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Cryopreservation of pre-hatch embryos of zebrafish *(Brachydanio rerio)*

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Abstract

The toxicity of five cryoprotectants—methanol, DMSO, glycerol, ethanediol and sucrose—on different development stages of zebrafish embryos was investigated. Embryos were exposed to a range of cryoprotectant concentrations for 30 min at room temperature. Post-heart beat stage embryos were more resistant to cryoprotectants than early embryonic stages. The maximum non-toxic concentrations of cryoprotectants on heart beat stage embryos were 2 M methanol, 2 M DMSO, 1 M glycerol, 2 M ethanediol and 0.5 M sucrose. Gradual stepwise addition did not reduce toxicity. Heart beat stage embryos survived 2 M methanol at room temperature for up to 5 hours, whilst DMSO and ethanediol were toxic after 3 and 1 hour exposure respectively. The effect of the nature and concentration of cryoprotectant, equilibrium time, and cooling rate were investigated during cooling to −30°C. Methanol was more effective than either DMSO or ethanediol for zebrafish embryo cryopreservation and 0.3°C/min was found to be the optimum cooling rate. Two-step addition of cryoprotectants, with the higher concentration of cryoprotectants added at 0°C, improved the results. The best embryo survivals obtained after cooling were 94% at −10°C, 72% at −15°C, 43% at −20°C, and 8% at −25°C, no embryos hatched following cooling to −30°C. Ice formation within the egg was found to be the main factor affecting survival of the embryos.

Keywords: Zebrafish, *Brachydanio rerio*, Cyprinidae, pre-hatch embryos, cryopreservation, cryoprotectant toxicity, freezing storage, ice formation.

La cryoconservation d'embryons avant éclosion de danio zébré, Brachydanio rerio.

Résumé

La toxicité de cinq cryoprotecteurs a été étudiée, méthanol, DMSO, glycérol, éthanediol et sucrose, sur différents stades de développement d'embryons de *Brachydanio rerio*, avec une gamme de concentrations en cryoprotecteurs pendant 30 min et à la température de la pièce. Les embryons au stade post-battements cardiaques sont plus résistants aux cryoprotecteurs que les stades embryonnaires plus précoces. Les concentrations maximums non toxiques sur les embryons au stade « battements cardiaques » sont de 2 M pour l'éthanol, 2 M pour DMSO, 1 M pour le glycérol, 2 M pour l'éthanediol et 0,5 M pour le sucrose. La toxicité n'est pas réduite avec une addition graduelle progressive. Les embryons au stade « battements cardiaques » survivent avec 2 M de méthanol, à la température de la pièce, jusqu'à 5 heures, tandis que le DMSO et l'éthanediol sont toxiques après respectivement 3 et 1 h d'exposition. L'effet de la nature et de la concentration des cryoprotecteurs, au temps d'équilibre, et du taux de congélation ont été étudiés durant une congélation à −30°C. Le méthanol est plus efficace que le DMSO et l'éthanediol pour la cryoconservation d'embryons de *Brachydanio* et le taux optimum de congélation a été de 0,3°C/min. Une addition de cryoprotecteurs effectuée en 2 fois, avec la concentration la plus importante ajoutée à 0°C, améliore les résultats. Les meilleurs taux de survie,

obtenus après congélation, sont de 94 % à −10°C, 72 % à −15°C, 43 % à −20°C et 8 % à −25°C, aucun embryon n'a éclos lors de l'étape de congélation suivante à −30°C. Le principal facteur affectant la survie des embryons est la formation de glace dans les œufs.

Mots-clés : *Brachydanio rerio*, Cyprinidé, embryon avant éclosion, cryoconservation, congélation, toxicité de cryoprotecteur, formation de glace.

INTRODUCTION

The successful cryopreservation of fish embryos would open new perspectives in fish culture and in the management of wild stocks for fish farming. Cryopreserved embryos would also be useful for ecotoxicology bioassays. Successful cryopreservation of sensitive cells, tissues and organs requires the use of cryoprotective agents. Methanol, dimethyl sulphoxide, glycerol, ethanediol and sucrose are the most commonly used cryoprotectants. All of them are reported as being moderately toxic (Asahina and Takahashi, 1978; Harvey, 1983; Willhite and Katz, 1984; Robertson and Lawrence, 1988). Thus, the toxicity of cryoprotectants limits the concentration that can be used and therefore limits the cryoprotective efficiency of these agents (Arakawa *et al.*, 1990). There is also evidence that cryoprotective agents, despite their benefits, can actually play a direct role in producing cryoinjury (Fahy, 1986). Relatively little work has been done on the toxicity of cryoprotectants on fish embryos. In the study reported here the toxicity of cryoprotectants on zebrafish embryos were examined prior to studies on the effect of cooling rate in cryopreservation.

