STUDIES ON CRYOPRESERVATION OF JAPANESE WHITING, *Sillago japonica* EMBRYOS

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ABSTRACT

Advances in research on cryopreservation of gametes and embryos of aquatic organisms remain elusive. Survival after cryopreservation has never been achieved with fish embryos and this failure has been attributed to the insufficient cryoprotectant permeation prior to cooling. The major constraint appears to be the formation of ice crystals during cooling, which causes damages resulting in cell death. The intrinsic biophysical properties of cells or embryos and the procedural steps used to prepare the suspension for cooling seem to be species-specific and, therefore, need to be empirically determined for every species of concern. While several freshwater fish species have been introduced for cryopreservation studies little information exists on marine species. In this regard, Japanese whiting (*Sillago japonica*), a small-sized, easy-to-rear, and prolific marine fish was considered as a suitable experimental material for the development of cryopreservation methods for fish embryos. Using Japanese whiting as an experimental marine teleost, the investigation described in this dissertation examines some of the problems that have hindered successful fish embryo cryopreservation. Thus, it provides insight on the effectiveness of some chemical and mechanical treatments to enhance the entry of cryoprotectants as well as identifies protocols that may yield the expected results.

The contents of this dissertation are described in a total of 6 chapters. The background, rationale and objectives of this research are described in chapter 1 whereas chapter 2 compares the suitability of cryopreservation protocols for embryos of Japanese whiting. The following two chapters focus on the development of techniques to promote cryoprotectant impregnation into the embryos. Chapter 5 deals with the development of cryoprotectant impregnation protocols for whiting embryos using information from previous experiments and chapter 6 summarizes the findings of the entire study.

The toxicity and permeability pattern of various cryoprotectant agents (CPAs) and the effectiveness of CPA mixtures to prevent ice formation during freeze-thawing of whiting embryos of different developmental stages were investigated and the results are described in Chapter 2. Exposure of gastrula, somites, tail elongation, and pre-hatching embryos to 10, 15, and 20% solutions of propylene glycol (PG), methanol (MeOH), dimethyl sulfoxide (DMSO), dimethylformamide (DFA), ethylene glycol (EG) and glycerol (Gly) in artificial sea water (ASW; 33 psu) for 20 min revealed that CPA toxicity for whiting embryos increased in the order of PG<DMSO<DFA<EG<MeOH<Gly. CPA permeability, estimated by proton nuclear magnetic resonance spectroscopy, showed the same trend as toxicity except for Gly, which was highly toxic but showed only moderate permeability. Stepwise (20% × 5 steps) impregnations with CPA mixtures of 20-25% PG with 10-15% DFA, DMSO, MeOH, or EG in ASW were well tolerated by the embryos. Embryos exposed to mixtures of PG with MeOH or EG also had lower nucleation temperature and less ice formation during slow and rapid cooling, respectively although none of the embryos survived. However, these embryos showed lower rates of morphological abnormalities after thawing than those impregnated with other CPAs. Tail elongation embryos were more tolerant to cryoinjuries than those of other developmental stages.

Chapter 3 deals with an investigation carried out to test the effectiveness of sugars (sucrose and trehalose), pronase and inorganic salts ($MgCl_2$ and $CaCl_2$) to enhance cryoprotectant uptake by fish embryos. In this study, embryos were exposed to trehalose or sucrose (3 min) and pronase (40 min) prior to impregnation with 10 and 15% DMSO in ASW for 20 min at 24°C. Embryos were also exposed to 10 and 15% DMSO in ASW or a solution of 0.25M $MgCl_2$ and 0.125M $CaCl_2$ in distilled water for 20 min at 24°C. The toxicity of all solutions was estimated from the hatching rates of the embryos and High Performance Liquid Chromatography (HPLC) was used to determine the amount of DMSO taken up by the embryos during impregnation. Exposure of embryos to trehalose (1M)

prior to impregnation with DMSO enhanced the uptake of the cryoprotectant by 40 – 45% in comparison to untreated controls without any deleterious effect on survival rates. On the other hand, pronase did not significantly promote the DMSO permeation into embryos. Interestingly, DMSO incorporation into the embryos was greatly (80 – 180%) enhanced in the presence of $MgCl_2/CaCl_2$ compared to ASW. The inorganic salts were not toxic to the embryos but, probably as a result of the enhanced DMSO uptake, caused decreases in survival relative to ASW. Somites stage embryos were more tolerant than tail elongation ones to DMSO both in ASW and $MgCl_2/CaCl_2$ solutions.

Chapter 4 examined the relative efficiency of ultrasound and electroporation for incorporation of CPA into the embryos of the Japanese whiting. This study was also conducted to assess embryo mortality resulting from various concentrations of DMSO and exposure to ultrasound and electroporation at different embryonic developmental stages. Hatching rates of embryos exposed to ultrasound (37.5 W/cm^2) for 1, 2, and 3 min in the presence of 10 and 20% DMSO solutions were comparable to that of only ultrasound treated embryos but decreased sharply at the concentration of 30%. DMSO content of embryos increased significantly during ultrasound exposure for 20% (internal concentrations of DMSO of 61 to 110 mM) and 30% (72 to 153 mM) DMSO but not for 10% DMSO (36 to 45 mM). On the other hand, embryos (somites stage) treated with electroporation at 100, 200, 300, and 400V in ASW had hatching rates similar (88 to 96%) to that of the control (95.5%) but those treated at 500V had only 47%. Hatching rates were similar for embryos exposed to electroporation at 300V in 10 and 20% DMSO solutions but reduced slightly for 30% DMSO. However, mass mortality was evident for embryos electroporated at 400 and 500V. The DMSO content of embryos (somites stage) electroporated at 300V in 10, 20, and 30% DMSO solutions were 45, 57, and 78 mM, respectively. Embryos treated with other voltages (400 and 500V) had further DMSO uptake but poor survival. This study also proved that cryoprotectant permeability is stage dependent since embryos at tail elongation stage were more permeable than those at the somites stage. Further, embryos (somites stage) pre exposed to 10% DMSO for 20 min followed by exposure to ultrasound and electroporation had increased DMSO uptake about 49 and 41%, respectively, with similar hatching rates.

Chapter 5 attempted to develop suitable cryoprotectant incorporation protocols for Japanese whiting embryos using a combination of chemical and mechanical treatments and the information on CPA solutions from Chapter 2. For this purpose, embryos were first dehydrated with trehalose for 3 min and then stepwise impregnated with various complex cryoprotectant solutions (in ASW or $MgCl_2$) for 20 min (5 steps of 4 min) and 10 min (5 steps of 2 min) and the hatching rates were observed. It was found that complex cryoprotectant solutions in $MgCl_2$ reduced the hatching rates as an indication of rapid permeation into embryos compared to solutions in ASW. Somites stage embryos were more tolerant to all complex CPA solutions than those in tail elongation stage. Among the complex cryoprotectant solutions, CS26 composed of 5% PG/DFA/DMSO, 10% MeOH and 15% EG (in $MgCl_2$) appears to be suitable for the vitrification of whiting embryos due to its low toxicity. Alternatively, pre exposure CS26 (in $MgCl_2$) followed by ultrasound and electroporation in the same solution also appears to be suitable for embryo vitrification. The findings of this study provide fundamental information on suitable impregnation protocols with applicability for embryos of this species and perhaps for other species as well.