Cryoprotectant agent toxicity in porcine articular chondrocytes

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Abstract

Large articular cartilage defects have proven difficult to treat and often result in osteoarthritis of the affected joint. Cryopreservation of articular cartilage can provide an increased supply of tissues for osteochondral allograft but cryoprotective agents are required; however, few studies have been performed on the toxicity of these agents. This study was designed to determine the order of toxicity of five commonly used cryoprotectant agents as well as interactions that occur between them. Isolated porcine articular chondrocytes were exposed to individual cryoprotectant agents and combinations of these agents at 1M and 3M concentrations for 5 minutes and 120 minutes. Cell viability was determined using membrane integrity dyes and a metabolic activity assay. Subsequently, a regression analysis based study was undertaken to extract the maximum amount of information from this data. Results of this study demonstrated that all 1M solutions were minimally toxic. The 3M solutions demonstrated varying toxicity after 120 minutes. Ethylene glycol and glycerol were less toxic than propylene glycol, dimethyl sulfoxide, and formamide. Combinations of cryoprotectant agents were less toxic than single cryoprotectant agents at the same concentration. This is the most comprehensive study investigating cryoprotectant agent toxicity in articular chondrocytes and has resulted in important information regarding the order of toxicity and interactions that occur between these agents.

Key Words

Articular cartilage, cryoprotectant agents, viability, statistical analysis, porcine, chondrocytes, cryopreservation

Introduction

Treatment techniques for articular cartilage (AC) defects continue to be investigated. Despite these efforts, large defects confound clinicians because of the inability to reproduce the complex matrix using tissue engineering modalities. Historically, treatment of large defects, including whole joints has been accomplished using fresh osteochondral allografts[1; 2]. This technically demanding procedure is made more difficult because of logistical issues that require transplantation to occur within 48-72 hours of harvest. These issues include timing of the surgical procedure (i.e. obtaining a donor at the time a patient requires the graft), size and contour matching on short notice, having the appropriate surgical team available for the transplantation procedure, and possibility of transmission of infectious diseases. To accommodate for some of these obstacles, hypothermic storage of allografts at 4°C has been attempted. Unfortunately, recent studies have demonstrated that the cells and matrix begin to deteriorate after 7-14 days in these conditions[3; 4; 5; 6]. Furthermore, regulatory conditions in some countries such as the United States require at least 14 days before clearance of this tissue for clinical use. Longer term storage of this tissue in a frozen state results in cell death and matrix damage with outcomes of frozen-thawed graft transplantations deteriorating after only two to five years[7]. It is with this background that cryopreservation of AC with maintenance of cell viability and matrix integrity has been investigated.

Cryopreservation of AC has proven extremely difficult. Initial standard cryopreservation (low cryoprotectant agent (CPA) concentration) techniques such as stepped cooling following by controlled freezing have been uniformly unsuccessful in maintaining cell

viability to any significant extent[8; 9; 10]. Furthermore, the formation of ice within the matrix alters its integrity[11] and that may have long term consequences on the transplanted tissue. More recently, vitrification has been investigated as a method to maintain cell viability while avoiding the alterations in the matrix. Standard single CPA vitrification has had some success in thin AC[12] but this has not been extended to thicker AC that is required in the clinical scenario. Multiple-agent vitrification has also had moderate success[13; 14; 15; 16] but, again, extension to the clinical realm has not been achieved due to difficulties in scaling up protocols.

Two important obstacles to the successful vitrification of clinically relevant thickness AC include CPA toxicity and permeation. The high concentrations of CPAs required to vitrify AC at realistic cooling rates are toxic to chondrocytes within minutes[17]. Interestingly, investigations into mechanisms of toxicity and the effects of high concentrations of CPAs (especially with respect to chondrocytes) are relatively few[15; 18; 19; 20; 21; 22; 23; 24]. To complicate matters, experiments on different tissues or cells result in different ordering of toxicity. Various studies in Zebrafish ovarian follicles[25], Abalone embryos[26], zebrafish germ cells[27], vascular endothelial cells[28], shrimp embryo and larvae[29] and mouse morula[30] have demonstrated that CPA toxicity is variable within different cells and different species with one CPA being the most toxic in one study but the least toxic in others. Fahy is one of the few investigators to attempt an evaluation of toxicity interactions between CPAs in AC[20]. He concluded that it was possible to predict solution toxicity based on the number of moles of water per molar quantity of CPA polar groups in solution. One significant

drawback to this work was the limited number of combinations investigated but they were able to show some positive interactions between CPAs indicating that adding two CPAs together resulted in less toxicity than a prediction based on the addition of the effects of the two individual CPA toxicities. This study was recently expanded to include other possible positive interactions[24]. Zhang e*t al.* performed a more comprehensive study in flounder that also showed positive interactions between different CPAs[31].

