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Overcoming a Permeability Barrier by Microinjecting Cryoprotectants into Zebrafish Embryos (*Brachydanio rerio*)

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The goal of this research was to examine the developmental effects on zebrafish embryos (*Brachydanio rerio*) when cryoprotectants were directly microinjected into the yolk. Our objectives were to: (i) determine the final concentration of propylene glycol (PG) and dimethyl sulfoxide (Me_2SO) that the embryos could tolerate without causing teratogenic effects; (ii) determine if the toxicity of Me_2SO could be reduced by the simultaneous presence of various proportions of amides; and (iii) examine whether this intracellular cryoprotectant incorporation could reduce the cryodamage to the yolk syncytial layer (YSL) after vitrification trials. The rationale for conducting these microinjection experiments was to overcome the permeability barrier of the YSL. Intracellular PG produced better survival than Me_2SO ($P < 0.05$). Embryos tolerated both 10- and 30-nl microinjections of PG, yielding final concentrations of 2.3 and 5.0 M within the yolk, resulting in 70 ± 3 and 35 ± 4% survival at day 5, respectively. In similar experiments with Me_2SO, survival was lower than PG at 60 ± 4 and 14 ± 4% at 2.4 and 5.2 M. Unlike other cellular systems, the presence of amides, specifically acetamide or formamide, did not reduce the toxicity of Me_2SO in zebrafish embryos ($P > 0.05$). During vitrification trials, we estimated a 25% dehydration of the yolk, yielding an effective PG concentration of 5.9 M. However, the incorporation of this vitrifiable concentration of PG was not sufficient to improve the postthaw morphology of the YSL ($P > 0.05$). Clearly, other factors need to be examined in establishing a successful vitrification protocol for zebrafish embryos.

Key Words: cryopreservation; teleosts; zebrafish; cryoprotectants; yolk syncytial layer; vitrification; propylene glycol; dimethyl sulfoxide; formamide; acetamide.

Fish embryos have never been cryopreserved. For the most part, this is due to their large embryo size (ca. 800 μm), a complex multicompartmental system (i.e., the yolk and the blastoderm), discrete membrane permeability properties that differ substantially between compartments (14), and a high sensitivity to chilling injury (12, 37). Successful cryopreservation of fish embryos would benefit the preservation of thousands of fish species. Most importantly, it would enable the establishment of genome resource banks. A frozen collection of embryos would act as an insurance policy to reduce the threat of extinction and aid in the preservation of rare and endangered fishes (34, 35), thus maintaining genetically valuable lines. Additionally, this technology would give aquaculturalists the opportunity to obtain frozen embryos during seasons when certain species do not naturally spawn.

In this study, we used the zebrafish embryo (*Brachydanio rerio*) as our model to understand the developmental effects that long-term exposure to cryoprotectant solutes would have on normal development. Zebrafish were selected because of their relative ease of maintenance, prolific production of embryos, and their well-characterized, rapid development (33). During early epiboly, the blastoderm cells envelop the yolk and the developing yolk syncytial layer (YSL) (4). The YSL creates a permeability barrier between the yolk and the blastoderm, which minimizes the entry and exit of most cryoprotectants (Fig. 1) (11). If zebrafish embryos are frozen without taking this cryoprotectant bar-

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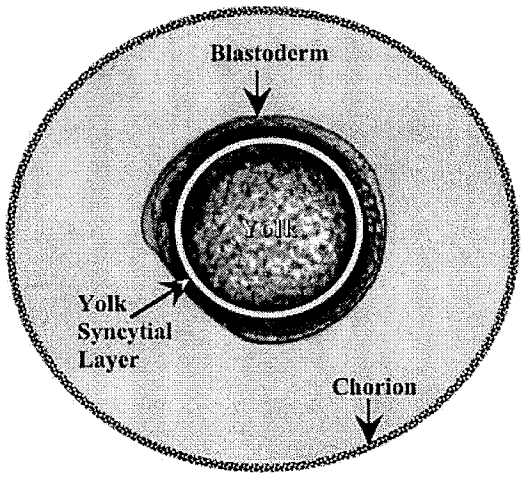

FIG. 1. The compartments of the zebrafish embryo are the yolk, blastoderm, yolk syncytial layer (YSL), and chorion. The YSL is ca. 10 μm thick and is depicted here as a white ring only to demonstrate its position between the yolk and the blastoderm. The chorion was enzymatically removed in all of the preparations in this paper.

rier into consideration, lethal damage to the embryo results (14).

