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Water and Cryoprotectant Permeability Characteristics of Isolated Human and Canine Pancreatic Islets

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Cryopreservation allows accumulation of the necessary islet transplantable mass as well as adequate time for tissue typing and infectious disease screening. Cryopreservation protocols may be optimized by modeling the osmotically induced volume excursions that occur during the addition and removal of cryoprotective agents (CPAs). To that end, three transport parameters were measured at 22°C in canine and human islets isolated by collagenase digestion and euroficoll purification: (i) the apparent hydraulic conductivity (L_p), (ii) the permeability coefficient of the CPA (P_s), and (iii) the associated reflection coefficient (σ). The parameters were determined by volumetric analysis of islets upon abrupt exposure to 1, 2, and 3 M dimethyl sulfoxide (DMSO), ethylene glycol (EG), glycerol (GLY), and propylene glycol (PG). The parameters were calculated using the Kedem-Katchalsky theory to describe islet volume excursion kinetics (assuming islets to be single equivalent osmotic units with the same volume and surface area of the actual islet) and a three-parameter curve fit was performed using the Marquardt-Levenberg method. It was determined that the permeability characteristics of pancreatic islets are species specific, and based upon the measured parameters, the highest P_s values for canine islets were observed following exposure to 2 M EG, and the highest P_s values for human islets were observed following exposure to 2 M PG. The permeability parameters were analyzed adjusting for islet radius using ANCOVA procedures to acquire least square means. For canine islets exposed to 2 M EG these values were determined to be 0.936 μm/min/atm, 2.47 μm/s, and 0.90 (for L_p, P_s, and φ, respectively) and for human islets exposed to 2 M PG the values were determined to be 1.56 μm/min/atm, 3.48 μm/s, and 0.85 (for L_p, P_s, and σ, respectively). These parameters were used in a model to calculate osmotically induced islet volumetric response upon addition/dilution of the optimum CPAs, taking into consideration critical volume excursion limits at which irreversible damage occurs.

Key words: Cryopreservation; Pancreatic islet; Permeability characteristics

INTRODUCTION

The clinical significance associated with the successful cryopreservation and banking of pancreatic islets has been well documented (8,19,31). Cryopreservation allows accumulation of the necessary islet transplantable mass from several donors, selection of specific HLA-matched tissue, and provides time for infectious disease screening and functional viability testing.

Current islet cryopreservation protocols require exposure to molar concentrations of plasma membrane permeating cryoprotective solutes. These protective solutes, or cryoprotective agents (CPAs), enable cells to survive freezing at cooling rates that allow internal osmotic equilibration by water efflux rather than damaging ice crystal formation (14). Permeating CPAs protect not by specific chemical action but by their colligative properties (14) to: (i) reduce the fraction of the solution frozen at a given temperature, (ii) suppress the salt concentration in solution, and (iii) reduce harmful cell shrinkage at a given temperature.

Pancreatic islets are composed of thousands of cells arranged in a roughly spherical shape. Therefore, it is important to note that referring to islet "permeability" or the change in islet volume (volume excursion) actually refers to the cumulative response of its constituent cells. It is possible, however, to define an "apparent permeability" based on the islet as an equivalent single osmotic unit with the same volume and surface area as

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that of the actual islet (29). Under these assumptions, the osmotically induced changes in volume of the equivalent osmotic unit (EOU) associated with the addition and removal of permeating cryoprotectants can be described theoretically using the biophysical equations derived from the methods of irreversible thermodynamics by Kedem and Katchalsky (6). This formalism can be used to describe the changes in volume of the EOU in relation to the fluxes of water and solutes providing islets act as ideal osmometers (demonstrate a linear response in a plot of volume vs. reciprocal of external osmolality) and if three transport parameters are known: (i) the apparent hydraulic conductivity (L_p), (ii) the permeability coefficient of the CPA (P_s), and (iii) the associated reflection coefficient (σ) (10).

Permeability is generally defined as the function relating chemical flux to chemical potential gradient. In the case of multiple chemical species fluxes, there is not only a permeability function for each species, but also a set of functions representing the interactions of the different fluxes (29). In the present study two fluxes are of primary interest: water and CPA. Because most cells are highly permeable to water as well as the CPAs under investigation, a coupled flow of both occurs. The dynamics of this coupled transport dictates cell volume and intracellular concentrations of CPA during the addition/removal portion of the cryopreservation process, and represents a fundamental role in the success of cryopreservation procedures (16).

Upon altering the CPA concentration in the external environment, the individual islet cells respond osmotically to these changes in solution composition. Theoretically, upon permeable CPA addition, cells within the islet could respond in one of three predictable ways: (i) if the cell membranes are more permeable to water than they are to the CPA (which is the case in all cell types studied to date), the cells will initially shrink and then swell to a new equilibrium volume, (ii) if the cell membranes are equally permeable to water and the CPA, then the cells will swell to a new equilibrium volume as the internal CPA concentration rises to reach equilibrium, (iii) if the cell membranes are less permeable to water than they are to the CPA, the cells will initially swell and then shrink to a new equilibrium volume (9).

