Characterization of a Major Permeability Barrier in the Zebrafish Embryo\textsuperscript{1}

Mary Hagedorn,\textsuperscript{2,3} F.W. Kleinhans,\textsuperscript{4} Dmitri Artemov,\textsuperscript{5} and Ulrich Pilatus\textsuperscript{5}

Reproductive Physiology Program,\textsuperscript{1} National Zoological Park, Smithsonian Institution, Washington, District of Columbia 20008

Physics Department,\textsuperscript{4} IUPUI, Indianapolis, Indiana 46202

Radiology Department,\textsuperscript{5} Johns Hopkins School of Medicine, Baltimore, Maryland 21205

ABSTRACT

Fish embryos represent a class of multicompartmental biological systems that have not been successfully cryopreserved, primarily because of the lack of understanding of how water and cryoprotectants permeate the compartments. We are using the zebrafish embryo as a model to understand these kinetics. Zebrafish embryos have two major compartments, the blastoderm and the yolk, which is surrounded by the multinucleated yolk syncytial layer (YSL). We determined the water and cryoprotectant permeability in these compartments using two methods. First, we measured shrink/swell dynamics in optical volumetric experiments. Zebrafish embryos shrank over time and did not re-expand while immersed in dimethyl sulfoxide (DMSO) or propylene glycol. Second, we measured DMSO uptake with diffusion-weighted nuclear magnetic resonance spectroscopy. DMSO uptake was rapid during the first few minutes, then gradual thereafter. We used one- and two-compartment models to analyze the data and to determine the permeability parameters. We found that the two-compartment model provided a better fit to the data. On the basis of this model and in the presence of DMSO, the yolk and blastoderm had very similar water permeabilities (i.e., 0.01 and 0.005 $\mu$m $\times$ min $^{-1}$ atm $^{-1}$, respectively), but they had different DMSO permeabilities separated by three orders of magnitude (i.e., $\leq 5 \times 10^{-6}$ and $1.5 \times 10^{-3}$ cm/ min, respectively). The low solute permeability of the yolk predicted that the yolk/YSL compartment should be more susceptible to cryodamage. To test this, the yolk, blastoderm, and YSL were examined at the ultrastructural level after vitrification. Only the YSL incurred significant damage after freezing and thawing ($p \leq 0.05$).

INTRODUCTION

Yolk-laden embryos of fish, reptiles, birds, and amphibians represent a class of multicompartmental biological systems that have not been successfully cryopreserved. Over the past 50 years, the fish embryo has presented a challenge to cryobiologists because of its interesting and complex physiology. Successful cryopreservation of fish embryos must address several important issues, including 1) the intrinsic biophysical properties of the cells (e.g., membrane permeabilities and osmotic tolerance limits) and 2) determination of the procedural cryopreservation steps, based on the cell’s biophysical properties, necessary to minimize cryodamage and maximize survival [1]. In this paper, we examine the kinetics of cryoprotectant permeability of the yolk and blastoderm compartments, and the effects of cryodamage on the fish embryo.

We are using the zebrafish embryo as a model to understand the cryobiological properties of a multicompartmental system. The zebrafish model is becoming one of the most important vertebrate models for the study of development and genetics. Transgenic and mutagenic studies of zebrafish are expected to play an important role in human health and disease. The human genome is being mapped, but the functionality of many of the genes is not known. On the other hand, the function of many of the genes in the zebrafish genome is known because of the mutational screens going on in a number of labs throughout the world. When the zebrafish genome is fully mapped (hopefully within the next few years), it will have great value, because the organization of the fish and human genomes has been relatively conserved throughout evolution [2]. Therefore, cryopreservation of this embryo would be a great service to the field. The zebrafish embryo is composed of two cellular compartments: a large yolk and the developing blastoderm (Fig. 1). The major component of the yolk is vitellinogen, a large phospholipid (approximately 400 kDa; [3]), stored in membrane-bound vesicles within the yolk. The major developmental stages important for this work are 100% epiboly to the 3-somite stage. We did not consider earlier developmental stages, because they are sensitive to chilling [4, 5] and are mechanically fragile, thus making them less attractive for cryopreservation. At the onset of development, the blastoderm divides and forms a cap of cells on the large yolk. At the 128-cell stage, epiboly begins as the blastoderm envelopes the yolk and ceases once the yolk is completely surrounded by blastoderm at 100% epiboly [6]. Underlying the blastoderm and covering the yolk is the yolk syncytial layer (YSL). This is a multinucleated layer (approximately 10 $\mu$m thick) of nonyolky cytoplasm that begins to develop at approximately the 1000-cell stage in the zebrafish embryo [7]. As the YSL develops, it replaces the thin (approximately 2 $\mu$m thick), nonnucleated yolk cytoplasmic layer [8]. The YSL begins to envelop the yolk ahead of the blastoderm, and it surrounds the embryo by approximately 50–75% epiboly [9]. The segmentation period follows. The stages within this period are identified by the number of muscle somites visible in the tail region from the 1- to the 26-somite stage.