Cryopreservation of fish eggs and embryos is proving to be a difficult problem in cryobiology. The eggs of most species are large and have a high yolk content, they are complex when compared to sperm cells and they also have very low permeability (Prescott, 1955; Loeffler and Lovstrup, 1970). Attempts to cryopreserve fish eggs or embryos have been conducted on herring (*Clupea harengus*), rainbow trout (*Oncorhynchus mykiss*), coho salmon (*Oncorhynchus kisutch*), brook trout (*Salvelinus* sp.), zebrafish (*Brachydanio rerio*), common carp (*Cyprinus carpio*), *Oryzias latipes* and red drum (*Sciaenops ocellatus*) with limited success (Zell, 1978; Whittingham and Rosenthal, 1978; Stoss and Donaldson, 1983; Zhang *et al.*, 1989). Eggs or embryos from all species have shown a tolerance to temperatures below 0°C but little success has been reported on cooling to −60°C and below. In this study, heart beat stage zebrafish embryos were used in experiments using conventional cryobiological methods and freezing damage was examined.

MATERIALS AND METHODS

Cryoprotectants

All the cryoprotectants were of the highest available purity. Dimethyl sulphoxide (99.5%), ethanediol (99.5%) and sucrose (AnalaR) were from BDH, Poole, UK. Methanol (ACS reagent) was from Aldrich, Poole, UK. Glycerol (99%) was from Sigma, Poole, UK.

Experiments on the toxicity of cryoprotectants

Zebrafish embryos were obtained from Luton College of Higher Education laboratory stock. Fertilized embryos at gastrula (7 h), heart beat (24 h) and pre-hatching (49 h) stages were used in the experiments. Newly fertilized embryos were washed and kept in a tray with carbon-filtered and aerated tap water (pH 8.1-8.3; dissolved oxygen 80±5%; temperature 26±0.5°C; hardness (expressed as $CaCO_3$) 150-200 mg/l) until they reached the stage for the toxicity test. Cryoprotectant solutions were prepared in carbon-filtered and aerated water, fresh solutions being used for each experiment.

One-step addition of cryoprotectants

Gastrula, heart beat and pre-hatching stage embryos were exposed to methanol, dimethyl sulphoxide (DMSO), glycerol or ethanediol at concentrations of 0, 0.5, 1, 2 and 4 M, and sucrose at concentration of 0, 0.5, 1 and 2 M for 30 min at room temperature (22°C) in a one-step addition, in tissue culture plates. One plate was used for each cryoprotectant in order to avoid any interference between the cryoprotectants. Toxicity trials were initiated by adding 1 ml of double-concentration cryoprotectants to the wells containing 10 fish embryos in 1 ml of carbon-filtered and aerated tap water using a pipette. 2 M sucrose was added directly to the cells. Carbon-filtered and aerated tap water (2 ml) was used for control cells. In another experiment, heart beat stage embryos were exposed to 2 M methanol, DMSO or ethanediol for up to 6 h at room temperature.

Stepwise addition

Heart beat stage embryos were exposed to increasing concentration of methanol, DMSO or ethanediol

by four steps (0.5, 1, 2 and 4 M) at room temperature. Embryos were exposed to each concentration for 15 min.

After an equilibration period, the contents of each well were removed and drained through a fine mesh (180 µm) sieve using a Pasteur pipette. The embryos retained on the sieve were thoroughly rinsed three times with carbon-filtered and aerated tap water. Embryos were then removed to beakers containing 100 ml of carbon-filtered and aerated tap water which was changed daily. Embryo-larvae survival was recorded and dead embryos removed every 24 h. No food was provided during the experiments. 7 days after fertilization, living embryo-larvae were counted in each replicate. Toxicity was evaluated in terms of percentage hatch and 7-day survival. For each experiment, 3 replicates of all cryoprotectants and concentrations with 10 embryos were used and the experiments were repeated 3 times.