Prevention of ice crystal damage in the cells or tissue is necessary for successful cryopreservation. There are two methods of cryopreservation, slow and rapid cooling. In slow cooling, cells are cooled at a controlled rate, resulting in an increase in the osmolality of the extracellular solution, which leads to cell dehydration (20). This process is facilitated by the addition of a cryoprotectant (20). The second method, vitrification, is a rapid freezing process which requires cells to be exposed to a viscous, highly concentrated vitrifying solution. This exposure significantly dehydrates the cell such that cytoplasmic and extracellular solutes vitrify (23, 27). Cells are dehydrated prior to cooling and then plunged directly into liquid nitrogen or other coolants. Due to the unusually high concentrations of cryoprotectants in the vitrification medium, ice crystals are inhibited and the solution goes directly into a glass or vitreous state (6, 17).

Vitrification appears to be an attractive method for cryopreservation of zebrafish embryos because it reduces the ultrastructural damage to various cells caused by intra- and extracellular ice crystal formation (16, 24) and removes the deleterious effects of chilling sensitivity (12, 37). Additionally, vitrification may be more suitable for multicellular organisms because the freezing protocol does not have to be optimized by cell type, as is the case in slow freezing (9). All of these issues were faced in attempting to cryopreserve the *Drosophila* embryo (*Drosophila melanogaster*) and were overcome by vitrification (21, 28, 29). Although vitrification has many advantages, selection of a vitrification solution is difficult, because the highly concentrated solutions have undesired toxic or teratogenic effects (6, 7, 9).

A number of studies have examined the toxicity of external cryoprotective agents on aquatic embryos. In many of these systems, either dimethyl sulfoxide (Me_2SO) or propylene glycol (PG) are the least toxic of various cryoprotectants tested in the following embryos: rainbow trout *Oncorhynchus mykiss,* medaka *Oryzias latipes,* and pejerrey *Odonthestes bonariensis* (30); red drum *Sciaenops ocellatus* (23); rotifer *Brachionus plicatilis* (32); rosy barb *Puntius conchonius* (1); blue mussel *Mytilus edulis* (31); and zebrafish embryos (13, 38). However, it was unknown what effect these two cryoprotectants might have when introduced intracellularly for longer periods of time (i.e., hours to days). This is because once the cryoprotectant is microinjected into the yolk, it has little permeability to exit (14). Fahy and colleagues have examined the toxic effect of Me_2SO on complex cellular systems. They found that the addition of an amide to Me_2SO mitigated its toxicity (3, 5, 6, 9). As a result, investigators have used amides to reduce the toxicity of Me_2SO in a variety of systems, such as rat kidney renal cortex (3), rabbit renal cortex (5, 6), porcine blastocysts (36), rat blastocysts (19), bovine oocytes (10), mouse oocytes (22, 24), and mouse embryos (23). Thus it seemed promising to use amides in zebrafish embryos.

The goal of this developmental research was to examine the teratogenic effects on zebrafish embryos (*B. rerio*) when cryoprotectants were directly microinjected into the yolk. Specifically, our objectives were to: (i) microinject PG, Me_2SO, or Me_2SO/amide combinations into the

yolk of zebrafish embryos at 100% epiboly, measuring toxic or teratogenic effects of these solutes on the normal development of the embryos to day 5; (ii) examine the ability of amides to reduce the toxicity of Me_2SO; and (iii) examine if cryoprotectant incorporation would improve the morphology of the YSL after freezing.

