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Abstract: Objective: Osteochondral allografting is an effective method to treat large osteochondral defects but difficulties in tissue preservation have significantly limited the application of this technique. Successful cryopreservation of articular cartilage (AC) could improve the clinical availability of osteochondral tissue and enhance clinical outcomes but cryopreservation of large tissues is hampered by a lack of knowledge of permeation kinetics within these tissues. This study describes the refinement and extension of a recently published technique to measure the permeation kinetics of cryoprotectant agents (CPAs) within porcine AC.

Design: Dowels of porcine AC (10 mm diameter) were immersed in solutions containing 6.5 M concentrations of four commonly used CPAs [dimethyl sulfoxide (Me2SO), propylene glycol (PG), ethylene glycol (EG) and glycerol] for different times (1 second, 1, 2, 5, 10, 15, 30, 60, 120, 180 minutes, 24 hours) at three different temperatures (4, 22, and 37 ☐ C). The cartilage was isolated and the amount of CPA within the matrix was determined.

Results: Diffusion coefficients (Me2SO = 2.4 - 6.2 x 10-6 cm2/s; PG = 0.8 - 2.7 x 10-6 cm2/s; EG = 1.7 - 4.2 x 10-6 cm2/s; and glycerol = 0.8 - 2.4 x 10-6 cm2/s) and activation energies (Me2SO = 4.33 kcal/mol, PG = 6.29 kcal/mol, EG = 3.77 kcal/mol, and glycerol = 5.56 kcal/mol) were determined for each CPA.

Conclusion: The results of this experiment provide accurate permeation kinetics of four commonly used CPAs in porcine articular cartilage. This information will be useful for developing effective vitrification protocols for cryopreservation of AC.

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Introduction

Large, focal articular cartilage (AC) defects greater than 1cm^2 result in joint degeneration with subsequent osteoarthritis[1,2]. Current cartilage regeneration techniques are unable to reproduce the complex architecture and consistent, successful long-term results are difficult to obtain. Transplantation of fresh or frozen allografts has been successful but clinical use is limited by 1) poor size and contour matching, 2) limited tissue availability, 3) possibility of infectious disease transmission, and 4) difficult operative timing for the patient, surgical team and surgeon. Successful cryopreservation of osteochondral tissue that maintains cell viability and matrix structure can eliminate these limitations and enhance the long-term clinical results of allograft transplantation for focal articular defects and possibly complete joint replacement.

Successful cryopreservation of biologic tissues has proven exceedingly difficult. Early attempts using controlled-cooling and stepped-cooling procedures were successful with cells in suspension and simple tissues but extension to more complex tissues such as AC has been fraught with failures despite occasional marginal success.[3-7] The failure of traditional cryopreservation techniques has resulted in investigations of vitrification for complex tissues with improved recovery.[8]

To achieve vitrification at practical cooling rates in large (>1 cm) tissues, high concentrations of cryoprotective agents (CPAs) are required. Unfortunately, these concentrations are toxic to the chondrocytes that reside within the cartilage matrix.[9-11] Therefore, to minimize chondrocyte

74 toxicity, we need to determine precise permeation kinetics of CPAs into AC matrix so that the
75 maximum amount of CPA permeation can be achieved with the minimum amount of exposure.
76
77 Recently we published a novel technique to measure dimethyl sulfoxide (DMSO) and propylene
78 glycol (PG) permeation into intact porcine AC.[12] The objective of the study presented herein
79 was to refine our technique and extend it to multiple CPAs in an effort to determine permeation
80 kinetics of suitable CPAs for the vitrification of AC. Specifically, we increased the number of
81 CPAs to four (DMSO, PG, ethylene glycol (EG), and glycerol) to provide a better idea of the
82 range of diffusion kinetics of these CPAs. In addition, we increased the number of measurement
83 time points with an emphasis on time points within the first hour as this is when the most rapid
84 permeation into the AC occurs. Finally, we incorporated a second weighing into the
85 experimental procedure to increase the accuracy of calculated quantities.
86
87

Methods and Materials

Osteochondral dowels (OCD, 10 mm diameter full thickness AC on 5-10 mm bone base) were harvested from the distal femoral condyles of sexually mature pigs of mixed gender. The pigs were sacrificed for meat consumption by a local packing plant (Sturgeon Valley Pork, St. Albert, AB, Canada). Porcine tissue was selected due to its similarity of thickness (2-4 mm) to human knee AC. Up to six OCDs were taken from the weight-bearing portion of each femoral condyle using a custom hand-held coring device and held at 4°C in 1x Dulbecco's phosphate-buffered saline (PBS) solution (pH 7.1) (Gibco Invitrogen, Carlsbad, CA).

