Elsevier Editorial System(tm) for Cryobiology
Manuscript Draft

Manuscript Number: CRYO-D-11-00101R2

Title: Cryoprotective agent toxicity interactions in human articular chondrocytes

Article Type: Research Paper

Keywords: cryopreservation; vitrification; chondrocytes; toxicity; cryoprotectant agents; interactions

Corresponding Author: Dr. Nadr M Jomha, PhD, MD, FRCS(C)

Corresponding Author's Institution: University of Alberta

First Author: Khaled A Almansoori, MBBCh

Order of Authors: Khaled A Almansoori, MBBCh; Vinay Prasad, PhD; Fraser Forbes, PhD; Garson K Law, MSc; Locksley E McGann, PhD; Janet A W Elliott, PhD; Nadr M Jomha, MD, PhD, FRCS(C)

Abstract: Background: Vitrification is a method of cryopreservation by which cells and tissues can be preserved at low temperatures using cryoprotective agents (CPAs) at high concentrations (typically ≥6M) to limit the harmful effects of ice crystals that can form during cooling processes. However, at these concentrations CPAs are significantly cytotoxic and an understanding of their toxicity characteristics and interactions is important. Therefore, single-CPA and multiple-CPA solutions were evaluated for their direct and indirect toxicities on chondrocytes. Methods: Chondrocytes were isolated from human articular cartilage samples and exposed to various single-CPA and multiple-CPA solutions of five common CPAs (dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), glycerol (Gy) and formamide (Fm) at both 6.0M and 8.1M concentrations at 0°C for 30 minutes. Chondrocyte survival was determined using a fluorescent cell membrane integrity assay. The data obtained was statistically analyzed and regression coefficients were used to represent the indirect toxicity effect which a specific combination of CPAs exerted on the final solution's toxicity.
Results: Multiple-CPA solutions were significantly less toxic than single-CPA solutions ($P<0.01$). The indirect toxicity effects between CPAs were quantifiable using regression analysis. Cell survival rates of approximately 40% were obtained with the four-CPA combination solution DMSO-EG-Gy-Fm. In the multiple-CPA combinations, PG demonstrated the greatest degree of toxicity and its presence within a combination solution negated any benefits of using multiple lower concentration CPAs.
Conclusions: Multiple-CPA solutions are less cytotoxic than single-CPA solutions of the same total concentration. PG was the most toxic CPA when used in combinations. The highest chondrocyte survival rates were obtained with the 6.0M DMSO-EG-Gy-Fm combination solution.

Cryoprotective agent toxicity interactions in human articular chondrocytes

KA Almansoori MBBCh[a]*, (almansoori@ualberta.ca)*
V Prasad PhD[b]*,(vprasad@ualberta.ca)*
JF Forbes PhD[b]*, (Fraser.Forbes@ualberta.ca)*
GK Law MSc[a]*,(gklaw@ualberta.ca)*
LE McGann PhD[c]*, (locksly.mcgann@ualberta.ca)*
JAW Elliott PhD[b]*, (janet.elliott@ualberta.ca)*
NM Jomha MD FRCS(C) PhD[a] *(njomha@ualberta.ca)*

a. Division of Orthopaedic Surgery, Department of Surgery, University of Alberta, Edmonton, AB T6G 2B7
b. Department of Chemical and Materials Engineering, University of Alberta, Edmonton, AB T6G 2V4
c. Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, AB T6G 2R7

Corresponding author:
NM Jomha
2D2.28 WMC, Department of Surgery
University of Alberta Hospital
Edmonton Alberta
Canada
T6G 2B7
Ph: 1 780 407 2816
Fax: 1 780 407 2819
Email: njomha@ualberta.ca

Abstract

Background: Vitrification is a method of cryopreservation by which cells and tissues can be preserved at low temperatures using cryoprotective agents (CPAs) at high concentrations (typically ≥6M) to limit the harmful effects of ice crystals that can form during cooling processes. However, at these concentrations CPAs are significantly cytotoxic and an understanding of their toxicity characteristics and interactions is important. Therefore, single-CPA and multiple-CPA solutions were evaluated for their *direct* and *indirect* toxicities on chondrocytes. ***Methods***: Chondrocytes were isolated from human articular cartilage samples and exposed to various single-CPA and multiple-CPA solutions of five common CPAs (dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), glycerol (Gy) and formamide (Fm) at both 6.0M and 8.1M concentrations at 0°C for 30 minutes. Chondrocyte survival was determined using a fluorescent cell membrane integrity assay. The data obtained was statistically analyzed and regression coefficients were used to represent the indirect toxicity effect which a specific combination of CPAs exerted on the final solution's toxicity. ***Results***: Multiple-CPA solutions were significantly less toxic than single-CPA solutions ($P<0.01$). The *indirect* toxicity effects between CPAs were quantifiable using regression analysis. Cell survival rates of approximately 40% were obtained with the four-CPA combination solution DMSO-EG-Gy-Fm. In the multiple-CPA combinations, PG demonstrated the greatest degree of toxicity and its presence within a combination solution negated any benefits of using multiple lower concentration CPAs. ***Conclusions***: Multiple-CPA solutions are less cytotoxic than single-CPA solutions of the same total concentration. PG was the most toxic CPA when used in combinations. The highest chondrocyte survival rates were obtained with the 6.0M DMSO-EG-Gy-Fm combination solution.

Key words: cryopreservation, vitrification, chondrocytes, toxicity, cryoprotectant agents, interactions

Introduction

Osteoarthritis (OA) results in a massive socioeconomic burden with significant individual implications. Arthritis is the leading cause of work disability in Canada, with a yearly economic cost of approximately $4.4 billion in Canada[1; 2] and $40 billion in the USA[3]. Thus, prevention and treatment of OA are of paramount importance to society. Although several methods are available to treat small articular cartilage (AC) injuries, treatment options for larger defects have been challenging. Cartilage transplantation is a potential treatment option for large cartilage defects, especially when considering the low immunogenicity and avascularity of the tissue[4]. Hypothermic storage at 4°C has had some success but the storage period is limited to 28-42 days because cell deterioration and death begins after 7-14 days in this condition[5; 6] making it difficult to establish a sufficient inventory of osteochondral tissue. Thus, before osteochondral transplantation becomes widely available, an effective method of storage and preservation is necessary to maintain the cartilage tissue's integrity and cellular viability. Cryopreservation offers a solution to this problem by which sub-zero temperatures are used to arrest the biochemical reactions responsible for the deterioration of stored cells and tissues.