MATERIALS AND METHODS

Animal Care and Culture

All animals were maintained for breeding as described in Westerfield (33). Briefly, 10–15 zebrafish were kept in 40-liter aquaria, with recirculated water maintained at a temperature of 28.5°C, a pH of 7.0, and a conductivity of 280 μS/cm. Animals were fed dried copepods and krill (Argent Laboratories, Redmond, WA, U.S.A.) twice daily and kept on an 11-h light: 13-h dark cycle. Females spawned in response to the photoperiod, producing ca. 50–100 eggs per female. Fertilized embryos were siphoned from the tanks and transferred into a collecting dish. The chorions were removed by enzymatic digestion using a 1% protease solution (Sigma Chemical Co., St. Louis, MO, U.S.A.) and maintained in embryo medium (EM), an ionic buffer solution (0.040 Osm) as described in Westerfield (33). Dechorionated embryos were placed in an incubator at 28.5°C and cultured until 100% epiboly. All solutions were made up in EM and experiments were carried out over many months.

Treatments and Solutions

Because two of the most nontoxic cryoprotectants do not permeate the yolk of the zebrafish (11), our goal in these experiments was to examine the intracellular toxicity of various cryoprotectants, at two concentrations, after they had been microinjected into the yolk. The intracellular toxicity was tested by microinjecting cryoprotectants into the yolk (n = 100). We tested PG, Me_2SO, or varying Me_2SO/amide combinations. Although the yolk is an anucleate cell that does not undergo cell division, it is actively metabolizing nutrients and providing the blastoderm with necessary components for proper growth and development. Each cryoprotectant was tested using both a 10- and a 30-nl injection. A matching number of control embryos were injected with EM. There were three treatment groups. The first treatment compared PG versus Me_2SO. Ten or 30 nl of a PG stock solution (13.6 M; Sigma Chemical Co.) or Me_2SO stock solution (14.1 M; Sigma Chemical Co.) was injected into the yolk, and then embryo development and viability were assessed at day 5. The second and third treatments examined the toxicity of Me_2SO in combination with different proportions from 0 to 100% (v/v) of either formamide or acetamide. Fourteen molar formamide (Sigma Chemical Co.) and acetamide (Sigma Chemical Co.) stock solutions were prepared by mixing 56 ml of formamide with 44 ml of EM and 24.81 g acetamide in a final volume of 30 ml EM, respectively. With these amide concentrations, it was possible to maintain an approximately 14 M cryoprotectant mixture for toxicity measurements, independent of the proportion of amide mixed with Me_2SO. The molar concentration of cryoprotectant within the yolk after injection was calculated using previously determined parameters for the water content of the yolk and the blastoderm (14) (Table 1).

Microinjection

A Flaming–Brown P-77 micropipette puller (Sutter Instrument Co., Novato, CA, U.S.A.) was used to pull microinjection pipette tips (aluminosilicate capillary tubing, 1.2 mm o.d. × 0.86 mm i.d.; FHC, Brunswick, ME, U.S.A.). A stereomicroscope at 1000× (Wild, Heerbrugg, Switzerland) was used to cut the micropipette tips to a uniform 20 μm diameter in order to ensure even volume delivery to all embryos. Solutions were loaded, along with a small quantity (<1 ng) of an indicator dye used for visual accuracy of injection technique (Fast Green FCF; Sigma Chemical Co.), into the pipette tips using a micropipette-filling device (MicroFil; World Precision Instruments, Sarasota, FL, U.S.A.). A pressure injector and backpressure unit (Applied Scientific Instrumentation Inc.,

TABLE 1
Zebrafish Embryo and Solution Parameters[a]

Parameter	Value	Comments
M_{Me_2SO}	14.1 M	Concentration of Me_2SO injected
M_{PG}	13.6 M	Concentration of PG injected
V_{CPAi}	10 nl	Volume of cryoprotectant injected
	30 nl	
V_y	87.1×10^6 μm^3	Volume of the yolk[b]
V_w	0.59	Fraction of water in the yolk[b]
V_{yw}	51.4 nl	Volume of yolk water[b]
c_0	1	Constant (assuming no dehydration)
c_{25}	0.75	Constant (assuming a 25% dehydration of the embryo while immersed in cryoprotectant solution)

[a] Experiments were carried out on embryos at 100% epiboly.
[b] Data from (14).