Each OCD was assigned to one of four CPA treatment groups [DMSO, PG, EG, glycerol (all at 6.5M, in 1x PBS)] and one of 11 incubation times (1 second, 1, 2, 5, 10, 15, 30, 60, 120, 180 minutes, 24 hours), as well as one of three temperatures (4, 22, 37 °C). Each combination of treatment, time, and temperature was repeated three times. In all, 396 OCDs were used.

The AC from each OCD was separated from the bone base by scalpel and the AC disc returned to 1x PBS for 5 min, then blotted lightly using Kimwipes® tissue (Kimberly-Clark, Roswell, GA) to remove excess fluid from the cartilage surface, weighed (W1), and immediately immersed in 5 ml of CPA solution for the specified incubation time at the specified temperature. After incubation, the disc was removed from the solution, blotted lightly and weighed for a second time (W2). The cartilage disc was then placed into a 35 x 10 mm cell culture dish (Corning Inc., Corning, NY) containing 4 mL of 1x PBS. The disc was ensured to be fully immersed in the PBS and the dish was sealed with Parafilm® (American National Can, Chicago,

IL) and held for 24 h at 22°C under dark conditions to allow the CPA within the AC disc to fully equilibrate with the surrounding PBS solution. After 24 h, the CPA/PBS solution was mixed using a pipettor and a 1 mL sample was taken into a 1.5 mL microcentrifuge tube (Thermo Fisher Scientific, Waltham, MA). From this sample, 50 µL was placed into a µOSMETTE™ micro-osmometer (Precision Systems, Natick, MA) to determine its osmolality (Osm).

Calculations

As the measured osmolalities were quite low, the immersion solution can be considered ideal and dilute.

The number of moles of CPA(n_s) in the surrounding solution was calculated as:

(1) $$n_s(mole) = \frac{[\pi_s - \pi_{PBS}](mosm/kg) \times 4\ mL \times \rho^*(g/mL)}{1000(mosm/osm) \times 1000(g/kg)}$$

where π_s = osmolality of the final solution, π_{PBS} = osmolality of the initial PBS solution, and ρ^* = density of water at 22°C (0.99777 g/mL).

The total number of moles of CPA(n_{total}) that had permeated into the cartilage is then given by:

(2) $$n_{total}(mole) = n_s(mole) + n_{inside\ cartilage}(mole)$$

Note: $n_{\text{inside cartilage}}$ was estimated to be negligible and was omitted from the calculations.

The weight of the CPA was calculated as:

(3) $$Wt_{CPA}(g) = n_{total}(mole) \times MW_{CPA}\left(\frac{g}{mole}\right)$$

The volume of CPA was then calculated as:

(4) $$V_{CPA}(mL) = \frac{Wt_{CPA}(g)}{Density_{CPA}\left(\frac{g}{mL}\right)}$$

Note: True (pure) densities for each CPA at corresponding temperatures were taken from Aspen-HYSYS v. 2004.2 simulation software, using the PRSV fluid package.

The amount of water within isotonic cartilage was measured to be 77.6 $\pm$ 0.5 % (S.E.) by mass in our previous study.[12] Assuming a constant dry weight percentage of 22.4 %, the dry weight of each AC disc was calculated as:

(5) $$Dry\ Weight(g) = W1(g) \times 0.224$$

The volume of water in the AC disc after each treatment condition was calculated as:

(6) $$V_{water\ in\ treated\ cartilage}(mL) = \frac{W2(g) - \{Dry\ Weight(g) + Wt_{CPA}(g)\}}{\rho\left(\frac{g}{mL}\right)}$$

Where ρ, the density of water, is 0.999973, 0.99777, and 0.99336 g/mL at 4, 22, and 37°C respectively.

Finally, the concentration of CPA that penetrated the AC disc was calculated to be:

158 (7) $$[CPA]\left(\frac{mol}{L}\right) = \frac{n_{total}(mole) \times 1000\left(\frac{mL}{L}\right)}{V_{CPA}(mL) + V_{water\ in\ treated\ cartilage}(mL)}$$

159

160 Note that this is the solution concentration (i.e. moles per fluid volume in cartilage and not moles
161 per cartilage volume).

162

163 *Statistical Analysis*

164 CPA concentration comparisons between temperatures were performed at each time point and
165 were evaluated by one-way ANOVA with Bonferroni post-hoc multiple comparison tests using
166 STATA 9.2 (StataCorp, College Station, TX). The level of significance was set at P=0.02.
167 Ninety-five percent confidence interval limits of CPA concentration at each time point and
168 temperature were also determined using STATA 9.2.

169

170

Results

The results for the four CPAs are shown on the graphs below (Figure 1). There were some consistencies between all four CPAs. For example, in all CPAs tested there was an initial rapid increase in CPA concentration within the cartilage matrix between 15-30 minutes after exposure. This slowed down over the next 90-180 minutes with a gradually approach to the original concentration of the surrounding solution. In all solutions, the early increase in CPA concentration within the matrix was most rapid at 37 °C and least rapid at 4 °C although the final concentrations were similar after 24 hours of exposure.