The efficacy of cryoprotectant permeation is crucial to formulating successful cryopreservation procedures. Each compartment may have different permeability to water and cryoprotectants. To prevent cryodamage, the compartment with the lowest permeability to water and cryoprotectant must be considered when formulating cryopreservation procedures. Much of the work to date on cryoprotectant permeability of the teleost embryo has not addressed the embryo’s multicompartmental nature. Harvey et al. [10] examined the permeability of zebrafish embryos at 50% epib-
We have examined the multicompartmental zebrafish embryo using a variety of techniques that can reveal the permeability response of the individual compartments [16, 17]. These experiments identified the YSL as a barrier to the movement of cryoprotectants into the yolk. Specifically, little or no DMSO or propylene glycol (PG) entered the yolk, but methanol did. Unfortunately, methanol may not be a suitable cryoprotectant for vitrification (a method needed because of the embryo’s chill sensitivity), whereas DMSO and PG may. Our previous studies [16] identified the existence and location of the permeability barrier to DMSO and PG, whereas the present study quantified the water and solute permeability parameters for the yolk and blastoderm. We used two methods to estimate the permeability parameters. First, we measured cryoprotectant-induced volumetric changes by light microscopic analysis and then cryoprotectant uptake with diffusion-weighted nuclear magnetic resonance (NMR) spectroscopy. Nonlocalized NMR spectroscopy was selected for this study instead of single-embryo NMR microscopy, as used previously [16], because a larger sample size could be used. This increased the signal-to-noise; therefore, smaller amounts of cryoprotectants could be detected within the embryos.

When a single cell is exposed to a hyperosmotic solute solution (e.g., 2 M DMSO or 2 M PG), water initially leaves the cell and solute enters the cell in response to the osmotic and solute gradients. Eventually, as the intracellular solute concentration rises, water re-enters the cell, producing the typical shrink-swell volume curves seen in many optical experiments. The rate at which the cell volume changes in response to the water and solute flux reflects the permeability of the cell membrane to water and solute. These parameters are characterized by the hydraulic or water permeability (L_w) and solute permeability (P_s), respectively. In order to determine permeability parameters for the multicompartmental zebrafish embryo from the NMR and optical data, it is necessary to have a model of the embryo. Initially, we considered a one-compartment model and then generalized to a more complex two-compartment model. For modeling purposes, we did not distinguish the YSL from the yolk and consider it part of the yolk compartment.

As stated earlier, all attempts to cryopreserve fish embryos have produced lethal damage. This analysis of the permeability parameters of the zebrafish embryo predicted that a major site of the lethal cryodamage would occur within the yolk compartment. Presumably, without sufficient cryoprotectant entering the yolk, damaging ice-crystals form. To test this hypothesis, we vitrified zebrafish embryos and examined the cellular ultrastructure of the cryodamage in the blastoderm and the yolk compartments.

MATERIALS AND METHODS

Maintenance of Animals

Animals were maintained according to the maintenance and culturing procedures described by Westerfield [6]. Briefly, 12–16 fish were kept in 5-L aquaria (temp = 28.5°C; pH = 7.0), illuminated according to a 12L:12D cycle, and fed dried copepods and krill (Argent Laboratories, Redmond, WA). Animals spawned in response to photoperiod, producing 50–60 embryos per female. Embryos were collected, dechorionated by enzymatic digestion, and maintained in embryo medium (a modified Hepes buffer at 0.040 Osm) as described in Westerfield [6]. All solutions were made up in this embryo medium. Because the devel-
Solute Uptake: NMR Spectroscopy

NMR data were acquired on an Omega 400 NMR (General Electric, Fremont, CA) spectrometer (equipped with GE Accustar actively shielded gradients up to 130 G/cm in three directions) using a specially designed probe for perfusion experiments with 5-mm NMR tubes. Embryos (each sample consisted of 20 embryos at 100% epiboly) rested as a monolayer on a porous filter (Fritware; Bel-Art Products, Pequannock, NJ, polyethylene sheets, 70-micron pore size, 3.2 mm thickness) placed in a bottom-cut 5-mm NMR tube (Fig. 2). Embryo medium was pumped through the NMR tube, perfusing the embryos from below at 0.3 ml/min with a peristaltic pump (Masterflex; Cole-Palmer, Vernon Hills, IL) for 20 min and was then changed to 2.0 M DMSO for 60 min. To follow any rapid changes in the embryos, localized spectra (two scans/spectrum, 1 sec repetition time) were acquired every 44 sec at two different cross-sectional locations in the NMR tube, one slice containing the embryos and the other in the solution just above the embryos (Fig. 2). 

