

Altering Fish Embryos with Aquaporin-3: An Essential Step Toward Successful Cryopreservation¹

M. Hagedorn,^{2,4} S.L. Lance,^{3,4} D.M. Fonseca,^{3,5,8} F.W. Kleinhans,⁶ D. Artimov,⁷ R. Fleischer,⁸
A.T.M.S. Hoque,⁹ M.B. Hamilton,¹⁰ and B.S. Pukazhenthil^{3,4}

Smithsonian Institution,⁴ National Zoological Park and Conservation and Research Center,
Washington, District of Columbia 20008

Department of Entomology,⁵ Walter Reed Army Institute of Research, Silver Spring, Maryland 20910-7500

Department of Physics,⁶ Indiana University-Purdue University Indianapolis, Indianapolis, Indiana 46202

Department of Radiology,⁷ Johns Hopkins School of Medicine, Baltimore, Maryland 21205

Smithsonian Institution,⁸ National Zoological Park, Department of Zoological Research and Molecular Genetics
Laboratory, Washington, District of Columbia 20008

Laboratory of Plasma Derivatives,⁹ Division of Hematology, Center for Biologics Evaluation and Research,
Food and Drug Administration, Bethesda, Maryland 20892

Department of Biology,¹⁰ Georgetown University, Washington, District of Columbia 20057

ABSTRACT

Fish populations are globally threatened by overharvesting and habitat degradation. The ability to bank fish embryos by cryopreservation could be crucial for preserving species diversity, for aquaculture (allowing circannual fish farming), and for managing fish models used in human biomedical research. However, no nonmammalian embryo has ever been successfully cryopreserved. For fish, low membrane permeability prevents cryoprotectants from entering the yolk to prevent cryodamage. Here, we present evidence of a membrane mechanism hindering cryopreservation of fish and propose a novel solution to this obstacle. Zebrafish (*Danio rerio*) embryos have rectifying membranes that allow water to leave but not to reenter readily. This feature may be an evolutionary trait that allows freshwater embryos to grow in hypoosmotic environments without osmoregulatory organs. However, this trait may also prevent successful fish embryo cryopreservation because both water and cryoprotectants must move into and out of cells. As a solution, we injected zebrafish embryos with mRNA for the aquaporin-3 water channel protein and demonstrated increased membrane permeability to water and to a cryoprotectant. Modeling indicates that sufficient cryoprotectant enters aquaporin-3-expressing zebrafish embryos to allow cryopreservation.

embryo

INTRODUCTION

Nonmammalian vertebrate embryos have not been successfully cryopreserved. To look for solutions to this prob-

lem, we used the zebrafish (*Danio rerio*) as a model to better understand the barriers preventing cryopreservation. During the freezing phase of cryopreservation, most internal cellular water must be replaced with an appropriate cryoprotectant (i.e., a solution that reduces freeze-related cell lysis while stabilizing membranes). After thawing, the cryoprotectant must exit and water must reenter the cells. Therefore, to successfully cryopreserve embryos, all cells must be permeable to both water and an appropriate cryoprotectant. An understanding of the specific membrane mechanisms affecting both water and cryoprotectant permeability is imperative. Factors that appear to complicate fish embryo cryopreservation include 1) a large overall size resulting in a low surface:volume ratio that can slow water and cryoprotectant efflux and influx, 2) the presence of compartments, such as the blastoderm and yolk, with different permeability properties, and 3) susceptibility to chilling injury [1–7].

We previously determined that a major permeability barrier in the zebrafish embryo is the yolk syncytial layer (YSL) [2–4]. The permeability barrier of the yolk compartment and the YSL physiologically prevents both the entry of some cryoprotectants and the exit of water. As a result of these permeability restrictions, conventional approaches to zebrafish embryo cryopreservation result in the lethal destruction of the YSL [4]. Although this barrier can be circumvented by microinjecting cryoprotectant directly into the yolk [8], the need to remove water from the large yolk compartment remains. Because of the large size (approximately 800 μm) and low water permeability of the zebrafish embryo, the dehydration time of the yolk and blastoderm is significantly longer than the reported values for the mouse [9], cow [10], and permeabilized *Drosophila* embryos [11]. Therefore, to achieve successful cryopreservation of the fish embryo, membrane permeability must be enhanced to allow a more rapid efflux and influx of water and cryoprotectants. We predicted that permeability could be facilitated by introducing major intrinsic proteins (MIPs) into the membranes of zebrafish embryos.

The MIP proteins are distributed in a variety of biological membranes throughout the plant and animal kingdom [12–19]. Generally, MIPs are transmembrane proteins that pass water, glycerol, and/or other small uncharged molecules. One important class of MIPs is the water channels, known as aquaporins (AQPs). AQPs, which are membrane-

¹This work was supported by grants to D.F. and R.F. from Friends of the National Zoo, to B.P. from the National Institutes of Health (K01 RR00135), and to M.H. from the National Institutes of Health (R01 RR08769), by the Maryland Sea Grant College, and by a private donation from Mrs. S. Sichel.

²Correspondence: M. Hagedorn, Department of Reproductive Sciences, National Zoological Park, Smithsonian Institution, 3001 Connecticut Ave., NW, Washington, DC 20008. FAX: 202-673-4377; e-mail: hagedornm@nzp.si.edu

³These authors contributed equally to this work.

