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Effects of ultrasound on permeation of cryoprotectants into Japanese whiting *Sillago japonica* embryos

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ABSTRACT

Cryopreservation of fish embryos requires the swift uptake of considerable amounts of cryoprotectant (CPA) but this process is hampered by the low permeability of the egg chorion. This study examined the relative efficiency of ultrasound to promote the incorporation of CPAs in two different embryonic developmental stages (somites and tail elongation) of Japanese whiting *Sillago japonica* and performed a preliminary cryopreservation trial using the best conditions determined during the study. Embryos tolerated ultrasound densities up to 37.5 W/cm^2 well for up to 3 min but had significant mortality at 50 W/cm^2. Hatching rates of somites embryos sonicated at 37.5 W/cm^2 for 1–3 min in 10 and 20% Me_2SO solutions were comparable (61–72%) to that of sonication in artificial seawater (65–86%) but decreased sharply at the concentration of 30% (0–55%); at similar conditions, tail elongation embryos had comparatively lower survival. Me_2SO content of sonicated embryos at the somites and tail elongation stages increased significantly by 58–191% and 27–123%, respectively, compared to controls exposed to Me_2SO without ultrasound. Pre-exposure to Me_2SO before sonication increased the CPA uptake further by 36% without impairing survival. A preliminary cryopreservation trial after ultrasound-mediated impregnation of somites embryos with a CPA solution containing 20% PG and 10% MeOH did not yield live embryos after freeze-thawing but resulted in a significant decrease of nucleation temperature and increase of the proportion of morphologically intact embryos after freeze-thawing. These results suggest that sonication might be useful for fish embryo cryopreservation although it may require combination with other techniques to enhance CPA permeation.

1. Introduction

Fish embryo cryopreservation could play an important role in providing continuous supply of seedlings for aquaculture production and the conservation of commercially important and endangered fish species. However, this goal has not been achieved yet for any fish species. The difficulty in controlling the dynamics of cryoprotectant (CPA) and water movement into and inside the highly structurally complex embryos is probably the major impediment to successful cryopreservation [9]. The large amount of yolk and the presence of a surrounding yolk syncytial layer are also considered as significant barriers for permeation of CPAs into fish embryos [10]. Moreover, fish embryos have a high water content which can crystalize during cooling and therefore cause structural damage.

Several approaches to promote CPA uptake into fish embryos including electroporation, microinjection, treatment with aquaporins, positive or negative pressurization, and dechorionation have been evaluated [10,11,24,27,28,31]. Most of them have led to enhanced CPA uptake but not enough to achieve successful cryopreservation. Therefore, the search is still on for alternative methods that could provide sufficient CPA uptake, either self-sufficiently or in combination with other approaches.

Cavitation level ultrasound has been used for drug delivery through the skin in mammals [14,16,18]. Mitragotri et al. [17] reported that oscillation and collapse of air bubbles during cavitation effectively disorganize the lipid bilayers, allowing larger molecules

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to diffuse through the skin. In fish, cavitation level ultrasound has been used to promote the transportation of calcein into larvae [1] and the permeation of CPA (methanol, MeOH) into embryos of zebrafish [2,29,32]. It has been reasoned that sonication removes the granular farinaceous layer and physically dislodge the pore plugs in both outer chorionic membrane and underlying syncytial layer, thus providing a pathway for CPA permeation [2]. In resume, knowledge on the effectiveness of ultrasound to promote CPA uptake in fish embryos is limited and concerns only demersal eggs from freshwater species. In this context, we examined the effectiveness of sonication to improve the permeability of the commonly used cryoprotectant Me_2SO into the embryos of whiting *Sillago japonica*, a marine species that spawns small pelagic eggs. Me_2SO was chosen in this study because it is relatively less toxic to whiting embryos than other common cryoprotectants [21] and because its concentration inside the embryos can be easily measured by High Performance Liquid Chromatography (HPLC) [30]. We also observed the possibility of embryo vitrification using the best conditions determined in this study.

2. Materials and methods

2.1. Animal care and collection of embryos

Mature Japanese whiting broodstock were collected from Tateyama Bay, Japan and kept in the Aquatic Animal Rearing Facilities of Tokyo University of Marine Science and Technology. Naturally spawned, fertilized eggs were collected almost daily from animals reared in 1200L recirculated water tanks kept at 25 °C and a photoperiod of 14 h light: 10 h dark [21]. Salinity of the rearing water was adjusted to 33 psμ with artificial sea water salt (Rei-Sea Salt G, Rei-Sea Salt Co. Ltd. Tokyo, Japan) and the animals were fed frozen krill two times a day. Two developmental stages, e.g. somites (14–16 somites; approximately 14 h after fertilization 25 °C; [19]) and tail elongation (23–24 somites; approximately 16 h after fertilization) were used in this study. Artificial seawater (ASW) obtained by dissolution of seawater salt (Rei-Sea Salt G) in distilled water (salinity and osmolality of 33 psμ and 991 mmol/kg, respectively) was used for the preparation of Me_2SO solutions (Wako, Osaka, Japan) and for incubation of embryos to monitor survival after the trials. All experiments were conducted according to the guidelines for the care and use of animals of Tokyo University of Marine Science and Technology.