Given the toxic effects of high concentrations of CPA agents required for vitrification and the variability between cells and species, it is important to investigate the toxicity of CPAs alone and in combination with respect to articular chondrocytes if successful vitrification of AC is to be achieved. Furthermore, determination of positive and negative interactions of the commonly used CPAs will allow for informed decisions regarding the combination of these agents in multiple-agent vitrification solutions that are becoming more relevant. The main objective of this experiment was to determine the order of toxicity of five commonly used CPAs. A secondary objective was to determine any positive and negative interactions between pairs of these CPAs.

Methods and Materials

Chondrocyte isolation

Thin slices of AC were harvested from the weight-bearing region of porcine distal femurs donated by a local slaughterhouse (Sturgeon Valley Pork, St. Albert, AB) and immersed in a petri dish containing 1X phoshate-buffered saline (PBS, pH 7.1, Invitrogen, Carlsbad, CA). The cartilage slices were washed once with sterile 1X PBS. A 1 mg/mL

collagenase solution was prepared using solid collagenase (Sigma-Aldrich, St. Louis, MO) dissolved in Dulbecco's Modified Eagle Media (DMEM F-12, Invitrogen, Carlsbad, CA) containing 1 % penicillin/Streptomycin (Invitrogen, Carlsbad, CA) and 10% Fetal Bovine Serum (FBS, ATCC**,** Manassas, VA). The PBS was removed and the collagenase solution was filtered through a Millex GP .22 µm (PES) syringe filter (Millipore, Bedford, MA) onto the petri dish. The cartilage matrix was digested on top of a shaker for 6 hours at 37°C and 5% CO_2. At the end of the digestion, the solution was filtered through a 40 µm cell strainer (Fisher Scientific, Waltham, MA) into a 50 mL centrifuge tube and centrifuged for 6 minutes at 400x *g* and 20°C (Eppendorf 5810 R, Eppendorf, Hamburg, DE) . The supernatant was removed, the pellet re-suspended in sterile 1x PBS and centrifuged again. The resultant supernatant was removed and the pellet was re-suspended in Dulbecco's Modified Eagle Media (DMEM F-12, Invitrogen, Carlsbad, CA) leaving chondrocytes without matrix. The cell density was determined using a Coulter Counter Z2 (Beckman Coulter, Fullerton, CA) and the chondrocytes were seeded at a concentration of 25,000 cells/100 µL in each well of a 96-well cell culture cluster (Corning, Corning, NY). The cluster was placed on a steady surface for 30 minutes at room temperature to allow the chondrocytes to attach to the bottom of the wells, and then incubated for 48 hours at 37°C and 5% CO_2.

Cryoprotectant solutions

The cryoprotectant agents used included dimethyl sulphoxide (Me_2SO), ethylene glycol (EG), propylene glycol (PG), glycerol (Gly) and formamide (Form). Using these agents, 5 single-CPA aqueous solutions; 20 two-CPA solutions (10 combinations in 2 different

ratios); and 15 three-CPA solutions were formulated. For the single-CPA and three-CPA solutions, total concentrations of 1 and 3 M were used. For the two-CPA solutions, data were only collected at total solution concentrations of 3M.

Fifty microlitres of media was gently removed from the initial volume of 100 µL using a sterile Vacupette 96 (Bel-Art, Pequannock, NJ) attached to a syringe. The wells were washed by adding and then removing 100 µL of DMEM-F-12. The CPA solutions were added stepwise to avoid excessive intracellular volume changes. For the 1M treatment, 50 µL of a 2M CPA solution was added to each well to achieve the desired concentration. For the 3M treatment, 50 µL of a 2M CPA solution was added; after 3 minutes 50 µL was removed; 50 µL of a 3M CPA solution was then added and removed after an additional 3 minutes. Finally, 50 µL of a 4M CPA solution was then added to achieve the desired concentration. For all treatments, the cell cluster was placed in an incubator at 37°C and 5% CO_2 for either 5 or 120 minutes.

