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Efficiency of osmotic and chemical treatments to improve the permeation of the cryoprotectant dimethyl sulfoxide to Japanese whiting (*Sillago japonica*) embryos

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Abstract

Insufficient cryoprotectant permeation is one of the major obstacles for successful fish embryo cryopreservation. The purpose of this study was to test the effectiveness of osmotic and chemical treatments to enhance cryoprotectant uptake by fish embryos. Japanese whiting *Sillago japonica* embryos at the somites and tail elongation stages were treated with hyperosmotic sugar solutions (1 M trehalose and sucrose) for 2–6 min, or a permeating agent (2–6 mg/mL pronase) for 30–120 min, and then impregnated with 10–15% DMSO in artificial sea water or aqueous solutions containing inorganic salts (0.125–0.25 M $MgCl_2$ and $CaCl_2$). The viability of the embryos after the treatments was estimated from hatching rates and the internal DMSO concentration was measured by HPLC. Treatment with trehalose for 3 min prior to impregnation with DMSO enhanced the uptake of the cryoprotectant by 45% without significantly affecting embryo viability, whereas pronase had no noticeable effect on cryoprotectant permeation. Incorporation of DMSO into the embryos was enhanced by 143–170% in the presence of 0.25 M $MgCl_2$ and 0.125 M $CaCl_2$ compared to sea water. A combination of treatments with trehalose and $MgCl_2$ was even more effective in promoting DMSO permeation (191% compared to untreated embryos). Tail elongation embryos were less tolerant of the treatments, but had higher DMSO impregnation. In conclusion, the use of trehalose (as dehydrating agent) and $MgCl_2$/$CaCl_2$ (as a vehicle during impregnation) greatly promoted cryoprotectant uptake and may be a promising aid for the successful cryopreservation of fish embryos.
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Keywords: Japanese whiting; Fish embryos; Trehalose; Pronase; $MgCl_2$; Cryoprotectant

1. Introduction

Cryogenic preservation of embryos could improve dramatically the efficiency of breeding programs for the conservation of valuable aquatic resources. In spite of this interest, substantial advancement in the research on cryopreservation of gametes and embryos of aquatic organisms remain elusive. For instance, survival after cryopreservation has never been achieved with fish embryos and this failure is generally attributed to insufficient cryoprotectant (CPA) permeation prior to cooling [1–4]. This leads to the formation of ice crystals during cooling, which causes damages and subsequently cell death. Vitrification, an approach based on the use of high concentrations of CPAs, offers considerable promise for simplifying and improving the cryo-

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preservation of cells because potential injury associated with ice formation in the suspension is obviated. Unfortunately, the methods developed for mammalian embryo vitrification can not be easily applied to the multi-compartment, structurally complex, and yolk-laden embryos of fish [5]. For example, it is commonly found that CPA permeation is slow and uneven, causing toxicity to some cells while in others their concentration is not sufficient to prevent cryoinjuries [6,7].

The key to successful cryopreservation appears to involve the development of methods for faster, even impregnation and removal of CPAs into these biological materials. Several innovative techniques including impregnation with CPA after dechorionation, under negative or positive pressure, after microinjection of aquaporins, or by direct delivery of the CPA into the embryos through microinjection [7–11] have been developed to accelerate the permeation of CPA into fish embryos. In a recent study, Martínez-Páramo et al [12] tested the incorporation of antifreeze protein (AFP) into zebrafish embryos. Some of these techniques showed promising results but none has actually led to successful cryopreservation. It is doubtful as to whether sophisticated and time consuming techniques would be feasible in practical situations; hence, development of cryopreservation methods should also take into consideration the cost, the ease, and the possibility of application in the field.