Received: 20 December 2001.

First decision: 23 January 2002.

Accepted: 17 April 2002.

© 2002 by the Society for the Study of Reproduction, Inc.

ISSN: 0006-3363. <http://www.biolreprod.org>

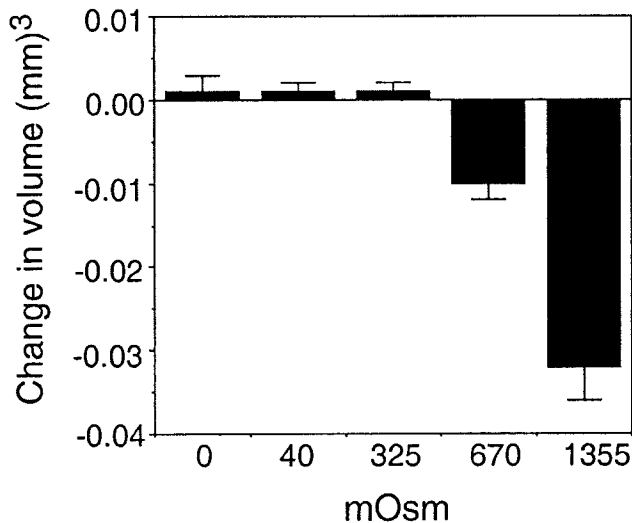


FIG. 1. Zebrafish embryos have rectifying membranes. Mean (SEM) volume change after 15 min in a particular solution are presented. Embryos experiencing a hypo- or isosmotic solution (≤ 300 mOsm) did not swell, whereas embryos experiencing a hyperosmotic solution (> 300 mOsm) became dehydrated.

bound homotetrameric water channel proteins, have been identified in the plasma membranes of many fluid-transporting epithelia and endothelia [20, 21]. AQPs play a crucial role in a cell's ability to rapidly respond to changing osmotic conditions. When AQP mRNA is microinjected into frog oocytes, the aquaporin proteins are formed and inserted into the oocyte membrane, thereby greatly increasing the water permeability of the oocyte [12, 22–24]. Insertion and expression of MIPs in embryos has not been accomplished, and could provide a solution to the impermeability of fish embryos.

Before attempting to modify zebrafish membrane permeability, we sought to understand selective membrane processes. The isosmotic point for zebrafish embryos is approximately 300 mOsm. Normally, vertebrate cells increase in volume (swell) under hypoosmotic conditions and decrease in volume (shrink) in hyperosmotic conditions. However, zebrafish embryos can develop normally in deionized water. The membrane mechanism responsible and the effects on the isosmotic value of zebrafish embryos are unknown. If the embryo isosmotic value were 40 mOsm (normal culture medium), they would differ from most vertebrate cells but would experience little damage in deionized water. However, if their cells had normal vertebrate osmolality (ca. 300 mOsm), they would be damaged by this treatment. One way to understand the isosmotic condition of an embryo is to test its volume behavior in nonpermeating solutions of various osmolalities.

Here, we report the results of experiments examining the permeability of natural and somatically altered zebrafish embryos. First, we investigated the osmotic properties of zebrafish embryos by measuring the volume changes of embryos under both hypo- and hyperosmotic conditions. Second, we determined whether AQPs exist in the native zebrafish embryo. Third, we introduced a fused mRNA of AQP3 and a reporter construct, green fluorescent protein (GFP), into early stage embryos and determined their effects on permeability in developing embryos. Previously, AQP3 mRNA inserted into frog oocytes increased several fold the permeability to both water and glycerol and to

other cryoprotectants [25, 26] such as propylene glycol (PG). We then modeled the response of AQP3-expressing embryos to changes in membrane permeability to assess whether these modifications were sufficient to proceed with development of a suitable vitrification protocol.

The efficacy of vitrification is important because this type of cryopreservation circumvents the high chill sensitivity of fish embryos [5]. Vitrification relies upon a highly concentrated solution of cryoprotectants that both permeate and dehydrate the embryo. This process is followed by rapid cooling (i.e., hundreds of degrees per minute) of the embryo suspension. The high concentration of external and internal cryoprotectant forms a stable glassy state.

MATERIALS AND METHODS

Maintenance of Animals

Zebrafish adults were maintained 12–16 fish per 5-L tank (28.5°C, pH 7.0), illuminated with a 14L:10D cycle, and fed dry pellets (Tropical Micropellets, Hikare, Japan) twice each day. The developmental temperature was 28.5°C, and all developmental stages were described according to the method of Hagedorn et al. [3, 4]. All solutions throughout the study were prepared using an embryo culture medium, which was a 10% Hanks buffer as described by Westerfield [27]. Prior to experimentation, all embryos were dechlorinated as previously described [3, 4]. Fish care met NIH animal care standards.

Osmotic Behavior of Zebrafish Embryos

We determined how water moved across the membrane in the native zebrafish embryo by measuring the volumetric water responses of embryos to a variety of hyper- and hypoosmotic solutions near to the fish's isosmotic point (Fig. 1). Embryos (100% epiboly, ca. 300 embryos/sample; $n = 3$) were packed into a tube, the interstitial water was removed, the cells were disrupted by freezing, and the contents were measured with a vapor pressure osmometer (Wescor, Logan, UT).