Eugene, OR, U.S.A.) was used to deliver the solutions into the embryo's yolk.

Successful injections were determined using the following criteria: a smooth lateral injection into the yolk with no invasion into the blastoderm, consistent distribution of injected material throughout the yolk, and no visible leakage from the injection site. Embryos that met these criteria were placed into a fresh agar coated petri dish with EM and penicillin–streptomycin–neomycin (0.15 mg/ml penicillin, 0.25 mg/ml streptomycin, 0.5 mg/ml neomycin; Sigma Chemical Co.) and were incubated at 28.5°C. Embryos were examined daily, necrotic embryos were removed, and intact embryos were washed with fresh EM and returned to the incubator.

Analysis of Surviving Animals

All embryos were examined and placed into one of two categories: (i) normal development to day 5 (surviving) or (ii) abnormal development at day 5 (scored as not surviving). In order to evaluate normal development, animals were assessed for the following normal morphological and physiological features: proper cardiac development, eye development, tail musculature (i.e., no deformities), the presence of pectoral fins, proper jaw morphology, and the presence of a functional swim bladder (33). Embryos that met all of these criteria were placed in the surviving category those that did not were placed in the nonsurviving category.

Vitrification and EM Analysis

Vitrification techniques destroy the morphology of the YSL (14), presumably because there is little or no cryoprotectant within the yolk or the YSL, resulting in cryodamage. Therefore, to determine whether increasing the concentration of cryoprotectant within the yolk would improve the morphology of the YSL, we vitrified embryos following precise vitrification methods (with slight modifications; 14). Briefly, embryos at 100% epiboly were microinjected with a 30-nl volume of PG ($n = 4$) or an equal volume of EM ($n = 6$) and incubated for 30 min to recover. After the cryoprotectant had been injected, the embryos were immersed in 2 M PG, 3 M PG, and 6 M PG (5 min each); transferred into 8 M PG; drawn into 0.25-ml straws; cooled in liquid nitrogen vapor (3 min); and plunged directly into liquid nitrogen (1 min). After freezing, embryos were removed, held at room temperature (22–23°C) for 10 s, and plunged into water (22°C) until thawed (approximately 10 s) and immediately diluted into 6 M PG (5 min) followed by 4 M PG (3 min). The embryos were not returned to isotonic conditions, but fixed at 4 M PG because they could not tolerate the osmotic gradient at lower concentrations and fell apart. In preparation for EM analysis, embryos were placed into a fixative (2.5% glutaraldehyde, 1% paraformaldehyde in cacodylate buffer) at 22°C and then at 4°C. Thin sections were cut from the center of the embryo, mounted onto copper grids, stained with lead citrate, and then examined under a transmission electron mi-

TABLE 2
Estimated Concentration of Yolk Intracellular Cryoprotectant after Microinjection

Cryoprotectant	10 nl	30 nl
No dehydration		
PG	2.3 M	5.0 M
Me_2SO	2.4 M	5.2 M
25% dehydration[a]		
PG	2.9 M	5.9 M
Me_2SO	3.0 M	6.2 M

[a] This is the estimate of the dehydration (loss of water) in the yolk during preparation for, but prior to vitrification.

croscope (Zeiss EM10 CA). Cellular morphology of the YSL was evaluated according to Hagedorn *et al.* (14) in five sections per embryo. A random area of the YSL was selected and scored according to the integrity of cells, the presence or absence of nuclear membranes, appearance of organelles, and the presence of yolk droplets.

Statistical Analysis

A one-way analysis of variance was performed to compare the relationship between the survival of each treatment: PG, Me_2SO, Me_2SO/amide, and their respective controls in both the 10-nl and the 30-nl injected groups. For the Me_2SO/amide treatments we used a multiple comparison procedure to examine differences between means (SAS, Ver. 6, 1989; SAS Institute, Cary, NC, U.S.A.). The Mann–Whitney U-test was used to compare the scored morphological criteria between the experimentals and controls in the vitrification trials.