Low temperature cooling

These experiments were confined to heart beat stage embryos since exposure of pre-hatch stage embryos to cryoprotectants was found to stimulate early hatching. Thus, heart beat stage embryos were treated with 2 M methanol for 1 or 2 h, 3 M methanol for 30 min or 1 h, 4 M methanol for 20 min, 2 M DMSO for 1 h and 2 M ethanediol for 30 min in one-step additions. After addition of cryoprotectants, embryos together with cryoprotectant solutions were placed in straws (MULTICOLOR™, 0.5 ml, Cassou, France) using a syringe; 20-30 embryos were placed in each straw. All pre-cooling treatments were conducted at room temperature. Following treatment in cryoprotectants, the straws were located in the chamber of a controlled rate cooler (KRYO 10, Series II, Planer Products Ltd., England) at a temperature of 20°C. Embryos were cooled to −10, −15, −20, −25 and −30°C at a range of cooling rates (0.1-0.75°C/min). Ice nucleation was induced in the suspending cryoprotectant at −7.5°C (2 M cryoprotectant treatment), −10°C (3 M cryoprotectant treatment) and −12.5°C (4 M cryoprotectant treatment) by touching the end surface of straws with a pair of tweezers previously cooled in liquid nitrogen. The straws were maintained for 10 min at ice-seeding point, and then cooled to various final temperatures.

The straws were either thawed immediately in a water bath (25°C, ~300°C/min) or at a slow rate of 10°C/min using the KRYO cooler and the embryos then placed in beakers, washed free of cryoprotectant and incubated in carbon-filtered tap water at a temperature of 26°C.

One-step addition cryoprotectant experiments were also carried out using an alcohol bath. 40-60 heart beat stage embryos were placed in a container with methanol (2 ml, 2 M), after equilibration time for 1 h, the container was placed in a alcohol bath at −5°C, embryos were cooled to −30°C with a cooling rate of 0.3°C/min, ice nucleation was introduced at −7.5°C. Embryos with ice formation within the egg were counted during cooling. This was an easy task since the eggs turned white and ice crystals could be identified if the eggs were examined under a microscope.

Two-step addition of cryoprotectants

Heart beat stage zebrafish embryos were treated with 2 M methanol for 0.5 and 1.5 h, and 3 M methanol for 0.5 h in a one-step addition at room temperature, and then embryos together with cryoprotectant were removed to a ice-water bath at 0°C. After equilibration for 15 min, the 2 M and 3 M methanol solutions was replaced with 3 M and 4 M methanol, respectively, pre-cooled on the ice-water bath. After 15 min, embryos, together with cryoprotectant solutions, were located in straws by means of a syringe. 20-30 embryos were placed in each straw and the straws were placed in the chamber of the controlled rate cooler at 0°C.

Embryos were cooled at rates ranging from 0.1 to 0.75°C/min. Ice nucleation in the cryoprotectant was induced at −10°C (with 3 M methanol treatment) or −12.5°C (4 M methanol treatment). After cooling, embryos were thawed in a 25°C water bath and kept in carbon-filtered tap water at a room temperature of 26°C. Both cooled and non-cooled controls were used. The experiments at each cooling rate were repeated three times.

Measurement uncertainties

Where measurements are quoted numerically then the standard deviation is indicated by the "±" values. In graphs, the standard deviation is shown by a bar.

RESULTS

Toxicity of cryoprotectants

The maximum concentrations of cryoprotectants that did not reduce hatch or survival for gastrula, heart beat and pre-hatch stages of zebrafish embryos exposed for 30 min at room temperature are given in table 1. Heart beat stage embryos exposed to 2 M methanol for up to 5 h, and DMSO for up to 2 h at room temperature developed normally. However, hatching and survival of embryos was significantly reduced when treated with ethanediol for 1 h (*fig.* 1). On a molar-equivalency basis, the toxicity of the tested cryoprotectants increasing in the order of methanol, DMSO, ethanediol, glycerol and sucrose. Post-heart beat stages of zebrafish embryos appeared to be more resistant to cryoprotectant than early embryonic stages.

Table 1. – Maximum concentrations of cryoprotectants that did not reduce hatching success or post-hatch survival for gastrula, heart beat and pre-hatch stages of zebrafish embryos.

