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Title: Dose-injury relationships for cryoprotective agent injury to human chondrocytes

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Abstract: Vitrification of articular cartilage (AC) could enhance tissue availability but requires high concentrations of cyroprotective agents (CPAs). This study investigated relative injuries caused by commonly used CPAs. We hypothesized that the in situ chondrocyte dose-injury relationships of five commonly used CPAs are nonlinear and that relative injuries could be determined by comparing cell death after exposure at increasing concentrations. Human AC samples were used from four patients undergoing total knee arthroplasty surgery. Seventy μm slices were exposed in a stepwise protocol to increasing concentrations of 5 CPAs (max=8M); dimethyl sulfoxide (Me2SO), glycerol (Gly), propylene glycol (PG), ethylene glycol (EG), and formamide (FM). Chondrocyte viability was determined by membrane integrity stains. Statistical analysis included t-tests and nonlinear least squares estimation methods. The dose-injury to chondrocytes relationships for all CPAs were found to be nonlinear (sigmoidal best fit). For the particular loading protocol in this study, the data identified the following CPA concentrations at which chondrocyte recoveries statistically deviated significantly from the control recovery; 1M for Gly, 4M for FM and PG, 6M for Me2SO, and 7M for EG. Comparison of individual means demonstrated that Gly exposure resulted in the lowest recovery, followed by PG, and then Me2SO, FM and EG in no specific order. The information from this study provides an order of damage to human chondrocytes in situ of commonly used CPAs for vitrification of AC and identifies threshold CPA concentrations for a stepwise loading protocol at which chondrocyte recovery is significantly decreased. In general, Gly and PG were the most damaging while DMSO and EG were among the least damaging.

Dose-injury relationships for cryoprotective agent injury to human chondrocytes

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Abstract

Vitrification of articular cartilage (AC) could enhance tissue availability but requires high concentrations of cyroprotective agents (CPAs). This study investigated relative injuries caused by commonly used CPAs. We hypothesized that the *in situ* chondrocyte dose-injury relationships of five commonly used CPAs are nonlinear and that relative injuries could be determined by comparing cell death after exposure at increasing concentrations. Human AC samples were used from four patients undergoing total knee arthroplasty surgery. Seventy μm slices were exposed in a stepwise protocol to increasing concentrations of 5 CPAs (max=8M); dimethyl sulfoxide (Me_2SO), glycerol (Gly), propylene glycol (PG), ethylene glycol (EG), and formamide (FM). Chondrocyte viability was determined by membrane integrity stains. Statistical analysis included t-tests and nonlinear least squares estimation methods. The dose-injury to chondrocytes relationships for all CPAs were found to be nonlinear (sigmoidal best fit). For the particular loading protocol in this study, the data identified the following CPA concentrations at which chondrocyte recoveries statistically deviated significantly from the control recovery; 1M for Gly, 4M for FM and PG, 6M for Me_2SO, and 7M for EG. Comparison of individual means demonstrated that Gly exposure resulted in the lowest recovery, followed by PG, and then Me_2SO, FM and EG in no specific order. The information from this study provides an order of damage to human chondrocytes *in situ* of commonly used CPAs for vitrification of AC and identifies threshold CPA concentrations for a stepwise loading protocol at which chondrocyte recovery is significantly decreased. In general, Gly and PG were the most damaging while DMSO and EG were among the least damaging.

Key Words: human, chondrocytes, injury, cryoprotective agents, vitrification, dose, cryopreservation

Introduction

Osteoarthritis is the leading cause of work disability in Canada, with an annual estimated economic productivity and long-term disability cost of $4.4 billion dollars[1,16]. When injured, articular cartilage (AC) has limited ability to repair itself and the injury may progress to disabling degenerative arthritis[36]. Due to the low immunogenicity and avascularity of cartilage tissue, osteochondral lesions can be treated with articular cartilage allograft transplantation[15]. However, in order to overcome transport, sizing, and availability challenges, an effective method for long-term preservation of these allografts must first be developed and optimized. Vitrification of AC is one method that can preserve this tissue indefinitely and involves the use of high concentrations of cryoprotective agents (CPAs) and rapid cooling rates[20,28,30]. One challenge is to limit the injury from the CPAs contained within the vitrification solution, while still obtaining an adequate tissue-concentration sufficient to vitrify.

Several factors affect CPA permeation into tissues including temperature, exposure time, solution agitation, and solution concentration[2,3,5,21,30,35]. However, a general limitation on vitrification of tissues such as AC has been the cellular injury of the CPA during the time required for tissue loading. Cryoprotective agents are categorized into either *permeating* (e.g. dimethyl sulphoxide, glycerol, ethylene glycol, formamide, etc.) or *non-permeating* compounds (e.g. sucrose, trehalose, etc), depending on their ability to penetrate the cell membrane. Because permeating CPAs can penetrate into the cell, they are generally more effective for vitrification but are also more toxic, relative to non-permeating CPAs[24,30]. The mechanisms and severity of these toxicities vary among the CPAs and their tissue targets[22,30]. For example, glycerol (Gly) and ethylene glycol (EG) affect cellular metabolism; dimethyl sulphoxide (Me_2SO) disrupts cell membrane ion channels; formamide (FM) is damaging to cellular cytoskeletal

architecture and function; and propylene glycol (PG), Me_2SO and FM, have been shown to exhibit genotoxic effects[6,8,17-19,26]. In fact, not only do different CPAs exhibit different degrees of toxicity on cells of the same cellular lineage, but the degree and order of toxicity among the CPAs from the most to least toxic, can be different for the same cell type (eg. chondrocytes) across different animal species[4,11,31,33-35].