RESULTS

In order to cryopreserve fish embryos successfully, it is necessary to overcome the permeability barrier of the YSL to most cryoprotectants. In this first set of experiments, we analyzed how much cryoprotectant the injected embryos could tolerate after injection into the yolk. Using conservative estimates for yolk volume dehydration, we calculated the final molar concentration of cryoprotectant present in the yolk (M_{CPAy}) after microinjection of 10 or 30 nl (Table 2).

$$M_{CPAy} = \frac{V_{CPAi} \times M_{CPA}}{(cV_{yw}) + V_{CPAi}},$$

where V_{CPAi} is the volume of cryoprotectant injected into the yolk, M_{CPA} is the molar concentration of the cryoprotectant injected, c is the fraction of water remaining in the embryo after dehydration treatment, and V_{yw} is the estimated yolk water volume.

Assuming no dehydration ($c = 1$), the 10-nl injections loaded the cell with 2.3 M PG and 2.4 M Me_2SO while the 30-nl injections loaded the cell with 5.0 M PG and 5.2 M Me_2SO (Table 2). However, if we calculated the intracellular cryoprotectant concentration within the yolk after moderate yolk dehydration (ca. 25% water removal which might occur prior to freezing in cryopreservation experiments; $c = 0.75$), then the cryoprotectant concentration within the yolk increased to 5.9 M PG and 6.2 M Me_2SO, for 30-nl injections of cryoprotectant (Table 2).

In our first treatment, we compared the toxicity of PG and Me_2SO. Because the YSL acts as a barrier for cryoprotectant exit, as well as entry (11), the concentration of injected cryoprotectant diminishes little with time. At the higher intracellular cryoprotectant concentrations, PG (5 M) was less toxic or teratogenic than Me_2SO (5.2 M) ($P < 0.05$), producing 2.5 times the survival for PG (35 ± 4%) compared to Me_2SO (14 ± 4%; Fig. 2A). However, the introduction of either cryoprotectant into the yolk reduced survival of the embryos, compared

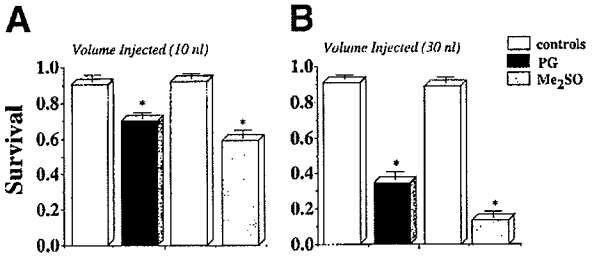

FIG. 2. Survival of zebrafish embryos after microinjection with either (A) 10-nl or (B) 30-nl volumes of PG or Me_2SO. An asterisk indicates a significant difference between the two PG and Me_2SO treatments ($P < 0.05$).