The temperature dependence of membrane transport, characterized by the activation energy (E_a) [15], can be used to distinguish between protein water channels and bilayer water transport. An E_a of < 6 kcal/mol is indicative of AQP-mediated water transport, and an E_a of > 10 kcal/mol is indicative of diffusion across the membrane bilayer [15]. We determined the E_a using an Arrhenius-type analysis and determined water permeability by measuring the volume change of embryos from 100% epiboly to three somites. Embryo volumes were held at constant temperature using a temperature controlled microscope stage (Linkam, Surrey, U.K.). The temperatures measured were 15°C, 22°C, and 29°C ($n = 5$ for each temperature), and measurements were made over 25 min with computer-aided videomicroscopy [3, 4] while the embryos were immersed in a nonpermeating hypertonic solution (0.35 M NaCl).

Construction and Translation of the AQP3-GFP Fused Protein

Using universal primers T7 and M13 forward, AQP3 cDNA was amplified by polymerase chain reaction (PCR) from pSPORT containing a rat AQP3 insert (generously donated by G. Frindt, GenBank accession NM031703). The product was purified with QIAquick columns (Qiagen, Valencia, CA) and served as template in a subsequent PCR. A second PCR was used to add specific sequences to the 5' and 3' ends of AQP3 to permit ligation to GFP contained in a T3TS plasmid. The forward primer was 5'-GATCAGATCTCAC CATGGGTCGACAGAAGG-3' containing a *Bgl*II site (underlined), followed by the 4-base pair (bp) Kozak sequence and then 13 bp matching the 5' end of AQP3. The reverse primer was 5'-GATCCTGCAGGATCTGCTCCTTGTGC-3', containing a *Pst*I site (underlined) and 16 bp complementary to the 3' end of AQP3. While adding a cut site, the reverse primer also served to remove the stop codon of AQP3. GFP was amplified from T3TS plasmids containing fused AQP1-GFP. The forward primer was 5'-GATCCTGCAGGTGAGCAAGGGCGAGG-3', containing a *Pst*I site (underlined) and 16 bp matching the 5' end of GFP. The reverse primer was 5'-GATCACTAGTTTACTTGTACAGCTCGTCCATGC-3', containing a *Spe*I site (underlined) and 23 bp complementary to the 3' end of GFP. Purified AQP3 amplicons were digested with *Pst*I and *Bgl*II, and purified GFP amplicons were di-

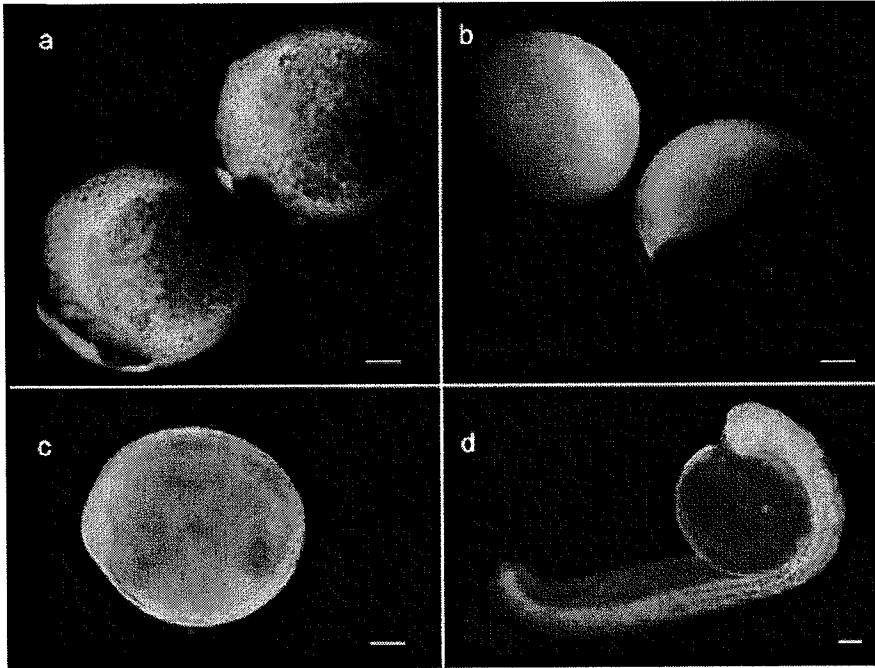


FIG. 2. Developmental expression of AQP3-GFP. During gastrulation, embryos expressed AQP3-GFP either in the yolk syncytial layer and blastoderm (a) or only in the blastoderm (b). Later in development at 100% epiboly, AQP3-GFP expression was evident throughout the embryo (c), and at 24 h, the expression is most dominant in the neuromusculature (d). As the embryos develop, the signal fades, and by 96 h it can only be seen in the lens (not shown). Bar = 100 μm .

gested with *Pst*I and *Spe*I. After digestion, the 3' end of AQP3 was ligated to the 5' end of GFP.