Two common methods of cryopreservation are controlled freezing and vitrification, and both have been successfully applied to single cell systems including semen, stem cells, and oocytes[7; 8; 9]. However, successful long term results have been limited when these techniques have been applied to more complex tissue like AC[10; 11; 12]. The primary obstacle of controlled freezing is the formation of ice crystals during the cooling process which results in molecular, mechanical, and osmotic cellular and tissue injuries[13; 14; 15]. Vitrification appears to exhibit greater survival rates for both

gonocytes[16; 17] and chondrocytes[18]. This process involves the use of cryoprotective agents (CPAs) at high concentrations (typically $\geq$6M for human chondrocytes) with a rapid cooling rate that limits the formation of ice crystals. Unfortunately, at the high concentrations required to achieve vitrification, the CPAs are cytotoxic and so the benefits of employing CPAs becomes undermined by their own toxicity[15; 19; 20; 21; 22].

In general, CPAs are categorized as *permeating* (eg. glycerol, ethylene glycol) or *non-permeating* (eg. sucrose, trehalose) based on their ability to penetrate the cell membrane layer. The mechanisms by which CPAs cross the cell membrane have not been completely established, but studies have shown that factors which include temperature, exposure time, solution agitation, and concentration can all affect their penetration into cells[23; 24; 25; 26; 27; 28; 29; 30; 31]. Membrane permeation enhances a CPAs ability to achieve successful cryopreservation but also makes them considerably more toxic relative to non-permeating CPAs[32]. The extensive experience and relative success with permeating CPAs were the reasons that this study focused exclusively on commonly used permeating CPAs.

Different CPAs exhibit various degrees and mechanisms of toxicity. Dimethyl sulfoxide (DMSO) has been investigated most thoroughly in different cell types and is shown to block Na^{+} and Ca^{2+} ion channels within cell membranes[33], alter cell membrane permeability[34], alter mitochondrial membrane potential[35; 36], induce cellular apoptosis[37], and inhibit telomerase activity[38]. Ethylene glycol (EG) has been shown to induce changes to the cell's metabolism by altering NAD/NADH ratios required for ATP production[39]; glycerol (Gy) is found to act on the glycerol phosphate

cycle to promote protein glycosylation and mitochondrial dysfunction[40]; and formamide (Fm) is shown to disrupt the cell's membrane, cytoskeleton and mitotic spindle architecture[41; 42]. Furthermore, several CPAs have also been found to exhibit genotoxicity effects; DMSO and formamide can denature DNA in calf thymus cells, while EG and propylene glycol (PG) can induce chromosomal injuries in hamster oocytes[41; 43]. Because of the different modes of toxicity, multiple CPAs at low-concentration are often combined into a 'cocktail' solution in order to limit their individual toxicities while at the same time obtaining an adequate total cryoprotective solution concentration needed for tissue vitrification[27; 44; 45; 46; 47].

When describing toxicity effects of a multiple-CPA solution, an important distinction is required; *direct* toxicity is the primary genotoxic or cytotoxic effects that a CPA has on a cell, while *indirect* toxicity describes the effect of the molecular interactions between CPAs within a solution which either enhance or reduce the respective individual CPA toxicities. An example of this indirect toxicity effect is demonstrated by the reduction in formamide's toxicity when DMSO is combined in the same solution[48]. Determination of CPA toxicity interactions is difficult due to the multiple combinations possible. The only comprehensive attempt on articular chondrocytes has been performed by our group using porcine chondrocytes. We used five different permeating CPAs in combinations up to 3M[21]. It was shown that toxicity interactions did occur and could be described using regression analysis. The current study was designed to evaluate the direct and indirect toxicity effects of five commonly used permeating CPAs [dimethyl sulfoxide, ethylene glycol, propylene glycol, glycerol and formamide] in human articular chondrocytes when placed into various combinations at

sufficient concentrations to vitrify[49]. We hypothesized that CPA direct and indirect toxicities of high concentrations of permeating CPAs could be determined and quantified in human articular chondrocytes.

Methods & Materials

Chondrocyte Isolation

After ethical approval from the University of Alberta's ethical review board, a total of 10 AC samples were obtained from donor patients undergoing elective total knee replacements and transported in a sterile phosphate-buffered saline solution (PBS, pH 7.1, Invitrogen, Carlsbad, CA) and tested within 24hrs. Only areas scored as Outerbridge[50] grade I or 0 were included. Cartilage samples were digested in 1mg/ml solution of collagenase-DMEM-antibiotic solution (Sigma, Cat# C-9891, Lot# 084K8613, 357 units/mg solid, St. Louis, MO: Dulbecco's Modified Eagle Media, DMEM F-12, Invitrogen, Carlsbad, CA: 1% Penicillin/Streptomycin, Invitrogen, Carlsbad, CA). This was performed over a period of 6 hours in an incubator at 37°C and 5% CO_2. The digested solution was filtered through a 40µm cell strainer (Fisher Scientific, Waltham, MA) and placed into a 50mL centrifuge tube and centrifuged for 6 minutes at 400x *g* and 20°C (Eppendorf 5810 R, Eppendorf, Hamburg, DE). The supernatant was then removed and the pellet was re-centrifuged and re-suspended in both sterile PBS and DMEM, eventually providing isolated chondrocytes without matrix. The cell number was determined using a Coulter Counter Z2 (Beckman Coulter, Fullerton, CA) and the chondrocytes were seeded onto a 96-well cell culture plate at a concentration of 25,000 cells in 100µL suspension of solution (DMEM F-12, 1%

Penicillin/Streptomycin, 10% fetal bovine serum). The plates were then placed inside an incubator for 48hrs (37°C, 5% CO_2) before they were exposed to the cryoprotective solutions.