Cryoprotectants	Concentration/M (%)		
Embryo stage	Gastrula	Heart beat	Pre-hatch
Methanol	2 (8.1)	2 (8.1)	2 (8.1)
DMSO	2 (14.2)	2 (14.2)	2 (14.2)
Glycerol	0.5 (3.7)	1 (7.3)	1 (7.3)
Ethanediol	1 (5.6)	2 (11.2)	2 (11.2)
Sucrose	0.5 (10.5)	0.5 (10.5)	0.5 (10.5)

Embryos were exposed to cryoprotectants at room temperature for 30 min. Percentages in brackets are on a volume basis.

Effect of cryoprotectants

The effects of different cryoprotectants on the survival of heart beat stage embryos cooled to −10, −15, −20 and −25°C are shown in *figure* 3. Methanol treated embryos showed consistently higher hatch percentages than those treated with either DMSO or ethanediol. Heart beat stage embryos without cryoprotectants did not survive at −10°C or below. The addition of 3 M methanol to heart beat stage embryos increased the hatch percentages when compared with 2 M methanol protection (*fig.* 4). The effect of different pre-cooling equilibration times are shown in *figure* 5. The extension of cryoprotectant equilibration using both one- and two-step additions increased hatch percentages at lower temperatures.

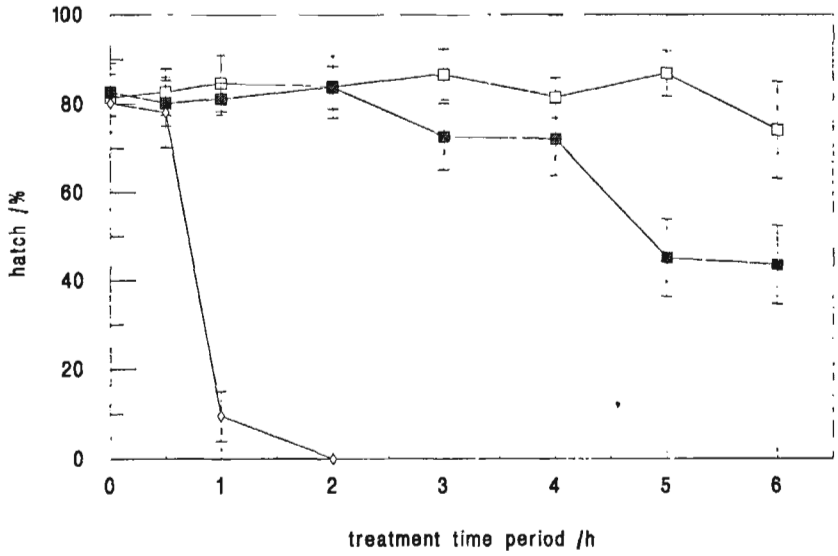

Figure 1. – Toxicity to heart beat stage zebrafish embryos at room temperature (22°C). Embryos were exposed to cryoprotectants in a one-step addition. ⊔ 2 M methanol; ■ 2 M DMSO; ◇ 2 M ethanediol.

Hatch and survival percentages of heart beat stage embryos were reduced significantly after they were exposed to methanol, DMSO or ethanediol from 0.5 to 4 M by four steps at room temperature with 15 min incubation for each concentration. The hatch percentages were control 83.5±11.6%; methanol 68.4±8.8% and DMSO 4.4±5.8%. There was no survival after ethanediol treatment.

Low temperature cooling

Effect of cooling rate

Embryo survival at different slow cooling rates (*fig.* 2) showed 0.3°C/min to be the optimum cooling rate from freezing point with methanol protection. Similar results were obtained with both one-step and two-step addition experiments.

Increasing methanol concentration to 3 M at 0°C generally increased embryo survival (*fig.* 6).

Effect of thawing rate

Comparison of both fast (approx. 300°C/min) and slow (10°C/min) thawing rates on the survival of heart beat stage embryos (*fig.* 7) showed that slow thawing did not improve the hatch percentage after cooling at 0.3°C/min.