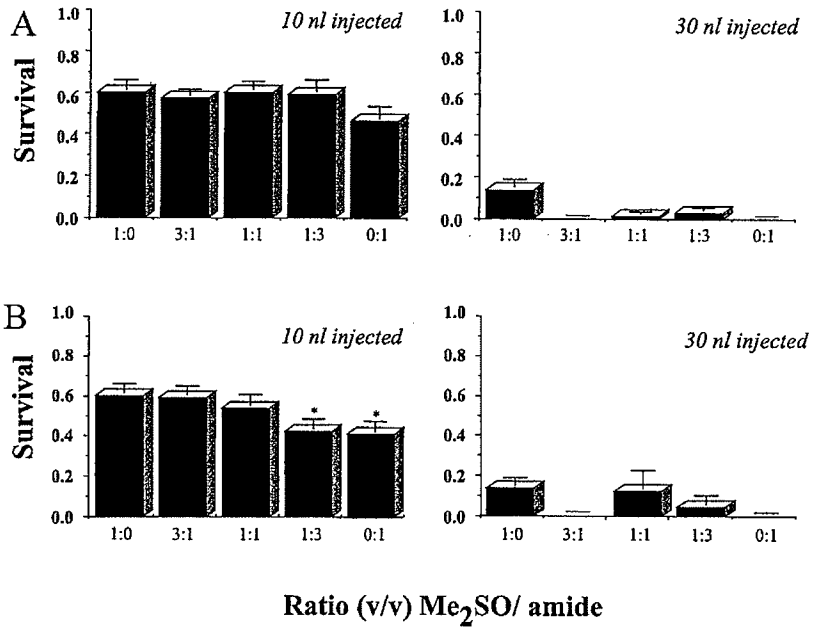

FIG. 3. Survival of zebrafish embryos after microinjection with 10 or 30 nl of a cryoprotectant mixture of (A) Me_2SO/acetamide or (B) Me_2SO/formamide. Me_2SO/amide proportions ranged from 4:0 to 0:4 (v/v). All solutions were ca. 14 M upon injection. An asterisk indicates a significant difference between pure Me_2SO (4:0) and Me_2SO/amide treatments.

to that of the controls ($P < 0.05$; Fig. 2). At the lower intracellular cryoprotectant concentrations, both PG (2.3 M) and Me_2SO (2.4 M) yielded good survival (70 ± 3% for PG; 60 ± 4% for Me_2SO) with PG, again proving to be less teratogenic than Me_2SO ($P < 0.05$; Fig. 2B). None of the cryoprotectant solutions used were immediately lethal to the embryos, suggesting that their effect became damaging over time.

We injected our control embryos with EM because deionized water caused osmotic problems leading to a blackened, distorted yolk. Our controls demonstrated that the damage to the embryos was not a result of increased yolk volume or mechanical disruption, with 92 and 89% survival for the 10- and 30-nl injection volumes, respectively ($P > 0.05$).

In our second and third treatments, neither acetamide nor formamide diminished the toxicity of the intracellular Me_2SO (Fig. 3). Using the 10-nl injection of Me_2SO/amide, we found the survival was similar (41–59%) to that of embryos treated with Me_2SO alone. However, a slight decrease in survival was observed as the proportion of formamide increased ($P < 0.05$; Fig. 3A), whereas no decrease in survival was observed with acetamide ($P > 0.05$; Fig. 3B).

Most importantly, we were interested in knowing whether the presence of cryoprotectants within the yolk might lead to an improvement in the morphology of the YSL after vitrification. PG was used in these experiments, both because it proved to be less damaging to the embryos than Me_2SO and for comparison with the previous YSL damage studies of Hagedorn *et al.* (14). The 30-nl volume injection of PG produced a concentration of 5.9 M under dehydrating conditions (Table 2). However, no difference was observed in the morphology of the YSL between the controls (injected with EM and frozen) and experimentals (injected with PG and frozen; Fig. 4; $P > 0.05$). In both treatments, mitochondria were damaged, ribosomes were scattered and disorganized, and the plasma membrane of the YSL was disrupted.

DISCUSSION

The YSL prevents sufficient incorporation of cryoprotectant into the yolk when placed in