The permeability characteristics of islets to water and CPAs are therefore not only of theoretical value, but are of practical importance because they determine the volume response of islets to the changing external osmotic conditions that occur during the addition and dilution of CPAs and during cooling and warming in the presence of ice. By combining this knowledge with experimentally defined osmotic volume excursion tolerance limits, injury avoidance strategies can be devised that may enhance current cryopreservation procedures.

The objective of the present study was to evaluate the dynamic volume responses of isolated human and canine islets following the addition of several cryoprotective additives, and to use this information to determine their apparent water (L_p) and CPA (P_s) permeability coefficients as well as a measure of the potential interaction between water and CPA flux (reflection coefficient or σ). The final goal was to determine, based on these parameters, which CPA would cause the least osmotic damage during addition and removal, and subsequently to develop a protocol for these procedures utilizing the least number of addition/removal steps. Because the canine model has been key in the development and optimization of human cryopreservation protocols, the species dependence of CPA permeability was also considered.

MATERIALS AND METHODS

Reagents

All tissue culture reagents were obtained from Gibco (Grand Island, NY) unless otherwise stated. Collagenase and DNAse for islet isolations were obtained from Boehringer Mannheim (Indianapolis, IN). EuroCollins solution was from Frisenius AG (Hamburg, Germany). Ficoll, dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), and glycerol (GLY) were obtained from Sigma Chemicals (St. Louis, MO). Cryoprotectant solutions were prepared volumetrically in isotonic M-199 tissue culture media.

Islet Isolation and In Vitro Culture

Pancreatic islets (108/species) were isolated from the pancreases of ($n = 3$) human cadaveric multiorgan donors and ($n = 3$) mongrel dogs using collagenase perfusion via the pancreatic duct, semiautomated dissociation, and purification using discontinuous gradients of Euro-Ficoll on a COBE 2991 Blood Cell Processor (5,11,23). Purified islets were cultured for 12–15 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in CMRL 1066 tissue culture medium supplemented with 10% fetal calf serum, 12.5 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Measurement of Islet Volume Responses

Three islets ranging from 50 to 200 μm in diameter were hand picked under a stereomicroscope (Nikon SMZU-10A, Melville NY) and deposited into a microperfusion chamber (33). Three islets were loaded in the chamber per each experimental condition, and each experiment was performed using islets from each of three human or canine isolations. Briefly, islets were placed onto a polycarbonate membrane (5-μm pores, Poretics, Livermore CA) at the bottom of a 20-μl microperfusion chamber that was prefilled with tissue culture medium. A 650-μl reservoir was filled with the CPA (1, 2, or 3

M DMSO, EG, PG, or GLY; 22°C), and a syringe was used to apply negative pressure to the chamber for the abrupt replacement of 20 chamber volumes of medium with that from the reservoir. The chamber was fixed under a compound microscope (Nikon Optiphot, Tokyo, Japan) equipped with a video camera (Optronics Engineering, Goleta, CA) to observe and a video recorder (Model HS-U560, Mitsubishi Electronics, Norcross,GA) to record the experiment. A PC-based video processing system (Haupauge Computer Works, Haupauge, NY) was used to digitize the recorded images. Commercial software (Sigma Scan/Image, Jandel Scientific Software, San Rafael, CA) was used to determine the cross-sectional area of the islets, which was then used to estimate the corresponding spherical volume. After an islet's kinetic volume excursion history had been recorded the tissue was stained with dithizone to ensure its endocrine origin. Area measurements were calibrated by measuring the cross-sectional area of spherical polystyrene beads (Coulter Corp., Hialeah, FL) with a diameter of 92.12 μm.

Following each experimental condition, the chamber was flushed with double distilled water, the membrane was replaced, and another aliquot of three human or canine islets was loaded into the system for the next experiment.

Determination of the Permeability Coefficients

The Kedem-Katchalsky method (6) describing passive coupled membrane transport derived from irreversible linear thermodynamics was used in the present study to determine the "apparent" permeability coefficients based on each islet as an equivalent single osmotic unit with the same volume and surface area as that of the actual islet (29). Briefly, for a ternary solution consisting of a permeable solute (CPA) and an impermeable solute (NaCl) and solvent (water) the total volume flux J_v and permeable solute flux J_s are represented as:

$$\frac{1}{A}\frac{dV}{dt} = J_v = L_p[(P^e - P^i) - RT(M_n^i) - \sigma RT(M_s^e - M_s^i)] \quad (1)$$

and

$$\frac{1}{A}\frac{dN_s^i}{dt} = J_s = (1-\sigma)\bar{m}_s J_v + P_s(a_s^e - a_s^i); \quad (2)$$

where V is the islet volume and N_s^i is the number of moles of CPA that has permeated the islet, P is the hydrostatic pressure, M is the osmolality, and a is activity (see Table 1 for explanations and definitions of terms). In the term $\bar{m}_s = \frac{m_s^e - m_s^i}{\ln m_s^e - \ln m_s^i}$ (where m is the molal concentration), the superscripts i and e refer to the internal islet or external islet as a compartment, respectively. L_p, P_s, and σ are the symbols for the effective hydraulic conductivity, effective permeability coefficient of the cryoprotective additive, and the reflection coefficient. The temperature and universal gas constant are given by T and R, respectively. In this analysis, osmolality and activity were approximated by setting the osmotic coefficient (ϕ_s) and activity coefficient (γ_s) to be unity, therefore:

