed by a media exchange and 3–4 h of free-swelling. IVDs were cultured for an additional 8 h and then dissected for cell viability measurement. Images of LIVE/DEAD™ staining showed a visible difference in the number of viable cells and their distribution in the tissue. We also observed cell death in the areas that were sliced by the scalpel (Figure 4). Viability was then quantified in the sagittal plane for all cryopreserved IVDs and compared with fresh unfrozen controls. There was no significant difference between the outer AF, inner AF and NP tissue regions (two-way ANOVA, $p = 0.40$). IVDs in treatment groups that were not subjected to compression showed mean viability of only 4 ± 4% and 3 ± 3% after being frozen in medium with and without CPA, respectively ($p < 0.001$ using a post hoc Tukey HSD test for each treatment vs fresh unfrozen control). IVDs frozen with DMSO+EG and compression had an average of 85 ± 11% cell viability (Figure 5), which was not significantly different from the fresh unfrozen controls with 89 ± 6% (post hoc Tukey HSD, $p = 0.29$). This demonstrates that both DMSO+EG and compression are required for successful IVD cryopreservation.

4 | DISCUSSION

This cryopreservation strategy capitalizes on the IVD's viscoelastic properties and convective transport. Speeding up CPA delivery limits toxic CPA exposure time and increases cell viability. Additionally, we increased the allotted exposure time by optimizing CPA formulation to reduce cytotoxicity. This resulted in 85% cell viability in all tissue compartments: the outer AF, inner AF, and NP. Whole IVD cryopreservation creates feasibility for allographic IVD transplantation and increases access to intact IVD culture for research and development of regenerative therapies. Furthermore, this work informs our basic science knowledge of transport phenomena in the IVD and solves a roadblock for the translation of future tissue-engineered IVDs.

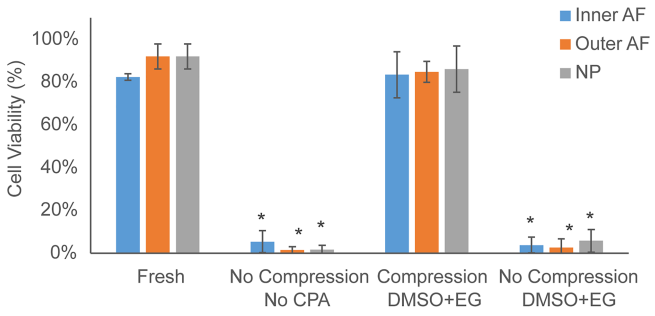

FIGURE 5 Mean cell viability in cryopreserved samples ($n = 5$) was improved with compression optimizing transport of DMSO into the IVD. Prior to cryopreservation, IVDs were treated with no compression and no CPA, with compression and with DMSO+EG, and no compression with DMSO+EG. Mean cell viability was measured from sagittal slices and compared against the slices taken from the fresh unfrozen samples. Significance was denoted by an * comparing treatment groups with the fresh unfrozen control (post hoc Tukey HSD, $p < 0.001$). There was no significance between tissue regions (two-way ANOVA, $p = 0.40$). Error bars are standard deviation.

4.1 | Transport mechanisms used to create novel cryopreservation protocol

CT examination of CPA penetration into unloaded IVDs showed full saturation taking over 72 h. Given a viable working time of 7.5 h in the presence of DMSO+EG, accelerating CPA transport was still essential. We compared two methods to improve transport in the IVD by (1) clearing transport pathways in the CEPs using PrimeGrowth™ and (2) capitalizing on convective transport through compression. PrimeGrowth™ is a proprietary solution that dissolves blood clots in the porous CEP post IVD harvest to improve solute transport, but our results do not show improved DMSO permeation.[24] Due to the proprietary nature of the product, we will not hypothesize mechanisms behind our result. Compressing the IVD prior to CPA addition, and then allowing it to swell, does drastically accelerate transport time. Compression decreases the IVD's height by expelling water and other solutes and decreasing the intradiscal hydrostatic pressure (Figure 6A).[26] In this work we utilized dynamic loading instead of static loading because of increased IVD height loss during dynamic loading for the same mean pressure.[27] The CPA is added when the IVD is at its minimum height, limiting the CPA exposure of the outer cells to only the following free-swelling time. When the load is removed the IVD free-swells, sucking in the dissolved CPA at a faster rate than diffusion would facilitate (Figure 6B), with >90% CPA saturation in ~5 h. The additional few hours of viable working time in the DMSO+EG solution allows for controlled freezing at a rate ~1°/min (~1.5 h), thawing (0.5 h), and compressing the disc to expel CPA and reduce concentrations to non-toxic levels (4 h).

