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Effect of Calcium Chloride on the Permeation of the Cryoprotectant Dimethyl Sulfoxide to Japanese Whiting *Sillago japonica* Embryos

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Summary

Cryopreservation of fish eggs and embryos is a highly desired tool to promote aquaculture production and fisheries resource management, but it is still not technically feasible. The failure to develop successful cryopreservation protocols for fish embryos is largely attributed to poor cryoprotectant permeability. The purpose of this study was to test the effectiveness of $CaCl_2$ to enhance cryoprotectant uptake by fish embryos. In this study, embryos (somites and tail elongation stages) of Japanese whiting *Sillago japonica* were exposed to 10 and 15% dimethyl sulfoxide (DMSO) in artificial sea water (ASW) or a solution of 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 was used to determine the amount of DMSO taken up during impregnation. The results showed that DMSO incorporation into the embryos was greatly (>50%) enhanced in the presence of $CaCl_2$ compared to ASW. $CaCl_2$ itself was not toxic to the embryos but, probably as a result of the enhanced DMSO uptake, caused decreases in survival of about 14-44% relative to ASW. Somites stage embryos were more tolerant than tail elongation ones to DMSO both as ASW and $CaCl_2$ solutions. The use of $CaCl_2$ as a vehicle for DMSO impregnation could be a promising aid for the successful cryopreservation of fish embryos.

Key words : Japanese whiting, embryo, cryopreservation, DMSO, $CaCl_2$, cryoprotectant, permeability

1. Introduction

Fish gamete and embryo cryopreservation are desirable techniques in aquaculture and fish resource management. Their major benefits for aquaculture include a reduction in the cost of seed production (because they obviate the need for broodstock fish rearing), the continuous supply of seeds for year-round production in farms, the easy exchange of germplasm between hatcheries, and their contribution to selective breeding[1]. By helping preserve pure genetic materials, these techniques can also help mitigate the loss of valuable germplasm due to environmental changes, pollution, overfishing, and genetic introgression between populations as a result of anthropogenic introductions. Unfortunately, successful cryopreservation of fish germplasm has

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been achieved only for sperm cells and isolated blastomeres (early cells of the embryos) of some species, while techniques for the cryopreservation of eggs and whole embryos have proved elusive.

The major constraint to the successful cryopreservation of fish eggs and embryos, as in other biological materials, appears to be the formation of ice crystals during cooling, which causes damages and results in the death of the cells. Vitrification, an ice-free cryopreservation technique using high cryoprotectant concentrations and rapid freezing rates, has been suggested as a tool to overcome this problem. However, though tremendous efforts have been pursuit in the last decades, cryopreservation is still not feasible either by slow cooling or vitrification. The insufficient impregnation of cryoprotectants into fish embryos is singled out as the major constraint for their cryopreservation[2-4]. This problem appears to be related to the fact that fish embryos have a low surface area to volume ratio, a thick chorion, and a complex, multi-compartmental structure. Because of this, it is very difficult to achieve a swift and uniform permeation of the cryoprotectants into the various compartments of fish embryos, resulting in toxicity to some cells while in others the concentration of cryoprotectants is not sufficient to prevent cryoinjuries[5,6].

Several protocols to accelerate the entry of cryoprotectants into fish eggs and embryos are being tested, such as the impregnation with cryoprotectant after dechorionation, under negative or positive pressure, after microinjection of aquaporins, or by direct delivery of the cryoprotectant into the embryos through microinjection[1,5,7,8,9]. Promising results have been obtained in some cases but it has not been possible so far to achieve successful cryopreservation. Moreover, the question remains as whether these sophisticated and time consuming techniques would be suitable for large scale operations. In order to address this problem, we are conducting experiments on the effectiveness of simple physical and chemical treatments to increase the permeation of cryoprotectants into the developing embryos.

In this context, the objective of the present study was to obtain information on the toxicity and effectiveness of $CaCl_2$ to promote the incorporation of dimethyl sulfoxide (DMSO) into marine fish embryos. We chose for this preliminary study the Japanese whiting, *Sillago japonica*, because it is small sized and spawns readily in captivity. DMSO was chosen as the cryoprotectant because it is relatively nontoxic to whiting embryos (Rahman et al., unpublished observations) and because its concentration in embryos can be accurately measured by High Performance Liquid Chromatography (HPLC)[10]. Finally, we focused on $CaCl_2$ because there is a possibility that calcium may interfere with transport mechanisms in biological membranes. To the best of our knowledge, this report provides the first evidence ever that $CaCl_2$ may help increase significantly the uptake of cryoprotectant by fish embryos.