As a result of the various mechanisms and complexities of CPA toxicity, the exact relationship between a CPA's dosing protocol (concentration and exposure time) and the resulting human chondrocyte viability is unknown. Dose-toxicity relationships are, in general, nonlinear[12]. We hypothesized that the *in situ* human chondrocyte dose-injury relationships for particular step-loading protocols are nonlinear and that the relative injuries of five commonly used CPAs could be determined by comparing cell death after exposure to increasing concentrations. To determine this, we exposed human chondrocytes *in situ* in AC slices to increasing concentrations of five commonly used permeating CPAs and measured the chondrocyte recovery. Cartilage slices with chondrocytes within their normal microenvironments (ie. *in situ*) were used because a previous study demonstrated that cryopreservation characteristics of cells change when they are released from their natural extracellular matrix[30].

Materials and Methods

Cryoprotective agent solution preparation

Five CPAs were used: dimethyl sulphoxide (Me_2SO), ethylene glycol (EG), propylene glycol (PG), glycerol (Gly) and formamide (FM) (Fisher Scientific Co., Ottawa, ON). Experimental solutions were prepared from 12 molar (M) stock solutions by diluting them with sterile X-VIVO [Lonza Group Ltd, Basel, Switzerland] to 1, 2, 3, 4, 5, 6, 7, and 8M concentrations. The 12M stock solutions were prepared by adding the required mass of CPA to

phosphate buffered saline to the required total solution volume. Experimental solutions were stored in a 4°C fridge for use within 24 hours of preparation. Each of the five CPAs were placed into a 96-well cell culture plate with increasing solution concentrations and labelled appropriately. This resulted in a total of 40 wells of increasing CPA concentrations at 1M increments. In addition, 16 wells were prepared containing just X-VIVO for use in dilution steps at the end of the protocols. All wells contained 1 mL of liquid.

Cartilage tissue isolation

After approval from the University of Alberta Research Ethics Board, a total of 4 AC samples were obtained from 4 donor patients undergoing elective total knee arthroplasty surgeries and transported in sterile phosphate-buffered saline [PBS, pH 7.1, Invitrogen, Carlsbad, CA]. Only AC samples graded as Outerbridge grade 0 or 1 were used[29,32]. Full thickness osteochondral dowels of 10 millimeter diameter were cored and the AC was cut (perpendicular to the articular surface) into 70µm thick slices using a vibrotome [The Vibratome Company, St. Louis, MO]. The slices were immersed in a petri-dish containing X-VIVO solution [Lonza Group Ltd, Basel, Switzerland] at room temperature, and used within 120 minutes of preparation. For each repeat of the experiment, sufficient numbers of slices were obtained from each donor to perform all treatment conditions as well as the positive and negative controls. Therefore, all four donors provided sufficient tissue to complete four independent repeats of the experiment.

Cryoprotective agent injury testing

Once the CPA solution concentrations were loaded into the 96-well plates and an adequate number of cartilage slices were obtained, testing of the above mentioned five CPAs were undertaken at room temperature (~21°C). Rather than directly placing the cartilage slices

into the respective final CPA testing concentrations, the slices were exposed to the CPAs in sequential stepped 1M increments to allow the cells time to equilibrate with their surrounding extracellular osmotic environments. This osmotic 'ladder' CPA exposure protocol is regularly used during vitrification protocols. Otherwise, directly placing a cartilage slice into a 6M or 8M CPA concentration solution can result in hyperosmotic-solution injury in addition to CPA-toxicity injury, and therefore confound our results. The ladder protocol involved immersing slices in a stepwise fashion beginning at 1M CPA solution (1 ml) for 5 minutes, before transferring slices to the next incremental solution (i.e. 2M) for another 5 minutes, and continuing until the CPA testing solution concentration was reached. Once a slice reached its intended testing concentration, it remained in that final testing concentration for a 10-minute period before the CPA was removed in a reverse stepwise manner down the same osmotic ladder by transferring the slices down 1M increments of descending CPA concentrations for 5 minutes each. Once the slices had been in the 1M concentration well for 5 minutes, they were then immersed in X-VIVO for 5 minutes and then removed and placed in a final X-VIVO well for a minimum of 5 minutes in order to wash any remaining CPA before being prepared for viability staining. The maximal length of CPA exposure was 80 minutes for the 8M solution (35 minutes to get to 8M, 10 minutes at 8M CPA testing concentration, and 35 minutes back down to 1M), after which the slices were washed once in an X-VIVO solution well for 5 minutes, and then transferred to a second X-VIVO solution well where the slice remained until a total experimental exposure time of 90 minutes was obtained. For all concentrations less than 8M, the slices were held in the second X-VIVO solution until the full 90 minutes was obtained. Positive controls involved placing two cartilage slices in a well containing X-VIVO solution for 90 minutes, while negative controls involved exposing the two cartilage slices to a solution containing 100% pure Me_2SO for 90 minutes.