Low diffusion-weighted spectra were obtained in the upper buffer slice to show the time course of the appearance of DMSO in the buffer. The rate at which DMSO signal appeared in the buffer slice placed a lower limit on the measurable embryo response time. Changes in intracellular DMSO were obtained from the high diffusion-weighted spectra from the lower slice (embryo slice) and were quantified by measuring the change in area under the spectral peaks of the DMSO. Since the intensity of diffusion-weighted signals depends on many factors, such as relaxation processes that may interfere with quantification of intracellular DMSO, we were not able to measure the absolute intracellular quantity of DMSO. However, the signal intensity (area under the signal) is proportional to the quantity of intracellular DMSO. These intensities were arbitrarily normalized, and then three separate NMR runs were averaged to yield the data presented here. In all experiments, the embryos were counted before introduction and after removal from the magnet to insure that changes observed were due to physiological changes and not due to the embryo number diminishing during the experiment. Initially, NMR experiments were performed using PG. Unfortunately, a signal of an endogenous (unidentified) compound within the embryos appeared at approximately the same position as PG. This caused large errors in quantifying intracellular PG, especially when low concentrations had to be determined, so the PG NMR experiments were terminated. The DMSO data were modeled as described below.

Permeability Parameters

The objective of the modeling was to determine the permeability parameters of the zebrafish embryo from the optical and NMR data. In the optical experiments, we measured the changing embryo volume ($V_e$), which depends on the flux of water and solute into or out of the embryo. In
the NMR experiments, we measured the (relative) quantity of solute taken up by the embryo as the number of moles of solute ($N_v$) in the embryo over time. The flux of water into a cell is determined by its water permeability or hydraulic conductivity ($L_p$). Experimentally, it is often found that $L_p$ depends on whether the osmotic driving gradient is produced by a permeating (e.g., DMSO) or nonpermeating (e.g., NaCl) solute. To distinguish between these two cases, we referred to the water permeability in the presence of a permeating solute as $L_p^p$. The flux of solute into the cell is determined by the solute permeability ($P_s$). The task was to relate the permeability parameters $L_p^p$ and $P_s$ to $V_c$ and $N_v$.

The embryo volume ($V_c$) is the sum of the water ($V_w$), solute ($V_s$), and solids ($V_{solids}$) volumes:

$$V_c = V_w + V_s + V_{solids} = V_w + M_i V_u \tilde{V}_s + v_b V_{co},$$

(1)

where $v_b$ is the fractional volume of the osmotically inactive cell contents, $\tilde{V}_s$ is the solute partial molar volume, and $V_{co}$ is the isotonic volume of the cell. Thus the problem was reduced to one of finding the water volume ($V_w$) and the solute content ($N_v$) of the embryo. These two quantities were given by a pair of coupled, differential equations that must be numerically integrated along with subsidiary defining equations. These equations are outlined below.

The water flux into a cell is given by:

$$dV_w/dt = L_p^p ART(M^e - M^s),$$

(2)

where $A$ is the cell surface area, $R$ and $T$ the gas constant and absolute temperature, respectively, and $M^e$ and $M^s$ are the intracellular and extracellular medium osmolality. The solute flux is given by:

$$dN_v/dt = P_s A (M^i - M^e),$$

(3)

where $N_v$ is the number of moles of solute in the cell, and $M^i$ and $M^e$ are the extracellular and intracellular solute osmolalities, respectively. Sometimes it is more convenient to express the solute flux in terms of the intracellular solute osmolality ($M^i = N_v/V_w$). Substituting $V_w, M^i$ for $N_v$ in equation 3 and utilizing equation 2 yielded:

$$dM^i/dt = P_s A (M^i - M^e) + L_p^p (A/V_w) T R T (M^e - M^s).$$

(4)

The external medium osmolality ($M^e$) was set by the experimental conditions:

$$M^e = M^e_n + M^e_f,$$

(5)

where $M^e_f$ is the external nonpermeating solute concentration and $M^e_n$ is the external permeating solute concentration (DMSO or PG). The intracellular osmolality $M^i$ was computed assuming a linear Boyle van’t Hoff cell response:

$$M^i = M^i_{peri}(V_{peri}/V_u) + M^i_f,$$

(6)

where the subscripts $n$, $o$, and $w$ refer to nonpermeating solute, initial ($t = 0$) conditions, and water, respectively.