After the experimental treatment and incubation, the 3M CPA solutions were removed stepwise, again to avoid excessive changes in cell volume as follows. 33 µL of the treatment solution was removed and replaced with an equal volume of DMEM-F12. After 3 minutes, 50 µL was removed and replaced with an equal volume of DMEM-F12. For both 1M and 3M treatments, the wells were then washed twice with 100 µL of DMEM-F12 to remove the remaining CPA.

Cell Viability

Metabolic Activity

Cell proliferation reagent was made by mixing WST-1 reagent (Roche, Basel, CH) in phenol red–free DMEM-F12 (5% penicillin/streptomycin, 10% FBS) at a concentration of 1µL/mL. The cell cluster wells were drained by inversion and 100 µL of the cell proliferation solution was added to each well. The cell cluster was incubated at 37°C and 5% CO_2 for 90 minutes. Following incubation, the absorbance was taken at 450 nm with a reference wavelength of 650 nm, using a SpectraMax Plus 384 spectrophotometer running SOFTmax PRO 5.3 software (Molecular Devices, Sunnyvale, CA).

Membrane Integrity

All excess DMEM-F12 solution was removed by inverting the well containing the cell cluster and chondrocyte nuclei were stained with 40 µL of Syto13/Ethidium Bromide (8.6 µM/8.6 µM in 1x PBS, Invitrogen, Carlsbad, CA / Sigma-Aldrich, St. Louis, MO). The cell cluster was incubated for 30 minutes at 20°C while protected from light and then imaged using a Nikon Eclipse TE 2000-U fluorescent microscope and NIS Elements software (v. F 2.30, Nikon, Tokyo, JP). Cell counts were determined using custom software (Viability 3.2.0.0, Cryolab, University of Alberta).

Statistical Analysis

In order to determine the primary toxicological effects of each cryoprotectant agent considered in this study and any interactions that arise from binary mixtures of these

CPAs, a regression analysis based study was undertaken. In particular, the data collected for the set of CPA mixtures described previously was used to estimate the parameters in a model of the form:

$$y = \alpha + \sum_{i=1}^{5} \beta_i x_i + \sum_{i=1}^{5} \sum_{j=i+1}^{5} \gamma_{ij} x_i x_j \qquad (1)$$

where y is the cell recovery expressed as a normalized fraction; α represents cell recovery for a control solution containing no CPAs; x_i is the concentration (M) of the i^{th} CPA; β_i is the coefficient for the primary effect of the i^{th} CPA; and γ_{ij} is the coefficient for the two factor interaction between the i^{th} and j^{th} CPAs.

The regression studies were conducted sequentially starting with an investigation of the primary effects of the CPAs. In the second stage, the interactions between pairs of CPAs were investigated. In each study, linear least squares regression was used to estimate the set of coefficients in the appropriate regression model. Then, a hypothesis test of the form:

$$H_0 : \{\alpha, \beta_i, \gamma_{ij}\} \equiv 0 \qquad (2)$$

was conducted (i.e. the estimated value for each coefficient was hypothesized to be indistinguishable from zero at a specified confidence level) at the 95% confidence level and using the estimated standard deviation for each coefficient estimate.

The results in Table 2 were produced using this regression analysis approach, using a modified version of Equation (1) where the interaction terms were omitted (i.e. was forced to be zero by truncating the equation after the second term). The results in Table 3 were produced using the full regression model given in Equation (1).

Statistical analysis was performed using Matlab R2009b software (The MathWorks Inc., Natick, MA).

Results

Cell viability was determined for single-CPA and three-CPA combinations at 1M and 3M. This was done after 5 min and 120 min (Figures 1 and 2). For two-CPA combinations, only data at 3M was obtained (graph not shown). All 1M solutions were minimally toxic after 5 min and 120 min exposure to chondrocytes (Figure 1). The majority of 3M solutions were minimally toxic after 5 min. Exposure to 3M concentrations of solutions for 120 min resulted in significant toxicity in a number of different single-CPA and three-CPA solutions (Figure 2). Similar results to those presented in Figure 2 were noted from 3M two-CPA solutions (data not shown).

Figure 1 here

Figure 2 here

Cell viability was measured using two methods including membrane integrity and metabolic activity to improve confidence that the cells were indeed viable. Table 1 lists the cell recovery using these two methods and ranks the different solutions based on cell viability. It can be seen that cell recovery was similar using these two methods.