Cryoprotective Solutions and Chondrocyte Exposure

Five CPAs were used to create single-, three-, four- and five- combination solutions (Table 1): dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), glycerol (Gy) and formamide (Fm). All twenty-one of the test solutions were prepared as 6M and 9M stock solutions using the various CPAs in equal concentrations and suspension media (DMEM F-12, 1% Penicillin/Streptomycin, and 10% fetal bovine serum) and pre-cooled to 4C° to decrease biochemical reactions[22]. The 96 well plates were removed from the incubator and placed on ice. Half of the volume of the wells were removed with a pipette and replaced with the 6M test solution on top of the adhered cells, thus creating a 3M solution environment in each well. After 5 minutes, 50 µL (half of the volume of the well) was removed and replaced with an equal volume of the 9M test solution, creating a final 6M environment. This stepwise increase in concentration was employed to minimize the potential osmotic effects on the cells. For the 8.1M treatment, an additional 70µL (70% of the volume) was removed and replaced with an equal volume of the 9M test solution, thus creating an 8.1M environment in each well. Wells containing only suspension media (DMEM/F12) and no CPAs were used as a positive control, and wells containing 100% DMSO (known to be cytotoxic to chondrocytes)[22] was taken as a negative control.

After 30 minutes of exposure, the CPAs were removed from the cells by the following process: 25% of the test solution was removed with a pipette and replaced with an equal volume of suspension media (DMEM/F12) and held for 5 minutes, creating an environmental concentration of 6M in the wells that were initially subjected to 8.1M CPA solutions. To further reduce the CPA concentration of all 6M solutions in a stepwise manner, 50% of the test solution was removed and replaced with an equal volume of suspension media. This was followed by two additional dilution steps removing 75% of the remaining CPA solution each time, resulting in a final CPA concentration of 0.1875M. There were 10 donors in total. Six donors were used for the 1- and 3-CPA combinations (32 wells – five 1-CPA solutions at both 6M and 8.1M, ten 3-CPA combinations at both 6M and 8.1M, one negative control and one positive control) and four donors were used for the 4- and 5-CPA combinations (14 wells – five 4-CPA combinations at both 6M and 8.1M and one 5-CPA combination at both 6M and 8.1M, one negative control and one positive control). Each donor provided one complete run of either 1- and 3-CPA combinations (32 wells) or 4- and 5- combinations (14 wells).

Evaluation of "direct" CPA toxicities via cell viability assays

To assess the level of toxicity of each of the CPA solutions, a membrane integrity assay was used to determine chondrocyte viability. A combination stain solution was prepared using Syto13/ethidium bromide (0.86µM/8.6µM, Invitrogen, Carlsbad, CA / Sigma-Aldrich, St. Louis, MO) in 1x PBS (Invitrogen, Carlsbad, CA). Syto13 is a cell membrane permeable nucleic acid specific stain which emits a green fluorescence upon excitation and ethidium bromide is a cell membrane non-permeable stain which is

specific for nucleic acids and emits a red fluorescence upon excitation. Therefore, cells with intact membranes block the ethidium bromide and emit a green fluorescence, while those with defective membranes allow the penetration of ethidium bromide and emit a red fluorescence. The wells of the 96-well plate were drained by inversion and 40µl of the combination stain solution was added to each of the wells (thus further reducing the CPA concentration in the wells to almost nil). The cells and stain were left to incubate for 30 minutes at room temperature (~21°C) in a dark environment. Chondrocyte membrane integrity was determined by fluorescent microscopy (Nikon Eclipse TE 2000-U fluorescent microscope, NIS Elements software [v. F 2.30, Nikon, Tokyo, JP]) and cell counts (live/dead) were made using a visual software program (Viability 3.2.0.0, Cryolab, University of Alberta)[51]. The chondrocyte survival rates were normalized and are reported as the number of intact (green-fluorescing) cells post-treatment divided by the number of intact cells in the self-control. This calculation was chosen as a conservative measure of cell survival that accounts for differences among samples in initial cell health and cell attachment properties.

Evaluation of "indirect" toxicities via Statistical Analysis

The following mathematical model was used for cell recovery, y.

$$y = \propto + \sum_{i=1}^{5} \beta_i x_i + \sum_{i=1}^{5} \sum_{j=i+1}^{5} \gamma_{ij} x_i x_j + \sum_{i=1}^{5} \sum_{j=i+1}^{5} \sum_{k=j+1}^{5} \emptyset_{ijk} x_i x_j x_k + \sum_{i=1}^{5} \sum_{j=i+1}^{5} \sum_{k=j+1}^{5} \sum_{l=k+1}^{5} \delta_{ijkl} x_i x_j x_k x_l \quad (1)$$

where x_i is the molar concentration of the i^{th} cryoprotective agent, α represents the expected control survival, and the symbols β, γ, Ø, and δ are the various regression

coefficients describing the strength of various direct and indirect toxicities of CPAs. The β_i coefficients, assume a linear dose-toxicity relationship for each CPA and describe the direct toxicity strength of each cryoprotectant i —that is, β_i indicates the amount that the cell recovery is reduced per molar increase in that CPA's concentration. The β_i coefficients are expected to have negative values as the presence of a single CPA generally reduces cell survival. The last three terms in Equation 1 represent higher order corrections to the control cell survival and direct toxicities, and are called "indirect toxicities". The coefficients γ, Ø, and δ represent the strength of the indirect toxicities or the additional contribution to cell recovery of a particular combination of CPAs above that which would be expected by considering the effect of each CPA individually. The last three terms in equation 1 involving these coefficients account for changes in solution toxicity due to interactions between CPAs in solution. The coefficients γ, Ø, and δ can be expected to be either negative or positive. Those combinations for which CPA–CPA interactions caused an increased level of solution toxicity have negative coefficients, while those combinations for which CPA–CPA interactions lead to a reduced level of toxicity have positive coefficients.

Using the data from the cell viability assays, the coefficients were identified using linear least squares multiple regression. Various models were tested, including models without indirect effects (i.e., neglecting the last three terms on the right hand side of equation 1), and models that only included terms up to 2^{nd} order and 3^{rd} order, respectively. However, the model that included all the terms including 4^{th} order terms in equation 1 best described the experimental data. After the regression was completed, hypotheses tests

were conducted to ensure that the estimated value of each coefficient was significant. The null hypothesis was that the coefficient was indistinguishable from zero at the 95% confidence level, and the tests were conducted using the standard deviation of each coefficient obtained from the regression. The tests established that each coefficient was significant and different from zero. The statistical analysis was performed using Matlab R2009b software (The MathWorks, Inc., Natick, MA).