136 ***Chondrocyte viability staining and viability quantification***

137 A dual fluorescent membrane integrity assay was used to determine the viability of the
138 chondrocytes within the slices. The slices were placed on a labeled microscope slide and stained
139 with 40µL of a combination stain solution of Syto13 and ethidium bromide stain (8.6µM/8.6µM,
140 Invitrogen, Carlsbad, CA / Sigma-Aldrich, St. Louis, MO). Syto13 is a cell membrane permeable
141 nucleic acid specific stain that emits a green fluorescence and ethidium bromide (EB) is a cell
142 membrane non-permeable stain specific for deoxy-ribonucleic acids that emits a red
143 fluorescence. Therefore, cells with intact cell membranes emit a green fluorescence due to Syto
144 13 uptake and EB exclusion, while those with defective membranes emit a red fluorescence as
145 EB is able to enter the cells. Once the combination stain solution was added to a cartilage slice,
146 the microscope slide was allowed to incubate for 20 minutes at room temperature (~21°C) in a
147 dark environment. Chondrocyte membrane integrity was determined using fluorescent
148 microscopy visualization using a fluorescence Nikon eclipse E600 with a dual filter (Omega
149 optical cube TE 2000 with an Excitation of 480/25 and Emission of 530/640 wavelengths) and
150 cell counts were obtained manually (Nikon Eclipse TE 2000-U fluorescent microscope, NIS
151 Elements software, v. F 2.30, Nikon, Tokyo, Japan). Finally, chondrocyte recovery for each slice
152 was normalized with respect to their own positive controls in order to account for losses due to
153 patient, environmental, and harvesting factors.

154 The method for calculating the normalized percentage recovery is given in Eq. (1). The green
155 cells represent intact (viable) cells and the red cells represent membrane disrupted (dead) cells.
156 The total number of cells within a slice is calculated by adding the green + red cells.
157

$$Normalized\ recovery =$$

$$\frac{\#\text{ green experimental cells }/(\#\text{ green experimental cells }+\ \#\text{ red experimental cells})}{\#\text{ green positive control cells}/(\#\text{ green positive control cells }+\ \#\text{ red positive control cells})}\ \text{x } 100$$

Equation 1

Data Analysis

The chondrocyte survival data were fit with a nonlinear (sigmoid) function by minimizing the sum of squared errors between the data and the fit over all CPA concentrations. In addition, t-tests were performed to evaluate the concentrations at which the mean normalized recovery differed significantly from 100% for each CPA. This analysis is specific to the stepwise protocol used.

A secondary analysis was performed in order to separately account for (or to deconvolve) the contribution of each individual CPA concentration being used in the multi-step protocol due to the stepwise concentration increases employed for the higher CPA concentrations. We measured the recovery at the end of multi-step protocols which are thus reporting the combined effect of exposure to the different concentrations. To account for this effect, the measured recovery at 3M, for example, can be described as the combination of 10 minutes exposure to 3M, 10 minutes exposure to 2M (5 minutes on the way up and 5 minutes on the way down), and 10 minutes exposure to 1 M. The recovery at the end of a multistep procedure can be found by multiplying the recovery of each step. Therefore, the chondrocyte data were analyzed again by fitting with a function composed of multiplied sigmoid functions for each concentration (as opposed to a single sigmoid function using *only* the final testing concentration as described for the first analysis). The fits obtained from this procedure are potentially extendable to other protocols which use a different stepwise increase in CPA concentration (e.g., when a 2M or 3M increment is used during the stepwise osmotic "ladder" CPA-addition protocol).

Results

The data obtained for the recovery (as defined in Equation 1) at each CPA concentration are shown in Figure 1. The abscissa (x-axis) represents the molarity of the CPA, while the ordinate (y-axis) represents the recovery. The data were fitted with sigmoidal functions of the form:

$$y = \frac{a}{1+e^{-b(x-c)}} \qquad \text{Equation 2}$$

where "y" represents the fractional recovery, "x" is the CPA concentration employed, and "a", "b", and "c" are the parameters obtained from the regression. The parameter "a" determines the range of the sigmoid function, and the parameters "b" and "c" determine its center and width, respectively. Since the chondrocyte recovery is a normalized fraction of the control and the recovery is 1 at 0M concentration for each of the CPAs, the parameter 'a' was set to 1. Plots are provided for each CPA (in the order Me_2SO, EG, FM, Gly, PG), with the graphs on the left displaying the sigmoid function fit to the data, and the graphs on the right displaying the same sigmoid function plotted against the mean of the data (Figure 1). 95% confidence intervals are provided for the fitted models in the graphs on the left side of the figure, and standard deviation error bars are provided for the means of the data in the graphs on the right side of the figure.