Effect of ice formation within the egg

Comparison of embryo survival after cooling at 0.3°C/min and the percentage of eggs within which ice formed during cooling at different temperatures showed that the pattern of these curves was similar (*fig.* 8). These results suggested that formation of ice

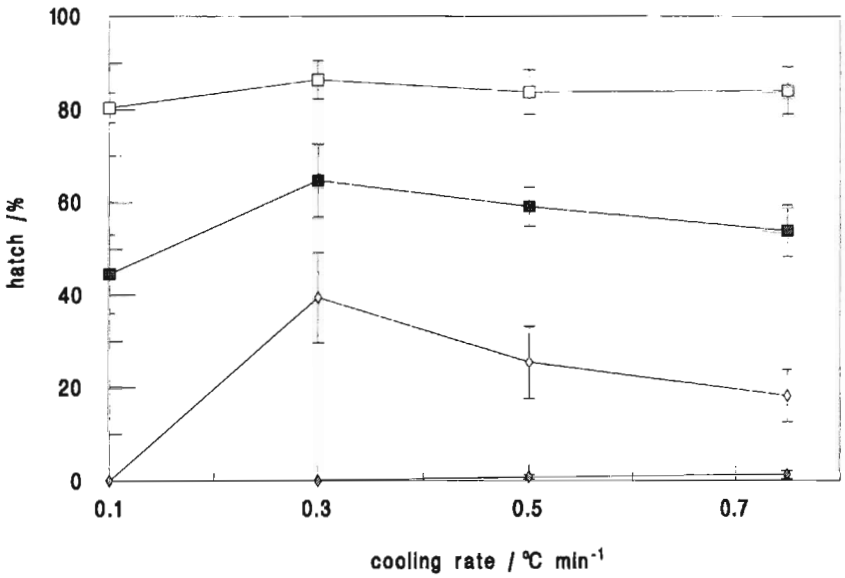

Figure 2. – Effect of cooling rate on survival of heart beat stage zebrafish embryos with two-step addition of methanol. Embryos were exposed to 2 M methanol at room temperature for 30 min followed by 15 min at 0°C, and then 2 M methanol was replaced with 3 M methanol for 15 min at 0°C. The embryos were then cooled to various temperatures. □ −10°C; ■ −15°C; ◇ −20°C and ◆ −25°C. Hatch percentage of control was 86.5%.

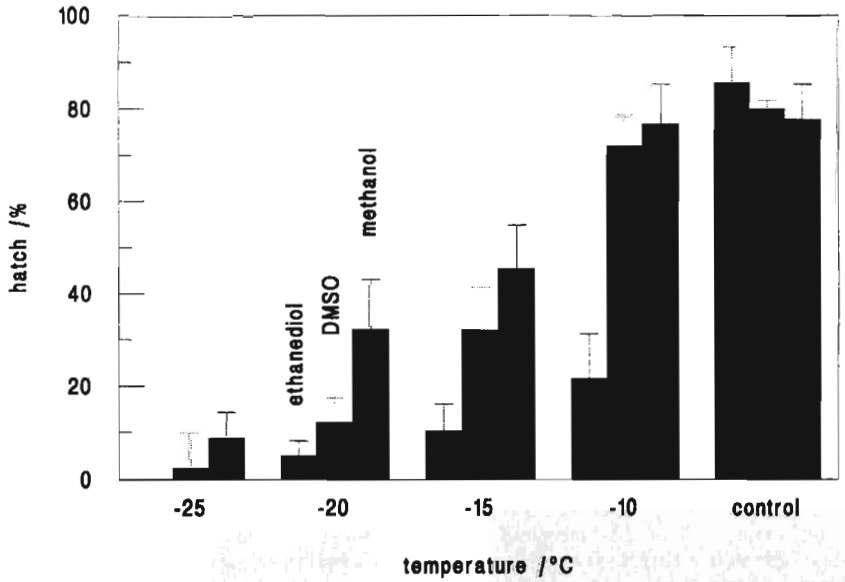

Figure 3. – Effect of 2 M cryoprotectants on survival of heart beat stage zebrafish embryos. Embryos were exposed for 1 h (methanol, DMSO) or 30 min (ethanediol) at room temperature. Cooling rate was 0.5°C/min.

within the egg was the main factor affecting the survival of embryos during freezing although other factors may affect viability.