Until now, this compression and free-swelling approach has not been utilized in cryopreservation of any organ. This novel method has potential applications in other avascular viscoelastic tissues, such as the meniscus, which suffers similar transport limitations to the IVD.[28] Additionally, researchers are developing tissue engineered whole IVD constructs to replace severely degenerated IVDs, which could also benefit from this method.[29]

4.2 | Implications for nutrient transport mechanisms in IVDs and exercise

IVD transport phenomena mechanisms discovered here are interesting beyond its cryopreservation applications as research also shows limited nutrient transport in the organ due to its geometry and

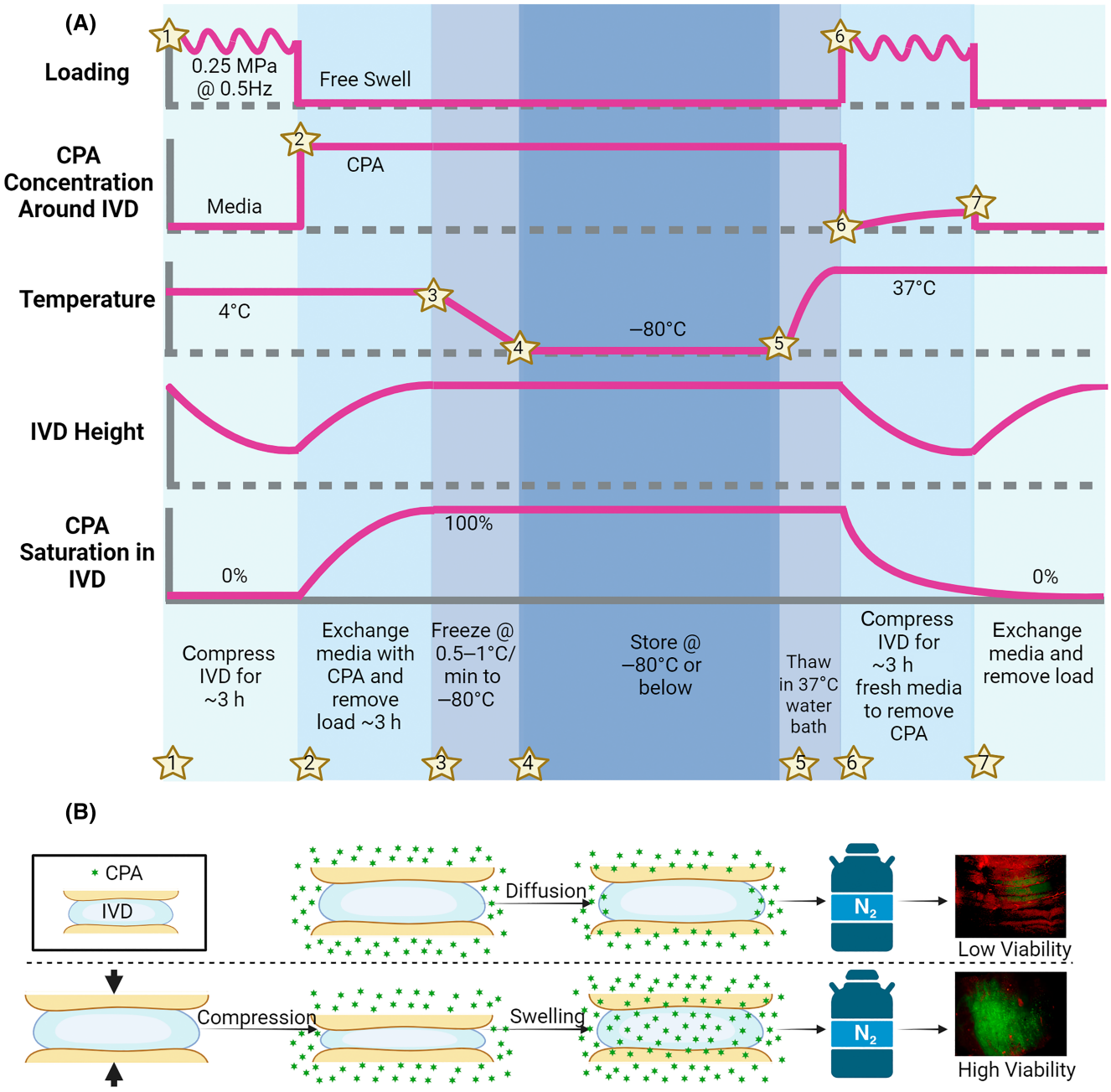

FIGURE 6 (A) Diagram of the physical mechanisms occurring during the compressive cryopreservation protocol (created with BioRender.com). The relationship between loading, CPA concentrations, temperatures, and IVD height are shown. Stars indicate actions taken by the researcher, while other phenomena are happening at that stage are consequences of the action. The actions are written at the bottom with their corresponding step number. (B) Shows a graphical representation of how swelling-mediated CPA delivery enables high cell viability after IVD cryopreservation.