We performed t-tests on the means of the data at each CPA concentration in order to identify the concentrations at which each of the CPAs became significantly damaging, i.e., at which the chondrocyte recoveries differed significantly from the positive control recovery. Based on the data, Gly deviated significantly from the positive control recovery at 1M, followed by FM and PG at 4M, Me_2SO at 6M, and EG at 7M. Furthermore, when comparing their individual

means at each of the concentrations, Gly demonstrated the lowest recoveries followed by PG. In fact, even at 1M concentration, Gly demonstrated a chondrocyte recovery of only 50.5% ± 12.7%. Recoveries for the remaining CPAs (Me_2SO, FM and EG) were not statistically distinguishable from each other at any of the 8 tested CPA concentrations (Table 1). Finally, even though FM demonstrated the most acute slope change in the relationship curve with increasing concentration, implying that increasing concentrations of FM had the greatest reductions in chondrocyte recovery, the confidence intervals between the slopes were broad enough that this observation could not be reported with statistical confidence (See parameter 'b' results and confidence intervals in Table 1).

Figure 2 provides another set of fits to the same data. In this case, a different method was employed to fit the data, with the idea of describing the data in terms of the combined effect of the differing concentrations (that the tissue was exposed to along its stepwise progression up and down the concentration ladder) along with the final testing CPA concentration. The effects of the decrease in recovery at each step are multiplied by each other to reach the final recovery. This relationship is summarized by Equation 3: If "y" represents the fractional recovery at a CPA concentration "x", then "y" is now represented by

$$y = \prod_{i=1}^{x} \frac{1}{1+e^{-b(i-c)}} \qquad \text{Equation 3}$$

where the concentration "i" takes values in steps of 1M from 1M to the final molar concentration "x" for each trial. This provides us with a method of describing the effect of all concentration exposures to the CPA during the addition and dilution steps, in addition to the final CPA concentration (whereas the initial analysis attributed all of the cell death to only one concentration; the final CPA testing concentration). Furthermore, because this method of

analysis considers the effect of the concentration steps, the corresponding fitted functions can be used to predict the recovery for protocols that use a different stepwise sequence of concentrations, as long as tissue is exposed to each concentration for a total of 10 minutes as was the case for the CPA steps in the experiments reported herein. If different exposure times are used, time would need to appear explicitly in any exposure–recovery relationship[12].

Table 2 provides the values of the parameters for the fitted functions that correspond to Figure 2. As in the previous analysis, the parameter "a" has been set to 1, which sets the normalized recovery to be 100% at 0M concentration. The results of this method provided similar graphs to the initial method. As mentioned previously, isolating the effect of each concentration provides us with the means to predict the recovery for other protocols where the stepwise progression in the concentration ladder may be modified or omitted.

Discussion

Cryoprotective agents at high concentrations are required for successful vitrification, but are known to be toxic to cells [13,14,20,30,35]. To limit the toxic effects of CPA exposure, advances in vitrification protocols include the use of combinations of CPAs at lower concentrations and stepwise addition/dilution of CPAs[4,10,25]. Given the standard approaches to vitrification, understanding the dose-injury relationship of each CPA in relation to human chondrocytes *in situ* is important to develop effective vitrification protocols for preservation of articular cartilage.

The primary analysis of our data, as seen in Figure 1, uses the final CPA concentration as the independent variable determining chondrocyte death. The CPA-dose to chondrocyte-recovery data was fit with a sigmoid function. Also, it was found that chondrocyte recovery levels

deviated significantly from the positive control recovery, at 1M CPA concentration for Gly, at 4M for FM and PG, at 6M for Me_2SO, and at 7M for EG. The secondary analysis, as seen in Figure 2, used a function that accounts for the stepwise CPA exposure in our testing protocol. This analysis attributes a portion of the injury to each incremental CPA-concentration exposure step within the protocol. The resulting fit function allows the results to be generalized to other protocols employing a stepwise CPA addition/dilution protocol (as long as exposure times are similar to those used here so that time need not be added as an extra variable in the dose–recovery relationship). The results are similar to those produced by the primary analysis indicating that chondrocytes *in situ* can be exposed for short periods of time to EG up to 6M, Me_2SO up to 4M, and FM up to 3M, without significant cell loss. On the other hand PG and Gly exposure resulted in significant chondrocyte death with relatively short exposure times and low CPA concentrations (e.g. 1-2M concentration). Thus, the results from this study showed that chondrocytes *in situ* tolerate and survive lower concentrations of some CPAs for short periods of time but become increasingly damaged at higher concentrations. This data provides an understanding of the maximal tolerable limits of CPA concentration after which there are significant reductions in chondrocyte recovery due to exposure to CPAs prepared and applied in the manner described. Furthermore, while this data provides a means of understanding and ordering CPA injury, it also deconvolves the injury effect due to each step in multi-step addition protocols. However, it is important to note that neither the temperature nor the time of exposure at each step were varied, so care must be taken before quantitatively applying these results to protocols that widely differ from those employed in this study. It is also important to recognize that these results are relevant to CPAs prepared in the manner described in this experiment – dilution from a stock solution without taking into account the effect of concentrating salts. This

manner was used in the experiment because it reflects the methods used in our successful AC vitrification protocol[20].