The objective of the present study was to obtain information on the effectiveness of osmotic and chemical treatments to promote the incorporation of dimethyl sulfoxide (DMSO), a commonly used CPA, into Japanese whiting *Sillago japonica* embryos. Routray et al [9] reported that medaka embryos dehydrated with trehalose prior to impregnation with CPA showed accelerated uptake of the CPA. Sugars are also used to remove the CPAs after thawing [13,14]. On the other hand, other studies used a protease solution to permeabilize shrimp and carp embryos and to remove the chorion of zebrafish embryos [7,15,16]. Recently, Rahman et al [17] showed in preliminary experiments that treatment with a simple inorganic salt solution (0.125 M $CaCl_2$) greatly enhanced the subsequent uptake of DMSO by whiting embryos compared to the routine exposure to artificial sea water (ASW). In this context, we investigated the toxicity and effectiveness of sugars (sucrose and trehalose), enzyme (pronase), and inorganic salts ($MgCl_2$ and $CaCl_2$) to promote the incorporation of DMSO into whiting embryos. The Japanese whiting is a small-sized, easy-to-rear, and prolific marine fish which constitutes a suitable experimental material for the development of cryopreservation methods for fish embryos. Likewise, DMSO was selected as the CPA as it is relatively less toxic to whiting embryos [18] and because its concentration in fish embryos can be measured easily by High Performance Liquid Chromatography (HPLC) [19].

2. Materials and methods

2.1. Collection of embryos

All animals were maintained for breeding as described in Rahman et al [18]. Briefly, whiting adults were reared in 1200 L re-circulated water tanks kept under a 14 h light and 10 h dark light cycle at 24 °C. Under these conditions, fertilized eggs were obtained almost daily. Embryos were collected with a net early in the morning and transferred to 1 L glass beakers with aerated sea water at 24 °C for incubation until the desired developmental stage. Embryos were examined periodically under a light microscope and staged according to the criteria of Oozeki and Hirano [20]. Two developmental stages, e.g. somites (14 to 16 somites; approximately 14 h after fertilization) and tail elongation (23 to 24 somites; approximately 16 h after fertilization) were used in the following experiments.

2.2. Toxicity of the osmotic and chemical treatments to embryos

Embryos at the two developmental stages (somites and tail elongation) were exposed to 1 M trehalose and sucrose for 2, 4, or 6 min, to 2, 4, and 6 mg/mL of pronase for 30, 60, 90, or 120 min, and to 0.125, 0.25, and 0.5 M $MgCl_2$ and $CaCl_2$ for 20 min. Sugar and pronase solutions were prepared in artificial sea water (ASW), obtained by dissolution of sea water salts (Rei-Sea Salt G, Japan) in distilled water to a salinity of 33 psu. The inorganic salt solutions were prepared in distilled water. Sucrose, trehalose, and $MgCl_2$ were purchased from Wako (Japan) while pronase (Type XIV from *Streptomyces griseus*) and $CaCl_2$ were purchased from Sigma-Aldrich (USA). The osmolality and pH of all solutions are shown in Table 1. Osmolality and pH were measured using the Wescor 5520 vapor pressure osmometer (Wescor, Logan, USA) and a Horiba B-212 pH meter (Horiba, Japan). Immediately after exposure to the chemicals, embryos were rinsed in ASW for 10 min and incubated in plastic Petri dishes containing 5 mL of ASW for observation of survival. Hatching was considered as an indication of embryo viability. All experiments were conducted at room temperature (ap-

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

Solutions	Osmolality (mmol/kg)	pH
ASW	911	7.5
0.125M $MgCl_2$ in DW	301	8.4
0.25M $MgCl_2$ in DW	624	8.5
0.5M $MgCl_2$ in DW	1288	8.8
0.125M $CaCl_2$ in DW	296	6.2
0.25M $CaCl_2$ in DW	659	6.1
0.5M $CaCl_2$ in DW	1378	6.4
10% DMSO in ASW	2250	7.8
15% DMSO in ASW	2894	8.4
10% DMSO in 0.125 M $MgCl_2$	1716	8.6
10% DMSO in 0.25 M $MgCl_2$	1992	8.6
15% DMSO in 0.125 M $MgCl_2$	2160	8.6
15% DMSO in 0.25 M $MgCl_2$	2440	8.6
10% DMSO in 0.125 M $CaCl_2$	1818	5.6
10% DMSO in 0.25 M $CaCl_2$	2110	5.5
15% DMSO in 0.125 M $CaCl_2$	2490	5.7
15% DMSO in 0.25 M $CaCl_2$	2800	5.7
1 M Trehalose	2010	4
1 M Sucrose	1640	4.2

DMSO, Dimethyl sulfoxide; ASW, artificial sea water; DW, distilled water.

proximately 24 °C). Each treatment consisted of five replicates with approximately 50 embryos each. Embryos not subjected to any treatment were used as controls.

