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Suitability of cryoprotectants and impregnation protocols for embryos of Japanese whiting *Sillago japonica* ☆

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ABSTRACT

The purpose of this study was to examine the suitability of cryoprotectant agent (CPA) impregnation protocols for the embryos of Japanese whiting (*Sillago japonica*), a small-sized, easy-to-rear, and prolific marine fish which may constitute a suitable experimental material for the development of cryopreservation methods for fish embryos. Our immediate goals were to assess the toxicity and permeability of various CPAs to whiting embryos of different developmental stages. Exposure of gastrula, somites, tail elongation, and pre-hatching embryos to 10%, 15%, and 20% solutions of propylene glycol (PG), methanol (MeOH), dimethyl sulfoxide (Me_2SO), 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 < Me_2SO < DFA < EG < MeOH < Gly. Relative 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. There were no marked differences in CPA tolerance between developmental stages except for a slight decrease in pre-hatching embryos. Stepwise (20% × 5 steps) impregnation with CPA mixtures of 20–25% PG with 10–15% DFA, Me_2SO, MeOH, or EG in ASW were well tolerated by the embryos. Overall, the results of toxicity and permeability suggest that PG, MeOH, and EG could be useful for the development of CPA solutions for whiting embryos.

The recent worldwide declines in natural fish stocks [20], largely resulting from overexploitation and anthropogenic changes in the environment, have stirred renewed interest in the creation of gene banks for wild aquatic organisms [14]. The ideal strategy for conservation of threatened and endangered species is through protection and/or restoration of their native habitats. Unfortunately, this requires a great deal of money and time as habitat restoration is clearly a slow process. One alternative is to maintain *ex situ* live or cryopreserved gene banks. Establishment of *ex situ* gene banks in the immediate term would ensure the maintenance of genetically pure fish stocks while "buying us the time" to improve habitat conditions for restocking. In addition, the rapid growth of the aquaculture industry has furthered the need for the timely production and delivery of fish seeds, a requirement which could be addressed also in the most effective manner by the cryogenic storage of gametes and embryos [9].

Methods for the cryopreservation of fish sperm have been developed for numerous species of inland water and marine fish. On the other hand, although tremendous efforts have been undertaken in the last decades, cryopreservation of eggs or embryos has made little progress. Fish embryos have a complex, multi-compartmental structure with several membranes. The yolk syncytial layer (YSL) surrounding the yolk is considered as the major obstacle for the permeation of cryoprotectant agents (CPAs) as well as their subsequent removal [12,13]. Insufficient internal concentration of CPAs results in physical damage to the embryos due to the formation of intra- and extra-cellular ice crystals, particularly during slow cooling and thawing. The low permeability of fish embryos to CPAs also prevents the application of vitrification, an ice-free cryopreservation technique which combines high CPA concentrations and rapid freezing rates [13,18,28]. Thus, the lack of a suitable CPA impregnation protocol is currently the greatest hurdle for successful cryopreservation of fish eggs and embryos [2]. CPA permeability and tolerance by the embryos varies with the type and concentration of the CPA, the impregnation protocol, as well as the species and developmental stage of the embryo, and these are all aspects that need to be optimized prior to cryopreservation [24,25].

In this report, we present the results of basic studies towards the development of cryopreservation protocols for the embryos

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of Japanese whiting *Sillago japonica*. The Japanese whiting is a Perciform species with high commercial value and is commonly found in the shallow coastal waters of Japan, Korea, China, Taiwan, and possibly the Philippines (www.fishbase.org). The major reproductive characteristics of this species include its small size as adult (c.a. 15–30 cm body length), natural spawning in captivity, high fecundity (20,000–80,000 eggs per adult female every second day) [16], the small, transparent, and pelagic embryos (diameter 0.65–0.71 mm), and the rapid embryonic development (hatching within 21 h at 24 °C) [21]. Thus, Japanese whiting could be a good model for the study of cryopreservation of fish embryos. In this context, the main goals of this study were to examine the toxicity and permeability of various CPAs to Japanese whiting embryos. In addition, this study was designed also to obtain preliminary information on the suitability of impregnation protocols and different embryo developmental stages for cryopreservation.