2. Materials and methods

2.1 Collection of embryos

Japanese whiting (*S. japonica*) broodstock was kept in 1200L recirculated water tanks at the fish rearing facilities of Tokyo University of Marine Science and Technology, Shinagawa Campus, in groups of about 5 females and 5 males. Fish were reared in artificial sea water (Rei-Sea Salt G, Japan) prepared to a salinity of 33 psu and were fed frozen krill twice daily to satiation. Water temperature and photoperiod were set to 24°C and 14L10D, respectively. Naturally spawned eggs were collected with a net and incubated until the desired developmental stage in water at the same salinity. Two developmental stages, e.g. somites (14 to 16 somites) and tail elongation (23 to 24 somites) were used in this study. Identification of the developmental stages followed Oozeki and Hirano[11].

2.2 Preparation of DMSO, $CaCl_2$, and washing solutions

Artificial sea water (ASW) obtained by dissolution of seawater salts (Rei-Sea Salt G, Japan) in distilled water (salinity of 33 psu) was used for the preparation of DMSO solutions and for incubation of embryos to monitor survival after the toxicity experiment. DMSO solutions were also prepared using a solution of 0.125M $CaCl_2$ in distilled water. A washing solution of 0.125 M sucrose in ASW water was used for rinsing the embryos prior to incubation in artificial sea water[4,12]. DMSO and $CaCl_2$ were purchased from Wako (Japan) and Sigma-Aldrich (USA), respectively. The osmolality and pH of all solutions were measured with a vapor pressure osmometer (Wescor 5520, Logan, USA) and pH meter (HORIBA B-212), respectively, and are summarized in Table 1.

2.3 Toxicity of DMSO and $CaCl_2$ to embryos

For determination of the toxicity of DMSO and $CaCl_2$, somites and tail elongation stage embryos were exposed to 0, 10 and 15% solutions of DMSO (v/v) in ASW and 0.125M $CaCl_2$ solution for 20 min at 24°C (Fig. 1). Immediately after exposure, embryos were rinsed in washing solution for 10 min and incubated in plastic Petri dishes containing 5 mL of ASW for observation of survival and hatching rates. Hatching was taken as an indication of embryo viability. Each treatment consisted of five replicates with approximately 50 embryos each. Embryos exposed to 0% DMSO in ASW were used as controls.

Table 1 Osmolality (mmol/kg) and pH of the experimental solutions.

Solutions	Osmolality (mmol/kg)	pH
Artificial Sea Water (ASW)	991	7.5
10% DMSO in ASW	2250	7.8
15% DMSO in ASW	2894	8.4
0.125M $CaCl_2$	296	6.2
10% DMSO in 0.125M $CaCl_2$	1818	5.6
15% DMSO in 0.125M $CaCl_2$	2593	7.7
Washing solution (WS)	1042	5.2