DISCUSSION

Toxicity of cryoprotectants

Tests showed that the toxicity of the cryoprotectants increased in the order of methanol, DMSO, ethanediol, glycerol and sucrose. These results are in agreement with Robertson and Lawrence's (1988) study on the toxicity of cryoprotectants to the embryos of red drum (*Sciaenops ocellatus*). Post-heart beat stages of zebrafish withstood higher concentrations of glycerol or ethanediol as was found by Robertson and Lawrence (1988). Harvey *et al.* (1983) reported that 1 M DMSO did not affect the percentage hatch of gastrula stage zebrafish embryos exposed for up to 1 h, whereas 1.5 and 2 M DMSO reduced embryo hatching significantly after 1 h exposure. In

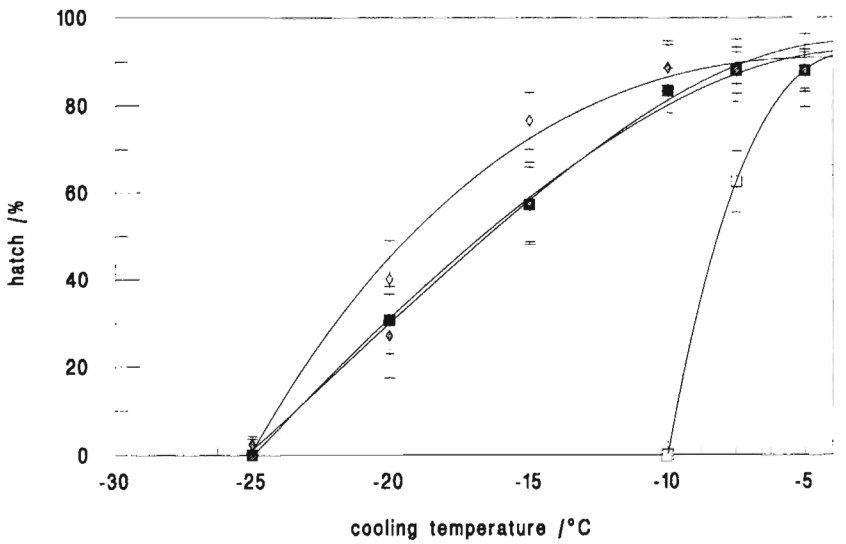

Figure 4. – Effect of methanol concentration on survival of heart beat stage zebrafish embryos. Embryos were exposed to different concentrations of methanol at room temperature in a one-step addition. Cooling rate was 0.3°C/min. □ no methanol; ■ 2 M, 1 h treatment; ◇ 3 M, 30 min treatment and ◆ 4 M, 20 min treatment.

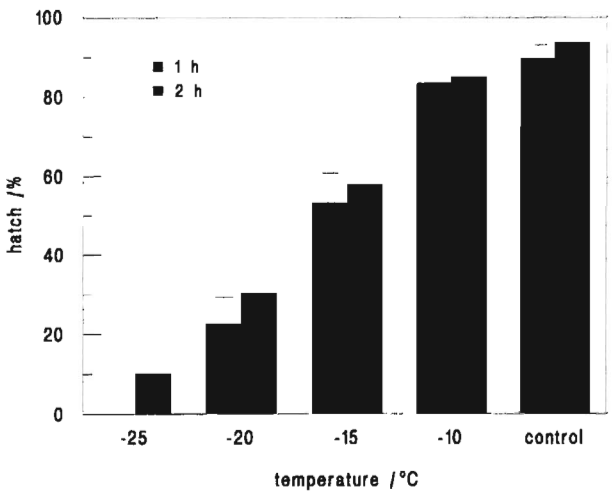

Figure 5. – Effect of equilibration time of 2 M methanol on survival of heart beat stage zebrafish embryos. Embryos were exposed for 1 or 2 h at room temperature in a one-step addition. Cooling rate was 0.3°C/min.

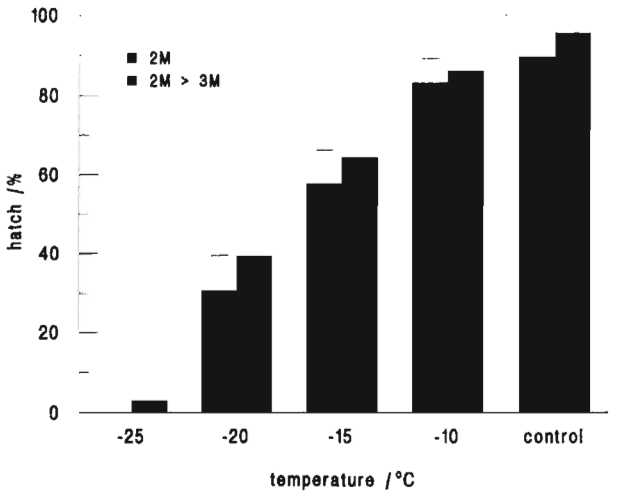

Figure 6. – Comparison of the effect of methanol on survival of heart beat stage zebrafish embryos using one- or two-step additions. For the one-step addition, embryos were exposed to 2 M methanol for 1 h at room temperature. For the two-step addition, embryos were exposed to 2 M methanol for 30 min at room temperature followed by 15 min at 0°C, and then 2 M methanol solution was replaced with 3 M methanol for 15 min at 0°C. Cooling rate was 0.3°C/min.