Previous studies have demonstrated CPA toxicities to be dose dependent[9,37]. Specific to articular cartilage, our group performed a study using Me_2SO on thin porcine articular cartilage slices similar to the current experiment[12]. That study showed that Me_2SO toxicity was concentration, temperature and time dependent. However, those results were limited to Me_2SO while this study explores four other CPAs as well. The significant decrease in cell recovery at concentrations greater than 4M in the current study for human articular cartilage was similar to the results from the porcine Me_2SO study that demonstrated a large decrease in cell recovery when the Me_2SO concentration increased from 3M to 5M. Also, that study had been the only identified study within the cryobiology literature which attempted to establish a dose–injury relationship of a CPA to chondrocytes *in-situ*. The current study has shown a non-linear (sigmoidal) relationship between final CPA concentration and chondrocyte recovery for all five CPAs tested. This suggests that the CPAs can be safely added without significant chondrocyte loss up to a specified concentration, after which there is a significant loss of cell viability. Determining the concentration at which chondrocyte viability acutely declines for a specific CPA has useful implications for vitrification protocol design and CPA selection.

Analysis of the data showed that Gly was the most damaging of the CPAs investigated. In addition, it is evident that the dose-injury response for Gly can also be fit equally well with an exponential curve; however, we have retained a sigmoidal fit for consistency with the other CPA dose-injury relationships. Even at exposure to 1M Gly, there was a significant decrease in chondrocyte recovery relative to its normalized control. This is in contrast to the remaining CPAs tested which all demonstrated similar significant declines in chondrocyte recovery but at

higher concentrations; FM and PG declining significantly at 4M, Me_2SO at 6M and EG at 7M. Overall, Gly and PG were clearly the most damaging of the tested permeating CPAs. Otherwise, due to the variability within the data, no statistical distinctions in damaging effects could be made between Me_2SO, FM and EG.

Previous CPA injury studies have identified variations in the damaging order of CPAs among various cell types, temperatures, and concentrations[5,22,23]. The results of this study using chondrocytes within their normal cartilage matrix (i.e. *in situ*) are somewhat different from our recently published study that examined CPA toxicities in isolated human chondrocytes[5]. In our earlier study on isolated human chondrocytes, we found that FM and Me_2SO were the most and least damaging CPAs, respectively. This differs from our current study that was not able to statistically distinguish whether FM, Me_2SO or EG were the least injurious CPAs. However, in our earlier studies with isolated chondrocytes, we did similarly find PG to be a relatively damaging CPA, most notably when employed within combination CPA solutions as described by others[4,5,7,27]. Interestingly, Gly was found to be the most damaging CPA in the current study, which contrasts with an earlier study using CPAs at lower concentrations on isolated porcine chondrocytes[22].

There are a several possible explanations for the reported differences in injury order of the CPAs. It has been shown that CPA toxicity varies between species and this could account for the porcine and human differences of Gly[4,11,31,33-35]. Furthermore, the current study was performed with cells in their native matrix (70µm thick slices), while the previous human chondrocyte study was performed with chondrocytes removed from the matrix[5]. The release of the cells from the native matrix requires that the cell-matrix attachments are broken and this may alter the chondrocyte's natural cell membrane properties, leaving them more vulnerable or

318 resilient to different CPA effects when compared to cells *in situ*[9]. Also, CPA permeation rates
319 through the AC matrix[21] and into cells[36] are different with Gly permeation being relatively
320 slow (compared with other CPAs) in both situations although the 70μm thick slices were chosen
321 to mitigate matrix permeation effects compared with whole cartilage. Cell membrane permeation
322 may be an important factor given the experimental protocol herein with short exposure times at
323 increasing concentrations. Furthermore, from the viewpoint of the fitted function, model
324 predictions are only valid for the exposure times and temperatures used within this data.
325 Therefore, the reported injury order of the CPAs may be inconsistent across studies which
326 employ different testing temperatures, chondrocyte environments (i.e. *in situ* or in suspension),
327 CPA exposure times, and species.

328 Cellular targets and toxicity mechanisms of CPAs differ, with some CPAs demonstrating
329 genotoxic effects that may require greater time for toxic effects to manifest[6,8]. This study
330 provided approximately 90 minutes of time for the cells to manifest signs of membrane injury,
331 which may differ from other study protocols. This type of evaluation may overestimate cell
332 viability by failing to capture terminally injured cells due to time delays in manifesting
333 apoptosis-induced membrane changes. Conversely, membrane integrity assays may
334 underestimate cell viability by including cells with leaky membranes as dead cells when these
335 membranes may be repairable. That said, membrane integrity assays are the standard method of
336 cell evaluation in cryopreservation of articular cartilage (and, in fact, in most cryopreservation
337 studies) and that is part of the reason we used them in this study. More importantly, we used
338 these stains to reliably evaluate cell recovery. Given that we had 46 samples per donor to
339 evaluate and we intended to control as many time variables as possible, it was not possible to use
340 multiple slices per treatment condition. This experiment used 70μm slices that contain

insufficient numbers of cells to make a metabolic assay useful. The 70μm slices were used to maintain the cells in situ but were thin enough to eliminate CPA permeation concerns. We could not use multiple slices per treatment condition due to the limited number of slices obtainable from each donor. Using one donor for all treatment conditions (repeated 4 times with 4 separate donors) allowed us to relate experimental results back to internal controls to control for human variation.