2.3. Toxicity of DMSO to embryos after the osmotic and chemical treatments

For determination of the toxicity of DMSO in combination with the osmotic and chemical treatments, we defined conditions based on the highest concentration and/or longest treatment duration that did not induce significant mortality (i.e., $< 20\%$) among those tested in the previous experiment. Thus, in this experiment, we impregnated embryos from the two developmental stages with 10 and 15% DMSO for 20 min at 24 °C after partial dehydration in 1 M trehalose for 3 min or treatment with pronase solution at 6 mg/mL for 40 min. We also impregnated embryos with DMSO simultaneously with exposure to 0.125 M $CaCl_2$ and 0.25 M $MgCl_2$ solutions. The choice of trehalose over sucrose was based on its ability to stabilize the membrane structure as well as to promote CPA permeability [9,21] but both sugars gave similar results on toxicity (see following section). The degree of dehydration of whiting embryos was not quantified, but the chorion was visibly shrunk after the treatment with trehalose, returning to normal within 10 min of immersion in the DMSO. In addition to these trials with single treatments, we also tested the effectiveness of exposure to DMSO in 0.25 M $MgCl_2$ solution after partial dehydration in trehalose. This combination was tested only with the somites stage embryos and a DMSO concentration of 15% at a temperature of 24 °C, but with variable impregnation times between 10 and 20 min. In these experiments, the number of embryos per treatment, number of replicates, and all procedures for incubating embryos and monitoring survival followed the methods described in the previous section.

2.4. Measurement of the DMSO concentration in treated embryos

The amount of DMSO taken up by the embryos in selected groups from the experiments (see section 3.3) was measured by High Performance Liquid Chromatography (HPLC). Immediately after impregnation, 30 embryos from each treatment were rinsed in ASW, blotted dry onto a tissue paper, and homogenized with 1 mL of 5% $HClO_4$ (Wako, Japan) for protein precipitation [22]. The homogenate was passed through a 0.45 μm membrane filter (Minisart RC 15, Germany) just before injection of a 20 μL aliquot into an HPLC system (Shimadzu Corp., Kyoto, Japan) equipped with a Bio-Rad Aminex HPX-87 column (7.8 mm $\times$ 300 mm). The mobile phase was 0.003 M H_2SO_4 and the flow rate was set at 0.6 mL/min. Each sample was measured in triplicate.

2.5. Statistical analysis

The results of all experiments were expressed as mean $\pm$ SD. The statistical significance of the treatments was analyzed by one-way ANOVA, followed by the Tukey's multiple comparison test. Differences with a probability value of $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Toxicity of the osmotic and chemical treatments to embryos

No significant reduction of hatching rates was observed until 2 min of exposure to both types of sugars in comparison to controls (Fig. 1). Further dehydration caused progressive reduction in hatching rates, which was tolerated better by somite stage embryos than those in the tail elongation stage. Embryos exposed to different concentration of pronase solutions for 30 min showed similar pattern of hatching rates and did not vary significantly compared to untreated embryos but hatching rates decreased steadily with increasing expo-

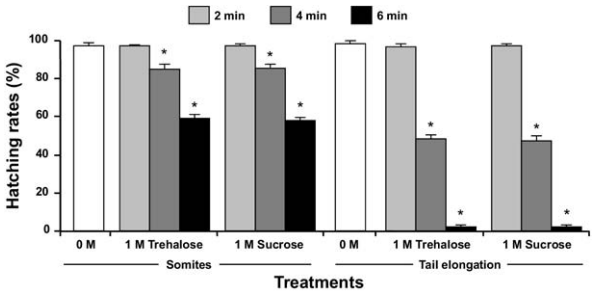

Fig. 1. Hatching rates of Japanese whiting (*Sillago japonica*) embryos (somites and tail elongation stages) exposed to 1 M sugar solutions (in ASW) for 2, 4, and 6 min at 24 °C. Thick and thin bars represent the mean and SD, respectively, of five replicates with approximately 50 embryos each. Bars with asterisks are significantly different from the respective controls.