This fused construct was ligated into the open reading frame of *Spe*I- and *Bgl*II-digested T3TS, transformed into ULTRA MAX DHFT-competent cells (Gibco BRL, Life Technologies, Gaithersburg, MD), and plated. Colonies were picked and replicated, and a portion was boiled to provide a crude extract template for a PCR with T3 and T7 universal primers. Resulting PCR products of approximately 2000 bp indicated AQP3-GFP inserts and were verified by sequencing purified PCR products using one-quarter scale BigDye Terminator Cycle Sequencing reactions (Applied Biosystems, Foster City, CA) on a Model 377 sequencer (PE Biosystems, Foster City, CA). Colonies containing complete AQP3-GFP constructs were grown overnight in 5-ml cultures, and plasmid DNA was isolated using QIAprep Spin mini preps (Qiagen). Plasmids were linearized with *Xba*I and transcribed with an m-CAP kit (m-Message Machine; Ambion, Austin, TX). The resulting capped RNA was cleaned with an RNeasy T3 promoter kit (Qiagen) and resuspended in 30 μl of RNase-free water. RNA was quantified by its optical density at 260 nm with a lambda spectrophotometer (PE Biosystems). All restriction endonucleases and the ligase were obtained from New England Biolabs (Beverly, MA).

Water and Cryoprotectant Permeability Measurements

We examined the change in permeability of PG in zebrafish embryos with and without expressed AQP3-GFP because PG is nontoxic and likely to be particularly useful for zebrafish cryopreservation [3, 6]. Embryos at the one- to eight-cell stage do not have a barrier between the yolk and the blastoderm, and therefore nucleic acids placed into the yolk are rapidly transported to the blastoderm cells [27, 28]. The yolks of these embryos were microinjected with 1.3 ng of AQP3-GFP mRNA (in a 2-nl volume of RNase-free water) or 2 nL of RNase-free water (controls) [22, 28, 29] and placed into sucrose medium (10% Hanks buffer supplemented with sucrose to 360 mOsm). After 2 h, GFP fluorescence was visible in both the yolk and blastoderm in >80% of the embryos ($n > 3000$). At this point, experimental embryos were sorted for GFP expression, and only GFP-positive embryos were used in further experiments.

Whole AQP3-GFP-treated and control embryos at 50% epiboly were immersed for 5–90 min in a 2 M ^{14}C PG solution made up in a sucrose culture medium. After treatment, all embryos were rinsed twice in 2 M PG in sucrose culture medium for 1–2 min, and then the intact embryos were placed on blotting paper and immersed in 3 ml of scintillation cocktail (Packard UltimaGold; Packard Institute, Meridan, CT) and analyzed with a scintillation counter (Beckman-Coulter, Fullerton, CA). Disintegrations per minute were measured and converted to concentration of PG based on a dilution curve generated from known amounts of radiolabeled

PG. To determine whether PG entered the yolk, we separated the blastoderm from the yolk with microscissors at 50% epiboly. Isolated blastoderm tissue forms a sphere that remains cohesive and stable in the sucrose medium for several hours. AQP3-GFP-treated and control blastoderm spheres were removed from the yolk, immersed in the ^{14}C PG for 60 min, and treated as above.

The change in the water permeability (L_p) and cryoprotectant permeability (P_s) was analyzed using Micromath Scientist software (Micromath Corp., Salt Lake City, UT) using a one-compartment model [3, 4]. L_p and P_s were determined by numerically integrating the coupled transport equations and adjusting L_p and P_s until a best least-squares fit to the experimental data was obtained. For the control embryos, we used our previously determined value of L_p ($0.004 \mu\text{m min}^{-1} \text{atm}^{-1}$) for native embryos [4] and obtained a P_s of $8.4 \text{E}^{-6} \text{cm/min}$. AQP3-GFP-treated embryos yielded a P_s of $5.0 \text{E}^{-5} \text{cm/min}$ and an upper limit for L_p of $0.01 \mu\text{m min}^{-1} \text{atm}^{-1}$, because L_p is only poorly constrained by the data. L_p is generally lower in the presence of cryoprotectants, especially for zebrafish embryos [4]. Therefore, for modeling purposes, we employed a conservative estimate for L_p of $0.01 \mu\text{m min}^{-1} \text{atm}^{-1}$. To better define L_p , we measured shrinkage of AQP3-GFP-treated embryos in hyperosmotic medium (860 mOsm made up of 360 mOsm sucrose medium plus 0.25 M NaCl) over 10 min, yielding an $L_p = 0.081 \mu\text{m min}^{-1} \text{atm}^{-1}$.

To estimate whether cryoprotectant moved into the yolk or merely bound to its surface, we immersed 50% epiboly embryos (AQP3-GFP-treated and controls) in ^{14}C PG for 90 min. Embryos were rinsed four times in 2 M PG/sucrose medium and then placed into a fifth 2 M PG/sucrose medium supplemented with 0.25 M NaCl. The yolks were cut with microscissors, the blastoderm and residual membranes were removed, and a yolk sample from each embryo (unknown amount) was removed with a pipette and placed onto a filter and analyzed with a scintillation counter, as above. We conducted the experiment twice but did not average the results because the amount of yolk sampled per embryo was unquantifiable.

