CRYOPROTECTANTS PROTECT MEDAKA (Oryzias latipes) EMBRYOS FROM CHILLING INJURY

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Abstract

This study was conducted to investigate the effect of six cryoprotectants (dimethyl sulfoxide (DMSO), glycerol (Gly), methanol (MeOH), ethylene glycol (EG), 1,2-propylene glycol (PG) and N,N-dimethylformamide (DMF) on the survival of medaka (Oryzias latipes) embryos at low temperatures (0 and -5°C). Firstly, the embryos at 8 to 16-cell stages were exposed to different concentrations (1 to 4 mol/L) of DMSO, Gly, MeOH, EG, PG and DMF for 40min at 26°C. After removal of the cryoprotectants (CPAs), the embryo survivals were assessed by their development into live fry following 9 day of culture. The results showed that the higher concentration of the CPA, the lower survival of the embryos; and that the toxicity of the six CPAs to medaka embryos is in the order of PG < MeOH = DMSO < Gly < EG < DMF (P<0.05). Secondly, based on the results obtained above, embryos at 8 to 16-cell stages or other stages were exposed to 2 mol/L of PG, MeOH or DMSO for up to 180 min at 0°C and up to 80 min at -5°C respectively. The 8 to 16-cell embryos treated with MeOH at low temperatures showed highest survival. Thirdly, when embryos at different stages were treated with 2 mol/L of MeOH at -5°C for 60 min, 16-somite stage embryos showed highest survival, followed by 4-somite, neurula, 50% epiboly, blastula, 32-cell and 8 to 16-cell embryos. These results demonstrated that PG had the lowest toxicity to medaka embryos among the six permeable CPAs at 26°C, whereas MeOH showed highest cryoprotective efficiency under chilling conditions and chilling injury decreased gradually with the development of medaka embryos.

Keywords: medaka embryo, cryoprotectant, toxicity, chilling injury, protective efficiency
INTRODUCTION

The development of feasible cryopreservation protocols for fish embryos has important applications in management of stocks in fisheries, facilitating the conservation of endangered populations of fish, creation of gene bank for fish species and bioassay in ecotoxicological research (18, 37). Therefore, cryopreservation of teleost embryos would have a great impact not only on the preservation of laboratory species, but also on aquaculture and the conservation of diverse aquatic species. Although the huge efforts made in the last decades have resulted in some progress, including improved slow freezing (14, 38) and vitrification procedures (5, 7, 16, 28) for enhancing fish embryo survival at low temperatures, and some specific techniques employed such as incorporation of antifreeze proteins into embryos (24, 26, 27), microinjection of CPAs into the yolk (2, 18), freezing of embryos with high-chilling resistance (27), increase membrane permeability of embryos or oocytes by dechorionation (12) and by incorporation of aquaporin-3 into the plasma membrane (6, 13), cryopreservation of fish embryo is, at present, an unsolved problem.

Medaka (Oryzias latipes) is an important experimental fish for developmental biology, genetics, and physiology studies (3). Although the blastomeres of medaka embryos had been successfully cryopreserved (31), embryo cryopreservation of this species has not been achieved. As an essential step towards low temperature storage of fish embryos, the chilling sensitivity of the embryos at zero and subzero temperature needs to be studied so successful nonfreezing storage protocol can be developed for this species. Valdez and others found that medaka embryos at the 2–4 cell stage were sensitive to chilling at 0°C or -5°C in Hanks solution without any CPA addition (33). As CPAs have been reported to protect embryos from chilling injury in zebrafish (36) and there is no information on the protective efficiency of CPAs to medaka embryos at zero and subzero temperatures, the aim of this study is to investigate the toxicity of CPAs to medaka embryos before studying their ability in reducing chilling injury of the embryos at zero and subzero temperatures.

In order to understand the effect of various CPAs on medaka embryos, in the present study, we studied the toxicity of six most commonly used permeable CPAs - dimethyl sulfoxide (DMSO), glycerol (Gly), methanol (MeOH), ethylene glycol (EG), 1,2-propylene glycol (PG) and N,N-dimethylformamide (DMF), to medaka embryos at 26°C before investigating the chilling sensitivity of medaka embryos in embryo medium (EM, a modified Hanks solution) containing CPA at 0°C and -5°C.

MATERIALS AND METHODS

Fish and Embryos

About 80 fish were kept in an aquarium of 30 cm × 20 cm × 30 cm (25°C, pH 7.0) in Beijing Fisheries Research Institute, under a 16-h light and 8-h dark cycle, and Brine Shrimp (Artemia cysts) larva were fed twice daily. All developmental stages described here are the same of those in Iwamatsu (17).