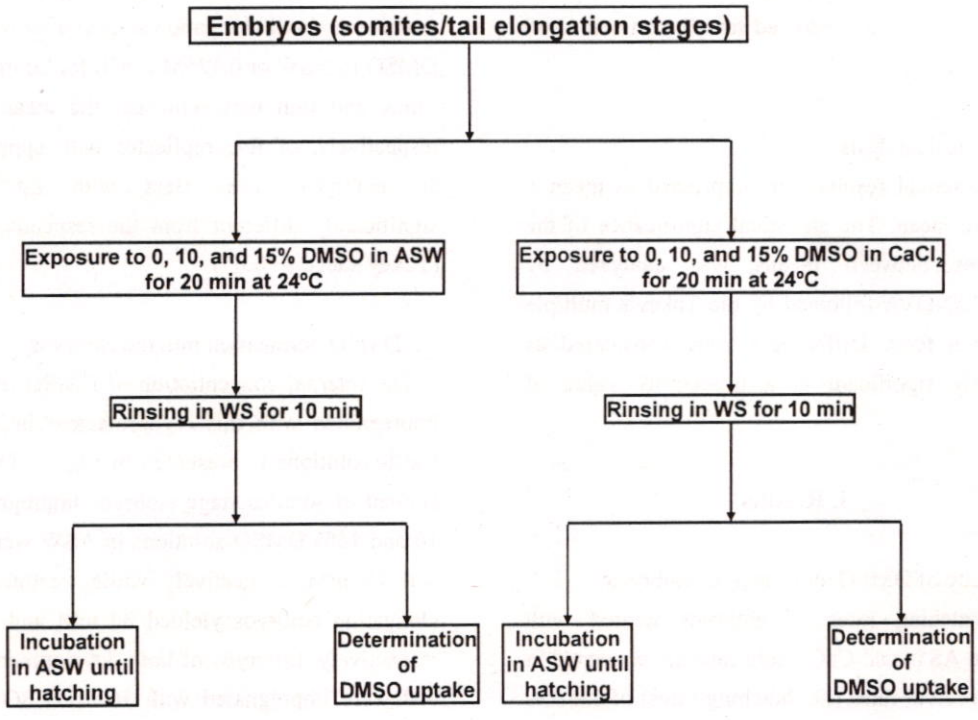

Fig. 1 Flow chart of experimental protocols.

2.4 Measurement of the DMSO concentration in embryos

The internal concentration of DMSO in embryos impregnated for 20 min with the same solutions of the toxicity trial was measured by High Performance Liquid Chromatography (HPLC). After impregnation, embryos were rinsed in ASW, carefully blotted dry onto tissue paper, and homogenized with 1 ml of 5% $HCLO_4$ (Wako Pure Chemical Industries LTD., Tokyo, Japan) for protein precipitation[13]. The homogenate was passed through a 0.45 μm membrane filter (Minisart RC 15, Germany) immediately before injection of a 20 μl aliquot into an HPLC system (Shimadzu Corp., Kyoto, Japan). The system consisted of a Bio-Rad Aminex HPX-87column (7.8 mm × 300 mm), Shimadzu LC-6A pump, CTO column oven at 35°C, SPD-6A detector at 210 nm, and a Chromatopac C-R6A unit. The mean diameter of the embryos (0.68 mm) was measured under a microscope before treatment and used for calculation of their volume and the internal concentration of DMSO. This calculation assumed the embryos to be spherical and no allowance was given for shrinkage or swelling during impregnation. Three replicates with 30 embryos each were conducted for all treatments and controls.

2.5 Statistical analysis

All numerical results were expressed as mean ± SD of the mean. The statistical significance of the differences between means was analyzed by one-way ANOVA followed by the Tukey's multiple comparison tests. Differences were considered as statistically significant at a probability value of $P<0.05$.

3. Results

3.1 Toxicity of DMSO and $CaCl_2$ to embryos

The hatching rates of embryos treated with DMSO in ASW and $CaCl_2$ solutions are presented in Fig. 2. Survival rates (i.e. hatching rates) of somites and tail elongation embryos exposed to 10% DMSO in ASW for 20 min were comparable to those of control embryos but decreased sharply at the concentration of 15%. Hatching rates of embryos exposed to the $CaCl_2$ solution in the absence of DMSO did not vary significantly (Tukey's test, $P>0.05$) compared to the controls at both developmental stages but decreased markedly in the presence of the cryoprotectant. For instance, the concentration of 15% DMSO in $CaCl_2$ was found to be extremely lethal to the embryos whereas that of 10% allowed about 80% and 50% survival in the somites and tail elongation stages, respectively. Somites stage embryos were more tolerant to both types (ASW and $CaCl_2$) of DMSO solutions than those in the tail elongation stage.

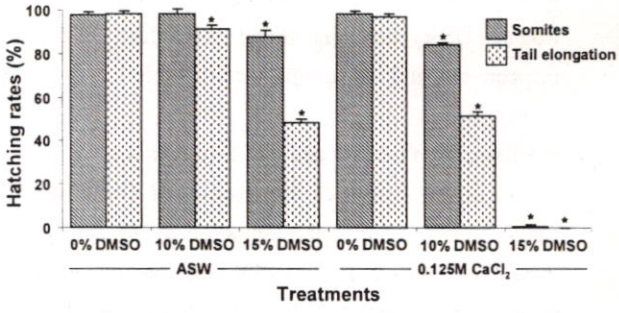

Fig. 2 Hatching rates of *Sillago japonica* embryos (somites and tail elongation stages) after exposure to DMSO in ASW or 0.125M $CaCl_2$ for 20 min at 24°C. Thick and thin bars represent the mean and SD, respectively, of five replicates with approximately 50 embryos each. Bars with asterisks are significantly different from the respective controls (Tukey's test, $P<0.05$).