$$a_s^i = m_s^i, \text{ and } M_s^i = m_s^i. \quad (3).$$

For the impermeable solute (NaCl), the internal osmolality is given by:

$$M_n^i = M_n^{(o)}\frac{V^{(o)} - V_b - V_s^{(o)}}{V - V_b - V_s}, \quad (4)$$

where V_b is the osmotically inactive islet volume and $V_s = N_s^i \cdot \bar{V}_s$ is the CPA volume ($\bar{V}_s$ is the partial molar volume of the CPA). The superscript *(o)* represents the initial values at $t = 0$. The values for V_{bp} used in this study were 0.47 for canine islets and 0.39 for human islets as determined previously (32). The relationship between and is given by:

$$n_s^i = (V - V_b - V_s)m_s^i \quad (5)$$

where $(V - V_b - V_s)$ denotes the volume of internal water.

By combining eqs. (1), (2), and (4), and assuming a hydrostatic pressure difference between the internal islet space and the external environment of zero, a pair of coupled nonlinear equations that describe the cell volume and amount of internal solute as a function of time are obtained:

$$\frac{dV}{dt} = L_p ART[(M_n^i - M_n^e) + \sigma(m_s^i - m_s^e)], \quad (6)$$

and

$$\frac{dm_s^i}{dt} = \frac{(1 + \bar{V}_s m_s^i)^2}{V - V_b}\left\{\left[\bar{m}_s(1-\sigma) - \frac{m_s^i}{(1 + \bar{V}_s m_s^i)}\right]\frac{dV}{dt} + AP_s\{(m_s^e - m_s^i)\}\right\}. \quad (7)$$

The Marquard-Levenberg curve-fitting method was used to fit eqs. (6) and (7) to the experimental data and determine the values for L_p, P_s, and σ (Fig. 1).

Theoretical Simulations

The interactive software package CellSim© V 1.0 was used to determine volume excursion in response to addition and dilution of the CPAs investigated. The cal-

Table 1. Definitions of Major Symbols and Abbreviations

Symbol or Abbreviation	Description	Units	Value
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid	none	none
HLA	Human Leukocyte Antigen	none	none
EOU	Equivalent Osmotic Unit	none	none
e,i	Superscripts: e = external to the islet, i = internal to the islet	none	none
s,n,w	Subscripts: s = solute, n = nonpermeating solute, w = water	none	none
L_p	hydraulic conductivity	$\mu m\ min^{-1}\ atm^{-1}$	parameter
P_s	solute permeability	$cm\ min^{-1}$	parameter
σ	reflection coefficient	none	none
T	temperature	K	273
A	islet surface area	μm^2	parameter
$V(t)$	islet volume	μm^3	variable
V_b	osmotically inactive islet volume	μm^3	parameter
V_{bp}	percentage of osmotically inactive volume	none	parameter
$V_w(t)$	volume of internal water	μm^3	variable
$n_s^i(t)$	moles of internal solute at time t	moles	variable
t	time	seconds	variable
M_n^{iso}	osmolality of initial (isotonic) nonpermeating internal salt	osm/kg H_2O	0.290
M_n^e	osmolality of external nonpermeating salts	osm/kg H_2O	0.290
M_n^i	osmolality of internal nonpermeating salts	osm/kg H_2O	variable
m	molality	mol/kg H_2O	variable
C	total solute concentration	g/100 g	variable
v_{10}	molar volume of water	$\mu m^3\ mol^{-1}$	1.8×10^{12}
$\bar{V}_s$	partial molar volume of solute	$l\ mol^{-1}$	0.069
R	the universal gas constant	$kcal\ mol^{-1}\ K^{-1}$	1.987×10^{-3}

culation was performed based on the values of V_{bp}, L_p, P_s, and σ independently in each species and CPA.

Data Analyses

To obtain a normal distribution, the original data were transformed by taking the natural log prior to analyses. These results were initially examined as a single population by standard four-way analysis of covariance approaches (ANCOVA) using the SAS program (SAS Institute Inc., Cary, NC). Sources of variation examined included CPA type, concentration, and species. A CPA by species interaction was also considered, and the nested variables of animal within species and islet within animal were included to control for natural biological variation.

The possibility that islet radius might have a statistically significant impact on the permeability parameters was considered; therefore, the study was designed to control for the anticipated variation by using ANCOVA. This method allowed for the comparison of response means that had been adjusted for islet radius (the covariate). More precise estimates of the effects of the independent variables were derived by including the covariate as a source of variation, thereby decreasing the estimated error variance (30).

Subsequent analyses treated the two species independently, and tested for the effects of CPA and CPA concentration on the effective hydraulic conductivity and CPA permeability coefficients. A three-way ANCOVA was used for this, which included animal nested within species to control for biological variation and the covariate islet radii to account for varying sizes.