Results

Single and combination CPA toxicities

The positive controls showed a cell survival rate of 54.9±2.7 (n=10) and the negative controls had cell survival rates of 0% (Figure 1). Overall, the single-CPA solutions were more toxic than the multiple-CPA solutions (Table 2). This finding was consistent in both the 6.0M and 8.1M concentrations. Furthermore, between the two concentrations, the 8.1M solutions resulted in significantly greater chondrocyte loss compared to the 6.0M solutions ($P<0.05$). Among the ***single***-CPA solutions, formamide was the most toxic agent and DMSO was the least toxic. However, when used in ***multiple***-CPA solutions, propylene glycol was found to be the most toxic CPA. In general, all solutions that contained PG resulted in a similar range of chondrocyte survival of between 0.05-5.09% (6.0M = 0.18 – 5.09%; 8.1M = 0.05 – 2.85%). However, CPA combination solutions where PG was excluded yielded significantly greater cell membrane integrity rates ($P<0.001$, Table 2). In fact, the average cell survival rates of the PG-excluded combination solutions ranged from 7.93-39.78% for the 6.0M solutions and 1.05-7.20% for the 8.1M solutions. The greatest chondrocyte survival rates

(approximately 40%) were obtained with the DMSO-EG-Gy-Fm combination solution, followed by the Gy-EG-Fm and Gy-DMSO-Fm solutions (Table 2).

Combination CPA "Indirect" Toxicity Effects

The interactions between the CPAs had measurable effects on a solution's toxicity; various CPA interactions resulted in either additive or subtractive effects on the final solution toxicity (Table 3). Two–CPA interactions increased the final toxicity of the solution above that expected from the single–CPA direct toxicities as evidenced by the negative values of the γ_{ij} coefficients. Three–CPA interactions decreased the final toxicity of the solution below that expected from the single–CPA direct toxicities and two–CPA indirect toxicities as evidenced by the positive values of the $Ø_{ijk}$ coefficients. Four–CPA interactions increased the final toxicity of the solution CPA above that expected from the single-CPA direct toxicities and two– and three–CPA indirect toxicities as evidenced by the negative values of the δ_{ijkl} coefficients. While some interactions resulted in antagonistic toxicity effects which reduced the CPA solution's final toxicity (eg. the 3-combination CPA solutions), other combinations resulted in synergistic toxicity effects which further increased the solution's toxicity (eg. the 2- and 4-combination CPA solutions). Of note, the values of the coefficients are not as significant as their ordinality in terms of grading the indirect toxicities of the different combinations in relation to one another.

Discussion

The ideal CPA solution for cartilage vitrification depends on determining the least toxic CPA solution as well as optimizing its tissue-permeability and efficacy in vitrification. However, in order to achieve this, a clear understanding of the direct and indirect toxicities is necessary to develop a safe and minimally toxic CPA solution. This study has attempted to investigate both the direct toxicity effects of CPAs in various CPA combinations, as well as the indirect toxicity effects of combination CPAs. The obtained coefficients provide us with an understanding of the CPA toxicities so that we can mathematically predict which combinations of CPAs will produce the lowest degree of toxicity without having to empirically test all possible combinations.

Surprisingly, the chondrocyte survival rates for the positive controls were approximately 60%. This suggests that environmental factors such as pre-existing condition of the cartilage, harvesting method and tissue processing were important in determining chondrocyte survival. Another important factor was chondrocyte exposure to the CPA solutions. Previous studies have shown that even though CPAs all share the similar function of preventing cryopreservation-induced injuries, their degree and mechanism of toxicity varies among species[24; 52; 53]. However, when we compare this data to our previous study which employed porcine chondrocytes, there are several important similarities: multiple-CPA solutions are less toxic than single-CPA solutions of the same concentration; higher concentration CPA solutions are more toxic; and PG is one of the most toxic CPAs[21].

An important concept presented within this study is that of direct and indirect toxicity effects. It has been shown that combining CPAs can result in lower toxicity when compared to a single CPA at the same total concentration[54; 55]. It has not been

305 determined whether the overall lower toxicity is due to the actual reduction of the
306 concentration of each individual CPA or interactions between the CPAs or a combination
307 of both of these mechanisms. As noted here, multiple-CPA solution toxicities are
308 *nonlinear* and the final CPA solution's toxicity is not equal to the sum of its individual
309 CPA toxicities. This nonlinear pattern of toxicity is best explained by the indirect toxicity
310 concept arising from molecular interactions between CPAs and their biochemical targets
311 and between the CPAs themselves. These interactions alter the activities and toxicities of
312 the CPAs within a combination and produce either synergistic (i.e. enhanced) or
313 antagonistic (i.e. reduced) effects on the final CPA solution's toxicity. The exact
314 mechanism of this property is unknown, but we propose some plausible mechanisms. For
315 example, PG may polarize another CPA's molecular charges and render that CPA more
316 toxic or potent. Considering PG's toxicity to the cell's cytoskeleton, it may interfere with
317 the mobilization of organelles and compounds necessary for repair and adaptation. Other
318 possibilities include a CPA compounding the mitochondrial dysfunction induced by
319 glycerol by inhibiting alternative energy sources for the cell, or the CPA may prevent the
320 mobilization of new membrane proteins needed for survival when a cell's membrane ion
321 channels are injured by DMSO. Regardless of the mechanism, the presence of indirect
322 toxicities due to CPA interactions within a combination does play a quantifiable role
323 which has been elucidated in this research.

324 Previous methods of describing and predicting CPA toxicities have focused on
325 quantifying the direct toxicities of CPAs based on their functional polar groups and
326 vitrification concentrations[56]. While the coefficients determined in the current study
327 are only meant to represent the indirect toxicity effects of a combination, their actual

values are not as significant as their ordinality in terms of grading a combination solution's indirect toxicity in relation to other combinations. Interestingly, all the 3-CPA combinations demonstrated positive coefficient values (i.e. interactions that reduce toxicity), while the 2- and 4-CPA combinations had negative coefficients (i.e. interactions which increased the solutions toxicity). One possible explanation is that the 3-CPA combination solutions may represent a cross-over point between toxicity due to CPA-CPA interactions (more likely to occur in the 2-CPA combinations due to the higher concentrations of the 2 CPAs involved) and the toxicity due to increased mechanisms of direct cellular injury (due to the greater overlap and number of cellular targets and toxicity mechanisms with 4-CPA combinations). A second explanation involves the toxicity relationships of the CPA's, which may be linear or non-linear (eg. curvi-linear or sigmoidal). In fact, even though a CPA's toxicity-dose relationship may be linear in isolation, when combined with other CPAs the relationship may become non-linear. Therefore, because our indirect toxicity model assumes a linear dose-toxicity relationship, by applying a linear least squares regression analysis to a 'curved' dose-toxicity relationship we may be over- or under-estimating the contributions of the CPA's indirect toxicity effects. That stated, other models were tested including models without indirect effects and models that included terms up the 2^{nd} order and 3^{rd} order. However, the model that included all terms including 4^{th} order terms in equation 1 best described the experimental data. Nevertheless, until further studies become available which better characterize the CPA-cell toxicity relationship, the current model can provide a preliminary framework to describe the indirect toxicity effects based on the 1-, 3-, 4- and

5-CPA data obtained, and provide us with an effective method for predicting which CPA interactions will optimize chondrocyte survival rates.