2.2. Effect of ultrasound on embryo survival

An ultrasonic processor (UP 50H; 50 W and 30 KHz; Hielscher Ultrasonics GmbH, Teltow, Germany) was fitted with a 0.5 mm sonotrode for sonic emission. In the first trial, embryos (somites stage) were sonicated in ASW at power densities of 0, 25, 37.5, and 50 W/cm^2 for 1, 2, and 3 min. This trial revealed that 50 W/cm^2 reduced the hatching rates of whiting embryos greatly (29–50%) compared to 25 W/cm^2 (76–86%) and 37.5 W/cm^2 (65–83%) (see Results and Fig. 1). Based on these results, 37.5 W/cm^2 was used for all subsequent trails. In the second trial, embryos at two developmental stages (somites and tail elongation) were sonicated for 1, 2, and 3 min in 10, 20, and 30% Me_2SO solutions (v/v in ASW). In the third trial, embryos (somites stage) were pre-exposed to 10% Me_2SO solution for 20 min before sonication in 20% Me_2SO solution for 3 min. Immediately after exposure, embryos were rinsed in ASW for 10 min and incubated in plastic Petri dishes containing 5 mL of ASW for observation of survival. Survival rates for each replicate were estimated from the percentage of embryos that successfully hatched. All experiments were conducted at room temperature (25 °C). Each treatment consisted of five replicates with approximately 100 embryos each. Embryos not treated with ultrasound were used as controls.

2.3. Effect of ultrasound on Me_2SO permeation in embryos

The internal concentration of Me_2SO in embryos (somites and/or tail elongation stages) from all trials was measured by HPLC (Shimadzu Corp., Kyoto, Japan) according to the methods developed by Suzuki et al. [30]. The HPLC system consisted of a Bio-Rad Aminex HPX-87 column (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 Me_2SO. Three replicates with 30 embryos each were measured for all treatments and respective controls.

2.4. Nucleation temperature and morphology of sonicated embryos after freeze-thawing

Embryos at the tail elongation stage were impregnated with a solution of 20% propylene glycol (PG) (Wako, Osaka, Japan) and 10% MeOH (Wako, Osaka, Japan) in ASW in (v/v) stepwise increments (20, 40, 60, and 80%) every 3 min and finally sonicated for 3 min in 100% CPA solution. We chose this CPA solution because our previous study revealed that it was relatively less toxic to whiting embryos than Me_2SO alone [21] and gave the highest rates of morphologically intact embryos after freeze-thawing (unpublished observations). Tail elongation stage embryos were chosen for this trial because our previous studies showed that this stage is more permeable to permeating cryoprotectants than somites stage embryos [22–24]. Immediately after treatment, each group of embryos was divided in two lots for attempted cryopreservation and determination of structural integrity after freeze-thawing and for measurement of nucleation temperature by Differential Scanning Calorimetry (DSC).

For attempted cryopreservation, embryos were loaded in 0.25 mL straws and plunged directly into LN_2 in a 2 L Dewar flask for 40–60 min. Embryos were then warmed at 40 °C for 5 s. Warming conditions were determined empirically in order to minimize the probability of recrystallization during warming. Immediately after warming, embryos were washed with ASW for several times and incubated in ASW for observation of morphological integrity and survival following Rahman et al. [24]. Briefly, embryos were carefully observed under a compound microscope at 10× magnification immediately after warming and categorized as “clearly damaged” or “apparently intact” when no obvious differences with untreated, live embryos at the same developmental stage could be identified. Each treatment consisted of nine replicates with approximately 30 embryos each. For DSC analysis, embryos were carefully blotted dry onto a filter paper after impregnation, sealed in aluminum DSC pans and loaded into the Diamond DSC instrument (PerkinElmer, Shelton, CT, USA). The sample was then cooled from 25 to −50 °C at 5 °C/min. Six to seven replicates with individual embryos were measured for each of the treatments and respective controls.

2.5. Statistical analysis

The statistical significance of the differences between means was analyzed by one-way ANOVA followed by the Tukey's multiple comparison test. All statistical analyses were performed using the Prism 5 software (GraphPad Software, Inc., La Jolla, California, USA). Values are expressed as means ± SD and are considered significant at $P < 0.05$.