Materials and methods

Broodstock maintenance and embryo collection

Mature Japanese whiting (*S. japonica*) broods were collected by angling from Tateyama Bay, Japan, and reared in 1200 L recirculated sea water tanks at the fish rearing facilities of Tokyo University of Marine Science and Technology. Rearing water was prepared to a salinity of 33 psu using artificial sea water salts (Rei-Sea Salt G, Japan) and dechlorinated tap water. Fish were stocked in groups of about 5 females and 5 males and were fed frozen krill twice daily (morning and evening) until satiation. Water temperature and photoperiod were set to 24 °C and 14L:10D, respectively, to simulate early summer conditions. Under these conditions, natural spawning occurred daily in the tanks, generally between 30 min and 3 h from dawn. The fertilized, buoyant eggs were collected as soon as possible after spawning and incubated until the desired developmental stage. In this study, four developmental stages, e.g. gastrula, somites (14–16 somites), tail elongation (23–24 somites), and pre-hatching (29–31 somites) were used. Developmental stages were determined following Oozeki and Hirano [21].

Preparation of CPA and washing solutions

Artificial sea water (ASW) obtained by dissolution of seawater salts (Rei-Sea Salt G, Japan) in distilled water (salinity and osmolality of 33 psu and 911 mmol/kg, respectively) was used for the preparation of CPA solutions and for incubation of embryos to monitor survival after toxicity trials. A 0.125 M sucrose solution (1042 mmol/kg) was used as washing solution for CPA removal [7,10] prior to incubation in artificial sea water. We confirmed that exposure to this solution for up to 10 min has no negative effects on whiting embryo morphology or viability.

Toxicity of CPAs to embryos

The toxicity of six CPAs [dimethylformamide (DFA), propylene glycol (PG), methanol (MeOH), ethylene glycol (EG), glycerol (Gly), and dimethyl sulfoxide (Me_2SO)] to Japanese whiting embryos was examined at four developmental stages (gastrula, somites, tail elongation, and pre-hatching). Me_2SO, MeOH, DFA, EG, and Gly were purchased from Wako (Japan) while PG was purchased from Sigma–Aldrich (USA). Embryos were exposed to CPAs at concentrations of 10%, 15%, and 20% (v/v) in ASW for 20 min at room temperature (24 °C). 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. Each treatment consisted of five replicates with approximately 50 embryos each. Embryos that were not exposed to the CPAs were used as controls.

Permeability of CPAs into embryos

The permeability of the CPAs into the embryos was analyzed by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy using embryos at the somites stage. For this purpose, embryos were incubated in 10% solutions of the six CPAs. This concentration was chosen because it was found in preliminary trials to be relatively non-toxic to whiting embryos. After 5, 10, 15, and 20 min impregnation, approximately 1000 embryos were blotted dry onto filter paper and placed in NMR sample tubes (5 mm diameter). The embryos inside the tube were gently squeezed with a glass insert tube to eliminate dead spaces between embryos. ^{1}H NMR spectra were recorded at 20 °C on a 400.13 MHz spectrometer (Avance II 400; Bruker, Rheinstetten, Germany). The resulting spectra were processed and analyzed using the Top Spin (Ver. 1.3) NMR software (Bruker) and the peak area ratio for water and CPA was calculated as weight fraction.

Toxicity of CPA mixtures and suitability of the impregnation protocol

Five CPAs were used to prepare twelve solutions of two CPAs each in ASW. Because PG and Gly were found to be the least and the most toxic to whiting embryos, respectively, PG was used in all solutions while Gly was avoided in all. The composition, osmolality, and pH of the solutions are summarized in Table 1. Osmolality (mmol/kg) and pH of the solutions were measured with a vapor pressure osmometer (Wescor 5520, Logan, USA) and pH meter (HORIBA F-211M-55G, Japan), respectively. Toxicity of the CPA solutions was tested during stepwise impregnation (four steps of 5 min or five steps of 4 min) of somites stage embryos at room temperature (24 °C). The four and five steps represented increases in CPA concentration of 25% and 20% per step, respectively, until 100%. This approach was used to determine the most suitable impregnation protocol as toxicity might depend on the rate of increase in the CPA concentration. After impregnation, embryos were treated as in the previous experiment for observation of survival. Five replicates, each consisting of approximately 50 embryos were used for each treatment. Untreated embryos were used as controls.

Statistical analysis

All numerical results were expressed as means ± SD. The statistical significance of the differences between means was analyzed by one-way ANOVA followed by the Tukey's multiple comparison

Table 1
Composition, osmolality, and pH of the cryoprotectant solutions used in this study

Cryoprotectant solution	Composition (%)*						Osmolality (mmol/kg)	pH
	PG	DFA	Me_2SO	MeOH	EG	ASW		
CS1	30	20				50	5270	7.61
CS2	25	15				60	4530	7.43
CS3	20	10				70	3180	7.35
CS4	30		20			50	7630	7.76
CS5	25		15			60	6360	7.54
CS6	20		10			70	5040	7.42
CS7	30			20		50	4280	7.39
CS8	25			15		60	3730	7.42
CS9	20			10		70	3240	7.38
CS10	30				20	50	8320	7.35
CS11	25				15	60	6970	7.32
CS12	20				10	70	5360	7.23

* PG, propylene glycol; DFA, dimethylformamide; Me_2SO, dimethyl sulfoxide; MeOH, methanol; EG, ethylene glycol; ASW, artificial seawater.

test. Differences were considered as statistically significant at a probability value of $P < 0.05$.