As early stage fish embryos have been reported to be most sensitive to chilling, early stage medaka embryos are used in the present study. Under our experimental conditions, when the developmental stage of medaka embryos is earlier than 8 to 16-
cell stage, it is difficult to collect and manipulate the embryos in large quantities, we therefore collected the 8 to 16-cell stage embryos from female medaka bearing embryo clusters after 2 h from the beginning of the light period. Later stage embryos were obtained following in vitro culture of 8 to 16-cell stage embryos at 26°C. The method for embryo separation was described by Valdez et al. (18). Briefly, the medaka was held on its back and the embryo cluster was collected gently using the surface of a net. In an embryo cluster, 20 to 30 embryos are usually present and attached to each other with long hairs on the chorion at the vegetal pole area. To separate the embryos, the attaching hairs were held with fine watchmaker forceps and were excised with microsurgery scissors under a dissecting microscope. This method for embryo separation minimizes embryo damage and microbial contamination.

**Chemicals**

All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise indicated.

**Toxicity test of various CPAs to medaka embryos at 26°C**

Four different concentrations (1 mol/L, 2 mol/L, 3 mol/L and 4 mol/L) of each of the six CPAs - DMSO, Gly, MeOH, PG, EG and DMF, made up with embryo medium (EM, a modified hanks solution), were tested in this experiment. One milliliter of each CPA solution was placed in a separate well of a tissue culture plate with 24 wells. About 30 embryos at 8-16 cell stage, washed with corresponding solution, were transferred into each of the wells. The embryos were kept in the solution at 26°C for 40 min. The embryos were then removed from the well and washed three times with freshwater. Then they were transferred into a culture dish for subsequent development with a change of freshwater every day. After 9-day culture, the normally developed embryos hatched. The hatching percentage was counted as the number of hatched embryos in relation to the total number of embryos in the dish and recorded. The experiment was replicated at least 3 times with different batches of embryos. A batch of control embryos was also cultured at 26°C along with every group.

**The protective efficiency of various CPAs to 8 to16- cell embryos at 0°C and -5°C**

Based on the results from the above experiments, the highest CPAs concentration without any effect on embryo survival (LD₀ concentration) was 2 mol/L for DMSO, MeOH and PG, respectively. Therefore in this experiment, 2 mol/L of each of the three CPAs (DMSO, MeOH and PG) were used to study the protective effect of the CPAs on embryos at 0°C. Thirty to forty embryos at 8-16 cell stage, following exposure to each solution at 26°C for 40 min, were transferred into 1.5 ml centrifuge tube containing 1 ml corresponding solution which had been kept on ice for 20 min. Following exposure at 0°C for 10, 20, 40, 80, 120 and 180 min respectively, solution containing embryos was put into a culture dish containing 50 ml freshwater in a 26°C water bath for 5 min. Embryos were then recovered and washed three times with freshwater. To assess survival, embryos were cultured in freshwater at 26°C in a culture dish, with a change of freshwater every day until they hatched. Control
embryos were treated using the same protocol in the EM. The experiment was replicated 8 times.

To examine the protective efficiency of 2 mol/L of each of the three CPAs (DMSO, MeOH and PG) at -5°C, embryos at 8 to 16-cell stages were used. After suspending in each solution at 26°C for 40 min, embryos were transferred into 1.5 ml centrifuge tube containing 1 ml corresponding solution which had been equilibrated to 0°C for 20 min beforehand. The tubes containing embryos were put into refrigeration room and cooled to -5°C. The samples were maintained at -5°C for 10, 20, 40 or 80 min respectively. The solution temperature was confirmed by a digit thermometer. Then 1 ml solution in the tube was put into a culture dish containing 50 ml freshwater in a 26°C water bath for 5 min. The embryos were then recovered and washed three times with freshwater, followed by culture in freshwater at 26°C in a culture dish until they hatched. For each treatment, 90 to 120 embryos were used. Control embryos were treated using the same protocol in the EM. The experiment was replicated 5 times.

The protective efficiency of MeOH to Medaka embryos at various developmental stages

Embryos at 8 to 16-cell (2 h 30 min post-fertilization, pf), 32-cell (3 h 30 min pf), blastula (6 h 30 min pf), 50% epiboly (17 h 30 min pf), neurula (1 d 1 h pf), 4-somite (1 d 8 h pf) and 16-somite (1 d 20 h pf) stages were tested in this experiment.