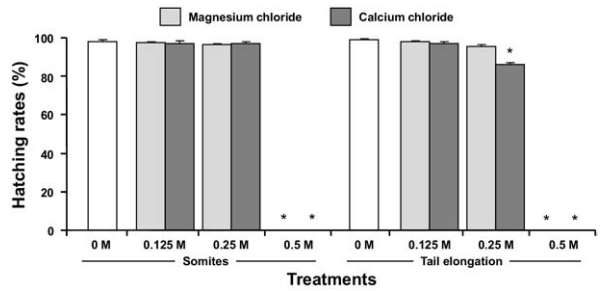

Fig. 3. Hatching rates of Japanese whiting (*Sillago japonica*) embryos (somites and tail elongation stages) exposed to different concentrations of inorganic salts (in distilled water) for 20 min at 24 °C. Thick and thin bars represent the mean and SD, respectively, of five replicates with approximately 50 embryos each. Bars with asterisks are significantly different from the respective controls.

sure time and pronase concentration (Fig. 2). Differences between developmental stages were negligible. The influence of inorganic salts on the hatching rates is shown in Fig. 3. The two lowest concentrations of both salts did not affect embryo viability regardless of the developmental stage except for a small but significant decrease in the survival rate at 0.25 M $CaCl_2$. None of the embryos survived the treatment with 0.5 M of $MgCl_2$ and $CaCl_2$ solutions.

3.2. Toxicity of DMSO to the embryos after the osmotic and chemical treatments

The 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% (Fig. 4). Somites embryos treated with trehalose and pronase prior to impregnation with DMSO had significantly lower survival rates than the controls at 15% but not 10% DMSO. On the other hand, both treatments caused significant losses at the tail elongation stage, particularly at the highest DMSO concentration. Hatching rates of somites embryos exposed to the two concentrations of DMSO in the presence of 0.25 M $MgCl_2$ did not vary significantly compared to the controls. However, they decreased significantly with 0.125 M $CaCl_2$, particularly at 15% DMSO. The toxicity of DMSO to tail elongation embryos was enhanced by treatment with inorganic salts as survival rates at 10 and 15% DMSO dropped to 90.89% and 48.21%, respectively.

3.3. DMSO concentration in osmotically and chemically-treated embryos

The uptake of DMSO by the embryos varied markedly with the developmental stage of the embryos and with the conditions of impregnation (Fig. 5). In general,

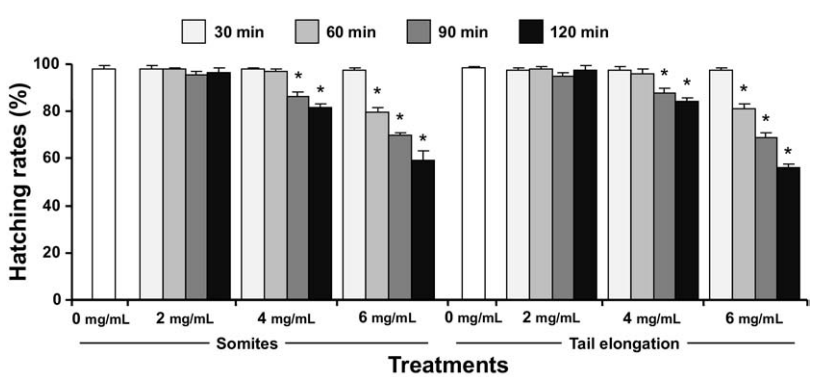

Fig. 2. Hatching rates of Japanese whiting (*Sillago japonica*) embryos (somites and tail elongation stages) exposed to different concentrations of pronase (mg/mL, in ASW) for 30, 60, 90, and 120 min at 24 °C. Thick and thin bars represent the mean and SD, respectively, of five replicates with approximately 50 embryos each. Bars with asterisks are significantly different from the respective controls.