our experiments, heart beat stage embryos withstood 2 M DMSO for up to 2 h. Post-closure of the plastopore stages (*i.e.* tail bud stage) have also been reported as the best stages for common carp embryo cryopreservation (Zhang *et al.*, 1989).

Low temperature cooling

The slow cooling rate (0.3°C/min) was optimum for heart beat stage embryos. Several workers have shown that slow cooling rates result in better survival of fish embryos after freezing. Harvey (1983) reported that survival of zebrafish blastoderms after freezing was markedly higher at cooling rates of 0.5 and 0.05°C/min than at 25°C/min. Studies by Zhang *et al.* (1989) on the cryopreservation of common carp embryos also showed some success with cooling rates less than 0.07°C/min.

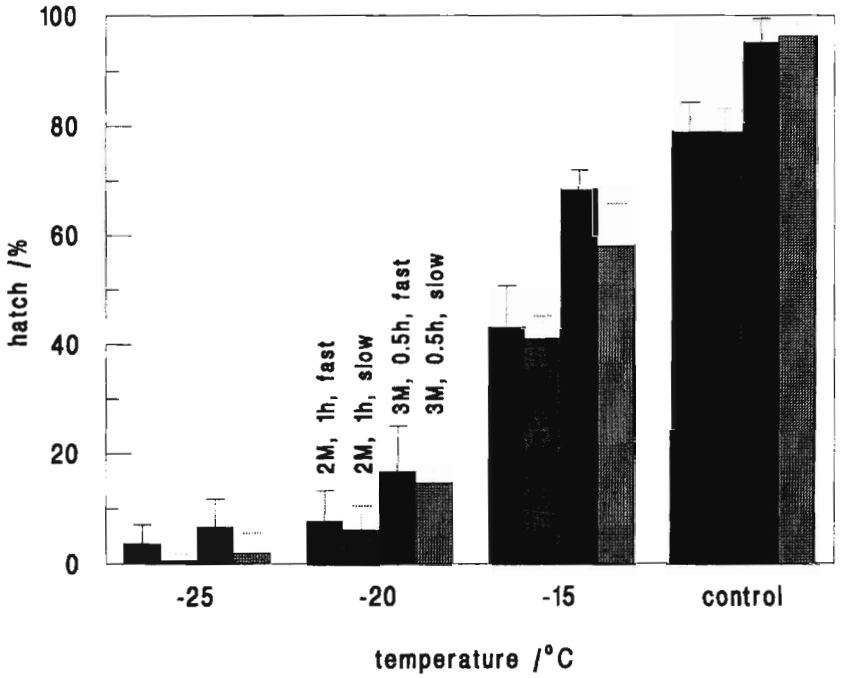

Figure 7. – Effect of thawing rate on survival of heart beat stage zebrafish embryos after cooling. Embryos were treated with 2 M and 3 M methanol for 1 h and 30 min respectively at room temperature before cooling. Fast thawing 300°C/min, slow thawing 10°C/min. Cooling rate was 0.3°C/min.