The cell volume ($V_c$) and intracellular solute ($N_v$) were modeled by simultaneously integrating equations 2 and 3 or equations 2 and 4. In the NMR experiments, only the relative solute concentration ($N_{sol}$) was measured; thus in equation 3, a scaling factor ($S_f$) must be introduced so that $N_{sol} = S_f \times N_v$. Integration was done using the Stiff Episode integrator in the program Scientist (MicroMath, Salt Lake City, UT). The permeability parameters $L_p$ and $P_s$ (and $S_f$ in the NMR experiment) were obtained by iteratively varying them until a good fit was obtained between the model calculations and the experimental observables, specifically $V_c, N_{sol}$, or $V_c$ and $N_{sol}$. Least-squares fitting was performed using the Powell variant of the Levenberg-Marquardt method as implemented in the program Scientist. In some cases, a wide range of $P_s$ values gave a satisfactory fit to the experimental data. In these cases, $P_s$ was manually varied to find the acceptable range of values as judged by visual evaluation of the fits. A number of fixed parameters appear

### Table 1. Parameters used for the one- and two-compartment model analyses of the NMR solute uptake and optical volume measurements; embryos were at approximately 100% epiboly for these experiments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$M_{e,n}$</td>
<td>0.300 Osm</td>
<td>Assumed cytoplasmic and yolk osmolality.</td>
</tr>
<tr>
<td>$M_{e,f}$</td>
<td>0.040 Osm</td>
<td>Osmolarity of external, nonpermeating embryo medium.</td>
</tr>
<tr>
<td>$M_{e}^i$</td>
<td>2.39 Osm</td>
<td>Osmolarity of external 2 M DMSO or 2 M PG.</td>
</tr>
<tr>
<td>$V_c$ (DMSO)</td>
<td>0.069 l/mol</td>
<td>Partial molar volume of DMSO.</td>
</tr>
<tr>
<td>$V_c$ (PG)</td>
<td>0.070 l/mol</td>
<td>Partial molar volume of PG.</td>
</tr>
<tr>
<td>$T$</td>
<td>294–296 K</td>
<td>Temperature of NMR and optical experiments.</td>
</tr>
<tr>
<td><strong>One compartment model parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_o$</td>
<td>0.2 mm$^3$</td>
<td>Volume of “standard” embryo.</td>
</tr>
<tr>
<td>$A$</td>
<td>1.65 mm$^2$</td>
<td>Area of “standard” embryo, assumed to be spherical.</td>
</tr>
<tr>
<td>$v_o$</td>
<td>0.36</td>
<td>Osmotically inactive fraction of embryo.</td>
</tr>
<tr>
<td>$L_{p}^o$</td>
<td>variable</td>
<td>Hydraulic conductivity in presence of solute.</td>
</tr>
<tr>
<td>$P_s$</td>
<td>variable</td>
<td>Solute permeability.</td>
</tr>
<tr>
<td><strong>Two compartment parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_o$</td>
<td>0.2 mm$^3$</td>
<td>Volume of “standard” embryo.</td>
</tr>
<tr>
<td>$f_o$</td>
<td>0.77</td>
<td>Fraction of embryo volume that is yolk.</td>
</tr>
<tr>
<td>$v_o$</td>
<td>0.23</td>
<td>Fraction of embryo volume that is blastoderm.</td>
</tr>
<tr>
<td>$A_y$</td>
<td>1.39 mm$^2$</td>
<td>Assumed surface area of yolk compartment.</td>
</tr>
<tr>
<td>$A_b$</td>
<td>1.65 mm$^2$</td>
<td>Surface area of blastoderm compartment.</td>
</tr>
<tr>
<td>$v_{by}$</td>
<td>0.41</td>
<td>Osmotically inactive fraction of yolk.</td>
</tr>
<tr>
<td>$v_{bb}$</td>
<td>0.20</td>
<td>Osmotically inactive fraction of blastoderm.</td>
</tr>
<tr>
<td>$L_{p}^y$</td>
<td>variable</td>
<td>Hydraulic conductivity of yolk compartment.</td>
</tr>
<tr>
<td>$L_{p}^b$</td>
<td>variable</td>
<td>Hydraulic conductivity of blastoderm compartment.</td>
</tr>
<tr>
<td>$P_s$</td>
<td>variable</td>
<td>Solute permeability of yolk compartment.</td>
</tr>
<tr>
<td>$P_{sb}$</td>
<td>variable</td>
<td>Solute permeability of blastoderm compartment.</td>
</tr>
</tbody>
</table>

*a The internal osmolality of the zebrafish is unknown. The embryos are held in embryo medium at 0.040 Osm. However, a number of arguments point to an internal embryo osmolality closer to 0.300 Osm. It is not understood how zebrafish embryos can regulate their internal osmotic environment while living in such a hypo-osmotic environment. Vapor pressure osmometer measurements suggest an osmolality of approximately 0.240 Osm (Hagedorn, unpublished data). We choose 0.300 Osm for these models to match the osmolality of a typical vertebrate cell.

*b This is determined by adding together the components of the two-compartment model ($v_o = f_o \times v_{by} + (1 - f_o) \times v_{bb}$).

*c These fractional compartmental volumes are taken from measurements of the yolk and blastoderm as a function of developmental stage [5].

*d At 100% epiboly, the embryo has an approximately spherical yolk compartment surrounded by a thin layer of blastoderm cells. The area of the blastoderm compartment is taken to be just the outer surface area of the embryo, assuming a spherical volume. The yolk compartment is a sphere of somewhat smaller size (containing 77% of the volume), with a surface area of 1.39 mm$^2$.