Results

Toxicity of CPAs to embryos

Hatching rates of embryos treated with the six CPAs varied markedly with the type and concentration of CPA (Fig. 1). Differences between developmental stages were not as marked as those between CPAs, but indicated that embryos at pre-hatching stage were slightly less tolerant to the CPAs than those at other stages. Embryos exposed to PG were the least affected followed by those exposed to Me_2SO, DFA, EG, MeOH, and Gly, in this order. Hatching rates dropped significantly (Tukey's test, $P < 0.05$) when the embryos were exposed to increasing concentrations of most CPAs. For example, no surviving embryos were observed after treatment with 15% and 20% of MeOH, EG, and Gly. Gly was also highly toxic to whiting embryos at the concentration of 10%.

Permeability of CPAs into embryos

NMR analysis of the internal concentration of CPAs in somites stage embryos indicated marked differences in the permeability pattern of the various CPAs (Fig. 2). All CPAs except PG showed a relatively large uptake in the first 5 min. Thereafter, EG, MeOH, and DFA continued to increase whereas Me_2SO and Gly remained nearly constant. PG was only detected after 15 min impregnation and after 20 min still had the lowest internal concentration among all CPAs. Overall, EG had the highest uptake rates followed by MeOH, DFA, Gly, and Me_2SO, in this order.

Toxicity of CPA mixtures and suitability of the impregnation protocol

The survival rates of somites stage embryos that were impregnated with CPAs in five steps of 4 min were higher than those impregnated in four steps of 5 min regardless of the CPA solution (Fig. 3). No significant reduction in hatching rate compared to the control (88.5%) was observed when embryos were impregnated in five steps with CS3 (89.6%) and CS6 (88.1%). CPA solutions

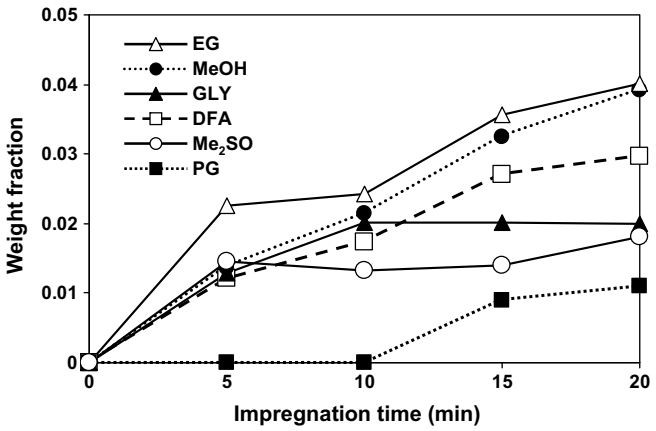

Fig. 2. Internal concentration (weight fraction) of cryoprotectant in *S. japonica* embryos impregnated with 10% solutions of six cryoprotectants at 24 °C. Each value was obtained by NMR analysis of a pool of about 1000 embryos.

CS1, CS2, CS5, CS8, CS9, and CS12 gave hatching rates between 3.4% and 80.9% while none of the embryos survived after exposure to CS4, CS7, CS10, and CS11.

Discussion

Successful cryopreservation of biological materials depends on the optimization of several steps from the selection of a suitable CPA to the post-thawing care of the materials, and its development is not a straightforward process as most of the steps involved are interdependent. The choice of an adequate CPA treatment, for example, must take into consideration its permeability and toxicity to the materials, physicochemical properties, and ultimately its ability to minimize or suppress the formation of ice crystals during cryopreservation. In this study, we examined the permeability and toxicity of six CPAs (DFA, PG, MeOH, EG, Gly, and Me_2SO) to whiting embryos. The results showed that PG was the least toxic among them followed by Me_2SO, DFA, EG, MeOH, and Gly and that only the first three CPAs were (partially) tolerated at concentrations