Embryos were exposed to 2 mol/L MeOH at 26°C for 40 min, and placed in 1.5 ml centrifuge tubes containing 1.0 ml of 2 mol/L MeOH which had been equilibrated to 0°C for 20 min previously. Then they were put into refrigeration room and cooled to -5°C, and maintained at this temperature for 60 min respectively. Based on our preliminary experiment, which showed that 48.0 to 62.9 % of the medaka embryos at 8 to 16-cell stage survived chilling at -5°C for 40 to 80 min, and previous report (39) that the living flounder embryos could be obtained after keeping them at -15°C for approximately one hour in CPAs solutions, we select 60 min as the chilling time to observe the effect of 2 mol/L MeOH on the survival of medaka embryos at various stages. After treatment, the embryos were warmed in a culture dish containing 50 ml freshwater in a 26°C water bath for 5 min, and washed three times with freshwater to remove MeOH. The embryos were then cultured to hatching at 26°C. Control embryos were treated using the same protocol in EM solutions. Experiments were repeated at least 4 times.

Statistics

The results were expressed as mean±S.D. Analysis of variance (ANOVA) was applied to determine significant differences among different groups in each test. Student–Newman–Keuls multiple range test for pair-wise comparisons was performed in all tests where significant differences were proved with ANOVA. Statistics were carried out by SPSS (version 11.5) for Windows. The statistical significant level was set at $P < 0.05$. 

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RESULTS

Toxicity of CPAs to medaka embryos at 26°C

In this experiment, we observed the survival of the medaka embryos after they were exposed in EM containing different concentrations of CPAs for 40 min at 26°C. The survival of embryos was assessed by their development to hatching. As shown in Figure 1, the toxicity of all six CPAs differed from each other, with PG showing the lowest toxicity, and DMF the highest toxicity when the concentration of 4 mol/L was used. The toxicity of CPAs increased in the order of PG < MeOH = DMSO < Gly < EG < DMF. The toxicity of all six CPAs increased with the concentration (Figure 1).

After exposure at 26°C to 1 to 2 mol/L DMSO, MeOH or PG for 40 min respectively, no significant differences ($P>0.05$) in the percentages of medaka embryo survival were observed when compared to the controls (Figure 1). When the embryos were exposed to 3 or 4 mol/L DMSO, MeOH or PG or 1 to 4 mol/L Gly, EG or DMF for 40 min, they were all damaged and showed lower survival percentages ($P<0.05$) when compared to the controls (Figure 1). No embryo survived to hatching after exposure to 3 mol/L or 4 mol/L DMF for 40 min. Therefore the highest CPAs concentration without detrimental effect on embryo survival ($LD_0$ concentration) was 2 mol/L for DMSO, MeOH and PG respectively.

(See Legend overleaf)
Figure 1. Toxicity of different CPAs to medaka embryos is illustrated as percentage of live embryos (which hatched 9 days later) after exposure to CPAs solution for 40 min mean ± S.D., n>3) at 26°C. Data superscripted by the different lower case letters from a to e are significantly different (P<0.05).

The protective efficiency of various CPAs to 8 to 16-cell embryos at 0°C and -5°C

In this study, 2 mol/L DMSO, MeOH and PG (LD₀ concentrations) were used for studying the effect of medaka embryo survival at 0°C and -5°C respectively. As shown in Figure 2, medaka embryo survival decreased as the exposure time at low temperature was prolonged in all groups. The protective efficiencies to medaka embryos of DMSO, MeOH and PG at zero and subzero temperatures were better (P < 0.05) than that of the control when the exposure time was more than 40 min. In addition, the protective efficiencies to embryos were directly related to various CPAs at different temperature. When the exposure time was prolonged (>40 min), addition of 2 mol/L MeOH in embryos medium showed the best protection, whereas PG gave the poorest results and DMSO in between when they were exposed at 0°C and -5°C respectively.

(See Legend overleaf)
Figure 2. Survival of medaka embryos at 8 to 16-cell stage after treatment at (a) 0°C and (b) -5°C in DMSO (2 mol/L, ⚫), MeOH (2 mol/L, ■), PG (2 mol/L, ▲) and Control (EM, ×) for various periods. Embryo survival was assessed by their hatching into live fries following 9 day of culture. The survival rate of non-chilled control embryos was 98.04±0.8%. Values are mean ± S.D. (n>5). a–d Values differ significantly in the same time point (P < 0.05).

The protective efficiency of MeOH to Medaka embryos at various developmental stages.

At both 0°C and -5°C MeOH showed the highest protective efficiency to 8 to 16-cell embryos among the three CPAs (Figure 2). Therefore in the following experiment, studies were carried out on the effect of 2 mol/L MeOH on the survival of medaka embryos at various stages. As shown in Figure 3, after exposure in 2 mol/L MeOH at -5°C for 60 min, the survival rate of embryos at 8 to 16-cell stage was the lowest (48.4±8.1%). The survival rate increased as development proceeded to 32 cell (50.6±8.6%), blastula (63.4±11.3%), 50% epiboly (72.8±16.4%), neurula (76.4±12.1%), 4-somite (82.5±7.2%) stages, and the highest rate (88.7±12.7%) was obtained for 16-somite embryos. Moreover, the survival rate of embryos exposed in MeOH were higher (P<0.05) than that of the control.