*e Hagedorn et al. [17], see text.
in the fitting equations, and their values are listed in Table 1. Some justifications for the fixed parameter values appear in the next section.

Typical initial volumes of 100% epiboly embryos (or slightly larger) in the optical experiments were observed to scatter approximately at 0.20 mm³. Therefore, all initial volumes were normalized to this value. Further, all data for a given experimental series were averaged before fitting to reduce noise and reduce the computational burden. In the NMR experiments, the data were first (arbitrarily) normalized, then averaged.

**Modeling the Zebrafish Embryo**

Two different structural models were used in fitting the data. The simpler was a one-compartment model assuming a spherical embryo of initial volume 0.20 mm³ with a fixed area of 1.65 mm² and an osmotically inactive fraction \(v_{by}\) of 0.36 (see below). A two-compartment model was also utilized in which the yolk and blastoderm were considered as separate, distinct compartments, of volume and water content appropriate to the 100% epiboly stage. For two compartments, the permeability equations must essentially be solved in duplicate. The fixed and variable parameters of this model are listed in Tables 1 and 2.

Some justification is required for our choice of fixed parameters in the two-compartment model (Table 1). Prior NMR studies [16] show that the yolk and blastoderm were responding differently to cryoprotectants, and this formed the basis for our decision to divide the embryo into a yolk and blastoderm compartment. The volumes and areas of the two compartments were determined from the morphometric data in previous papers [5, 17]. The most difficult parameter to determine was the osmotically inactive fraction of the yolk \(v_{by}\) and of the blastoderm \(v_{bb}\). For the blastoderm, we used our estimate of \(v_{bb} = 0.2\) from Hagedorn et al. [17]. However, depending on the method of measurement, we obtained \(v_{by}\)'s for the yolk ranging from 0.41 to 0.78 [17]. To help chose an appropriate value, we turned to the PG optical shrink data. In some experiments with exposure times up to 225 min in 2 M PG, the embryos were observed to shrink in a linear fashion by 40–50% (data not shown). This large shrinkage requires a large percentage of water (or a small percentage of inactive volume) in the zebrafish embryo. For this reason, we used the lowest yolk value, \(v_{by} = 0.41\), suggested by our earlier experiments. Combining the compartment volumes and \(v_{by}\) data yields an overall osmotically inactive volume \(v_{bb}\) for the embryo of 0.36 (see Table 1).

In either case, it must be understood that the permeability parameters determined are phenomenological parameters characterizing the average response of a very complex structure, and caution should be used in attributing them to any specific membrane. Because of the complexity of this system and the required approximations, none of the determined permeability parameters are considered to be accurate to better than a factor of two.

**EM Analysis**

We examined the type of cryodamage evident at the level of the electron microscope in various compartments of the zebrafish embryo after vitrification. We chose vitrification as a method because zebrafish embryos are chill-sensitive [4, 5], and the rapid rate of cooling used to vitrify may ultimately increase embryo survival. Embryos at 100% epiboly were immersed into 2 M PG for 2.5 h, then 6 M PG for 2–4 min, drawn into 0.25-ml straws, cooled in liquid nitrogen vapor for 3 min, then plunged into liquid nitrogen for 1–2 min. To thaw, straws were held horizontally at room temperature (22–23°C) for 10 sec, then plunged into room temperature water until thawed (approximately 30 sec). Afterwards, the embryos were diluted by steps into 3 M, 2 M, then 1 M PG for 20 min each. Control embryos were exposed to all chemicals but were not subjected to freezing. To prepare for EM, both experimental and control embryos (n = 3 each) were placed in a fixative (2.5% glutaraldehyde, 1% paraformaldehyde in cacodylate buffer) 10 min after thawing for 2–3 h at 22°C, and then for 1–3 h at 4°C. Embryos were stored for 24 h in cacodylate buffer at 4°C, fixed in 1% osmium tetroxide in cacodylate buffer for 1 h, en bloc stained with 2% uranyl acetate for 1 h, then dehydrated in a series of alcohols (70, 70, 80, 90 and 100, 100, 100% ethyl alcohol) for 5–6 min each, followed by propylene oxide (1:1, 1:2, and 1:3 for 5–6 min each) and 100% propylene oxide for 40 min. Embryos were infiltrated with Spurr’s plastic resin (for 18 h), embedded in fresh plastic, and placed in a 70°C oven for polymerization. Sections were cut at 600–800 Å and mounted onto copper grids that were stained for 1.5 min in lead citrate, and then examined using a Zeiss EM10 CA transmission electron microscope.