Statistical Analysis

All statistical analyses were conducted using StatView 4.5 (SAS Institute, Cary, NC) for the Macintosh. We used nonparametric tests for all comparisons because of small sample size and unknown underlying distributions. Kruskal-Wallis tests were employed when two or more groups were being compared. To compare uptake of PG over time for experimental groups and controls, we used simple regression statistics and then computed F values to compare the slopes of two regressions.

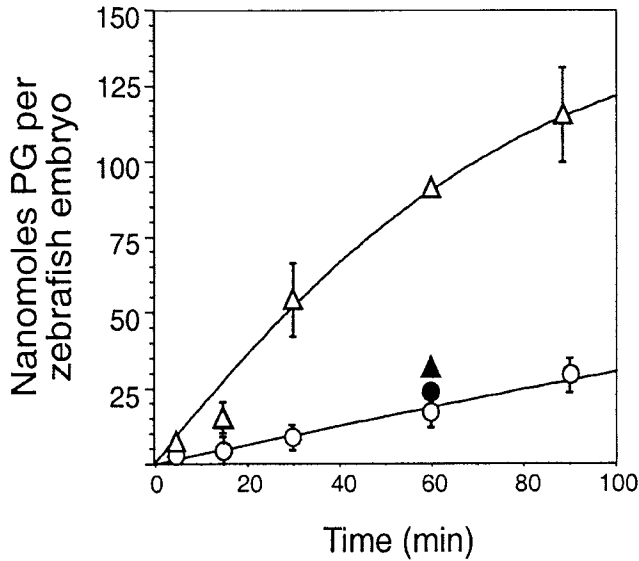


FIG. 3. Uptake of cryoprotectant into zebrafish embryos. AQP3-GFP-treated embryos (open triangles) display higher transport of the ¹⁴C PG than do the control embryos (open circles). Each open symbol is the mean ± SEM of three to five samples (n = 10–25 embryos/sample). Where the SEM bar is missing, the SEM was less than the size of the symbol. AQP3-GFP-treated (solid triangle) and control (solid circle) blastodermal spheres show approximately the same ¹⁴C PG uptake. Each solid symbol is the average of two samples, n = 20–30/sample. Thus, most of the ¹⁴C PG uptake in the whole embryo is present in the yolk. Lines connecting the open symbols represent least-squares fit to the data.

RESULTS

When exposed to a variety of different osmotic solutions, zebrafish embryos did not respond in a typical vertebrate fashion. Normal vertebrate cells increase in volume

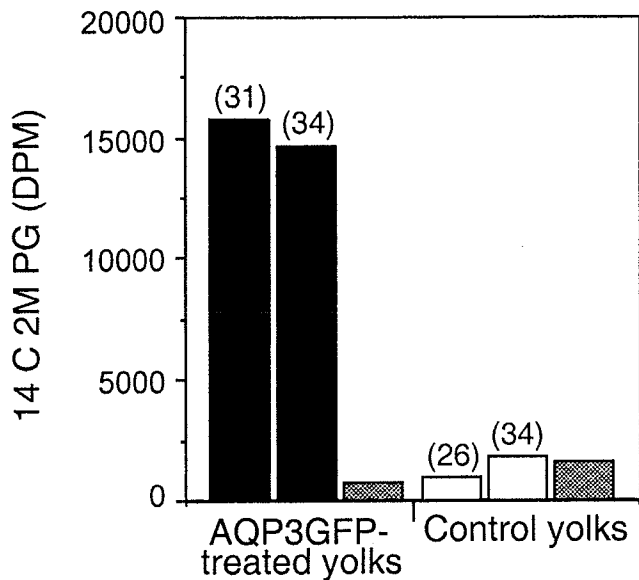


FIG. 4. Radiolabeled cryoprotectant is transported into the yolk. AQP3-GFP-treated yolk samples (solid bars) have a higher uptake of ¹⁴C PG than do the control yolk samples (open bars), suggesting that PG entered the yolk. Numbers in parentheses represent sample sizes. Shaded bars represent the background radioactivity in the final wash of the experimental and control groups.

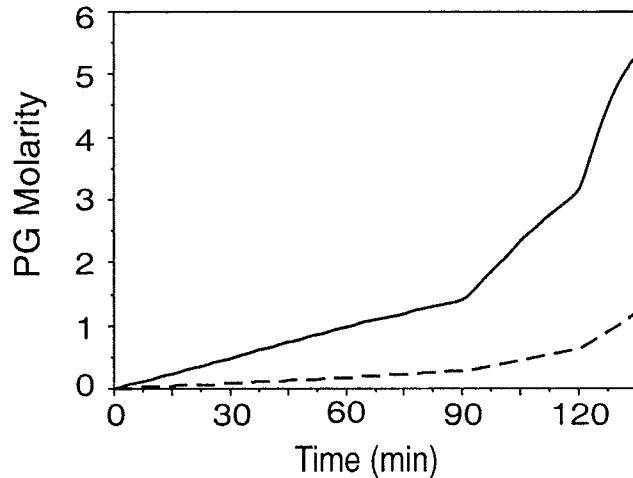


FIG. 5. Simulated response of embryos to a cryoprotectant loading protocol for vitrification. Knowing the permeability parameters (determined in Fig. 3), the cell response to vitrification solutions as a function of time may be modeled. Using the above determined permeability parameters, we simulated the embryo response to a cryoprotectant loading protocol in which embryos are incubated in solutions of 2, 4, and 6 M PG for 90, 30, and 15 min, respectively. Using this protocol, the control embryos (dashed line) only reached an internal concentration of about 1.2 M PG after 135 min, whereas the AQP3-GFP-treated embryos (solid line) reached an internal concentration of 5.4 M PG, well within the range of cell vitrification.