[Figure 1]

While permeation of individual CPAs was generally greater at higher temperatures at incubation times of 1 hour or less, the differences were only statistically significant when comparing data at 4 ºC and 37 ºC, between incubation times of 5 – 15 minutes (Table 1). The exceptions were with glycerol at 2 minutes and propylene glycol at 10 minutes which showed a significant difference between 4 ºC and 22 ºC, and between 22 ºC and 37 ºC respectively. Furthermore, the CPA concentration for all agents after 3 hour incubation at 37 ºC was lower than that of the other two temperatures (Table 1).

[Table 1]

Fitted data

The geometry of the AC discs was approximated as a perfect cylinder with thickness and diameter estimated at 2 mm and 10 mm respectively. Since the peripheral area of the disc (cut edges) is not negligible compared to the top and bottom areas, and the disc of cartilage was exposed to the solution on all sides, the numerical solution to the diffusion problem was performed in 2-D (axial and radial dimensions).

Analytical solutions of Fick's Second Law for diffusion into uniform discs are available in the literature.[13] Skelland (1974) expressed the solution of the 2-D radial and axial diffusion in a cylinder as the 1-D solution of diffusion in an infinite slab (axial) multiplied by the 1-D solution of diffusion in a cylinder (radial), for which the solutions are available from Newman[14]:

Slab:

$$\frac{C_{A0} - \bar{C}_A}{C_{A0} - C_A^*} = 1 - \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} exp\left(\frac{-D(2n+1)^2 \pi^2 t}{4a^2}\right) \quad (8)$$

Cylinder:

$$\frac{C_{A0} - \bar{C}_A}{C_{A0} - C_A^*} = 1 - \frac{4}{R^2} \sum_{n=1}^{\infty} \frac{1}{b_n^2} exp(-Db_n^2 t) \quad (9)$$

Where C_{A0} , $\bar{C}_A$, C_A^* , a and R are initial average concentration, average concentration after time t, boundary condition concentration, half thickness of the axial dimension (if the slab is exposed from both sides, or full thickness when slab is exposed only from one side), and radius of the cylinder, respectively. D is the diffusion coefficient and b_n's are the roots of the zero-order

Bessel function of the first kind, $J_0(b_n) = 0$. Representing the right hand side of the equations above with *f*(slab) and *f*(cylinder), the 2-D average concentration increase in discs over time can be expressed as:

$$\frac{C_{A0} - \bar{C}_A}{C_{A0} - C_A^*} = f(slab) \times f(cylinder) \quad (10)$$

$$= \left[1 - \frac{8}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(2n+1)^2} exp\left(\frac{-D(2n+1)^2\pi^2 t}{4a^2}\right)\right] \times \left[1 - \frac{4}{R^2}\sum_{n=1}^{\infty}\frac{1}{b_n^2} exp(-Db_n^2 t)\right]$$

The initial concentration of CPA in the tissue, C_{A0}, was taken to be zero and the diffusion coefficient, D, was considered constant throughout the cartilage. As can be seen in Figure 1, the increase in concentration reached a plateau at 24 hr, which was different from the bath solution concentration; hence, the boundary conditions C_A^* were represented by the value of concentration at the final time point, i.e. 24 hr. For comparison, the same calculation was made using 6.5 M as the boundary condition, i.e. C_A^* =6.5 M. The value of D was found that minimized the sum of squared errors between the model prediction and the experimental data of Figure 1. The best fits for D are reported in table 2 as the diffusion coefficients.

[Table 2]

Arrhenius plots

The temperature dependencies of the diffusion coefficients for the four CPAs are shown in Fig. 2, where the natural logarithm of fitted D values is plotted against the inverse of temperature. The Arrhenius equation describes the temperature dependence of diffusion coefficient as:

(11) $$D = Aexp\left(-\frac{E_a}{RT}\right)$$

Where E_a is the activation energy, R is the universal gas constant and A is the prefactor (DMSO = 6.21581 x 10^{-07}, EG = 6.21581 x 10^{-07}, Gly = 6.21581 x 10^{-07}, PG = 7.30465 x 10^{-06}). The activation energies were calculated based on the best fitted line to the three data points and were: $E_{a,\ DMSO}$ = 4.3 ± 1.9 kcal/mol, $E_{a,\ PG}$ = 6.29 ± 0.02 kcal/mol, $E_{a,\ EG}$ = 3.8 ± 0.7 kcal/mol, $E_{a,\ Glycerol}$ = 5.6 ± 1.2 kcal/mol (based on the 24-hour boundary condition).