avascularity.[14] Studies have hypothesized that low nutrient supply and metabolite clearance are factors propagating the cycle of degeneration, as biomass production is limited by the energy consumed by the cells.[14] Additionally, one barrier to regenerative therapies using growth factors or cell implantation is the necessity for an increased glucose supply to support additional hungry mouths or increased appetites, which is not always feasible due to transport limitations inherent to the IVD.[30] One potential mechanism to promote endogenous regeneration or supplement regenerative therapies is to increase the supply of nutrients to the disc. Loading has been proposed as a noninvasive mechanism to improve nutrition via exercise or traction therapy.[15] Through decades of research, there has been a lack of consensus about the effects of loading. Still, most recent studies support loading as a positive influence on nutrient concentrations in the disc, though the mechanism is unclear.[31] This study not only supports the idea that loading increases molecular transport in the disc but that it is precisely the decompression following a period of loading that creates an influx of solutes, like a sponge being squeezed and submerged in

the sink. The viscoelasticity of the IVD makes the ideal loading and offloading cycle necessitate several hours, but it could be practically implemented as an exercise and rest program in patients. The effect size of the solute influx is proportional to the percent IVD height change. Discs with more degeneration, less glycosaminoglycan, and therefore lower osmotic pressures will, unfortunately, experience less of an effect from loading and offloading but likely still see some benefit.

4.3 | Decreased toxicity

IVD cells are vulnerable to CPA-induced damage instigating apoptotic pathways, accumulating DNA damage, and compromising the membrane's integrity.[32] Our results demonstrated complete cell death after 6 h exposure to 10% DMSO and 10% PG in the monolayer culture and poor cell viability in the alginate beads at 8 h, reflecting results obtained by other researchers.[17] We compared literature cytotoxicity values for various CPA cocktails, including DMSO, ethylene glycol (EG), glycerol, and propylene glycol (PG). Interestingly, DMSO alone can be toxic at low concentrations of 5%–8%, upregulating interleukin-6 and increasing reactive oxygen species.[33] However, mixing DMSO with other CPAs reduces its necessary concentration in the solution, and thus deceases its toxicity.[33,34] This study reported better cell viability when DMSO was mixed with EG compared to PG (Figure 4), which is consistent with studies in other cell types.[13]

4.4 | Storage temperature

In this study, IVDs were stored at −80°C for 1 week. While previous studies have demonstrated high viability after storage of mammalian cells at −80°C for years,[35] degradation during storage at −80°C is possible, and liquid nitrogen theoretically enables more stable long-term storage. We do not anticipate any challenges translating our cryopreservation method to storage in liquid nitrogen since the critical temperature zone for cell damage is above −80°C. By the time the sample is cooled to −80°C, nearly all the extracellular water has already been converted to ice, water transport across the cell membrane has slowed to negligible levels, and molecular crowding transforms the cell cytoplasm into a glassy phase.[36] Thus, further cooling below −80°C is unlikely to cause cell damage, even when tissues are transferred directly from −80°C to liquid nitrogen without a controlled cooling rate.

4.5 | Limitations

The rewarding results of this study come with some limitations. First, this study was performed in bovine IVDs and the intended application is human specimens. Factors such as IVD size, extracellular matrix composition, and bone density vary between species and affect transport,[14,37,38] though bovine discs are relatively large and dense. In addition, this study lacked analyses on cell phenotype, morphology, gene expression, and protein levels, which can be impacted during cryopreservation.[39–41] It will be necessary to study if more subtle changes are occurring than cell death. Mechanical integrity of the cryopreserved specimens should also be checked, though non-cryoprotected IVDs have not shown mechanical damage from freezing in other studies.[42] Finally, this study was limited to storage −80°C for 1 week. It will be important in future studies to confirm IVD cell viability after longer-term storage.

Despite the limitations of this study, we successfully improved cell viability in intact cryopreserved bovine IVDs from previous protocols. An important application of our results is creating a human IVD organ bank to improve access to viable specimens.

5 | CONCLUSION

Biobanking cryopreserved IVDs could significantly improve access to intact specimens for researchers doing whole organ culture and clinicians exploring clinical applications of whole IVDs. In this study, we described an innovative method to improve IVD cryopreservation through controlled compression and decompression cycles. Bovine IVDs were preserved in a medium containing 10% DMSO-10% ethylene glycol for over 1 week. We systematically demonstrated how incorporating convective transport and a low-cytotoxic CPA was important in preventing cryoinjury to IVD cells, and creating a successful protocol. Post-thaw cell viability across the disc improved dramatically from 4% to 85% when dynamic compressive loading followed by free-swelling was utilized, importantly with no significant difference from fresh unfrozen controls. In addition to overcoming logistical challenges in whole IVD utilization, this work identified the primary mechanism for increased solute transport due to loading, which is convective transport of solutes when fluid influx occurs during free-swelling post-loading. We hope this study will support current research efforts and future clinical applications.

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STATEMENTS AND DECLARATIONS

The authors MBG, WS, and AZH have a patent pending related to this work.

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