RESULTS

The raw mean values (±SEM) obtained for L_p, P_s, and σ by fitting the theoretical curve to the observed data (averaged over all replicates) (Fig. 1) are represented in Table 2 (canine islets) and Table 3 (human islets). Initial statistical analyses indicated that the dependent variables L_p and P_s differed significantly between species (p = 0.0299, 0.0015, respectively). Although σ values did not vary significantly between species ($p > 0.05$), the islet volume response is primarily determined by the values of L_p and P_s; therefore, the two groups were subsequently subjected to an advanced analysis separately.

Canine Islets

Only CPA concentration had a significant ($p = 0.001$) effect on the values for L_p; CPA type did not ($p = 0.192$) (Fig. 2). Values for L_p were also determined to vary sig-

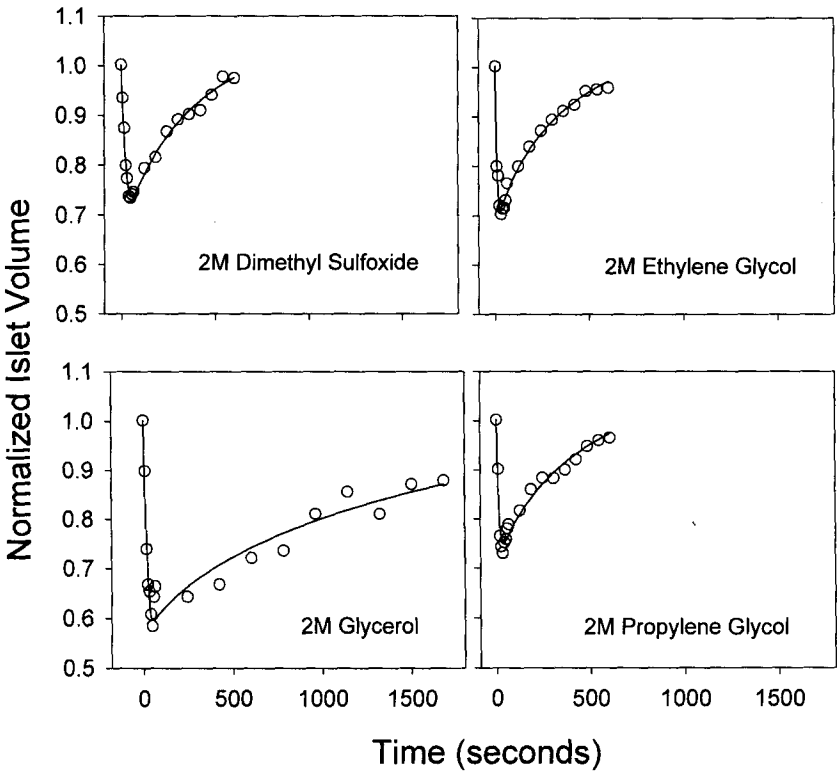

Figure 1. Changes in (normalized) canine islet volume as a function of time following the abrupt addition of CPA. The permeability parameters were obtained by fitting the theoretical curve (—) to the experimentally determined data points (○).

Table 2. Water and Cryoprotectant Permeability Coefficients of Canine Islets (Means ± SEM)

CPA and Concentration	Radius (μm)	L_p (μm/min/atm)	P_s (cm/min) ($\times 10^{-3}$)	σ (no units)
Dimethyl sulfoxide				
1 M	58.4 ± 6.2	0.70 ± 0.07	1.6 ± 0.30	0.86 ± 0.06
2 M	52.8 ± 5.6	0.57 ± 0.09	1.2 ± 0.16	0.75 ± 0.06
3 M	66.0 ± 6.8	0.50 ± 0.06	1.5 ± 0.21	0.70 ± 0.05
Ethylene glycol				
1 M	60.7 ± 6.9	0.88 ± 0.08	2.2 ± 0.21	0.91 ± 0.04
2 M	66.5 ± 6.9	0.60 ± 0.09	3.6 ± 0.99	0.75 ± 0.06
3 M	67.5 ± 2.7	0.45 ± 0.03	2.1 ± 0.51	0.75 ± 0.05
Glycerol				
1 M	65.2 ± 5.4	0.74 ± 0.09	0.24 ± 0.030	1.00 ± 0.00
2 M	51.2 ± 4.1	0.49 ± 0.06	0.16 ± 0.030	0.97 ± 0.01
3 M	52.7 ± 3.3	0.49 ± 0.11	0.13 ± 0.020	0.91 ± 0.04
Propylene glycol				
1 M	56.5 ± 3.8	0.83 ± 0.07	1.9 ± 0.18	0.79 ± 0.05
2 M	62.6 ± 5.0	0.54 ± 0.05	1.9 ± 0.19	0.76 ± 0.06
3 M	73.9 ± 6.4	0.60 ± 0.08	2.0 ± 0.19	0.81 ± 0.06

Table 3. Water and Cryoprotectant Permeability Coefficients of Human Islets (Means ± SEM)