Table 1. Comparison of cell viability using membrane integrity and metabolic assays.

3M	Membrane Integrity		Metabolic Activity	
	Rank	Recovery (%)	Rank	Absorbance
EG-Me_2SO-Gly	1	94	2	1.03
GLYC-GLYC-GLYC	2	92	4	0.98
EG-EG-EG	3	91	1	1.07
Gly-Me_2SO-Form	4	91	3	1.01
EG-Gly-Form	5	90	5	0.98
EG-PG-Gly	6	87	9	0.84
Gly-PG-Form	7	85	7	0.92
EG-Me_2SO-Form	8	85	8	0.89
Gly-Me_2SO-PG	9	82	6	0.94
EG-PG-Form	10	79	10	0.7
EG-Me_2SO-PG	11	72	11	0.67
Me_2SO-Me_2SO-Me_2SO	12	44	12	0.54
FORM-FORM-FORM	13	43	15	0.36
Me_2SO-PG-Form	14	33	13	0.46
PG-PG-PG	15	14	14	0.37

To extract the maximum amount of information from this data, a statistical analysis was performed. Using a linear regression model from all the data demonstrated the toxicity of individual CPAs to be as follows: PG, Me_2SO, and Form exhibited greater toxicity than EG and Gly. This was consistent for all three levels of combinations.

When considering the data from single-CPA solutions (Table 2), EG and Gly were non-toxic at these concentrations for these time exposures. PG tended to be more toxic than Me_2SO and Form. The data from the two-CPA solutions (Table 3) suggested that all combinations had a positive effect (i.e. decreased toxicity). In fact, all Me_2SO combinations were non-toxic or close to non-toxic. The least significant interactions were noted when two CPAs from the same set of most toxic (Me_2SO, PG, Form) or least toxic (EG, Gly) were mixed. Adding EG or Gly appeared to mitigate toxicity. The rank order of toxicity was the same in the single- and two-CPA solutions.

Table 2. Table showing statistical analysis of cell viability results considering solutions containing only 1 CPA.

Considering 1 CPA only			
Sample	Coefficient		Std Dev
Control	1.11		0.06
Me_2SO	-0.22		0.03
PG	-0.31		0.03
EG	-0.07		0.03
Gly	-0.069		0.03
Form	-0.22		0.03

Table 3. Table showing statistical analysis of cell viability results considering mixtures containing 2 CPAs.

Considering 2 CPAs			
Sample	Coefficient		Std Dev
Control	1.12		0.03
Me_2SO	-0.25		0.03
PG	-0.31		0.03
EG	-0.09		0.03
Gly	-0.09		0.03
Form	-0.23		0.03
Me_2SO + PG	0.05		0.03
Me_2SO + EG	0.08		0.03
Me_2SO + Gly	0.07		0.03
Me_2SO + Form	0.05		0.03
PG + EG	0.13		0.03
PG + Gly	0.14		0.03
PG + Form	0.02		0.03
EG + Gly	0.02		0.03
EG + Form	0.08		0.03
Gly + Form	0.11		0.03

Discussion

Development of effective vitrification solutions will require more detailed knowledge about CPA toxicity with respect to the specific cell types within the tissue being cryopreserved. There is scant information regarding either toxic effects of currently used CPAs on articular chondrocytes, or the effects of combining CPAs. This study demonstrated that toxicity of specific CPAs could be determined and that interactions do exist between CPAs. Two methods of cell viability were used and they provided very similar results. The membrane integrity assay was evaluated directly (each cell counted) while the WST assay was evaluated indirectly (colorimetric assay); therefore, the

statistical analysis was performed using the membrane integrity data only. The objectives of the study were to investigate the rank order of chondrocyte toxicity of each of the five common CPAs and any interactions that exist between them.