3.2 DMSO permeation into the embryos

The internal concentration of DMSO in embryos impregnated with this cryoprotectant in ASW and $CaCl_2$ solutions is presented in Fig. 3. The DMSO content of somites stage embryos impregnated with 10 and 15% DMSO solutions in ASW were 46 mM and 78 mM, respectively while treatment of tail elongation embryos yielded 80 mM and 134 mM, respectively. Embryos of both developmental stages that were impregnated with 10% DMSO in $CaCl_2$ had significantly higher internal concentrations of

this cryoprotectant (123.7 mM and 165 mM for somites and tail elongation stages, respectively) than those impregnated in ASW solutions. Results for 15% DMSO in $CaCl_2$ solution were disregarded because of the high mortality observed at this concentration.

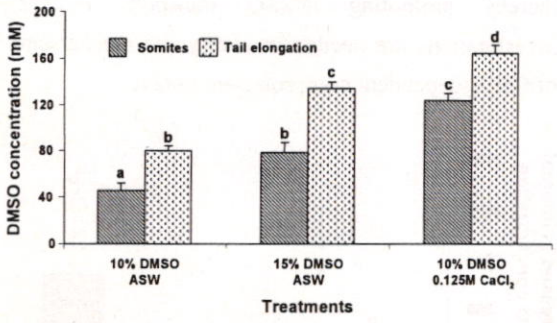

Fig. 3 DMSO concentration of *Sillago japonica* embryos (somites and tail elongation stages) exposed to DMSO in ASW or $CaCl_2$ for 20 min at 24°C. Thick and thin bars represent the mean and SD of three replicates with approximately 30 embryos each. Bars with different letters are significantly different (Tukey's test, $P<0.05$).

4. Discussion

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 results of this study provide novel insight on the effectiveness of $CaCl_2$ to enhance the entry of cryoprotectant in Japanese whiting embryos.

4.1 Toxicity of DMSO and $CaCl_2$ to embryos

Cryoprotectant tolerance varies with the type and concentration of the cryoprotectant, the impregnation protocol, as well as the species and developmental stage of the embryo[7,10]. In this study, DMSO tolerance by the embryos was markedly affected by the impregnation protocol, in particular the concentration, vehicle (solvent) of DMSO, and the developmental stage of the embryos. For example, DMSO in ASW was well tolerated by embryos at a concentration of 10% and a minimum of 48% of the embryos also survived the concentration of 15%. Survival of embryos in DMSO solutions prepared with 0.125M $CaCl_2$, on the other hand, was remarkably lower. At the concentration of 15%, for instance, all tail elongation embryos died and only <1% of the somites stage embryos survived. The concentration of 10% was tolerated better, particularly by the somites embryos, but still produced lower survival rates than the same concentration in ASW. However, the results of this study clearly indicate that increased mortality among embryos impregnated with DMSO in 0.125M $CaCl_2$ was not the result of $CaCl_2$ toxicity, as this salt alone had negligible effect on survival compared to the ASW controls, but the result of enhanced DMSO permeation (see the following section). Interestingly, embryos at the somites stage tolerated DMSO better than those at the tail elongation stages. Chorion permeability increases with development[7,14,15,16] and this fact, which probably resulted in higher DMSO uptake (see the following discussion), could be the reason for the lower cryoprotectant tolerance observed in tail elongation embryos. This stage-dependent cryoprotectant tolerance must be considered in future studies on the cryopreservation of whiting embryos.