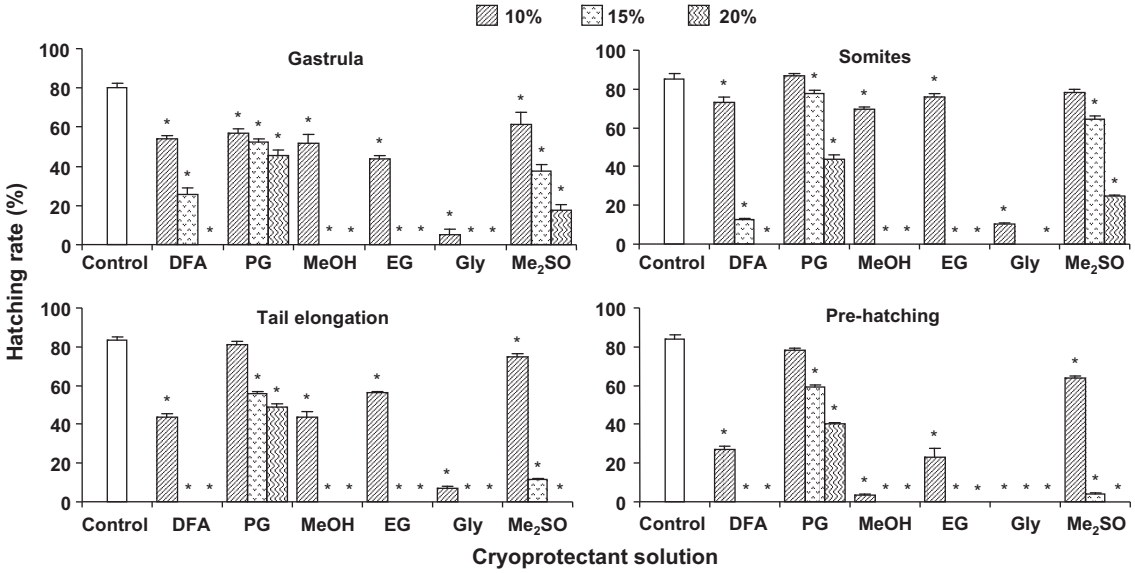

Fig. 1. Hatching rates of *S. japonica* embryos at four developmental stages that were exposed to different cryoprotectants and cryoprotectant concentrations (indicated in the panels). Data shown as mean and SD of five replicates with approximately 50 embryos each. Solutions with asterisks have significantly different hatching rates from those of the controls (Tukey test, $P < 0.05$). DFA, dimethylformamide; PG, propylene glycol; MeOH, methanol; EG, ethylene glycol; Gly, glycerol; and Me_2SO, dimethyl sulfoxide.

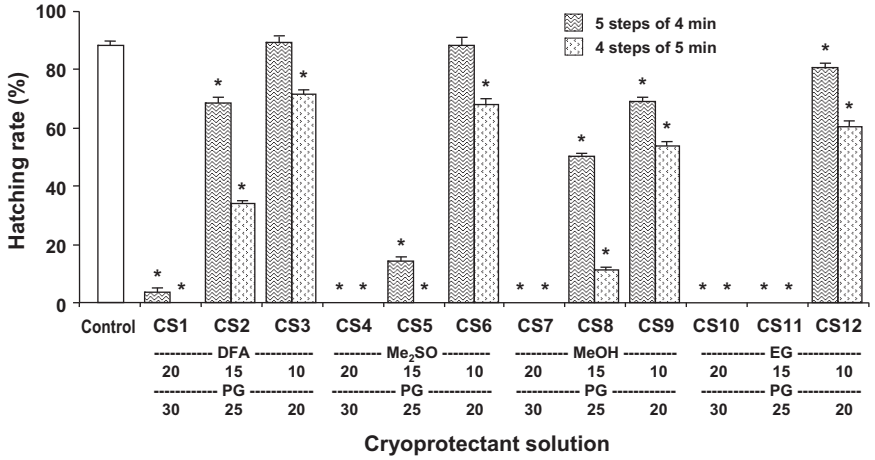

Fig. 3. Hatching rates of *S. japonica* embryos (somites stage) exposed to different cryoprotectant solutions and impregnation protocols. Data shown as mean and SD of five replicates with approximately 50 embryos each. The composition of the solutions (% v/v) is indicated under the bars. Solutions with asterisks have significantly different hatching rates from those of the controls (Tukey test, $P < 0.05$).