DISCUSSION

In our study, the toxicity of six CPAs to medaka embryos increased in the order of PG < MeOH = DMSO < Gly < EG < DMF. Similar results were also observed in
flounder embryos (PG < MeOH < DMSO < Gly < EG) (39), sea perch embryos (PG < MeOH < Gly < EG < DMSO) (32) and zebrafish embryos (MeOH < DMSO < EG < Gly) (37). The toxicity differences may due to the differences of the fish species, developmental stages, egg quality or sperm quality (1,20,21). Dinnyés et al. (9) reported that the toxicity to carp embryos increased in the sequence of MeOH, DMSO and Gly for morula and half-epibody stages, whereas the order of MeOH, Gly and DMSO was found for heartbeat stage, thus the toxicity level of a CPAs to the embryos at different developmental stages may be different (4).

Furthermore, toxicity of CPAs to embryos may be related to its molecular weight (MW) and the membrane permeability (12,25). Generally speaking, the lower MW, the higher permeation of the CPAs to the membranes (25). On the other hand, the CPA could modify the membranes including direct alteration of membrane bilayers and stearic interactions with bound proteins on the external surface, thus altering the membrane permeability (12,15). Although the MW of PG (76.1) is not the smallest among the six CPAs, it may have penetrated the embryos sufficiently without causing toxic effect. However, in zebrafish, it has been reported that PG does not penetrate zebrafish embryo at 100% epiboly, three somite and six somite stages sufficiently (12). Further study should be conducted to confirm the hypothesis.

In the present study, at 0℃ and -5℃, DMSO, MeOH and PG were effective in protecting medaka embryos from chilling injury, and MeOH was found to have better protective efficiency to medaka embryos than DMSO and PG. The results were similar to the observations by Zhang et al. (36) in zebrafish embryos at zero and subzero temperatures. The protective efficiency is not in direct reverse proportion to toxicity of a given CPA in this study – that is, the highest toxicity of CPA may not show the lowest protective efficiency to the embryos and vice versa. The intrinsic nature of the CPA reactions to the embryos under different condition is indeed complicated, hence the relationship needs further research. The relative effectiveness of MeOH as a CPA is thought to be due to its rapid penetration and low toxicity (37) unconditionally freezing. This phenomenon was also verified in flounder by Zhang et al., who suggested that when MeOH was added to any of other CPA solutions, better protective efficiency for flounder embryos at -15℃ could be obtained when compared with the addition of PG (39). Further studies are needed on the mechanisms of the effectiveness of MeOH in protecting fish embryos from chilling injury.

The survival of chilled fish embryos is temperature-dependent. Previously Valdez et al. (33) reported that the survival rate of 2–4 cell medaka embryos chilled in Hanks solution at 0 and -5℃ were 43% and 29% respectively. In our study it was found that survival rate of 8 to 16-cell embryos chilled in 2 mol/L MeOH at 0 and -5℃ for 40 min were 79.6% and 62.9% respectively (Figure 2). Although the embryo stage (2-4 cell vs. 8 to 16-cell embryos) differed between the two studies, significantly higher embryo survival rates were obtained from the present study when MeOH was used. The chilling sensitive temperature has been reported to be determined by the lipid profile of the membrane as previous studies showed that changes in composition of membrane lipids in mammalian gametes alter the phase transition temperature therefore reducing the chilling sensitivity (34).

The medaka embryos at early developmental stages were more sensitive to chilling than those at late-developmental stages (Figure 3). This finding was similar to that obtained by Valdez et al. (33) with medaka embryos treated in hanks solution. Stage-dependent chilling sensitivity has also been reported in many other species of
fish embryos, including zebrafish (12,36), fathead minnows (8), brown trout (23), rainbow trout (11), goldfish (22), carp (9,19,29) red sea bream (30), olive flounder (30), multicolorfin rainbowfish (30) and red drum (10). As development proceeds in fish embryos, it is expected that the composition of yolky lipids changes which may result in the alteration of the phase transition temperature of the yolky lipids (33). Therefore the stage-dependent differences in fish embryo sensitivity to chilling may be closely related to this change. Other reasons for the extent of stage-dependent chilling sensitivity might be related to the changes in cell and tissue types, the number of embryonic cells, effectiveness of repair mechanisms, and enzymatic reactions (9, 27).

In summary, among the six permeable CPAs tested, PG has the lowest toxicity to medaka embryos whereas MeOH is the most effective in protecting madaka embryos from chilling injury. The study also showed that the chilling injury decreases gradually as embryo development stage increases. The finding from this study on the effectiveness of MeOH in reducing chilling injury in fish embryos would have important implications in aquaculture where embryos have to be transported to different locations at reduced temperatures (35).

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