CPA and Concentration	Radius (μm)	L_p (μm/min/atm)	P_s (cm/min) ($\times 10^{-3}$)	σ (no units)
Dimethyl sulfoxide				
1 M	66.3 ± 4.6	0.86 ± 0.07	2.5 ± 0.23	0.89 ± 0.06
2 M	91.6 ± 6.5	0.67 ± 0.04	2.2 ± 0.26	0.80 ± 0.04
3 M	62.7 ± 2.5	0.41 ± 0.03	1.2 ± 0.15	0.79 ± 0.05
Ethylene glycol				
1 M	69.8 ± 6.17	1.38 ± 0.19	2.1 ± 0.24	0.85 ± 0.07
2 M	83.1 ± 2.98	0.80 ± 0.03	2.3 ± 0.26	0.87 ± 0.05
3 M	79.5 ± 6.14	0.65 ± 0.07	3.3 ± 0.74	0.63 ± 0.04
Glycerol				
1 M	66.9 ± 4.8	0.96 ± 0.41	0.32 ± 0.02	0.95 ± 0.04
2 M	54.7 ± 5.6	0.56 ± 0.16	0.42 ± 0.014	0.94 ± 0.05
3 M	53.2 ± 2.6	0.45 ± 0.04	0.14 ± 0.02	0.92 ± 0.02
Propylene glycol				
1 M	60.2 ± 6.3	1.24 ± 0.12	3.3 ± 0.53	0.87 ± 0.06
2 M	59.9 ± 7.0	1.84 ± 0.46	4.2 ± 0.91	0.87 ± 0.05
3 M	54.8 ± 5.9	0.78 ± 0.10	3.0 ± 0.28	0.91 ± 0.04

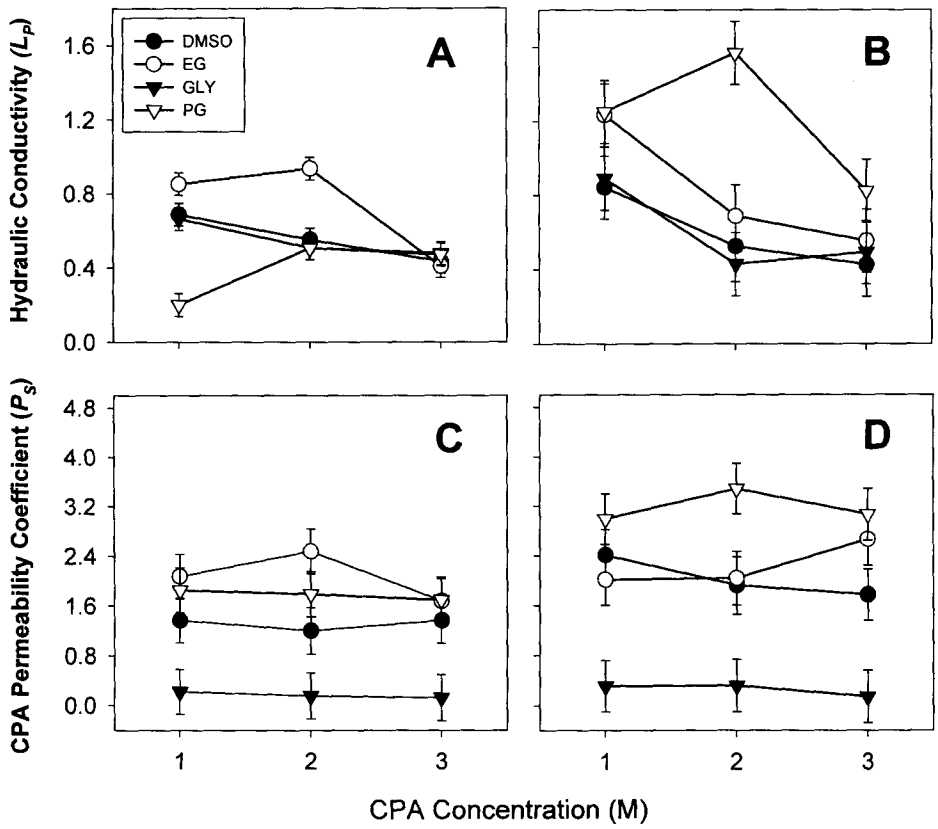

Figure 2. Least square means for the effects of CPA type and CPA concentration on (A, B) hydraulic conductivity (L_p) (μm/min/atm) and (C, D) CPA permeability (P_s) [cm/min ($\times 10^{-3}$)] in canine and human islets, respectively. Error bars are derived from nontransformed least square means.

nificantly ($p = 0.0338$) among individual animals, as well as among islets of varying radii ($p = 0.0001$).

The P_s values were determined to vary significantly ($p = 0.0001$) among the different CPAs investigated but were concentration dependent ($p = 0.1445$) (Fig. 2). Averaged over all concentrations, EG had the highest permeability coefficient (P_s), followed by PG, DMSO, then GLY [2.04, 1.77, 1.31, and 0.16 cm/min ($\times 10^{-3}$), respectively] (Fig. 3).