To evaluate whether the cellular morphology of the frozen and nonfrozen embryos was similar, thin sections were made at three positions (i.e., through the center of the embryo and 200 μm to each side, see Fig. 1B). Then, on each thin section, three areas including the blastoderm and YSL were randomly chosen and scored for intact cells, nuclear membranes, the presence and normal appearance of various organelles (e.g., mitochondria, Golgi bodies, endoplasmic reticulum, etc.), and the presence of yolk droplets (Fig. 1C).

In the yolk, the presence of intact membranes and smooth yolk material was assessed. Each intact class of organelle or membrane was given one point for its presence in the tissue. A maximum of 10 points was possible in the blastoderm and the YSL, and two points in the yolk. Mean scores from the yolk, YSL, and blastoderm of each treatment were analyzed with a two-tailed t-test and then graphed. Representative areas of the specimens were photographed and printed.

**RESULTS**

**Biophysical Data: Volumetric and NMR**

When zebrafish embryos were exposed for long periods of time (i.e., 60 min) to 2 M DMSO or PG, their light microscopic volumes exhibited an approximately linear decrease over time without any re-expansion (Fig. 3). The steady volume decrease observed over the long duration of the experiments suggested that the embryo experienced mostly dehydration with little to no solute permeation. At some point during the dehydrating events, the embryonic structure began to lose integrity. For example, after embryos were immersed in 2 M DMSO for 90–120 min, the blastoderm began to shed cells, and a rupture of the yolk in that area followed shortly thereafter.

NMR spectroscopy allows the solute uptake to be measured directly. When embryos were perfused with a 2 M DMSO solution, DMSO uptake was rapid during the first few minutes, then gradual thereafter (Fig. 4). The response time of the DMSO perfusion system was determined by measuring the NMR signal rise-time in the buffer slice (positioned above the embryos, data not shown). The rise-time
of the DMSO signal from 10% to 90% was 2.3 min. This imposed a lower limit on the attainable time resolution of the embryo response to DMSO exposure.

The NMR data suggested some embryo permeability to DMSO, whereas the light microscopic data suggested little embryo permeability to DMSO. At first glance, this presented a somewhat contradictory picture of the permeability within the embryo. In order to estimate the permeability parameters, we analyzed these data with a one- and two-compartment model.

**One-Compartment Model**

The NMR and optical data were first analyzed with a one-compartment model (Tables 1 and 2) to determine whether this would yield a useful phenomenological model of the embryo. The optical volumetric data gave a water permeability \( (L_p^w) \) for 2 M DMSO of 0.007 \( \mu m \times \text{min}^{-1} \text{atm}^{-1} \) and an upper limit to the solute permeability \( (P_s) \) of 4 \( \times \) 10\(^{-3} \) cm/min (Fig. 5A; Table 3). In contrast, the NMR DMSO-uptake data gave a water permeability \( (L_p^w) \) of 0.03 \( \mu m \times \text{min}^{-1} \text{atm}^{-1} \) and a \( P_s \) of 0.003 cm/min (Fig. 5B). Although good fits were obtained in each case (Fig. 5), the optical and NMR permeability parameters did not agree. Comparing the resultant permeability values (Table 3) reveals a 4-fold difference in \( L_p^w \) values and at least a 75-fold difference in \( P_s \) values between the optical and NMR one-compartment fits. Another way to illustrate this discrepancy was to simultaneously fit the optical and NMR data with a single set of parameters (Fig. 5C). This resulted in an intermediate pair of permeability parameters that yielded a poor fit to both the optical and NMR data.

Analysis of the optical volumetric data for embryos immersed in 2 M PG (data from Fig. 3) yielded similar water and solute permeabilities similar to those of DMSO (fit not shown; Table 3).

**Two-Compartment Model**

The optical and NMR data were also analyzed using a two-compartment model, the blastoderm being one compartment, and the yolk, the second. By fitting these data simultaneously, sufficient constraints were obtained to fit values for \( L_p^w \) of the yolk and blastoderm and \( P_s \) of the blastoderm, and to find an upper limit for \( P_s \) for the yolk. In the presence of 2 M DMSO, the yolk and blastoderm had very similar water permeabilities (i.e., 0.01 and 0.005 \( \mu m \times \text{min}^{-1} \text{atm}^{-1} \), respectively) but different solute permeabilities separated by three orders of magnitude (i.e., \( \approx 5 \times 10^{-6} \) and 1.5 \( \times \) 10\(^{-3} \) cm/min, respectively; Fig. 6, Table 3). Empirically, it was found that the permeability parameters for the yolk were largely fixed by the volumetric data, whereas those for the blastoderm were fixed by the solute uptake data.