[Figure 2]

Discussion

Successful vitrification of AC may be possible using combinations of CPAs as demonstrated in other tissues such as embryos.[15,16] Unfortunately, until precise permeation kinetics of each CPA are known, the time of exposure to achieve adequate permeation to ensure vitrification will be an estimation that may exacerbate the toxic effects of each CPA or alternatively compromise vitrification effectiveness.

The results of this experiment provide the diffusion coefficients of four commonly used CPAs in porcine AC (DMSO, EG, PG, glycerol). The diffusion coefficients for DMSO and PG determined in this experiment were extremely close to those in our previous experiment[12] (DMSO = 2.4 - 6.2 x 10^{-6} cm^2/s compared to 2.0 – 3.5 x 10^{-6} cm^2/s; PG = 0.8 – 2.7 x 10^{-6} cm^2/s compared to 0.63 – 1.8 x 10^{-6} cm^2/s depending on temperature). Our results are also consistent with coefficients published by other groups for DMSO and glycerol.[17,18] We are confident with our results because this experiment is an improvement to our previously published technique[12] by increasing the number of recorded early time points during the rapid permeation phase and by taking a second weight to account for weight changes during the process caused by water movement.

Subsequent to determining accurate diffusion coefficients, we calculated activation energies for each CPA. These values were marginally higher than reported in our previous work[12] (DMSO = 4.33 kcal/mol and PG = 6.29 kcal/mol compared to previous results of DMSO = 1.52 kcal/mol and PG = 2.43 kcal/mol). This difference is likely due to the increased accuracy of our new

technique with more time points in the early rapid phase of CPA permeation and the inclusion of a second weight measurement. The activation energies presented here are higher than those reported by Muldrew[19] (0.95 kcal/mol) but those results were obtained using only two temperature values. The current results indicate slightly more temperature dependence of permeation than previously found. Knowledge of the activation energy for each CPA will enable prediction of permeation rates at any temperature thereby enhancing mathematical modeling of vitrification protocols.

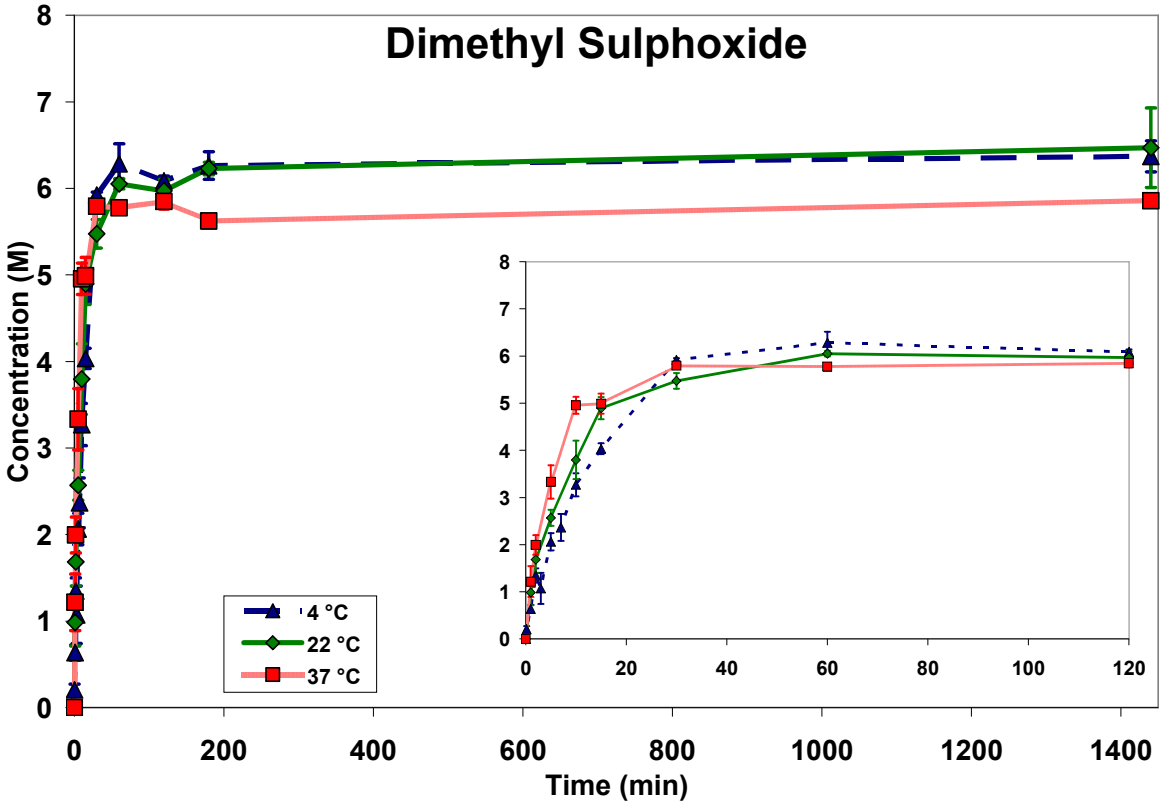
Vitrification of AC has proven exceedingly difficult because high concentrations of CPAs are required and human tissues can not be cooled and warmed rapidly enough to use nontoxic concentrations. To overcome this, combinations of CPA have been used to vitrify other tissues as well as AC with moderate success.[20-25] These advances have come using empirical testing without statistical and mathematical methodology to facilitate efficient protocol optimization. This study was designed to establish fundamental parameters on permeation kinetics that can be used to scientifically develop vitrification protocols to effectively preserve AC. In conclusion, this study has provided accurate permeation kinetics for DMSO, EG, PG, and glycerol that can be used for mathematical modeling of vitrification protocols.