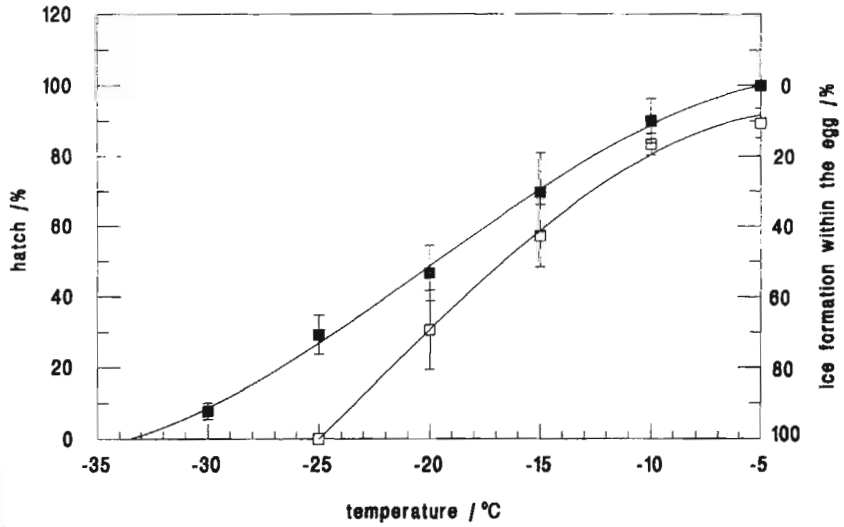

Figure 8. – Effect of ice formation within the egg on survival of heart beat stage zebrafish embryos. Embryos were treated with 2 M methanol for 1 h at room temperature in a one-step addition before cooling. Cooling rate was 0.3°C/min (□ hatch after cooling; ■ ice formation within the egg).

Methanol treated embryos showed consistently higher hatch percentages than those treated with DMSO or ethanediol, possibly due to its fast penetration and relatively low toxicity. Harvey and Ashwood-Smith (1982) reported that the penetration of methanol was significantly faster than either DMSO or glycerol in the eggs of salmonid fish and also that the rate of cryoprotectant penetration reflects their molecular weights, with the smallest molecules penetrating most quickly. Harvey *et al.* (1983) also reported that the fertility of supercooled salmonid ova was relatively high with methanol protection.

The cooling studies showed that the higher concentration of cryoprotectant (3 M) and the longer

equilibration time (2 h) increased hatch percentages even though the toxicity studies showed this concentration to be detrimental to hatching. This is in agreement with Harvey (1983) who showed that embryos may need to be loaded with cryoprotectant at concentrations that are known to have toxic effects. Sufficient equilibration time is needed to allow cryoprotectants penetration as confirmed by Harvey and Ashwood-Smith (1982).

Increasing the methanol concentration at 0°C generally increased embryo survival. These results agreed with the general supposition that ice formation within the egg results from seeding by extraovular ice rather than by spontaneous crystallization within the egg (Mazur, 1977; Harvey and Ashwood-Smith, 1982). Consequently, the prevention of ice formation in the surrounding medium with a stepwise increase in cryoprotectant concentration as cooling progresses is considered appropriate (Pegg, 1988; Harvey and Ashwood-Smith, 1982).

Harvey's (1983) studies on cooling of embryonic cells, isolated blastoderms, and intact embryos of zebrafish to −196°C showed that the warming rate was important in determining survival. Fast warming (200°C/min) was inferior to warming at an intermediate rate (43°C/min) when blastoderms were cooled at 0.5°C/min to −25°C; there was no improvement when samples were warmed more slowly (2°C/min). However, in the work reported here, the slow warming rate (10°C/min) was inferior to rapid warming (300°C/min).

The comparison of embryo survival after cooling and the percentage of embryos in which ice formed within the egg during cooling showed that the pattern of these curves was similar. This indicated that ice formation within the egg was probably the main factor limiting the survival of the embryos. The difference between the curves is probably due to other factors such as normal mortality of the embryos and cryoprotectant toxicity. As with mammalian embryos (Rall and Fahy, 1985) it is essential to prevent ice formation in order to achieve successful cryopreservation of fish embryos.

CONCLUSION

Toxicity tests of cryoprotectant on zebrafish embryos at different development stages showed post-heart beat stages to be the least sensitive to cryoprotectants. On a molar-equivalency basis methanol was less toxic to zebrafish embryos than DMSO, glycerol or ethanediol.

Heart beat stage embryos were chosen for use in cooling experiments which also showed that methanol was more effective than DMSO or ethanediol for zebrafish embryo cryopreservation.

The best survival values achieved, with 3 M methanol protection and a cooling rate of 0.3°C/min, were −10°C, 94%; −15°C, 72%; −20°C, 43% and −25°C, 8%. No embryos hatched after cooling to −30°C.

Cooling of zebrafish embryos by conventional methods showed that ice formation within the egg was inevitable even when embryos were cooled with a high concentration of cryoprotectant and at a slow rate. Methods of avoiding ice formation within the egg need to be developed in order to achieve high viability in fish embryo cryopreservation.

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