Conclusions

The dose-injury relationships of Me_2SO, FM, Gly, PG, and EG are all nonlinear in nature and have been described by a sigmoid function. Chondrocytes are able to endure different relatively low concentrations of selected CPAs before demonstrating a significant decrease in chondrocyte recovery at higher CPA concentrations. Gly was found to have the lowest chondrocyte recovery, followed by PG, and the remaining three CPAs (FM, Me_2SO, and EG) were not statistically distinguishable at the higher concentrations. Also, when evaluating for CPA concentrations at which significant changes occur, Gly demonstrated a significant change in chondrocyte recovery at 1M, followed by FM and PG at 4M, Me_2SO at 6M, and EG at 7M after the exposures performed in this experiment. An understanding of these dose-injury relationships between the individual CPAs and chondrocytes will provide a more accurate method of selecting CPAs and their potentially safe concentrations when developing vitrification protocols.

Tables

Table 1: Parameters of the fitted sigmoid function and their confidence intervals for each CPA

CPA	Parameter 'b' in sigmoid function	95% Confidence interval for b	Parameter 'c' in sigmoid function	95% Confidence interval for c
Me_2SO	-1.27	[-1.99 -0.56]	5.59	[5.09 6.09]
EG	-0.91	[-1.32 -0.50]	5.83	[5.30 6.36]
FM	-1.46	[-2.08 -0.84]	5.06	[4.73 5.39]
GLY	-0.80	[-1.20 -0.41]	1.15	[0.55 1.74]
PG	-1.07	[-1.67 -0.47]	3.38	[2.80 3.96]

Table 2: Parameters off the fitted combined sigmoid function (Equation 3) and the confidence intervals for each CPA when the combined effect of all concentrations is taken into account.

CPA	Parameter 'b' in sigmoid function	95% Confidence interval for b	Parameter 'c' in sigmoid function	95% Confidence interval for c
Me_2SO	-1.50	[-2.47 -0.53]	5.84	[5.35 6.32]
EG	-3.47	[-8.29 1.34]	6.45	[5.77 7.14]
FM	-2.82	[-4.27 -1.37]	5.45	[5.19 5.72]
GLY	-1.15	[-1.53 0.76]	2.10	[1.81 2.40]
PG	-2.70	[-6.07 0.66]	3.77	[3.27 4.28]

Figure Legends

Figure 1: Normalized recovery versus final CPA concentration. The graphs on the left show the sigmoid function fit and the data (with 95% confidence interval of the fit shown). The graphs on the right show the same sigmoid function plotted against the *means* of the data (with standard deviation error bars of the data shown).

Figure 2: *Means* of the measured recovery for each CPA (the same data as in Figure 1) and the fitted response based on Equation 3, accounting for the combined effect of all concentrations to which the slice was exposed. Error bars on the data represent one standard deviation.