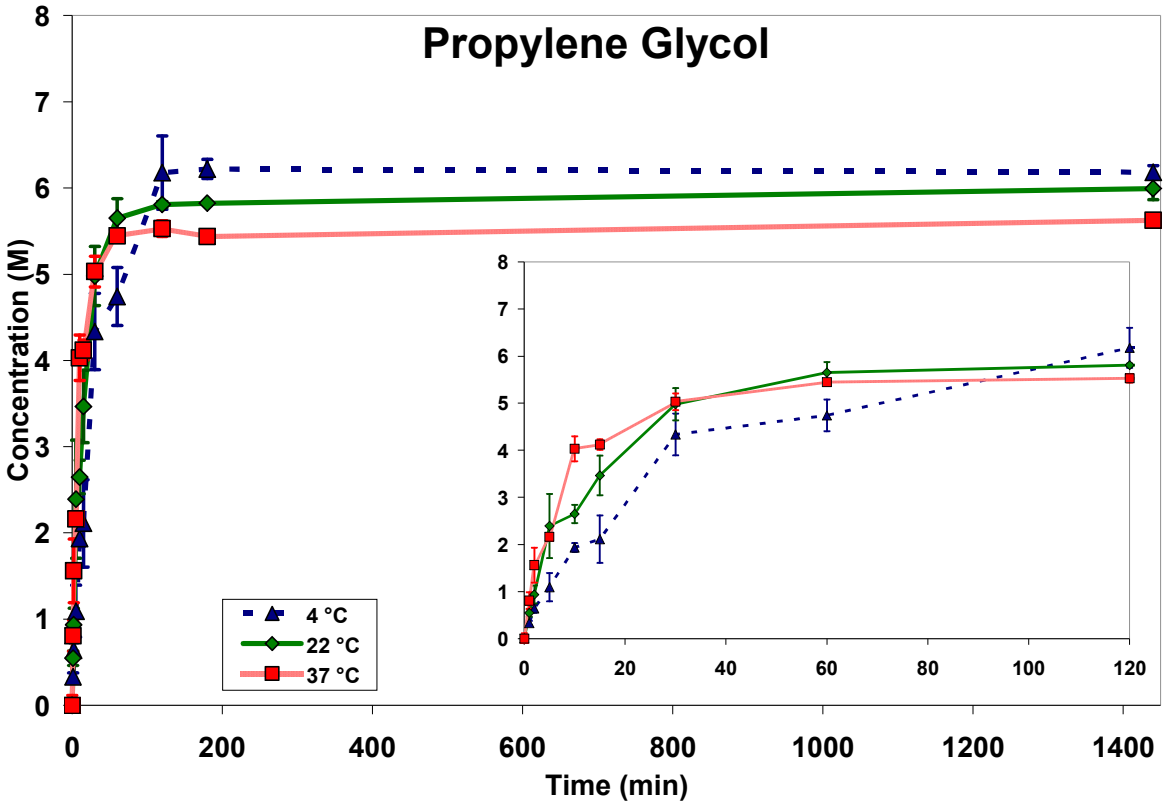
References

1. Anderson AF, Pagnani MJ. Osteochondritis dissecans of the femoral condyles. Long-term results of excision of the fragment. Am J Sports Med 1997;25:830-834
2. De Smet AA, Ilahi OA, Graf BK. Untreated osteochondritis dissecans of the femoral condyles: prediction of patient outcome using radiographic and MR findings. Skeletal Radiol 1997;26:463-467
3. Bujia J, Kremer D, Sudhoff H, et al. Determination of viability of cryopreserved cartilage grafts. Eur Arch Otorhinolaryngol 1995;252:30-34
4. Jomha NM, Lavoie G, Muldrew K, Schachar NS, McGann LE. Cryopreservation of intact human articular cartilage. J Orthop Res 2002;20:1253-1255
5. Muldrew K, Hurtig M, Novak K, Schachar N, McGann LE. Localization of freezing injury in articular cartilage. Cryobiology 1994;31:31-38
6. Ohlendorf C, Tomford WW, Mankin HJ. Chondrocyte survival in cryopreserved osteochondral articular cartilage. J Orthop Res 1996;14:413-416
7. Smith AU. Survival of frozen chondrocytes isolated from cartilage of adult mammals. Nature 1965;205:782-784
8. Pegg DE, Wang L, Vaughan D. Cryopreservation of articular cartilage. Part 3: The liquidus-tracking method. Cryobiology 2006;52:360-368
9. Elmoazzen HY, Poovadan A, Law GK, et al. Dimethyl sulfoxide toxicity kinetics in intact articular cartilage. Cell Tissue Bank 2007;8:125-133
10. Fahy GM. Prevention of toxicity from high concentrations of cryoprotective agents. In: Pegg DE, Jacobsen IA, N.A. H eds, Organ preservation, basic and applied sciences. Lancaster: MTP Press; 1982:367-369
11. Fahy GM, Wowk B, Wu J, et al. Cryopreservation of organs by vitrification: perspectives and recent advances. Cryobiology 2004;48:157-178
12. Sharma R, Law GK, Rekieh K, et al. A novel method to measure cryoprotectant permeation into intact articular cartilage. Cryobiology 2007;54:196-203
13. Skelland AHP. Diffusional Mass Transfer. New York: Wiley-Interscience; 1974