Although NMR PG-uptake data could not be obtained, it was still possible to do a two-compartment analysis of the volumetric PG data. As noted above, the volumetric data primarily reflect the response of the yolk. Thus, the yolk permeability parameters are only weakly constrained by the NMR solute uptake data. We made the assumption that the blastoderm water and solute permeabilities in the presence of PG are less than or equal to twice those found for DMSO. With these assumptions, a two-compartment fit to the volumetric PG data for the yolk compartment yielded slightly smaller water and solute permeabilities for PG than for DMSO (Table 3).
embryos were examined and scored at the ultrastructural level to identify areas of the embryo that were damaged. Control embryos showed little or no damage to the blastoderm, yolk, or YSL as a result of the chemical treatment (i.e., cell structures were intact and showed normal morphology; \( p \geq 0.05 \); Fig. 8). In contrast, experimental embryos exhibited altered morphology only in the YSL (\( p \leq 0.05 \)). Specifically, after freezing, many of the organelles in the YSL were destroyed, and the cellular membrane was destroyed (usually in the area of blastopore closure); some YSL nuclei were present, but the chromatin was congested, and the nuclear membrane was not always intact (Fig. 9). Morphologically, the yolk was not affected by freezing, as the yolk membranes were intact and the yolk contents appeared to be normal. We concluded that cryodamage within the yolk may be tolerated. However, damage to the YSL (the only other major organizing structure related to the yolk) may destroy the integrity of the yolk/YSL compartment, thus leading to the lethal effects observed after thawing.

**DISCUSSION**

Using a one-compartment model (Fig. 5), we found that no single set of parameters simultaneously fit the optical and NMR data. However, a two-compartment model provided a better fit to the data (Fig. 6). In the presence of DMSO, the yolk and blastoderm compartments had similar water permeabilities but substantially different solute permeabilities. The yolk compartment was essentially impermeable to DMSO (\( P_s \approx 5 \times 10^{-6} \) cm/min), whereas the blastoderm compartment had a “typical” membrane solute permeability (\( P_s = 1.5 \times 10^{-3} \) cm/min). This readily explains the different behavior seen in the DMSO uptake and volumetric experiments. In the volumetric experiments, when the blastoderm cells were exposed to 2 M DMSO, they experienced a rapid shrink/swell response. This is not
evident in the data, because the shrink/swell response was most likely small and occurred quickly (i.e., between the first and second experimental data points). Since the yolk compartment is essentially impermeable to DMSO, it showed a slow, steady loss of water to the hyperosmotic DMSO environment, yielding a steady decrease in volume, which dominated the volumetric data. In the NMR uptake experiments, primarily the blastoderm was permeable to the DMSO, and the data reflect this response. Initially there was a rapid influx of DMSO due to the large solute gra-

<table>
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<th>Table 3. Permeability parameters of zebrafish embryo exposed to 2 M DMSO at 21°C (NMR experiments), 22–23°C (optical experiments), and 2 M PG at 22–23°C (optical experiments): one- and two-compartment permeability results $L_p$ and $P_s$ of the optical volume versus time and the NMR uptake versus time data.</th>
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<tbody>
<tr>
<td><strong>Experiment</strong></td>
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<tr>
<td>One-compartment⁶</td>
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<tr>
<td>Optical volume</td>
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<td>Optical volume</td>
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<tr>
<td>Two-compartment⁷</td>
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<td>NMR &amp; optical</td>
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<td>Optical volume⁹</td>
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⁶ The dimensions of $L_p$ are micrometers × min⁻¹ atm⁻¹ and of $P_s$ are cm/min.
⁷ Model: one-compartment, two-parameter model.
⁸ Model: two-compartment (yolk and blastoderm), two parameter model.
⁹ The values of the blastoderm permeability ($L_{pb}$ and $P_{sb}$) could not be calculated and were assumed to be less than twice the values found in the DMSO experiments.
FIG. 8. Histogram quantifying the number of ultrastructural elements in 3-somite embryos after exposing embryos to a solution-control or cryoprotectant, then freezing, and thawing. Only the YSL in the frozen sample showed a loss of structural elements (*$p < 0.05$), whereas the ultrastructure of the blastoderm and the yolk appeared the same in both treatments ($p \geq 0.05$).

FIG. 9. After freezing and thawing, the ultrastructure of the YSL was destroyed. No clear YSL layer remained as the ribosomal material from the YSL was scattered throughout the blastoderm (B) and yolk (Y). However, the areas not in direct contact with the YSL appeared normal. Most membranes and nuclei (N) of the blastoderm (in the upper part of the figure) and yolk (area not shown) remained intact (bar, lower right $= 1 \mu m$).
to $1.2 \times 10^{-3}$ cm/min [23]. Because the yolk is completely surrounded by the blastoderm at 100% epiboly, it might be argued that the low apparent solute permeability of the yolk compartment simply results from the surrounding layer of blastoderm cells, which block ready access of the extracellular medium to the yolk. However, if this were the case, we would expect the yolk compartment to have a very low water permeability as well. This suggests that the blastoderm does not create the solute permeability barrier around the yolk compartment, but the YSL does.