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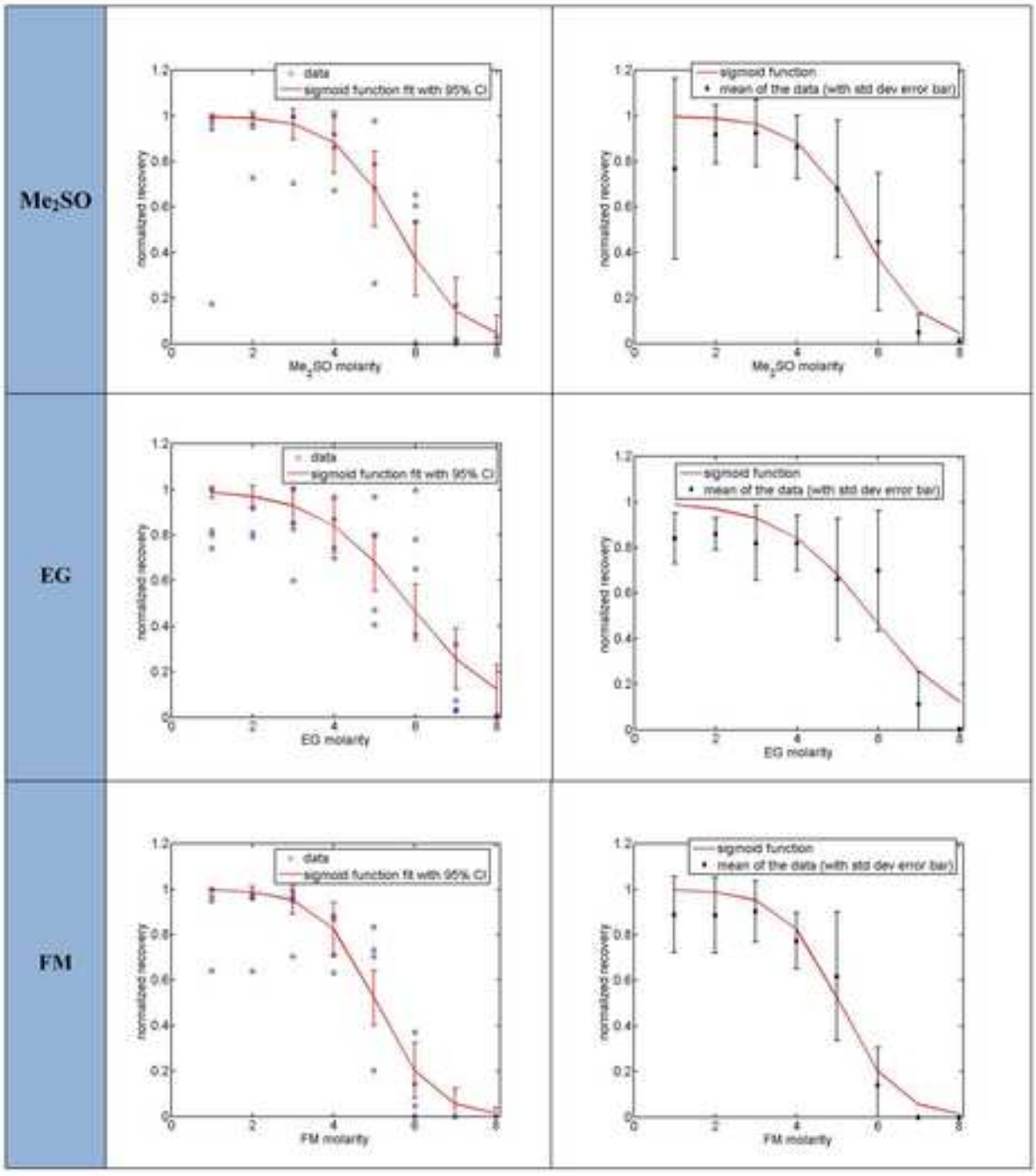

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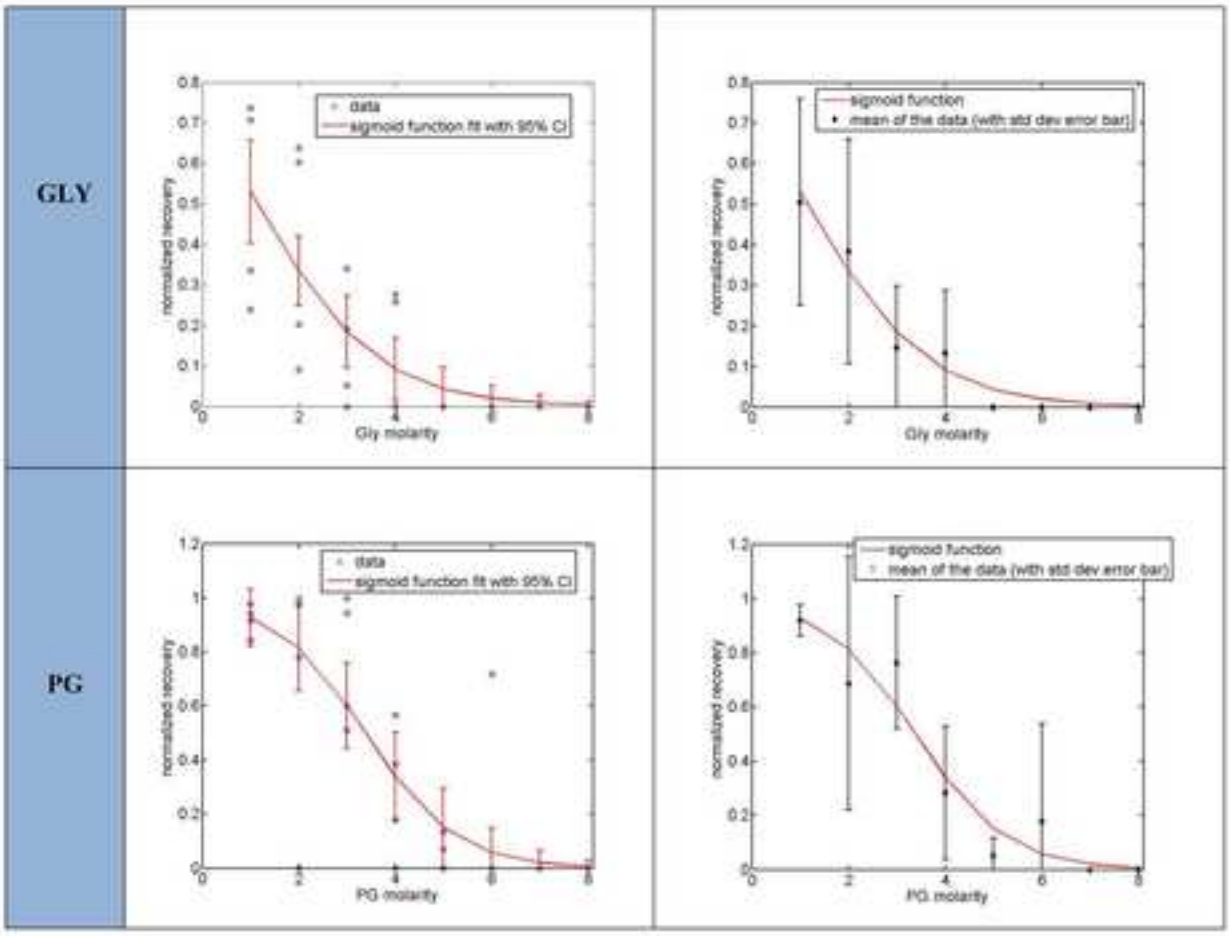