14. Newman AB. The drying of porous solids diffusion calculations. Transactions of the American Institute of Chemical Engineers 1931;27:310-333
15. Elliot K, Whelan J eds. The freezing of mammalian embryos. Amsterdam: Elsevier; 1977
16. Leibo SP. Fundamental cryobiology of mouse ova and embryos. In, The freezing of mammalian embryos. Amsterdam: Ciba Foundation Symposium; 1979:69-97
17. Carsi B, Lopez-Lacomba JL, Sanz J, Marco F, Lopez-Duran L. Cryoprotectant permeation through human articular cartilage. Osteoarthritis Cartilage 2004;12:787-792
18. Pegg DE. Cryobiology of cells in situ: Experiments with ovine articular cartilage. Cryobiology 1998;37:381-382
19. Muldrew K, Sykes B, N. S, McGann LE. Permeation kinetics of dimethol sulfoxide in articular cartilage. Cryo-letters 1996;17:311-340
20. Fujita T, Takami Y, Ezoe K, et al. Successful preservation of human skin by vitrification. J Burn Care Rehabil 2000;21:304-309
21. Kurata H, Takakuwa K, Tanaka K. Vitrification of hematopoietic progenitor cells obtained from human cord blood. Bone Marrow Transplant 1994;14:261-263
22. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. Nature 1985;313:573-575

23. Song YC, Hagen PO, Lightfoot FG, et al. In vivo evaluation of the effects of a new ice-free cryopreservation process on autologous vascular grafts. J Invest Surg 2000;13:279-288
24. Guan J, Urban JP, Li ZH, et al. Effects of rapid cooling on articular cartilage. Cryobiology 2006;52:430-439
25. Song YC, An YH, Kang QK, et al. Vitreous preservation of articular cartilage grafts. J Invest Surg 2004;17:65-70

Figure 1: Four graphs demonstrating permeation for DMSO, PG, EG, glycerol at three different temperatures over time. Each graph shows all data for each temperature as well as an inset graph for the first 60 minutes of exposure to illustrate the permeation rate during the steepest part of the CPA uptake.