The incorporation of the coefficients into a CPA's toxicity profile provides a more accurate framework for predicting and describing the toxicity characteristics of CPAs, so that as the data is expanded to include variations in concentrations and further combinations, the model will become more accurate. Nevertheless, considering that the regressions from the data are based on a 'best-fit' model, conclusions regarding physical or biochemical properties and reactions cannot be reliably inferred without first testing the accuracy of the model.

With regards to the single-CPA solution data, we found that formamide was the most toxic of the single CPA solutions and this finding is similarly reported in other studies[57; 58]. However, when analyzing the direct and indirect multiple-CPA toxicity data, we find that PG was the most toxic CPA. In fact, propylene glycol has been found to be a significantly toxic CPA across a variety of species[26; 44; 59] , and when placed in the context of our data, we find that combinations which excluded propylene glycol had significantly better chondrocyte survival rates ($P<0.01$). The mechanisms by which PG exhibits its toxicity is still not completely established; however a few studies have already demonstrated its harmful effects on cell membrane permeability, mitochondrial activity, thymidine incorporation into DNA and lysosomal activation[42; 60; 61]. Furthermore, when analyzing PG within the context of the indirect toxicities, we find that all of the two-combination CPA solutions which included PG had the most negative coefficients, suggesting that when compared to the other CPAs tested, PG significantly enhances the toxicity of a combination solution. Therefore, its inclusion within a

multiple-CPA solution for isolated chondrocytes should be avoided. Nevertheless, the reason for which formamide is found to be the most toxic CPA when in isolation and PG the most toxic in combination is likely related to the indirect toxicity concept where PG may not be the most toxic CPA, but its toxic properties enhance the toxic effects of surrounding CPAs. For this reason, PG is a harmful CPA to isolated chondrocytes when used in a multiple-CPA solution.

Overall, similar to other reports, we determined that multiple-CPA solutions are less toxic than the single CPA solutions[21; 47; 48] , and therefore a larger range of combinations of CPA solutions should be tested. Of course, many other factors may also be adjusted which include variations in individual CPA concentrations within the solution, the role of the order of CPA addition, the temperature of introduction, and the exposure time. Furthermore, considering the more toxic nature of permeable CPAs, there may be benefit to employing a balanced combination of non-permeable and permeable CPAs[23; 59; 61]. Importantly, toxicity effects may be altered by removing the cells from their native tissue and results may differ with cells *in situ*[54]. This study used chondrocytes isolated from the matrix to avoid permeation issues but cells *in situ* may react differently than cells in solution. Thus, it is important to relate these results specifically to isolated chondrocytes as opposed to those still in the matrix. It is possible that 5 minutes exposure is insufficient time. We believe this is unlikely due to the published permeation rates of DMSO, PG and glycerol into chondrocytes[62; 63] but it should be noted that these rates were at 20°C. Only the permeation rate of EG into chondrocytes has not been recorded. In many of the combinations, there was very little cell recovery. Even more informative data may be achieved by using lower

concentrations (we used these high concentrations in order to relate the results to vitrification) or to expose the cells for a shorter period of time. Nevertheless, the results of this study have helped emphasize the possibility of reducing a CPA solution's toxicity by using a selective combination of CPAs with good toxicity interactions.

Conclusions

Multiple-CPA solutions are less cytotoxic than single-CPA solutions of the same total concentration. CPAs exhibit direct and indirect toxicities, where specific molecular interactions can either enhance or reduce the toxicities of other CPAs within a combination solution. For chondrocytes from human AC, formamide was the most toxic CPA when only single-CPA solutions were used. Propylene glycol was the most toxic of the permeating CPAs tested when included in multiple-CPA solutions and its inclusion within a combination resulted in significantly higher indirect toxicities. Of the combination solutions tested, the 6.0M DMSO-ethylene glycol-formamide-glycerol combination solution resulted in the highest human AC chondrocyte survival of approximately 40%.

Acknowledgements

This research was funded by the Canadian Institutes of Health Research (MOP 93805). JAWE holds a Canada Research Chair in Thermodynamics.

Figure Legend

Figure 1: Digitized images of plates containing, A: Positive control; B: Negative control; and C: DMSO-EG-Gy-Fm solution that demonstrated the highest cell recovery of all solutions tested. Fluorescent staining by Syto13 (green = intact membranes) and ethidium bromide (red = disrupted membranes). Images were taken at 4x magnification. Scale bars represent 100 µm.

Table 1. CPA solutions*:

Single-CPA solutions				
1. DMSO	2. EG	3. PG	4.Gy	5.Fm

Three-CPA Combination solutions			
6. EG-DMSO-PG	7. EG-DMSO-Gy	8. EG-DMSO-Fm	9. EG-PG-Gy
10. EG-PG-Fm	11. EG-Gy-Fm	12. Gy-DMSO-PG	13. Gy-DMSO-Fm
14. Gy-PG-Fm	15. DMSO-PG-Fm		

Four-CPA Combination solutions		
16. DMSO-PG-EG-Gy	17. DMSO-PG-EG-Fm	18. DMSO-PG-Gy-Fm
19. DMSO-EG-Gy-Fm	20. PG-EG-Gy-Fm	

Five-CPA Combination solutions
21. DMSO-PG-EG-Gy-Fm

*DMSO (dimethyl sulfoxide), EG (ethylene glycol), PG (propylene glycol), Gy (glycerol), Fm (formamide)