The values for the reflection coefficient varied significantly among concentrations ($p = 0.0321$) but not among CPA type ($p = 0.3712$). The least square means for the effects of concentration on σ were 0.99, 0.773, and 0.777 for 1, 2, or 3 M CPA, respectively.

Human Islets

There was a significant effect of CPA type and concentration ($p = 0.001$) on hydraulic conductivity (L_p) (Fig. 2). There was also a significant difference among islets of varying radii ($p = 0.0029$), and a significant interaction between CPA type and concentration ($p = 0.007$). There was not a significant variation among individual humans ($p > 0.05$). Partitioning of the main effect of CPA on the effective hydraulic conductivity (averaged over concentration) indicated that (L_p) was highest in PG, followed by EG, GLY, and DMSO (1.732, 0.7777, 0.5735, and 0.5690 μm/min/atm, respectively).

Averaged over all concentrations, PG had the highest permeability coefficient (P_s), followed by EG, DMSO, then GLY [3.49, 2.56, 1.98, and 0.29 cm/min ($\times 10^{-3}$), respectively] (Fig. 3). There was a significant effect of CPA type ($p = 0.0001$) and concentration ($p = 0.011$) on (P_s) values (Fig. 2). There was also a significant interaction between CPA type and concentration ($p = 0.007$) (Fig. 2).

There was no effect of CPA concentrations ($p = 0.056$) or CPA type ($p = 0.191$) on reflection coefficient (σ) values. However, there was a significant effect of islet radii ($p = 0.01$) on all parameters.

Theoretical Simulations

Using the experimentally determined permeability coefficients, osmotically induced volumetric responses of islets were modeled (considering islets as EOUs) taking into consideration critical volume excursion limits at which irreversible damage occurs (34). It was determined in this study that, based on the permeability characteristics of the tissue, the optimum CPA for use in cryopreserving canine islets is EG and the optimum CPA for human islets is PG. Least square mean values for the permeability parameters were used to allow generalization of response for islets with varying radii. For comparison, DMSO (the most common CPA used in islet cryopreservation) addition/dilution was also modeled for each species, and the resulting volumetric consequences were plotted on the same graphs (Fig. 4). In the case of the canine model, it is important to note that

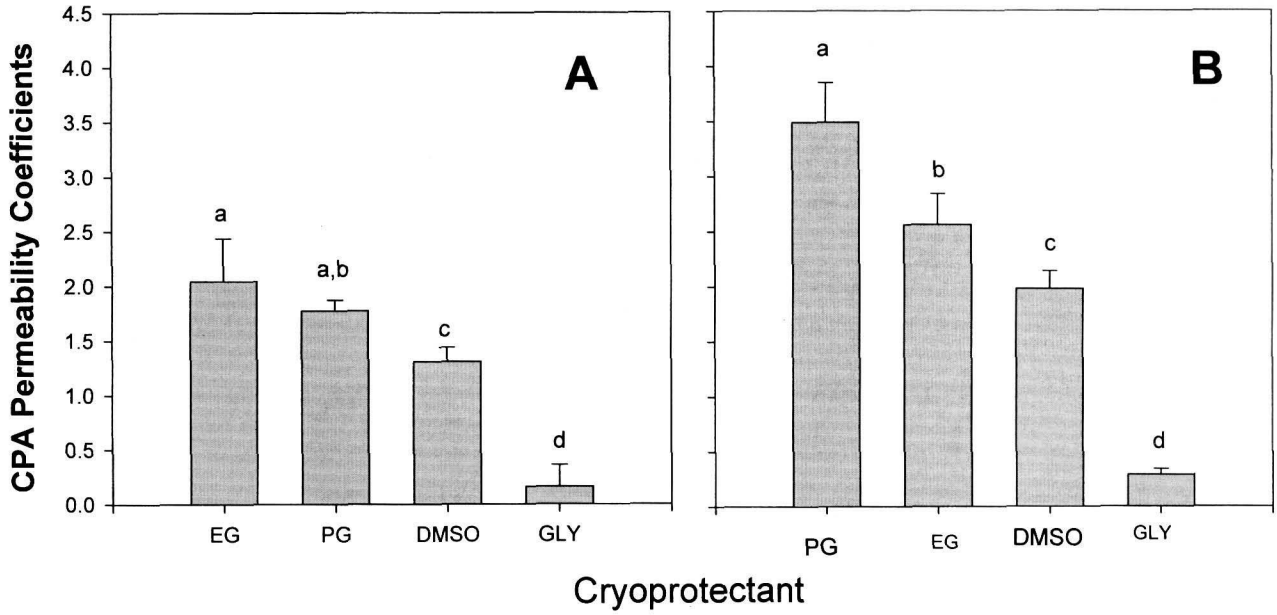

Figure 3. Comparison of cryoprotectant permeabilities for (A) canine and (B) human islets (averaged over CPA concentration). Numerical values are expressed in cm/min ($\times 10^{-3}$), error bars are derived from nontransformed SEM. Values with different superscripts are significantly different ($p < 0.05$).