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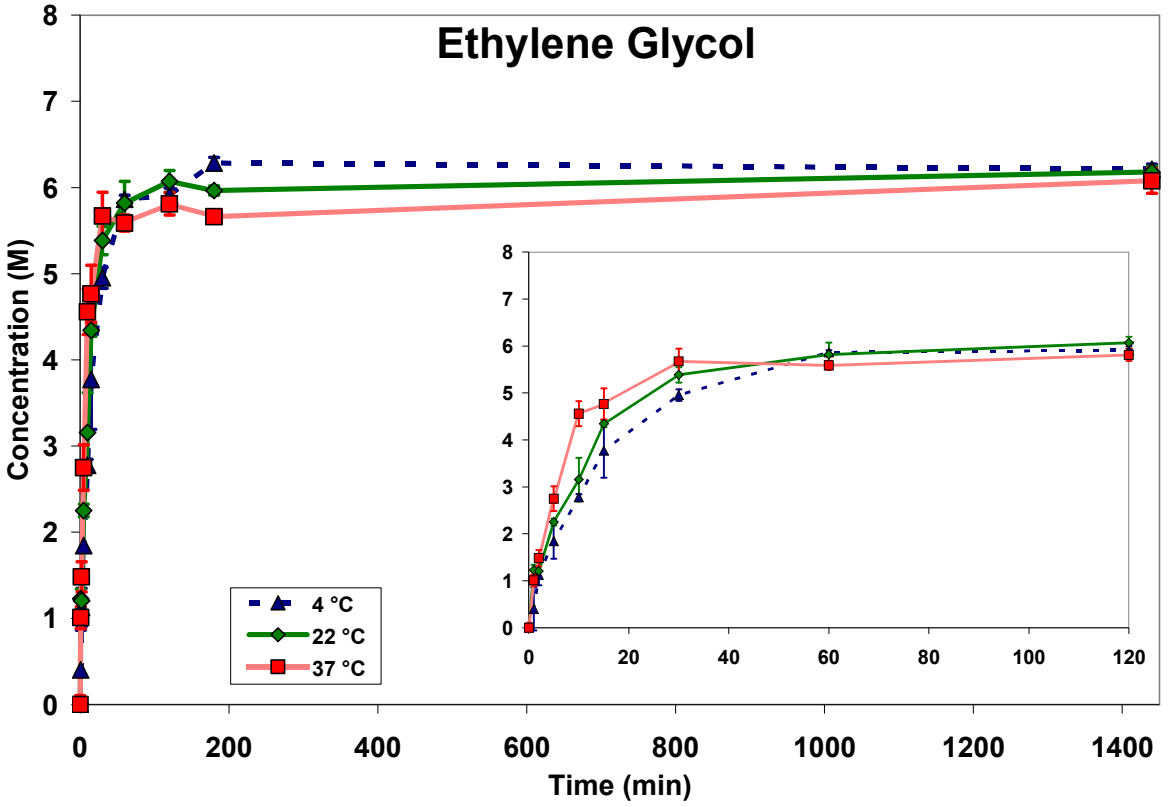
Concentration (M)
Time (min)
4 °C
22 °C
37 °C


Ethylene Glycol
Concentration (M)
Time (min)
4 °C
22 °C
37 °C

368

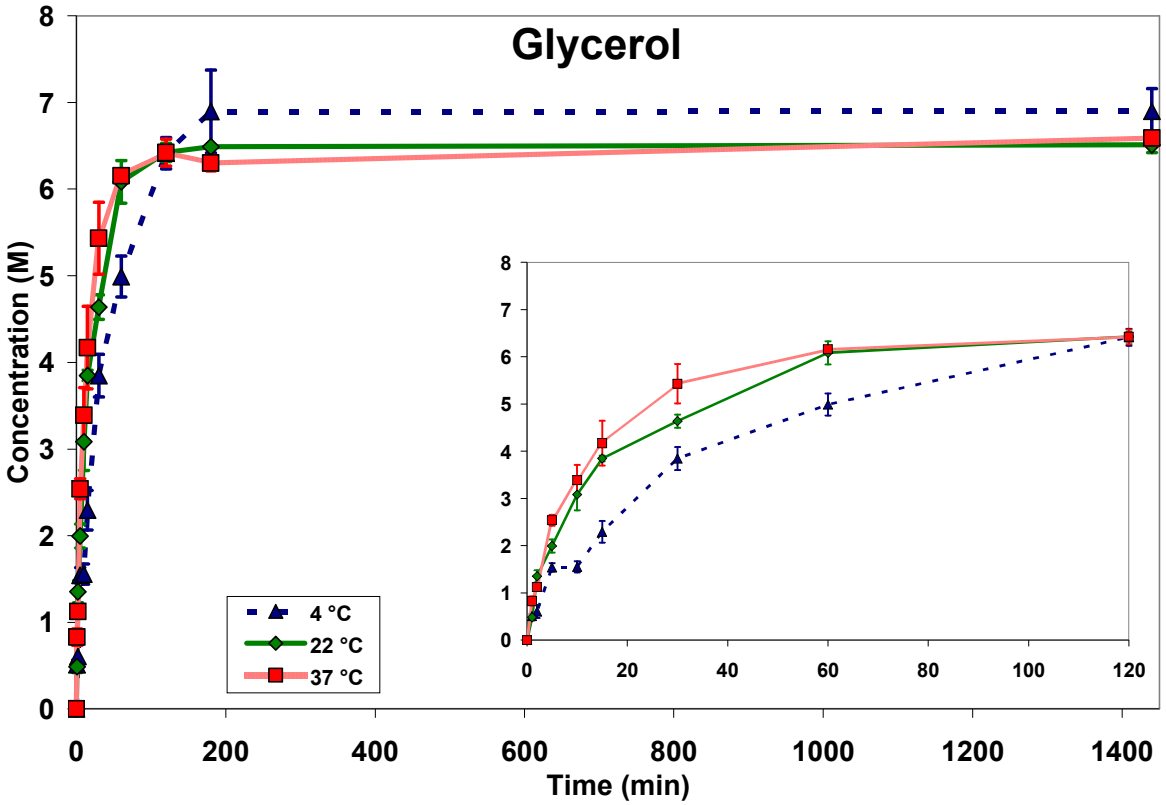

Glycerol
Concentration (M)
Time (min)
4 °C
22 °C
37 °C
0
1
2
3
4
5
6
7
8
0
200
400
600
800
1000
1200
1400
0
20
40
60
80
100
120

Table 1. Concentration of individual CPAs in articular cartilage at each time point and temperature level. Superscripts a-r denote 18 comparisons that yielded statistical significance by one-way ANOVA with Bonferroni Multiple Comparison Test, $P < 0.02$.