Table 2: CPA solutions and chondrocyte survival rates

	6.0M		8.1M	
Solution	% Survival	SE	% Survival	SE
D-D-D	3.00	0.01	0.97	0.00
P-P-P	0.10	0.00	0.03	0.00
E-E-E	0.34	0.00	0.09	0.00
G-G-G	0.19	0.00	0.06	0.00
F-F-F	0.02	0.00	0.01	0.00
G-E-D	7.93	0.04	4.32	0.01
G-D-F	24.03	0.05	5.07	0.04
G-E-F	28.43	0.03	2.86	0.04
G-E-P	0.20	0.00	0.05	0.00
G-P-F	0.18	0.00	0.09	0.00
E-D-F	9.09	0.01	1.05	0.01
G-D-P	0.45	0.00	0.17	0.00
E-P-F	0.20	0.01	0.70	0.00
E-D-P	0.23	0.00	0.08	0.00
D-P-F	0.25	0.00	0.07	0.00
D-P-E-G	0.41	0.00	0.20	0.00
D-P-E-F	0.48	0.00	0.24	0.00
D-P-G-F	5.09	0.03	2.85	0.00
D-E-G-F	39.78	0.07	7.20	0.09
P-E-G-F	0.60	0.00	0.35	0.00
D-P-E-G-F	3.25	0.01	1.45	0.01
100% DMSO	0.03	0.00	0.02	0.00

*D = dimethyl sulfoxide, P = propylene glycol, E = ethylene glycol, G = glycerol, F = formamide

Table 3: Coefficient toxicities of CPAs

Coefficient	Agent / Combination[±,¶]	Value	Std. Deviation	Notes
α	Control*	48.27	.06072	*Positive control containing suspension media only.*
β	D	-6.58	.02582	*Single-CPA solution's direct toxicity effects*
	P	-6.97	.02580	
	E	-6.80	.02581	
	G	-6.83	.02581	
	F	-6.81	.02580	
γ_{ij}	D + P	-0.8809	0.02301	*Multiple-CPA solution's indirect toxicity effects; the coefficient represents the toxicity effects due to the respective CPA interactions within a corresponding combination*
	D + E	-0.6754	0.02300	
	D + G	-0.2992	0.02303	
	D + F	-0.6231	0.02303	
	P + E	-1.0707	0.02319	
	P + G	-1.0333	0.02319	
	P + F	-0.9299	0.02322	
	E + G	-0.8731	0.02332	
	E + F	-0.6976	0.02307	
	G + F	-0.1326	0.02306	
	---	---	---	
$Ø_{ijk}$	D+P+E	1.0787	0.02074	
	D+P+G	0.9308	0.02074	
	D+P+F	0.1022	0.02074	
	D+E+G	0.9966	0.02073	
	D+E+F	1.1393	0.02074	
	D+G+F	1.7797	0.02074	
	P+E+G	1.2734	0.02074	
	P+E+F	1.1535	0.02074	
	P+G+F	0.9322	0.02074	
	E+G+F	2.3360	0.02074	
	---	---	---	
δ_{ijkl}	D+P+E+G	-0.5773	0.0167	
	D+P+E+F	-0.6301	0.0168	
	D+P+G+F	-0.1047	0.0168	
	D+E+G+F	-0.1185	0.0169	
	P+E+G+F	-0.1358	0.0180	

[±] Positive coefficients represent interactions which reduced the final solution's toxicity, while those with negative coefficients represent interactions which increased the final solutions toxicity.

[¶]D = dimethyl sulfoxide, E = ethylene glycol, P = propylene glycol, G = glycerol, and F = formamide

* DMEM F-12, 1% Penicillin/Streptomycin, and 10% fetal bovine serum

References

[1] Arthritis, Public Health Agency of Canada, 2010.

[2] C. Health, Health Canada; Economic Impact of Illness in Canada 1998. Ottawa: Public Works and Government Services Canada Catalogue # H21-136/1998E, (2002).

[3] A.F. Praemer, and S. Rice, Musculoskeletal conditions in the United States, American Academy of Orthopaedic Surgeons, Rosemont, IL, 1999.

[4] D.W. Jackson, and T.M. Simon, Chondrocyte transplantation [see comments]. Arthroscopy 12, (1996) 732-738.

[5] S.T. Ball, D. Amiel, S.K. Williams, W. Tontz, A.C. Chen, R.L. Sah, and W.D. Bugbee, The effects of storage on fresh human osteochondral allografts. Clin Orthop Relat Res, (2004) 246-52.

[6] R.J. Williams, 3rd, J.C. Dreese, and C.T. Chen, Chondrocyte survival and material properties of hypothermically stored cartilage: an evaluation of tissue used for osteochondral allograft transplantation. Am J Sports Med 32, (2004) 132-9.

[7] K.G. Brockbank, Effects of cryopreservation upon vein function in vivo. Cryobiology 31, (1994) 71-81.

[8] M.L. Check, D.J. Check, J.H. Check, R. Long, and M. Press, Effect of shortened exposure time to the critical period for ice crystal formation on subsequent post-thaw semen parameters from cryopreserved sperm. Arch Androl 32, (1994) 63-67.

[9] L.E. McGann, A.R. Turner, M.J. Allalunis, and J.M. Turc, Cryopreservation of human peripheral blood stem cells: optimal cooling and warming conditions. Cryobiology 18, (1981) 469-472.

[10] N.M. Jomha, G. Lavoie, K. Muldrew, N.S. Schachar, and L.E. McGann, Cryopreservation of intact human articular cartilage. J Orthop Res 20, (2002) 1253-5.

[11] T. Malinin, H.T. Temple, and B.E. Buck, Transplantation of osteochondral allografts after cold storage. J Bone Joint Surg Am 88, (2006) 762-70.

[12] C. Ohlendorf, W.W. Tomford, and H.J. Mankin, Chondrocyte survival in cryopreserved osteochondral articular cartilage. J Orthop Res 14, (1996) 413-416.

[13] G.M. Fahy, T.H. Lilley, H. Linsdell, M.S. Douglas, and H.T. Meryman, Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms. Cryobiology 27, (1990) 247-68.

[14] B. Han, and J.C. Bischof, Direct cell injury associated with eutectic crystallization during freezing. Cryobiology 48, (2004) 8-21.

[15] D.E. Pegg, Principles of cryopreservation. Methods Mol Biol 368, (2007) 39-57.