in hypoosmotic solutions and decrease in volume in hyperosmotic solutions [30]. The zebrafish embryos in a solution of ≤300 mOsm were unchanged in volume, whereas those in solutions of >300 mOsm decreased in volume (Fig. 1). Specifically, embryos immersed in hypoosmotic solutions (0 mOsm and 325 mOsm) showed the same volume as did control embryos (40 mOsm) (Kruskal-Wallis $H_2 = 0.95$, not significant). However, those embryos in the hyperosmotic solutions (670 mOsm and 1350 mOsm) and control embryos (40 mOsm) were significantly different in volume (Kruskal-Wallis $H_2 = 15.19$, $P < 0.05$) (Fig. 1). Thus, an asymmetry or rectification exists in zebrafish embryo membranes that allows water to leave readily but not to reenter.

Although zebrafish have a complex membrane mechanism controlling water movement, we verified that the embryos do not have AQPs. We examined the way water moves across the fish membranes at various temperatures and measured an E_a of 22 kcal/mol in zebrafish embryos. This indicated passive membrane diffusion rather than AQP-mediated transport.

We successfully introduced mammalian AQP3 into the zebrafish embryo. The AQP3 cDNA was fused to the GFP cDNA. GFP provided a phenotypic marker rapidly identifying embryos expressing the fusion product (AQP3-GFP). During development the AQP-GFP begins to be expressed within a few hours, as is evident by the strong green fluorescence in the blastoderm (Fig. 2, a and b). The blastoderm cells continue to divide, producing the YSL. At 100% epiboly, the RNA is still active, producing a bright fluorescent glow throughout the embryo (Fig. 2c). This glow suggests that the pores are uniformly distributed throughout the embryo at this stage. As the embryo develops for another 12 h, this pattern of growth and distribution of the water channels is maintained (Fig. 2d). Based on anatomical, developmental, and physiological criteria discussed previously

[3, 4], we produced normal larvae that expressed AQP3-GFP in somatic tissue throughout the embryo for up to 4 days. The long-term effect of these modifications on survival and reproduction is of crucial importance for the success of cryopreservation, and studies examining the full developmental effects are underway.

In exposing embryos to radiolabeled PG, our experiments demonstrated that AQP3-GFP-expressing embryos experienced higher rates of PG uptake than do control embryos (Fig. 3). Linear regression models revealed significant effects of time on amount of PG uptake into the embryos for both controls ($F_{(1,13)} = 23.23$, $P = 0.0003$, $R^2 = 0.61$, $Y = 2.30 + 0.28X$) and experimental embryos ($F_{(1,10)} = 88.77$, $P = 0.0001$, $R^2 = 0.89$, $Y = -1.79 + 1.37X$). However, the slopes of the two lines are significantly different ($F_{(1,23)} = 9.36$, $P < 0.01$), revealing the higher rate of uptake in AQP3-GFP-expressing embryos. To understand how L_p and P_s changed in the AQP3-GFP-treated embryos, ^{14}C PG uptake data were fit using our permeability model. These data suggest that the L_p and P_s of AQP3-GFP-treated embryos are approximately 6 times and 2.5 times greater than those values for the controls, respectively.

To cryopreserve embryos, the cryoprotectant must permeate both the blastoderm and the yolk. The AQP3-GFP-expressing spheres, control spheres, and control intact embryos displayed similar levels of ^{14}C PG uptake (Fig. 3). Although the blastoderm was expressing AQP3-GFP, its permeability to PG was virtually equal to the natural permeability of the control blastoderm. Therefore, the additional ^{14}C PG observed in the intact AQP3-GFP-treated embryos should represent the amount transported into the yolk. We further verified this result by directly measuring the amount of ^{14}C PG entering yolk samples (Fig. 4).

The changes that we observed in cryoprotectant and water permeability of the AQP3-GFP-treated embryos are modest. However, after modeling the embryo response, even modest changes in permeability produced great changes over time. After 135 min, the PG concentration determined in our model for AQP3-GFP-treated embryos was 5.4 M, well within the range found necessary for vitrification of cells (Fig. 5). These predictive models will aid in designing future toxicity and vitrification trials.

DISCUSSION

These results demonstrate that zebrafish embryos possess unique membrane properties that may inhibit cryopreservation but that expression of AQPs may overcome this obstacle. The zebrafish membrane may be the first true example of a rectifying membrane. Rectifying membranes were previously described in RBCs [31], but those results are now considered measurement error [32]. Currently, the physiological mechanism for membrane rectification is unknown. We hypothesize that the presence of rectifying membranes is a fundamental feature that allowed the radiation of fish into freshwater. Vertebrates first evolved in the ocean [33, 34], and many have an isosmotic point of around 300 mOsm. As a result, freshwater fish are faced with a continuous swelling stress. Osmoregulation in adult fish is regulated by the skin, gill, and kidneys [35]; however, freshwater embryos do not have these organs. The presence of rectifying membranes could allow embryos to osmoregulate by restricting water transport into cells. Clearly, additional studies on membrane permeability of other freshwater fishes are needed to determine whether rectifying membranes are widespread throughout the group.