⩾15%. Results for other fish species show some parallels but also marked differences with those for whiting. Chen and Tian [7] reported that PG and MeOH were less toxic to flounder embryos (*Paralichthys olivaceus*) than Me_2SO and DFA and that no embryos survived treatment with EG and Gly at a concentration of 20%. Working with turbot (*Scophthalmus maximus*) embryos, Cabrita et al. [4] showed that embryos tolerated Me_2SO better than MeOH and EG, a fact also reported by Chereguini et al. [8]. On the other hand, studies on gilthead sea bream (*Sparus aurata*) showed that EG was the least toxic to embryos among four CPAs (EG, Me_2SO, MeOH, and PG) [5]. The substantial differences reported between species emphasize the need to test CPA tolerance on a species-by-species basis.

The results of our NMR analysis, although not sufficient to clarify the exact distribution of the CPAs inside the highly compartmentalized embryos (see [12]), point to an intriguing correlation between the relative permeability of the various CPAs and their toxicity. For instance, low embryo survival was associated with high permeability for EG and MeOH whereas the opposite was found for PG and Me_2SO. DFA, in turn, gave intermediate results for both toxicity and permeability. The only exception to this pattern was Gly, which had moderate permeability but was highly toxic. As pointed out by Harvey and Ashwood-Smith [15], in general the degree of permeation of a CPA is inversely related to its molecular weight. The results of our study with six CPAs and those obtained by Edashige et al. [10] with four (MeOH, PG, EG, and Me_2SO) provide broad support to this assumption. Nevertheless, the dynamics of CPA permeation is also related to the presence of structural (membrane) barriers in the embryos, as can be inferred from the report of Hagedorn et al. [12] that MeOH was able to permeate the entire zebrafish embryo whereas Me_2SO and PG did not. Thus, it might be worth investigating to what extent the differences in CPA toxicity reported among fish species can be explained by the presence of specific structural barriers to particular CPAs rather than toxicity itself. The present results also suggest that, in the absence of other less toxic and more effective CPAs, successful cryopreservation of whiting (and probably of fish embryos in general) will depend on the development of methods to mitigate the toxic effects of current ones.

One approach to mitigate CPA toxicity is to impregnate embryos faster, so the materials can be cooled to cryogenic temperatures before toxicity causes critical damage [24]. Indeed, several kinds of mechanical and chemical treatments are being tested for this purpose [2,3,23,26,27]. A complementary strategy is to use a mixture of CPAs at low individual concentrations rather than a high concentration of a single substance [6,22]. In this study, we tested the toxicity of various CPA mixtures to whiting embryos. PG was used in all combinations, even though it had low permeability, because of its low toxicity (this study, [7]) and because it forms glassy solids at relatively low concentrations [12]. This analysis revealed that solutions containing 10% of DFA (CS3), Me_2SO (CS6), MeOH (CS9), and EG (CS12) in addition to 20% PG were well tolerated by the embryos. Solutions CS2 and CS8, which contained 15% of DFA and MeOH, respectively, as well as 25% PG also allowed survival of a significant part of the embryos. Thus, our results support the assumption that CPA mixtures help overcome the toxicity of individual CPAs. Ongoing studies with mixtures of up to five CPAs have given promising results (Rahman et al., unpublished observations) and substantiate further the applicability of this strategy.

We also examined the effect of the developmental stage of the embryos on their survival during impregnation with the CPAs. CPA tolerance in fish embryos is often found to be stage-dependent, decreasing in the youngest and oldest embryonic developmental stages compared to intermediate ones [1,4,7,19,24]. Interestingly, our toxicity test with four developmental stages of whiting, including the gastrula stage, did not reveal marked differences between stages except for a slight decrease in tolerance by pre-hatching embryos. This result agrees with the observation that pre-hatching turbot embryos were less tolerant to CPAs than those at the tail elongation stage [4]. Another aspect of the cryopreservation protocol examined in this study was the method of CPA impregnation. The addition of CPAs causes the release of water from the cells as the concentration equilibrium is maintained, and the resulting dehydration can be lethal if it occurs too rapidly [17]. Thus, most studies currently adopt a gradual, stepwise protocol for CPA impregnation in order to reduce toxicity and osmotic stress to embryos [4,7,11]. We compared two methods and confirmed that impregnation in five steps of 4 min allowed higher survival of whiting embryos than four steps of 5 min for all tested solutions. Future studies should test the suitability of increasing further the number of steps.

In summary, this study provides for the first time information on the toxicity and relative permeability of six CPAs (Gly, MeOH, EG, Me_2SO, DFA, PG) to embryos of Japanese whiting (*S. japonica*) and on suitable impregnation protocols. From the viewpoint of toxicity and permeability, PG, MeOH, and EG appear to be the most promising among them. Ongoing studies should help clarify the amount of cryoprotection afforded to whiting embryos by the different CPAs and further optimize the impregnation protocols.

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