There is a major difference in the predictions that the one- and two-compartment models make regarding cryopreservation protocols. We modeled the time required for DMSO to reach two thirds of its final concentration (approximately 2 M) within the embryo and yolk for the one- and two-compartment models, respectively, using the upper limits of the solute permeability given in Table 3. The one-compartment model predicted a minimum time of 2.2 h for the embryo to reach the projected DMSO concentration. This is a reasonable treatment time given the constraints of the rapidly developing embryo. However, the two-compartment model predicted a minimum of 9 h for the yolk to reach the projected concentration. This is an unreasonable treatment time, because of toxicity and the large change in development that the embryo would experience by the end of the test period. Clearly, the predictions of the two-compartment model will impose some important constraints on future cryopreservation protocols.

Previously, we measured the water permeability of the zebrafish embryo in the absence of cryoprotectants using optical volumetric methods and a one-compartment model [17]. The value of $L_p$ we obtained at the 100% epiboly stage was $0.05 \, \mu m \times \text{min}^{-1} \cdot \text{atm}^{-1}$, and may be compared with our one-compartment analyses of the DMSO and PG optical volumetric data (Table 3). This revealed that $L_p$ dropped by an order of magnitude in the presence of either DMSO or PG and is consistent with a growing body of evidence suggesting that $L_p$ is modified in the presence of permeating cryoprotectants for a variety of membrane types [5, 24, 25]. Clearly, for most cryobiological purposes, it is important to measure membrane water permeability in the presence of proposed cryoprotective solutes. Other investigators have also measured the water permeability of fish embryos in the presence of cryoprotectants. Zhang and Rawson [15] report zebrafish $L_p$ of 0.10 and 0.17 $\mu m \times \text{min}^{-1} \cdot \text{atm}^{-1}$ in the presence of PG and methanol, respectively. Their permeabilities are more than an order of magnitude larger than our similarly obtained, optical volumetric results. Some of this may be due to a difference in modeling parameters or in the osmolality of the buffer used to make the solutions. Nevertheless, in the presence of solutes, their $L_p$s appear to be substantially larger than those obtained here.

Previously, we identified the YSL, and not the yolk itself, as the barrier to the movement of cryoprotectants into the yolk in a previous study [16]. In this study, we have characterized a permeability barrier in the yolk compartment of the zebrafish embryo, but what are the consequences of this barrier to cryopreservation? At the ultrastructural level, the most obvious consequence of our vitrification protocol was a destruction of the YSL surrounding the yolk. This is not to suggest that other, more subtle changes did not occur in the blastoderm and the yolk after the tissue was frozen and thawed. For example, the mere presence of ultrastructural elements within a tissue does not mean they are present in sufficient quantity for normal cell function. A study of serial sections quantifying the number of ultrastructural elements per area of tissue may have shown a clear difference in the blastoderm tissue of the controls and frozen embryos. Additionally, the high concentration of cryoprotectants used in vitrification (approximately 6 M or greater) could have caused a disruption of cytoskeletal elements. Using fluorescent antibodies, Hotamisilgil et al. [26] observed a disruption in the actin filaments of the mouse embryo after exposure to 4 M ethylene glycol for more than 10 min. Hypothetically, if little or no cryoprotectant reached the yolk because of the impermeable YSL, ice-crystals may have formed, leading to some type of damage in both the yolk and YSL regions. However, these more subtle issues are moot, because the test vitrification protocol caused the complete destruction of the YSL.

Our ultrastructural experiments suggested that cryodamage led to the death of the embryos, in part because of insufficient cryoprotectant entering the yolk/YSL region. Isotope-uptake experiments have quantified how little cryoprotectant enters the blastoderm, and the yolk in particular. Harvey et al. [10] measured a DMSO concentration in the whole, dechorionated zebrafish embryo of 100 mM after 60 min in 1 M DMSO. After incubating carp embryos in 1.5 M isotope-labeled DMSO for 2 h, Magnus et al. [27] extracted yolk fractions with a micropipette and found approximately 100 mM DMSO. All of these values are well in line with the predictions that the two-compartment model made about the permeability of the zebrafish yolk compartment to DMSO after 2 h (approximately 150 mM) and lends support for the two-compartment model when considering the yolk specifically.

Now that we have characterized and quantified the permeability parameters in the zebrafish embryo, we must examine how we can overcome this permeability barrier to successfully cryopreserve fish embryos. The YSL is a common morphological feature in all teleost fish and will be an important consideration for cryopreservation in most threatened species and for important cultured species, such as catfish, salmonids, tilapia, sturgeon, and striped bass. It is clear that exposure alone may not allow enough cryopro-

![Graph](image-url)
tectant to enter the yolk for successful cryopreservation. A means for getting cryoprotectant into and past the YSL barrier is required. The vast arsenal of molecular biological methods created for transfecting cells, such as the introduction of pores in the YSL through chemical (e.g., antibiotics) or physical (e.g., electroporation) means, may help overcome this permeability barrier, thus opening the door for successful cryopreservation protocols for fish embryos.

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