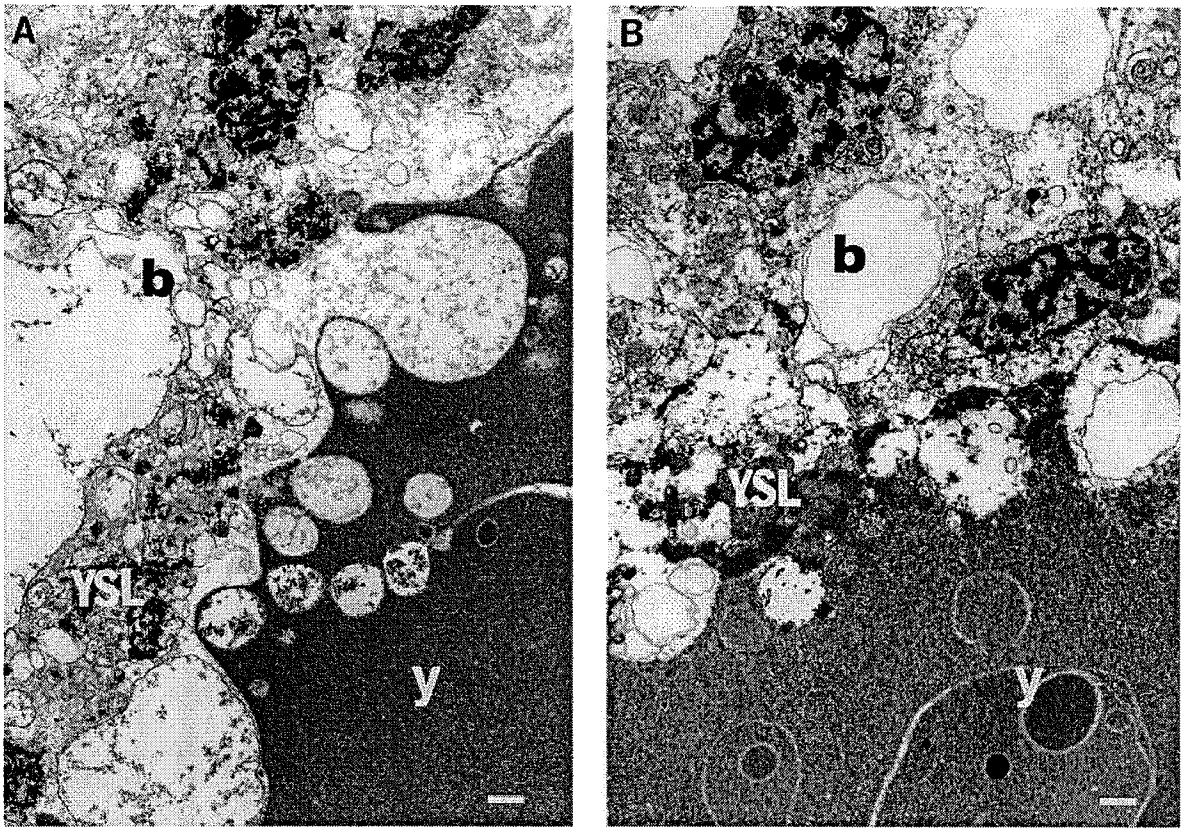

FIG. 4. Adding a potentially vitrifiable concentration of cryoprotectant to the yolk of the zebrafish is not sufficient to protect the yolk syncytial layer (YSL) from cryodamage. Electron micrographs of (A) control embryo (injected with embryo medium) and (B) experimental embryo (injected with 5.9 M PG; see Table 2) show identical damage to the YSL ($P > 0.05$). The blastoderm (b) is in the upper portion and the yolk (y) is in the lower portion of both images. Bar, 1 μm.

solution (11). To overcome the YSL permeability barrier, we directly microinjected cryoprotectants into the yolk. Our objective was to find a balance between providing adequate cryoprotection for the yolk, without causing lethal or teratogenic damage to the cell.

Zebrafish embryos can tolerate up to 5.0 M PG incorporated into the yolk and still show over 30% survival. Therefore, we found PG to be a better choice for possible vitrification of zebrafish embryos, because it is less damaging than Me_2SO. Zhang and Rawson (38) also suggest PG as a preferable external cryoprotectant for vitrification of zebrafish embryos, because they tolerated exposure to a 3 and 5 M PG solution for 30 min. However, their experiments were carried out with the chorions intact, and there is some question as to how much cryoprotectant actually permeated this layer.

Although the permeability of the YSL is very low to the exit of cryoprotectants at 100% epiboly (14), we do not know how the permeability of this syncytium changes with further development. Moreover, cells differentiate and epidermis forms, most likely changing the permeability of the embryo substantially. The developing embryo can tolerate the presence of cryoprotectants in the yolk, presumably because the rapidly dividing blastoderm is not exposed to the same high concentration as the nondividing yolk. Once the cryoprotectant is injected, it diffuses evenly throughout the yolk (ca. 15 min; 11). The blastoderm cells, on the other hand, slowly absorb small yolk droplets over many days. This process prevents immediate exposure of blastoderm cells to the cryoprotectants and, instead, introduces small amounts of the cryoprotectant over an extended period of time.