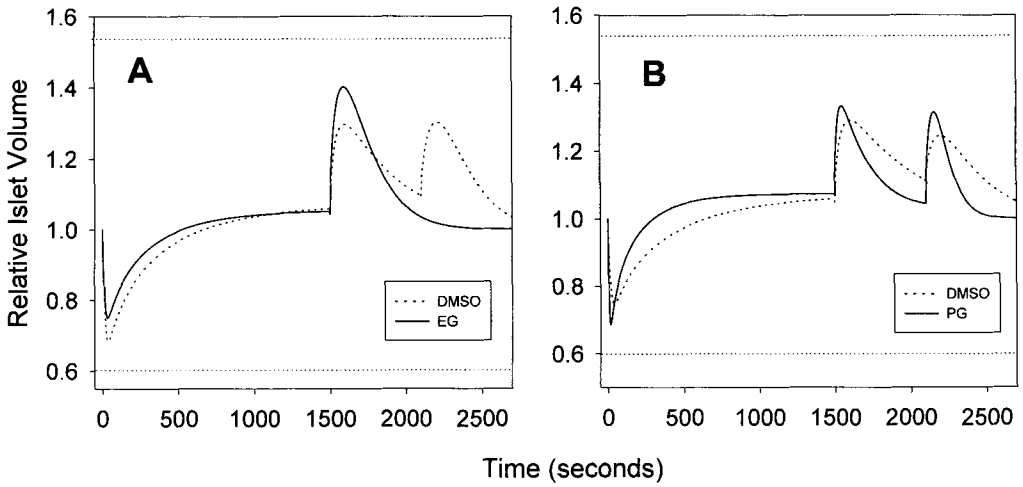

Figure 4. Simulation of cryoprotectant addition and removal processes developed from computer models based on the experimentally determined permeability characteristics. The horizontal dotted lines represent the minimum and maximum osmotically induced volume excursion tolerance limits as determined in a separate study (31). (A) Simulation of relative canine islet volume upon addition and removal of 1.5 M dimethyl sulfoxide (DMSO) or ethylene glycol (EG). For both CPAs a single addition step is osmotically tolerated (0–1.5 M); however, EG may be removed in a single step as well. DMSO requires a two-step (1.5–0.6–0 M) dilution process as indicated. (B) Simulation of relative human islet volume upon a single addition (0–1.5 M) step for each CPA, two-step (1.5–0.5–0 M) dilution of DMSO and two-step (1.5–0.65–0 M) dilution of propylene glycol (PG). For both CPAs a single addition step is osmotically tolerated in conjunction with a two-step dilution process; however, islets equilibrate faster with PG than DMSO, allowing shorter CPA exposure time prior to cooling. All simulations were based on least square mean values (adjusting for islet radius).

islets equilibrated in EG stayed within tolerable range even with a single dilution step. Consistent with this theoretical phenomenon, this has been empirically observed in rat pancreatic islets (7). In the case of the human model, the rapid transport of PG allows faster equilibration than DMSO and hence a shorter exposure time to the nonphysiological concentrations of the solute.

DISCUSSION

Since the serendipitous discovery of the cryoprotective properties of glycerol for spermatozoa by Polge, Smith, and Parkes (18), and the discovery of the cryoprotective abilities of DMSO by Lovelock and Bishop in 1959 (12), cryopreservation techniques based on permeating CPAs have been developed for many cells types, including red blood cells, lymphocytes, hematopoietic stem cells, spermatozoa, and embryos. Indeed, the use of these protective additives has allowed much flexibility in the cryopreservation process. For example, human red blood cells need to be cooled at a rate of approximately 1000°C/min for optimal survival without any permeating CPA present (14). In the presence of 3.3 M (30%) GLY, however, survival of this cell type remains around 90% over a several hundred fold range in cooling rates (15). To fully reap the benefits of using such protective solutes, however, requires quantitative values for the transport properties of the specific combination of tissue and CPA of interest to avoid mechanically damaging volume dynamics during the CPA introduction and removal (3,10).

Hydraulic Conductivity (L_p) in the Presence of CPA

Cell membrane hydraulic conductivity is commonly described as the mechanical filtration coefficient, or the velocity of water moving through the membrane per unit pressure difference (6). A high L_p is desirable to allow a rapid water efflux/influx to balance the effects of the high osmotic gradients that occur due to the addition or dilution of the CPAs. The presence of a CPA has been shown to modify the hydraulic conductivity of the cellular membranes in RBCs (13,17,27), lung fibroblasts (24), rat kidney brush border membrane vesicles (28), and human sperm (4). For these reasons, the L_p was measured separately in the presence of each CPA at each concentration for each species.

For canine islets, CPA concentration had the most significant effect on L_p. Though not statistically significant over all CPA concentrations, in 1 and 2 M EG (the initial working concentrations that would be necessary for equilibrium freezing) L_p was highest (least square mean 0.852 and 0.936 μm/min/atm, respectively).

For human islets, both CPA concentration and type

had significant effects on L_p. In all concentrations measured, in the presence of PG L_p had the highest values (least square means 1.25, 1.57, 0.82 μm/min/atm for 1, 2, and 3 M solutions, respectively). In addition, controlling for other explanatory variables (including concentration), PG had the highest L_p (least square mean of 1.17 μm/min/atm).