4.2 DMSO permeation into the embryos

The results of this study clearly indicate that the DMSO content of the embryos increased with increasing concentration of DMSO in the impregnation medium. This fact was also reported by Magnus *et al.* [17], Suzuki *et al.* [10], and Cabrita *et al.* [14] for carp, medaka, and turbot embryos, respectively. We impregnated somites embryos with 1.4 M (10%) and 2.1 M (15%) DMSO in ASW and observed internal concentrations of 46 and 78 mM, respectively, after 20 min while Cabrita *et al.* [14] reported much lower values for turbot embryos impregnated under similar conditions (5.3 and 11.8 mM for 1.5 M and 2 M, respectively). On the other hand, much higher permeability was detected in rainbow trout and pejerrey embryos (400 and 600

mM, respectively) when embryos were impregnated in 2M DMSO[10]. The reason for the differences reported among species is not known but could be related to the presence of species-specific cryoprotectant barriers, particularly in the comparison between marine and freshwater species, and perhaps also to differences in the chemical and physical properties of the medium of impregnation (see the following discussion on ASW vs. $CaCl_2$). We also noted a clear effect of the developmental stage of the embryos on their permeability to DMSO, as discussed above. Depeche and Billard[18] suggested that this might be due to an increase in the diameter of the chorion canals during development, which may have favored DMSO permeation.

The major finding of this study was that a simple salt solution (0.125M $CaCl_2$) greatly enhanced the uptake of DMSO by whiting embryos. Thus, exposure to this solution accelerated the permeation of DMSO by 62 and 51% for somites and tail elongation stages, respectively, in comparison to DMSO in ASW. It is noteworthy that the internal concentration of DMSO in embryos treated with 10% of the cryoprotectant in $CaCl_2$ was significantly higher than that in embryos treated with 15% in ASW for both developmental stages. In fact, the permeation enhancement was such that all embryos died when treated with 15% DMSO in $CaCl_2$. To the best of our knowledge there is no comparable information on the use of inorganic salts such as $CaCl_2$ to increase the permeation of cryoprotectants into the embryos of fish or any other species. In this context, we can not yet provide an explanation for the accelerated DMSO uptake by the treated embryos. One possibility is that the enhanced uptake of DMSO is simply the result of passive absorption in the presence of an osmotic gradient since the osmolality of 0.125M $CaCl_2$ solution is about 1/3 that of ASW (Table 1). To test such a possibility, we compared the uptake of cryoprotectant in embryos exposed to isosmotic solutions of 10% DMSO prepared with 0.125M $CaCl_2$ and diluted (33.33%) ASW. This trial confirmed that $CaCl_2$ treatment induced a several-fold increase in DMSO uptake compared to the ASW control and showed that this uptake is not simply due to the lower osmotic pressure of the medium (Fig. 4). One alternative is that the presence of Ca^{+2} ion alters the polarity of pore channels in the vitelline membrane of the embryos or the binding ability of carrier proteins, thereby promoting DMSO transport. Further investigations are needed to clarify the mechanism of $CaCl_2$-dependent cryoprotectant uptake.

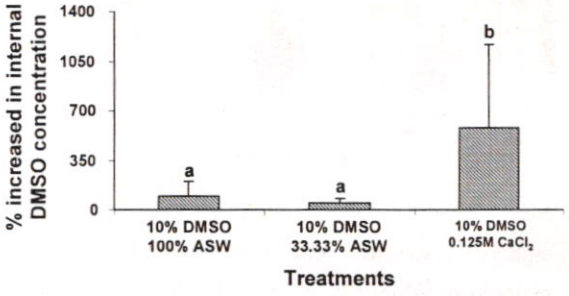

Fig. 4 Percent increased in internal DMSO concentration of *Sillago japonica* embryos (somites stage) exposed to 10% DMSO in 33.33% ASW (osmolality: 1790 mmol/kg) or 0.125M $CaCl_2$ (osmolality: 1818 mmol/kg) compared to 10% DMSO in 100% ASW (osmolality: 2250 mmol/kg). Impregnation was performed for 20 min at 24°C. Thick and thin bars represent the mean and SD of three replicates with approximately 30 embryos each. Bars with different letters are significantly different (Tukey's test, $P<0.05$).

5. Conclusions

Cryopreservation of fish embryos requires an optimal distribution of cryoprotectants inside all embryo compartments to prevent intra-embryonic ice formation and cryoinjuries during cooling. This study revealed that the use of an inorganic salt ($CaCl_2$) greatly promoted DMSO uptake by Japanese whiting embryos and this finding could be instrumental for the development of cryopreservation protocols for the embryos of this and other species. Further studies must be conducted to try other salts, optimize the salt concentration, and determine the most suitable conditions (time and

temperature) of impregnation to maximize cryoprotectant uptake and embryo survival.

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