Regardless of their current or historic role, the presence of rectifying membranes combined with the YSL (restricting both water and cryoprotectant transport) may explain the difficulty encountered when trying to cryopreserve fish embryos with conventional methods. By modifying the membranes with AQP3-GFP, we were able to change the permeability of the membrane to both water and cryoprotectants. Although these changes are modest, we believe they are sufficient for cryopreservation. Our results indicate that radiolabeled PG diffused into the yolk. Our previous studies [2–4] indicated that the YSL blocked cryoprotectant entry into the yolk. Thus, our results suggest that AQP3-GFP channels within the YSL now permit the movement of cryoprotectants into the yolk. This finding is crucial because one of the main barriers to fish embryo cryopreservation is that little or no cryoprotectant can cross the YSL, and cryodamage results [2–4]. Clearly, this barrier has been overcome with these methods.

Although water channels have been described and studied in frog oocytes [15, 22], the use of these channels in embryos and the application of these techniques to cryopreservation have not been previously described and will have a profound effect on genome resource banking. Using mRNA to modify the fish's membranes produces transient expression in somatic cells that will have no impact on the germline. These types of somatic changes are ideal for genomic resource banking because only the stored embryos in the bank will bear these modifications.

The cryopreservation of teleost embryos will greatly affect the conservation of rare or threatened species, medical research, and aquaculture. For conservation, the development of frozen embryo technology would permit the creation of ex situ populations to preserve genetic diversity and assist in the prevention of extinctions in natural populations [36–38]. The zebrafish has become a widely used vertebrate model in medical research for developmental and genetic studies. Genetically characterized strains of zebrafish are being used to understand gene function, with applications to human disease. These genetic lines are maintained in live culture and thus are susceptible to loss or modification and are expensive to maintain. Fish is also an essential protein source for much of the world's population. Many fish species used in aquaculture spawn only once each year. The ability to routinely cryopreserve fish embryos would contribute to more efficient, cost-effective, and year-round fish production. This technology could aid in the ex situ conservation of the almost 46 000 species of fish, birds, amphibians, and reptiles that have yet to be cryopreserved.

ACKNOWLEDGMENTS

We thank P. Mazur, G.M. Preston, D. Erwin, and P. Agre for discussion, C. McIntosh for technical assistance, R. Wilkerson for the use of laboratory space, S. Ekker, J. Larson, and B.J. Baum for the generous gift of plasmids and for technical advice, G. Frindt for the generous gift of the AQP3, D. Wildt for reviewing the manuscript, and three anonymous reviewers for improving earlier versions of the manuscript.

REFERENCES

1. Rall WF. Advances in the cryopreservation of embryos and prospects for the application to the conservation of salmonid fishes. In: Cloud JG, Thorgaard GH (eds.), *Genetic Conservation of Salmonid Fishes*. New York: Plenum; 1993: 137–158.
2. Hagedorn M, Hsu EW, Pilatus U, Wildt DE, Rall WF, Blackband SJ. Magnetic resonance microscopy and spectroscopy reveal kinetics of cryoprotectant permeation in a multicompartmental biological system. *Proc Natl Acad Sci U S A* 1996; 93:7454–7459.
3. Hagedorn M, Kleinhans FW, Freitas R, Liu J, Hsu E, Wildt DE, Rall