CPA Permeability (P_s)

Historically, DMSO has been the conventional CPA used in islet cryopreservation protocols over a wide range of species (7,12,19,20). However, recent studies have indicated a possible toxic effect of DMSO upon exposure to rat islets (25,26), and there has been very little work on comparative cryoprotectant permeabilities in canine or human islets.

The results of the present study indicated that for canine islets, EG had the highest overall effective permeability, and there was not a statistically significant concentration dependence among any of the CPAs.

In human islets, as well, there was a clear difference in permeability rates for the different CPAs, and an interaction between CPA and concentration was detected (increasing concentrations of DMSO lowered its P_s while increasing concentrations of EG increased its P_s). Over all concentrations, however, PG had the highest permeability coefficient [least square means 3.0, 3.5, and 3.1 cm/min ($\times 10^{-3}$) for 1, 2, and 3 M PG, respectively].

Reflection Coefficient σ

In the case of single cells, the reflection coefficient is a measure of the selectivity of the plasma membrane (16). A membrane is assumed to be an ideal, semipermeable membrane when $\sigma = 1$ and all of the solute (in the present case CPA) is "reflected" from the membrane while only solvent (in the present case water) passes through. If some of the CPA penetrates the membrane along with water, a complex coupled flow occurs and part of the solute penetrates and is not reflected (there is a solute–solvent interaction; $\sigma < 1$).

In the present study, the values of the reflection coefficient appeared CPA concentration dependent but not CPA dependent for canine islets and marginally for human islets, though a distinct pattern was not apparent.

Within the Kedem-Katchalsky formalism, a solute–solvent interaction implies that the solute and solvent are using (at least in part) the same "channel" (or other membrane "pathway") for transport and consequently there is a physical interaction of the two fluxes (6). In the case of whole islets modeled as EOUs the exact meaning of the concentration dependence is difficult to explain, and σ was poorly constrained by the experimental data. This is typical in experiments of this nature in which volumetric response is primarily determined by the values of L_p and P_s and to a lesser extent by σ (4). It should be noted, however, that it cannot be inferred from these data whether there are solute–solvent interactions in islet cellular membranes, and the estimates of the hydraulic conductivity and CPA permeability also are not representative of single islet cell membrane permeability coefficients. By using this formalism, however, it was possible to make accurate phenomenological predictions of islet volumetric response to CPA addition and removal without the need for complex tissue models requiring the determination of more parameters (e.g., single islet cell permeability measurements, extracellular capillary space, effects of gap junctions on permeability, etc.). These phenomenological predictions may be combined with empirically determined volume excursion tolerance data to optimize these procedures.

Recently, it has been suggested that the addition of trehalose, a disaccharide found at high concentrations in a wide variety of organisms that are capable of surviving complete dehydration (2), to solutions of 2 M DMSO greatly enhances the in vivo function of human adult or fetal islets (1). In that study, the total insulin content of frozen–thawed adult or fetal human islets both prior to and 3 months following transplant into nude mice appeared nearly identical to noncryopreserved preparations. Although the mechanism for the enhanced efficacy of the trehalose/DMSO mixture was not determined, it was noted that trehalose does form glasses more efficiently than other sugars, and the authors suggested that vitrification may have played a role in the enhanced survival. Based on the results of the current study, efficacy using this method may be further enhanced if PG were used instead of DMSO in conjunction with trehalose. In addition to being less osmotically stressful to human islets, if the mechanism of the improved cryopreservation is indeed related to vitrification, this may also be enhanced by using propylene glycol, as it has been previously reported as a better glass-forming compound than DMSO (22).

CONCLUSION

This investigation represents a quantitative approach to the study of pancreatic islet cryopreservation. Numerous biophysical parameters have been determined and combined with previously established data for use in a theoretical model to describe the osmotic events that occur upon exposure and subsequent removal of CPAs from canine and human islets at 22°C. Based on the observed permeability characteristics (analyzed controlling for islet radius) EG and PG were determined to be the optimum CPAs for use in canine and human islets, respectively, because their high effective permeability values allow efficient addition and removal while maintain-

ing critical volume excursion limits. These values (L_p and P_s), the associated reflection coefficient (σ), and the model were used to simulate islet volumetric response to allow development of optimal exposure and removal protocols for each case. The model predicts volume excursion using 2 M concentrations for each CPA for both species. This concentration was chosen because: (i) the most widely used protocols [developed by Rajotte et al. (20,21)] rely on 2 M concentrations of DMSO, and (ii) least square means for L_p and P_s were highest for the respective CPAs at that concentration. Future work evaluating the effects of temperature on the determined permeability parameters of these CPAs may be used for further model development to simulate water volume, water flux in and out of the islet, intra- and extracellular solute concentrations, and the probability of ice nucleation during the cooling and warming procedures of islet cryopreservation. This will allow development of optimal cooling and warming rates that can be added to the optimized CPA addition/dilution strategies to further enhance islet cryo-survival.

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