[16] E.M. Kolibianakis, C.A. Venetis, and B.C. Tarlatzis, Cryopreservation of human embryos by vitrification or slow freezing: which one is better? Curr Opin Obstet Gynecol 21, (2009) 270-4.

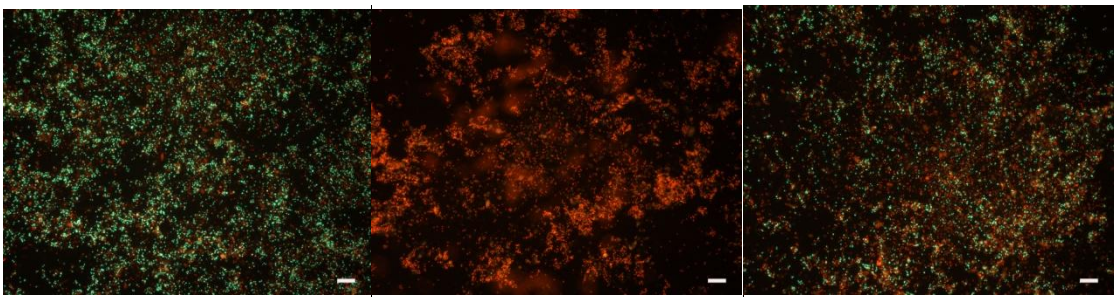
[17] K.E. Loutradi, E.M. Kolibianakis, C.A. Venetis, E.G. Papanikolaou, G. Pados, I. Bontis, and B.C. Tarlatzis, Cryopreservation of human embryos by vitrification or slow freezing: a systematic review and meta-analysis. Fertil Steril 90, (2008) 186-93.

485 [18] K.G. Brockbank, Z.Z. Chen, and Y.C. Song, Vitrification of porcine articular
486 cartilage. Cryobiology 60, (2010) 217-21.
487 [19] G.M. Fahy, D.I. Levy, and S.E. Ali, Some emerging principles underlying the
488 physical properties, biological actions, and utility of vitrification solutions.
489 Cryobiology 24, (1987) 196-213.
490 [20] G.M. Fahy, B. Wowk, J. Wu, J. Phan, C. Rasch, A. Chang, and E. Zendejas,
491 Cryopreservation of organs by vitrification: perspectives and recent advances.
492 Cryobiology 48, (2004) 157-78.
493 [21] N.M. Jomha, A.D. Weiss, J. Fraser Forbes, G.K. Law, J.A. Elliott, and L.E.
494 McGann, Cryoprotectant agent toxicity in porcine articular chondrocytes.
495 Cryobiology 61, (2010) 297-302.
496 [22] H.Y. Elmoazzen, A. Poovadan, G.K. Law, J.A. Elliott, L.E. McGann, and N.M.
497 Jomha, Dimethyl sulfoxide toxicity kinetics in intact articular cartilage. Cell
498 Tissue Bank 8, (2007) 125-33.
499 [23] R.H. Hammerstedt, J.K. Graham, and J.P. Nolan, Cryopreservation of mammalian
500 sperm: what we ask them to survive. J Androl 11, (1990) 73-88.
501 [24] J.M. Blanco, G. Gee, D.E. Wildt, and A.M. Donoghue, Species variation in osmotic,
502 cryoprotectant, and cooling rate tolerance in poultry, eagle, and peregrine falcon
503 spermatozoa. Biol Reprod 63, (2000) 1164-71.
504 [25] J.M. Blanco, J.A. Long, G. Gee, A.M. Donoghue, and D.E. Wildt, Osmotic tolerance
505 of avian spermatozoa: influence of time, temperature, cryoprotectant and
506 membrane ion pump function on sperm viability. Cryobiology 56, (2008) 8-14.
507 [26] J.M. Blanco, J.A. Long, G. Gee, D.E. Wildt, and A.M. Donoghue, Comparative
508 cryopreservation of avian spermatozoa: benefits of non-permeating
509 osmoprotectants and ATP on turkey and crane sperm cryosurvival. Anim Reprod
510 Sci 123, (2011) 242-8.
511 [27] R. Fabbri, Cryopreservation of human oocytes and ovarian tissue. Cell Tissue Bank
512 7, (2006) 113-22.
513 [28] S.R. Lyu, W.T. Wu, C.C. Hou, and W.H. Hsieh, Study of cryopreservation of
514 articular chondrocytes using the Taguchi method. Cryobiology 60, (2010) 165-76.
515 [29] R. McClean, W.V. Holt, Y.P. Zee, A. Lisle, and S.D. Johnston, The effect of
516 cryoprotectant on kangaroo sperm ultrastructure and mitochondrial function.
517 Cryobiology 57, (2008) 297-303.
518 [30] Y. Yamaji, D.M. Valdez, Jr., S. Seki, K. Yazawa, C. Urakawa, B. Jin, M. Kasai,
519 F.W. Kleinhans, and K. Edashige, Cryoprotectant permeability of aquaporin-3
520 expressed in Xenopus oocytes. Cryobiology 53, (2006) 258-67.
521 [31] H.Y. Elmoazzen, J.A. Elliott, and L.E. McGann, The effect of temperature on
522 membrane hydraulic conductivity. Cryobiology 45, (2002) 68-79.
523 [32] M. Kasai, and T. Mukaida, Cryopreservation of animal and human embryos by
524 vitrification. Reprod Biomed Online 9, (2004) 164-70.
525 [33] S.W. Jacob, and J.C. de la Torre, Pharmacology of dimethyl sulfoxide in cardiac and
526 CNS damage. Pharmacol Rep 61, (2009) 225-35.
527 [34] B. Bakar, E.A. Kose, S. Sonal, A. Alhan, K. Kilinc, and I.S. Keskil, Evaluation of
528 the neurotoxicity of DMSO infused into the carotid artery of rat. Injury, (2011).
529 [35] J. Vondracek, K. Soucek, M.A. Sheard, K. Chramostova, Z. Andrysik, J.
530 Hofmanova, and A. Kozubik, Dimethyl sulfoxide potentiates death receptor-