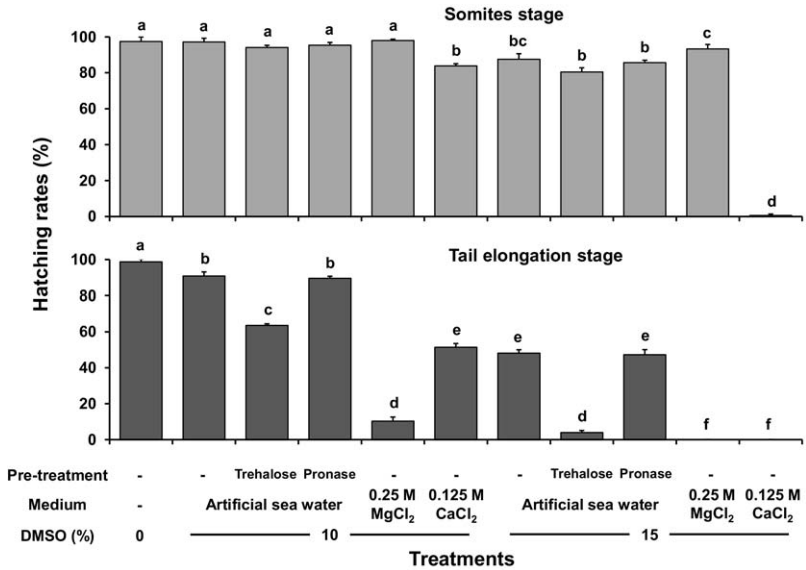

Fig. 4. Hatching rates of Japanese whiting (*Sillago japonica*) embryos (somites and tail elongation stages) after exposure to 0, 10, or 15% DMSO for 20 min following or in conjunction with various treatments. Treatments with 1 M trehalose and 6 mg/mL pronase had durations of 3 and 40 min, respectively. Thick and thin bars represent the mean and SD, respectively, of five replicates with approximately 50 embryos each. Bars with different letters are significantly different.

impregnation with 15% DMSO was associated with higher internal concentrations of the CPA than with 10%. In both developmental stages and DMSO concentrations, the CPA content of embryos treated previously with trehalose and pronase was comparable to those of embryos exposed directly to DMSO in ASW. An exception was the impregnation with 15% DMSO after trehalose treatment, which produced a remarkable in-

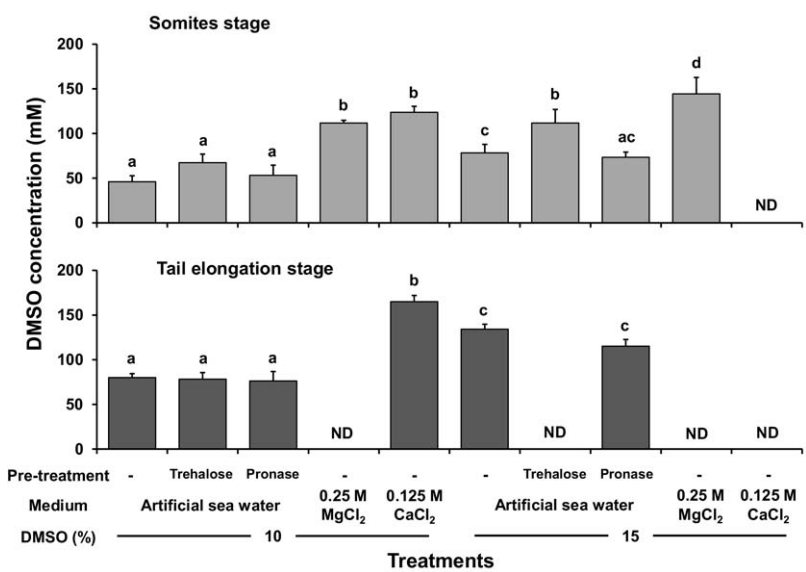

Fig. 5. Internal DMSO concentration of Japanese whiting (*Sillago japonica*) embryos (somites and tail elongation stages) after exposure to 10 or 15% DMSO for 20 min following or in conjunction with various treatments. Treatments with 1 M trehalose and 6 mg/mL pronase had durations of 3 and 40 min, respectively. Thick and thin bars represent the mean and SD, respectively, of three replicates with approximately 30 embryos each. Bars with different letters are significantly different. ND, Not determined.

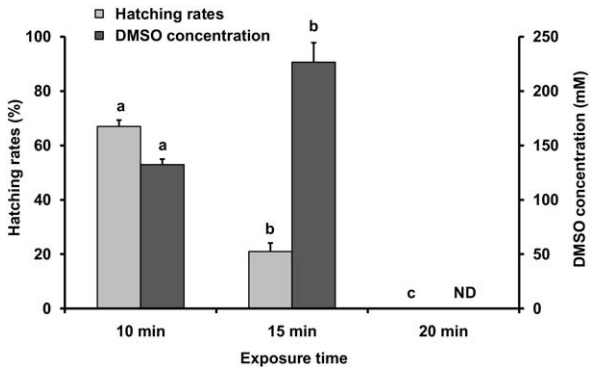

Fig. 6. Changes in hatching rates and internal DMSO concentration of Japanese whiting (*Sillago japonica*) embryos (somites stage) that were treated with 1 M trehalose for 3 min followed by exposure to 15% DMSO in 0.25 M $MgCl_2$ for 10, 15, and 20 min. Thick and thin bars represent the mean and SD, respectively, of three replicates with approximately 30 embryos each. Bars with different letters are significantly different. ND, Not determined.