The raw data from the different CPA solutions (Figures 1 and 2) showed that CPA solutions of 1M for up to 120 minutes are minimally toxic. As the concentration increased to 3M and the time increased to 120 minutes, toxicity occurred in a number of solutions including Me_2SO, PG, Form, and most combinations containing at least two of these three CPAs. Surprisingly, EG and Gly showed little toxicity at 3M over this time period and most of the combinations that included EG and Gly also showed little toxicity. In fact, EG, Gly and EG-Me_2SO-Gly solutions were three solutions that did not demonstrate significant toxicity after 120 minutes. This suggested that solution combinations containing EG and Gly should be considered for vitrification solutions in AC cryopreservation. This was consistent with the results in mouse morulae[30] and shrimp embryo and larvae[29] that demonstrate the least toxicity using EG. Conversely, other studies in flounder[31], fish germ cells[27], abalone embryos[26] and zebrafish follicles[25] demonstrated increased toxicity with either EG or Gly. Thus, for porcine articular chondrocytes, EG and Gly are the least toxic CPAs but these results may not be applicable to other types of cells.

The data from single-, and two-CPA solutions consistently demonstrated that PG, Me_2SO, and Form induced greater toxicity than EG and Gly. Further ordering was not possible based on the available data. This result is interesting because Me_2SO is the most

commonly used CPA for many tissues including AC, yet it is not the least toxic. The common use of Me_2SO is likely due to historical reasons (one of the first accepted CPAs) and it is the CPA with the most information about its toxicity and mechanism of action. Me_2SO may still be useful as the two-CPA solutions containing Me_2SO were either non-toxic or close to non-toxic despite the toxicity of Me_2SO itself suggesting positive interactions with other CPAs. This may be the explanation behind the relatively successful cryopreservation achieved using VS 55, a solution that contains the three most toxic CPAs (Me_2SO, PG, and Form)[32]. This also supports Fahy's conclusion that there is a positive interaction when Me_2SO is combined with formamide[20; 24].

Another important finding from this experiment was that adding two CPAs together is less toxic than one CPA at the same final concentration, irrespective of what CPAs were used. This can be seen from Table 3 that shows all single CPA solutions exhibit a negative coefficient while all 2-CPA component solutions have positive coefficients indicating less toxicity. One difficulty in determining the exact nature of this interaction was that EG and Gly were not toxic at the concentration and time used, therefore, we can not conclusively say that adding them to other CPAs is beneficial. It was also shown that if a more toxic CPA has to be used (e.g. Me_2SO, PG, or Form), then EG or Gly should be added to reduce or mitigate that toxicity because these two CPAs have been shown to have the least toxicity of the five examined and have larger positive coefficients when combined with more toxic CPAs (Table 3).

One limitation of this study was that it was performed at a maximum concentration of 3M due to considerations of osmotic stress when adding each CPA. This study was not designed to develop a vitrification solution for AC; therefore, these results may not extrapolate to higher concentrations required for vitrification (approximately 8M). This experiment did not investigate the mechanism of toxicity, but the end result (viability) of chondrocyte exposure to CPAs. This experiment was performed using cells removed from the matrix. It is well documented that cells removed from the matrix do not respond the same as cells *in situ*; therefore, it is possible that this information is not completely applicable to cells *in situ*. We performed the experiment in this manner to eliminate confounding variables such as CPA permeation in the matrix. Finally, the results of this experiment are likely not applicable to other cell types for the reasons mentioned previously.

In conclusion, this is the most comprehensive study investigating CPA toxicity in chondrocytes. It was determined that EG and Gly were the least toxic CPAs to isolated porcine articular chondrocytes when used individually. Furthermore, when CPAs are combined at lower concentrations, there was a positive effect (i.e. less toxicity) when compared to a single CPA at the same concentration. When adding CPAs together, it is likely best to make the additional CPA either EG or Gly.

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Figure Captions

Figure 1 Title. 1M Toxicity at 37°C

Figure 1. Cell recovery after exposure to 1M (single and three-CPA) solutions for 5 min and 120 min as determined by membrane integrity stains..The controls are absolute values while the experimental solutions have been normalized to their own internal controls taken from the same tissue. The negative control of 100% Me_2SO at the right of the x axis and had 0% recovery. There was minimal difference between any solution.

Figure 2 Title. 3M Toxicity at 37°C

Figure 2. Cell recovery after exposure to 3M (single and three-CPA) solutions for 5 min and 120 min as determined by membrane integrity assays. The solutions are listed in order of descending recovery after 120 min. The controls are absolute values while the experimental solutions have been normalized to their own internal controls taken from the same tissue. The three-CPA solution containing EG-Me_2SO-Gly resulted in the highest cell viability. The single-CPA solutions of Gly and EG also had the highest viability. The negative control of 100% Me_2SO is at the right side of the x axis and had 0% recovery.