CPA	DMSO (M) (± 95% C.I.)			PG (M) (± 95% C.I.)			EG (M) (± 95% C.I.)			Glycerol (M) (± 95% C.I.)		
Temp	4ºC	22ºC	37ºC	4ºC	22ºC	37ºC	4ºC	22ºC	37ºC	4ºC	22ºC	37ºC
Exposure Time												
1 sec.	0	0	0	0	0	0	0	0	0	0	0	0
1 min.	0.64 (0.38)	0.98 (1.16)	0.33 (1.40)	0.33 (0.19)	0.55 (0.36)	0.81 (0.78)	0.40 (0.49)	1.23 (1.98)	1.01 (0.54)	0.51 (0.23)	0.49 (0.29)	0.83 (0.41)
2 min.	1.35 (0.66)	1.68 (1.19)	1.99 (0.89)	0.64 (0.38)	0.94 (0.82)	1.56 (1.59)	1.13 (0.05)	1.21 (0.96)	1.48 (0.75)	0.60[l] (0.59)	1.35[l] (0.55)	1.13 (0.24)
5 min.	2.06[a] (0.58)	2.57 (0.74)	3.33[a] (1.52)	1.09 (1.29)	2.39 (2.94)	2.16 (0.87)	1.89 (0.34)	2.25 (1.62)	2.75 (1.14)	1.54[m] (0.39)	1.99 (0.60)	2.54[m] (0.50)
10 min.	3.27[b] (1.05)	3.80 (1.75)	4.96[b] (0.78)	1.94[d] (0.40)	2.65[e] (0.84)	4.03[d,e] (1.13)	2.78[i] (2.00)	3.16 (0.30)	4.56[i] (1.13)	1.55[n] (0.50)	3.08 (1.42)	3.39[n] (1.37)
15 min.	4.03 (0.50)	4.90 (1.01)	4.99 (0.91)	2.11[f] (2.18)	3.47 (1.80)	4.12[f] (0.49)	3.78 (0.31)	4.34 (2.49)	4.77 (1.41)	2.29[o] (0.98)	3.85 (0.26)	4.17[o] (2.05)
30 min.	5.92 (0.16)	5.47 (0.71)	5.80 (0.21)	4.34 (1.91)	4.98 (1.47)	5.03 (0.76)	4.95 (0.70)	5.38 (0.52)	5.67 (1.18)	3.85 (1.05)	4.64 (0.61)	5.43 (1.79)
60 min.	6.28 (1.00)	6.05 (0.26)	5.78 (0.28)	4.74 (1.44)	5.65 (0.52)	5.45 (0.15)	5.86 (1.08)	5.82 (0.21)	5.59 (0.40)	4.99[p] (1.01)	6.08 (1.06)	6.16[p] (0.31)
120 min.	6.09 (0.17)	5.97 (0.58)	5.85 (0.37)	6.18 (1.83)	5.81 (0.15)	5.53 (0.39)	5.91 (0.55)	6.07 (0.36)	5.81 (0.55)	6.41 (0.78)	6.42 (0.45)	6.42 (0.67)
180 min.	6.26[c] (0.68)	6.23 (0.31)	5.62[c] (0.29)	6.22[g] (0.48)	5.82 (0.07)	5.44[g] (0.29)	6.29[j,k] (0.23)	5.97[j] (0.28)	5.66[k] (0.21)	6.38 (5.20)	6.49 (0.09)	6.30 (0.38)
24 h	6.37 (0.77)	6.47 (1.98)	5.86 (0.28)	6.18[h] (0.33)	5.99 (0.56)	5.63[h] (0.19)	6.21 (0.30)	6.18 (0.26)	6.08 (0.63)	7.27[q,r] (1.12)	6.51[q] (0.39)	6.59[r] (0.28)

Table 2. Diffusion coefficients (x 10^{-10} m^2/s *or* 10^{-6} cm^2/s)

	6.5 M boundary condition			**24 hr conc. boundary condition**		
	4°C	**22°C**	**37°C**	**4°C**	**22°C**	**37°C**
DMSO	2.4	3.0	4.5	2.6	3.1	6.2
EG	1.7	2.3	3.4	2.0	2.7	4.2
GLY	1.0	1.8	2.4	0.8	1.8	2.3
PG	0.9	1.6	2.2	0.8	1.6	2.7

Figure 2. Arrhenius plot showing the temperature dependence of the diffusion coefficients for each CPA.