- WF. Water distribution and permeability of zebrafish embryos, *Brachydanio rerio*. *J Exp Zool* 1997; 278:356–371.
4. Hagedorn M, Kleinhans FW, Artemov D, Pilatus U. Characterization of a major permeability barrier in the zebrafish embryo. *Biol Reprod* 1998; 59:1240–1250.
 5. Zhang T, Rawson DM. Studies on chilling sensitivity of zebrafish (*Brachydanio rerio*) embryos. *Cryobiology* 1995; 32:239–246.
 6. Zhang T, Rawson DM. Feasibility studies on vitrification of intact zebrafish (*Brachydanio rerio*) embryos. *Cryobiology* 1996; 33:1–13.
 7. Zhang T, Rawson DM, Jongebloed WL. Further studies on zebrafish (*Brachydanio rerio*) embryo membranes: permeability and ultra-structure. *Cryo Lett* 1998; 65.
 8. Janik M, Kleinhans FW, Hagedorn MM. Overcoming a permeability barrier by microinjecting cryoprotectants into zebrafish embryos (*Brachydanio rerio*). *Cryobiology* 2000; 41:25–34.
 9. Leibo SP. Water permeability and its activation energy of fertilized and unfertilized mouse ova. *J Membr Biol* 1980; 53:179–188.
 10. Ruffing NA, Steponkus PL, Parks JE. Osmotic behavior, hydraulic conductivity, and incidence of intracellular ice formation in bovine oocytes at different developmental stages. *Cryobiology* 1993; 30:562–580.
 11. Lin TT, Pitt RE, Steponkus PL. Osmotic behavior of *Drosophila melanogaster* embryos. *Cryobiology* 1989; 26:453–471.
 12. Agre P, Preston GM, Smith BL, Jung JS, Raina S, Moon C, Guggino WB, Nielsen S. Aquaporin CHIP: the archetypal molecular water channel. *Am J Physiol* 1993; 265:F463–F476.
 13. Chrispeels MJ, Agre P. Aquaporins: water channel proteins of plant and animal cells. *Trends Biochem Sci* 1994; 421–425.
 14. King LS, Agre P. Pathophysiology of the aquaporin water channels. *Annu Rev Physiol* 1996; 58:619–648.
 15. Verkman AS, Van Hoek AN, Ma T, Frigeri A, Skach WR, Mitra A, Tamarappoo BK, Farinas J. Water transport across mammalian cell membranes. *Am J Physiol* 1996; 270:C12–C30.
 16. Wintour EM. Water channels and urea transporters. *Clin Exp Pharmacol Physiol* 1997; 24:1–9.
 17. Lee MD, King LS, Agre P. The aquaporin family of water channel proteins in clinical medicine. *Medicine* 1997; 76:141–156.
 18. Heymann JB, Agre P, Engel A. Progress on the structure and function of aquaporin 1. *J Struct Biol* 1998; 121:191–206.
 19. Verkman AS. Physiological importance of aquaporins: lessons from knockout mice. *Curr Opin Nephrol Hypertens* 2000; 9:517–522.
 20. Wellner RB, Hoque ATMS, Goldsmith CM, Baum BJ. Evidence that aquaporin-8 located in the basolateral membrane of rat submandibular gland acinar cells. *Pflug Arch Eur J Physiol* 2000; 441:49–56.
 21. Hurley PT, Ferguson CJ, Kwon TH, Andersen ML, Norman AG, Steward MC, Nielsen S, Case RM. Expression and immunolocalization of aquaporin water channels in rat exocrine pancreas. *Am J Physiol Gastrointest Liver Physiol* 2001; 280:G701–G709.
 22. Preston GM, Carroll TP, Guggino WB, Agre P. Appearance of water channels in *Xenopus* oocytes expressing red cell CHIP28 protein. *Science* 1992; 256:385–387.
 23. Yang B, Verkman AS. Water and glycerol permeabilities of aquaporins 1–5 and MIP determined quantitatively by expression of epitope-tagged constructs in *Xenopus* oocytes. *J Biol Chem* 1997; 272:16140–16146.
 24. Ishibashi K, Kuwahara M, Gu Y, Tanaka Y, Marumo F, Sasaki S. Cloning and functional expression of a new aquaporin (AQP9) abundantly expressed in the peripherical leukocytes permeable to water and urea, but not to glycerol. *Biochem Biophys Res Commun* 1998; 244:268–274.
 25. Echevarria M, Windhager EE, Frindt G. Selectivity of the renal collecting duct water channel aquaporin-3. *J Biol Chem* 1996; 271:25079–25082.
 26. Edashige K, Sakamoto M, Kasai M. Expression of mRNAs of the aquaporin family in mouse oocytes and embryos. *Cryobiology* 2000; 40:171–175.
 27. Westerfield M. *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Brachydanio rerio)*. Eugene, OR: University of Oregon Press; 1993.
 28. Hyatt TM, Ekker SC. Vectors and techniques for ectopic gene expression in zebrafish. *Methods Cell Biol* 1999; 59:117–126.
 29. Hoque AT, Liu X, Kagami H, Swaim WD, Wellner RB, O'Connell BC, Ambudkar IS, Baum BJ. Construction and function of a recombinant adenovirus encoding a human aquaporin 1-green fluorescent protein fusion product. *Cancer Gene Ther* 2000; 7:476–485.
 30. Guyton AC. *Textbook of Medical Physiology*, 6th ed. Philadelphia, PA: W.B. Saunders; 1981.
 31. Finkelstein A. *Water Movement Through Lipid Bilayers, Pores, and Plasma Membranes: Theory and Reality*, vol. 4. New York: John Wiley; 1987.
 32. Farmer R, Macey R. Perturbation of red cell volume: rectification of osmotic flow. *Biochim Biophys Acta* 1970; 196:53–65.
 33. Shu DG, Chen L, Han J, Zhang XL. An Early Cambrian tunicate from China. *Nature* 2001; 411:472–473.
 34. Holland ND, Chen JY. Origin and early evolution of the vertebrates: new insights from advances in molecular biology, anatomy and paleontology. *Bioessays* 2001; 23:142–151.
 35. Romer AS, Parsons TS. *The Vertebrate Body*, Philadelphia, PA: W.B. Saunders; 1977.
 36. Ballou JD. Potential contribution of cryopreserved germ plasma to the preservation of genetic diversity and conservation of endangered species in captivity. *Cryobiology* 1992; 29:19–25.
 37. Wildt DE. Genetic resource banking for conserving wildlife species: justification, examples and becoming organized on a global basis. *Anim Reprod Sci* 1992; 28:247–257.
 38. Wildt DE, Seal US, Rall WF. Genetic resource banks and reproductive technology for wildlife conservation. In: Cloud JG, Thorgaard GH (eds.), *Genetic Conservation of Salmonid Fishes*. New York: Plenum; 1993: 159–173.