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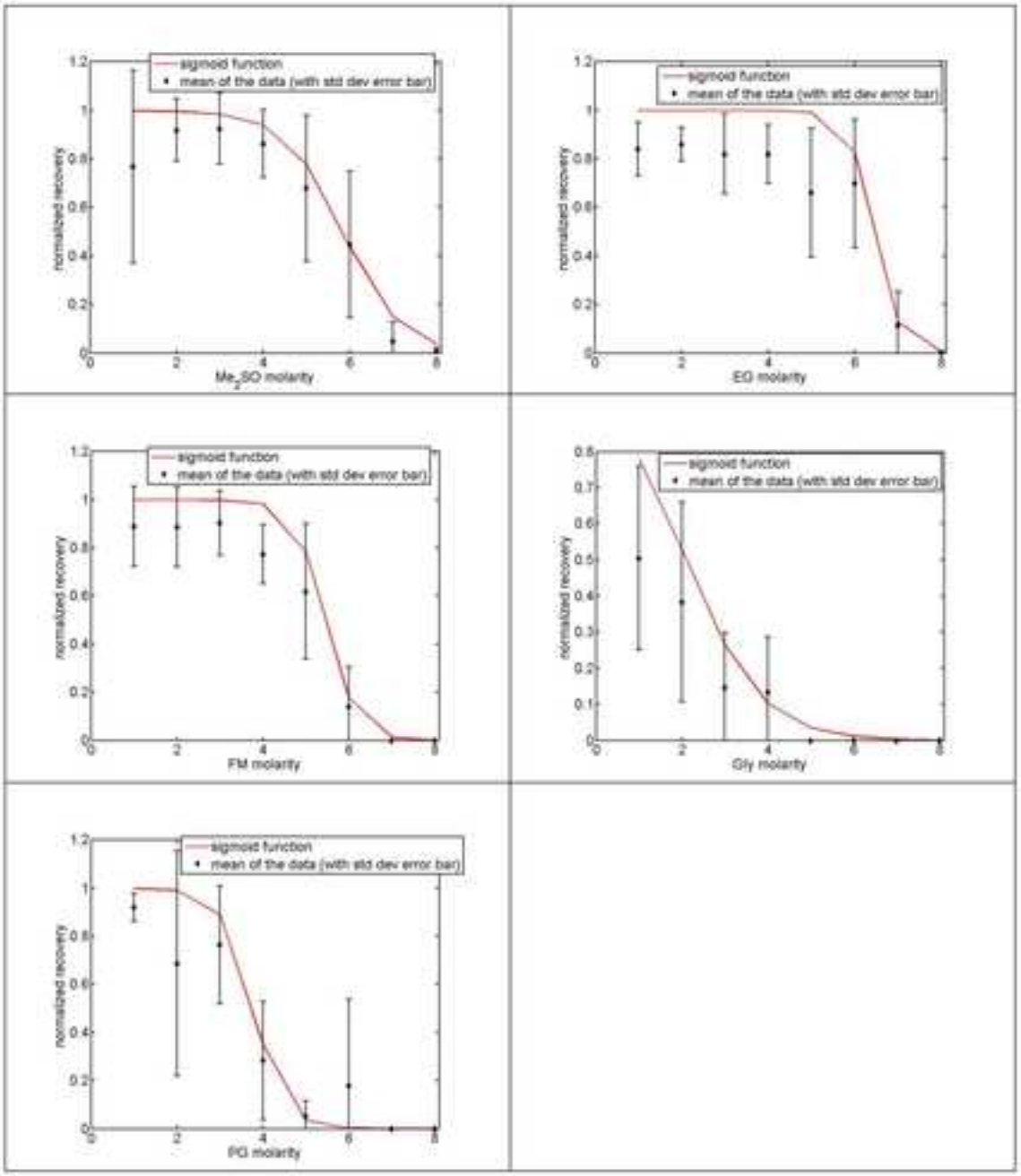

Conflict of Interest

The authors have no conflict of interest.

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