crease in the DMSO concentration of somites stage embryos (DMSO content of tail elongation embryos was not determined because of low survival). Embryos of both developmental stages that were impregnated with DMSO in $MgCl_2$ and $CaCl_2$ solutions had significantly higher internal concentrations of this cryoprotectant than those impregnated in ASW solutions with or without pre-treatment with trehalose or pronase. For instance, the DMSO content of embryos treated with 10% DMSO in inorganic salt solutions were 2–3 fold those in other treatments and a 2-fold increase was also noted with 15% DMSO in 0.125 M $CaCl_2$. The DMSO content of embryos could not be measured in some inorganic salt treatments because of the high mortality associated with these treatments (see Fig. 4). In the trial with a combination treatment of trehalose for 3 min followed by 15% DMSO in 0.25 M $MgCl_2$, somites stage embryos had decreasing hatching rates and increasing DMSO content with exposure time (Fig. 6). Maximum DMSO content of embryos was recorded after 15 min (227 mM) for a survival rate of 21%. Exposure for 20 min at these experimental conditions was found to be lethal to whiting embryos.

4. Discussion

Fish embryos contain a large amount of water which may result in ice formation and cryoinjuries during the cooling and warming processes. In this regard, removal of most of the free water within the embryos and the achievement of sufficient permeation of CPAs into the embryos are crucial to prevent intracellular ice formation and to aid successful cryopreservation [23]. Large molecules such as sugars generally have limited permeation through biological membranes and can be used to induce dehydration of cells and tissues or to help remove CPAs after thawing [13,24,25]. Routray and colleagues [9] demonstrated that short exposure of medaka embryos to a hyperosmotic solution of the disaccharide trehalose prior to impregnation with DMSO enhanced their uptake of the CPA by 20–40%. They assumed that partial dehydration facilitated the uptake of the waterborne DMSO during impregnation. This study tested the ability of two sugars, sucrose and disaccharide trehalose to promote the uptake of DMSO in whiting embryos. Embryos tolerated well 1 M solutions of the two sugars for 2 min and further exposure steadily reduced embryo viability. However, in the presence of DMSO solutions, hatching rates remained similar compared to the control or were reduced only slightly when somites embryos were pre-exposed to trehalose for 3 min prior to impregnation with 10 or 15% DMSO. Moreover, we also observed enhanced CPA uptake (46 and 43% for 10 and 15% DMSO, respectively) in partially dehydrated somites embryos in comparison to embryos that were not pre-exposed to trehalose. Thus, osmotic manipulation with trehalose appears to be a relatively safe [9,26] and efficient method to promote impregnation of fish embryos with CPAs.

The influence of pronase on embryo survival and CPA permeation into whiting embryos was also observed in the present study. Embryos at both developmental stages tolerated pronase up to a concentration of 6 mg/mL for up to 30 min, whereas exposure times over 60 min induced a dose-dependent decrease in survival rates. Other authors have also used a pronase solution to permeabilize shrimp [15], carp [16], and zebrafish [7] embryos and did not notice any deleterious effects on embryos. However, Cabrita et al [27] reported lower hatching rates in turbot embryos after prolonged exposure to pronase. In this study, embryos treated with pronase prior to impregnation with DMSO solutions (in ASW) did not show enhanced CPA uptake compared to untreated control groups, which was consistent with data obtained by Cabrita et al [27] and Robles et al [28] for turbot and winter flounder embryos, respectively. However, those authors noticed that although pronase did not improve the permeation of CPA into the embryos, it did facilitate the elimination of the CPA during the process of washing. Hence, although for a different reason that tested in this study,

pronase treatment might still be beneficial for the cryopreservation of fish embryos.