Once incorporated by the blastoderm cells, these cryoprotectants are permeable, and it is possible that they exit the cells via diffusion (11). Additionally, Klyachko *et al.* (18) found that there is an uneven distribution of enzymes present in early developing embryos of bony fish (*Misgurnus fossilis*). The blastoderm possesses almost 80% of the enzymes necessary for proper development. Since enzymes are often the target of cryoprotectant toxicity (1, 2, 3, 9), we speculate that the high tolerance of the yolk to cryoprotectant is due, in part, to the lower enzymatic activity within the yolk.

Since Me_2SO was more damaging to zebrafish embryos than PG, we combined it with varying proportions of acetamide and formamide to try and mitigate its toxicity. Baxter and Lathe (3) found that different amides reduce the toxicity of Me_2SO. They concluded that Me_2SO causes damage to rat renal cortex slices by activating fructose-1,6-diphosphatase (FDPase), an enzyme that disrupts glycolysis. They theorized that in combining Me_2SO with amides there is a "protective effect" by which the amide and Me_2SO complex with each other, as opposed to interacting individually with polar side groups on native proteins, such as lysine. This masking of Me_2SO subsequently inhibits the activation of FDPase. On the other hand, Fahy and colleagues (9) suggest that the protective nature of these amides is actually a result of reducing the concentration of Me_2SO needed to cryopreserve these cells. Our results show that both acetamide and formamide in all proportions tested were equally, if not more, teratogenic than Me_2SO alone for zebrafish embryos. In fact, in agreement with Fahy *et al.* (9), we found that as the proportion of formamide to Me_2SO was increased in the 10-nl injections, we saw a decrease in embryo survival. Therefore, Me_2SO by itself, or in combination with amides, is not suitable for use at high concentrations with zebrafish embryos.

When considering vitrification, the following suggestions have been made by a number of authors for an optimal protocol: (i) choose a cryoprotectant that is the least toxic; (ii) apply "toxicity neutralizers" when possible (3, 5, 6, 8); (iii) minimize embryo exposure time to the solution (6, 23); (iv) remove cryoprotectant immediately after thawing (9, 23, 25); and (v) expose embryos to cryoprotectants at lower temperatures (3, 6). In trying to formulate a protocol for zebrafish embryos based on these recommendations, we found: (i) PG to be the least teratogenic cryoprotectant at high concentrations; (ii) the application of amide "toxicity neutralizers" did not aid in survivorship of zebrafish embryos; (iii) microinjection directly into the yolk greatly reduced the time necessary to load the embryo with cryoprotectant; (iv) because the YSL is relatively impermeable, we could not remove the cryoprotectant after thawing; and (v) we did not lower the culturing temperatures because zebrafish do not develop properly at lower temperatures due to microtubule disruption (15).

Physically overcoming the permeability barrier of the YSL and incorporating a high concentration of cryoprotectant into the zebrafish embryos is an important step toward successful vitrification. Presuming a low permeability of the YSL during further development, the fact that these fish can tolerate high cryoprotectant concentrations within their yolk for up to 5 days without killing 100% of the treated embryos is remarkable. Most cells cannot tolerate intracellular cryoprotectants for more than tens of minutes, much less days. This great tolerance for these potentially toxic chemicals bides well for future vitrification trials. However, the vitrification process is complex. With standard freezing rates, unless there is sufficient cryoprotectant within the cell, failure to cryopreserve these embryos is inevitable. In previous work (14), we used an unoptimized vitrification method to analyze the type of cellular damage that occurs when insufficient cryoprotectant is in the yolk of zebrafish. In the present experiments, we were interested in determining if this one step, incorporating cryoprotectants intracellularly, would rescue the morphology of the YSL. Our results show that simply incorporating the cryoprotectant alone is not sufficient and that opti-

mization of the vitrification process is also important (e.g., altering the dehydration times, and the freezing and thawing rates). We believe that microinjection holds great promise for the future, and it along with an optimized vitrification process may ultimately lead to successful cryopreservation of the first fish embryo.

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