mediated apoptosis in the human myeloid leukemia U937 cell line through enhancement of mitochondrial membrane depolarization. Leuk Res 30, (2006) 81-9.
[36] T. Zampolla, E. Spikings, T. Zhang, and D.M. Rawson, Effect of methanol and Me2SO exposure on mitochondrial activity and distribution in stage III ovarian follicles of zebrafish (Danio rerio). Cryobiology 59, (2009) 188-94.
[37] R.K. Koiri, and S.K. Trigun, Dimethyl sulfoxide activates tumor necrosis factoralpha-p53 mediated apoptosis and down regulates D-fructose-6-phosphate-2-kinase and lactate dehydrogenase-5 in Dalton's lymphoma in vivo. Leuk Res 35, (2011) 950-6.
[38] S. Sharma, E. Raymond, H. Soda, E. Izbicka, K. Davidson, R. Lawrence, and D.D. Von Hoff, Dimethyl sulfoxide (DMSO) causes a reversible inhibition of telomerase activity in a Burkitt lymphoma cell line. Leuk Res 22, (1998) 663-70.
[39] D. Jammalamadaka, and S. Raissi, Ethylene glycol, methanol and isopropyl alcohol intoxication. Am J Med Sci 339, (2010) 276-81.
[40] A.R. Hipkiss, Proteotoxicity and the contrasting effects of oxaloacetate and glycerol on Caenorhabditis elegans life span: a role for methylglyoxal? Rejuvenation Res 13, (2010) 547-51.
[41] M. Aye, C. Di Giorgio, M. De Mo, A. Botta, J. Perrin, and B. Courbiere, Assessment of the genotoxicity of three cryoprotectants used for human oocyte vitrification: dimethyl sulfoxide, ethylene glycol and propylene glycol. Food Chem Toxicol 48, (2010) 1905-12.
[42] K.M. Morshed, S.K. Jain, and K.E. McMartin, Propylene glycol-mediated cell injury in a primary culture of human proximal tubule cells. Toxicol Sci 46, (1998) 410-7.
[43] G. Bonner, and A.M. Klibanov, Structural stability of DNA in nonaqueous solvents. Biotechnol Bioeng 68, (2000) 339-44.
[44] J. Ali, and J.N. Shelton, Design of vitrification solutions for the cryopreservation of embryos. J Reprod Fertil 99, (1993) 471-7.
[45] A.M. Dalimata, and J.K. Graham, Cryopreservation of rabbit spermatozoa using acetamide in combination with trehalose and methyl cellulose. Theriogenology 48, (1997) 831-41.
[46] F. Lahnsteiner, The effect of internal and external cryoprotectants on zebrafish (Danio rerio) embryos. Theriogenology 69, (2008) 384-96.
[47] A. Lawson, H. Ahmad, and A. Sambanis, Cytotoxicity effects of cryoprotectants as single-component and cocktail vitrification solutions. Cryobiology 62, (2011) 115-22.
[48] G.M. Fahy, Cryoprotectant toxicity neutralization. Cryobiology 60, (2010) S45-53.
[49] A.D. Weiss, J.F. Forbes, A. Scheuerman, G.K. Law, J.A. Elliott, L.E. McGann, and N.M. Jomha, Statistical prediction of the vitrifiability and glass stability of multi-component cryoprotective agent solutions. Cryobiology 61, (2010) 123-7.
[50] E.C. Rodriguez-Merchan, and P. Gomez-Cardero, The outerbridge classification predicts the need for patellar resurfacing in TKA. Clin Orthop Relat Res 468, (2010) 1254-7.

[51] N.M. Jomha, P.C. Anoop, J.A.W. Elliott, K. Bagnall, and L.E. McGann, Validation and reproducibility of computerised cell-viability analysis of tissue slices. BMC Musculoskelet Disord 4, (2003) 5.
[52] J.P. Barbas, and R.D. Mascarenhas, Cryopreservation of domestic animal sperm cells. Cell Tissue Bank 10, (2009) 49-62.
[53] H. Yang, M. Norris, R. Winn, and T.R. Tiersch, Evaluation of cryoprotectant and cooling rate for sperm cryopreservation in the euryhaline fish medaka Oryzias latipes. Cryobiology 61, (2010) 211-9.
[54] L.H. Campbell, and K.G.M. Brockbank, Cryopreservation of porcine aortic heart valve leaflet-derived myofibroblasts. Biopreservation and Biobanking 8, (2010) 211-217.
[55] G.M. Fahy, Cryoprotectant toxicity neutralization. Cryobiology, (2009).
[56] G.M. Fahy, B. Wowk, J. Wu, and S. Paynter, Improved vitrification solutions based on the predictability of vitrification solution toxicity. Cryobiology 48, (2004) 22-35.
[57] T.H. Dresser, E.R. Rivera, F.J. Hoffmann, and R.A. Finch, Teratogenic assessment of four solvents using the Frog Embryo Teratogenesis Assay--Xenopus (FETAX). J Appl Toxicol 12, (1992) 49-56.
[58] V. Vuthiphandchai, S. Chomphuthawach, and S. Nimrat, Cryopreservation of red snapper (Lutjanus argentimaculatus) sperm: effect of cryoprotectants and cooling rates on sperm motility, sperm viability, and fertilization capacity. Theriogenology 72, (2009) 129-38.
[59] I.A. Nascimento, M.B. Leite, M.M. Sampaio de Araujo, G. Sansone, S.A. Pereira, and E.M. do Espirito Santo, Selection of cryoprotectants based on their toxic effects on oyster gametes and embryos. Cryobiology 51, (2005) 113-7.
[60] M. Hagedorn, E. Hsu, F.W. Kleinhans, and D.E. Wildt, New approaches for studying the permeability of fish embryos: toward successful cryopreservation. Cryobiology 34, (1997) 335-47.
[61] M. Hagedorn, F.W. Kleinhans, D.E. Wildt, and W.F. Rall, Chill sensitivity and cryoprotectant permeability of dechorionated zebrafish embryos, Brachydanio rerio. Cryobiology 34, (1997) 251-63.
[62] X. Xu, Z. Cui, and J.P. Urban, Measurement of the chondrocyte membrane permeability to Me2SO, glycerol and 1,2-propanediol. Med Eng Phys 25, (2003) 573-9.
[63] L.E. McGann, M. Stevenson, K. Muldrew, and N. Schachar, Kinetics of osmotic water movement in chondrocytes isolated from articular cartilage and applications to cryopreservation. J Orthop Res 6, (1988) 109-115.

Figure 1



A B C

***Statement of Funding**

This research was funded by the Canadian Institutes of Health Research which did not have any role in the design and performance of this study.

Conflict of Interest

The authors have no conflicts of interest.