The most interesting finding of this study was that simple salt solutions greatly enhanced the uptake of DMSO by whiting embryos. Thus, we confirmed a preliminary report that $CaCl_2$ enhanced the DMSO permeability of whiting embryos compared to ASW [17] and found that another salt, $MgCl_2$, had similar effects. Somites stage embryos exposed to a 10% DMSO solution in 0.25 M $MgCl_2$ and 0.125 M $CaCl_2$ had their permeation of DMSO enhanced by 144% and 169%, respectively, in comparison to ASW. It is noteworthy that the internal concentration of DMSO in embryos treated with 10% of the CPA in $MgCl_2$/$CaCl_2$ was significantly higher than that of embryos treated with 15% in ASW at both developmental stages. To the best of our knowledge, there is no comparable information on the use of inorganic salts such as $MgCl_2$ and $CaCl_2$ to increase the permeation of CPAs into the embryos of fish or any other species. In a previous study [17], we examined the possibility that the enhanced DMSO uptake might be the result of passive absorption in the presence of an osmotic gradient since the osmolality of a 0.125 M $CaCl_2$ solution is about 1/3 that of ASW (see Table 1). However, this possibility was ruled out, since embryos exposed to DMSO in sugar solutions isosmolar to 0.125 M $CaCl_2$ did not show enhanced uptake [17]. It must be noted that the salt solutions we tested contain much higher concentrations of the divalent ions than sea water. For instance, natural sea water contains 2450 mg/L Mg^{+2} and 587 mg/L Ca^{+2} [29] whereas 0.25 M $MgCl_2$ and 0.125 M $CaCl_2$ contain 6078 mg/L Mg^{+2} and 5010 mg/L Ca^{+2}, respectively. Thus, future studies should examine other possibilities such as that Mg^{+2}/Ca^{+2} could alter the polarity of pore channels in the vitelline membrane of the embryos or the binding ability of carrier proteins, and thereby promote DMSO transport.

It is well known that cryoprotectant tolerance varies with the type and concentration of the CPA, the impregnation protocol, as well as the species and developmental stage of the embryos [9,18,19,27,30]. Many of these observations also apply to the results of this study. For example, 10% DMSO in ASW was well tolerated by the embryos and at least 48% of them survived at the concentration of 15%. However, the survival of embryos impregnated with DMSO in 0.25 M $MgCl_2$ and 0.125 M $CaCl_2$ was remarkably lower than in ASW, particularly for the tail elongation stage. The increased mortalities among embryos impregnated in the presence of salts was not the result of their toxicity, as 0.25 M $MgCl_2$ and 0.125 M $CaCl_2$ solutions *per se* had negligible effect on survival, but probably the result of the enhanced DMSO content of embryos impregnated with this protocol. Likewise, that tail elongation embryos had comparatively higher DMSO content after impregnation and lower survival than those at the somites stage in almost all treatments probably reflected a difference in chorion permeability, which generally increases with development [9,27,28,31]. This stage-dependent CPA tolerance must be considered in future studies on the cryopreservation of whiting embryos but it will be obviously counterproductive to choose less permeable developmental stages to maximize survival if it means less CPA content after impregnation. Hence, it is likely that success will come from the use of combinations of several CPAs as opposed to high concentrations of a single one [18] and by performing impregnation and actual cryopreservation faster, before the CPAs become toxic to the cells [9].

In this context, this study examined the efficiency of a combination of two methods, viz. pre-treatment with trehalose prior to impregnation with DMSO in 0.25 M $MgCl_2$. The results clearly pointed to increased DMSO uptake compared to each treatment when they were run separately. For example, somites embryos treated for 20 min with 15% DMSO in ASW, 15% DMSO in 0.25 M $MgCl_2$, and 15% DMSO in ASW after exposure to 1 M trehalose had internal DMSO concentrations of 78, 144, and 112 mM, respectively. In contrast, embryos impregnated with 15% DMSO in 0.25 M $MgCl_2$ after exposure to trehalose had 132 mM DMSO after only 10 min and up to 227 mM after 15 min. The increased DMSO permeation was likely the cause of the mass mortality after 20 min of exposure. These findings suggest possible ways to speed up CPA impregnation, and perhaps also AFP impregnation [12], and could become instrumental for the development of cryopreservation protocols for the embryos of this and perhaps other fish species. Further investigations are also needed to clarify the mechanism of $MgCl_2$/$CaCl_2$-dependent CPA uptake. In addition, studies must be conducted to determine the most suitable conditions of impregnation to maximize CPA uptake and embryo survival.

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