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Simple Procedure for Sperm Cryopreservation in the Larvacean Tunicate *Oikopleura dioica*

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The larvacean tunicate *Oikopleura dioica* is an attractive organism for studies of the development, evolution, and physiology of chordates, showing considerable promise for genetic approaches given its short life cycle of five days. To facilitate future genetic studies, the development of protocols for the maintenance of individual strains is essential. Here we report a simple and practical protocol for the cryopreservation of sperm using liquid nitrogen (–196°C) and dimethyl sulfoxide (DMSO) as a protective agent. The quality of the frozen-thawed sperm was evaluated in terms of fertilizing ability and subsequent development of the fertilized eggs. We examined several parameters to optimize the efficiency of cryopreservation, such as the concentration of DMSO, the method for acclimation of sperm to DMSO before freezing, and for placing sperm in liquid nitrogen, as well as the pH of the seawater used in resuspending the thawed sperm. We confirmed that viable sperm were recovered after preservation for more than one year. In addition, mature animals, and even a subsequent generation, were obtained from eggs fertilized by the cryopreserved sperm. The present procedure seems to be simple and sufficiently practical for maintenance of future established lines of *O. dioica* using frozen sperm.

Key words: *Oikopleura dioica*, larvacean, appendicularian, sperm cryopreservation, fertilization

INTRODUCTION

Appendicularians (larvaceans) are planktonic animals (Phylum Chordata, Subphylum Tunicata) that retain a swimming tadpole shape throughout their life. Together with ascidians, they are the closest relatives of the vertebrates. *Oikopleura dioica* is distributed in coastal regions throughout most of the world, and is characterized by a simplified life habit and anatomical organization. *O. dioica* is the only dioecious tunicate species reported to date. Male and female gametes are released after a five-day developmental period, after which the adult animals die.

O. dioica is a candidate model organism for several reasons (reviewed in Nishida, 2008). In particular, it has a short life cycle of only 5 days at 20°C (Fenaux, 1998a); inland and multi-generation culture methods have been established (Paffenhöfer, 1973; Bouquet et al., 2009); the animal is transparent throughout development; the juvenile and adult anatomies have been comprehensively described (Fenaux, 1998b); it has a tiny genome of only 72 Mb, a draft sequence of which is now available (Seo et al., 2001); and, morphogenesis and cell lineages during embryogenesis are well described (Stach et al., 2008; Fujii et al., 2008). These features make *O. dioica* an attractive organism for studies of chordate development, evolution, and physiology, offering considerable promise for genetic investigations.

To facilitate future genetic approaches, the development of practical protocols for the maintenance of individual strains is essential. It is not easy to maintain many strains of transgenic and mutant lines. Therefore, for various animal species, including aquatic animals as well as human and domestic animals, procedures for cryopreservation of sperm have been devised (Mounib, 1978; Alvarez and Storey, 1993; Bailey et al., 2000; Suquet et al., 2000; Holt, 2000). Sperm cryopreservation facilitates storage of sperm of specific strains for long periods, and their subsequent use for artificial insemination.

We have begun to develop methods for the genetic manipulation of *O. dioica* in order to establish a model organism comparable in utility to *Drosophila* and *C. elegans*, taking advantage of its short life cycle. One of these steps has been to develop a practical protocol for the cryopreservation of *O. dioica* sperm, with reference to previous methods reported for the ascidian, *Ciona savignyi* (Moody et al., 1999; Hendrickson et al., 2004). Here we report a simple and practical protocol for cryopreservation of *O. dioica* sperm using liquid nitrogen and DMSO as a cryoprotectant, for which we have examined several parameters to optimize its efficiency.

MATERIALS AND METHODS

Specimens of the appendicularian *Oikopleura dioica* were reared through generations at our inland laboratories (Spada et al., 2001; Nishida, 2008; Bouquet et al., 2009). The wild animals were collected at Tanabe Bay off Shirahama, Wakayama, Japan, by scooping surface seawater. The animals were sorted, identified

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under a dissection microscope, and reared in 5-liter beakers containing artificial seawater ("Rohto Marine", REI-SEA, Tokyo, Japan). They were fed with the microalgae *Isocrysis galbana* and *Rhinomonas reticulata*, the diatom *Chaetoceros calcitrans*, and the cyanobacterium *Synechococcus* sp., as described previously (Spada et al., 2001; Bouquet et al., 2009). *O. dioica* reared in the laboratory (18–22°C) grows for about five days after fertilization and matures dioeciously. We prepared several beakers, each containing animals at a different growth stage, which allowed almost daily use of the mature animals.

Sexually mature males and females were discriminated visually (Nishino and Morisawa, 1998) and placed into dishes. A single mature female was isolated into a 40-mm dish containing ~3 ml of artificial seawater (ASW), after 2–3 washes by transfer of the animal into fresh ASW to avoid chance fertilization by sperm transferred from the culture. The female was then allowed to spawn naturally. The eggs were utilized for the experiment within four hours after spawning. A single male was also washed and placed in a small 80–150 μl droplet of ASW on the bottom of a dish. Evaporation of the water drop was prevented by covering the top of the dish. The male shed sperm in the droplet, which enabled us to obtain a condensed sperm suspension originating from a single male. The concentration of sperm in seawater of the 100 μl droplet was in the range from 3×10^5 to 5×10^6 spermatozoa/μl. The sperm suspension was then processed for cryopreservation within 2 hours.

Our starting strategy for freezing the sperm of *O. dioica* was based on the previously reported method used for the ascidian, *Ciona savignyi* (Moody et al., 1999; Hendrickson et al., 2004), as follows (see Fig. 3). (i) Twenty microliters of the sperm suspension was mixed with 80 μl of dimethyl sulfoxide (DMSO; Sigma, Biotechnology performance certified grade, D2438) solution (2.5–35% mixture with ASW) in a 0.5-ml cryotube. (ii) The cryotube was placed into liquid nitrogen and stored there. (iii) For recovery, the sample was removed from the liquid nitrogen and thawed at 35°C in a water bath for 40 seconds. We then added 200 μl of ASW to the cryotube, and the entire volume of the sample was suspended in a plastic dish containing 2 ml ASW and 50 unfertilized eggs. (iv) The number of eggs (of a total 50) that exhibited first cleavage was counted to derive a score for the efficiency of fertilization success. In a series of experiments, each differing in a single parameter, the sperm suspensions obtained from a single male were preserved in different cryotubes under various experimental conditions, and the corresponding fertilization efficiencies were evaluated using eggs obtained from a single female.

RESULTS AND DISCUSSION

Acclimation to DMSO before freezing

We first examined whether storage of sperm on ice or at room temperature (20°C) was optimal for acclimating the sperm suspension to 10% DMSO for 5 min prior to transfer to liquid nitrogen (see Fig. 3). Immediately after addition of DMSO ASW, sperm suspensions were put on ice or kept at room temperature for 5 min, then placed into liquid nitrogen. Sperm samples and unfertilized eggs were obtained from a single male and female pair. After freezing and thawing of the sperm samples, 50 unfertilized eggs were inseminated, and the numbers of fertilized eggs that had initiated the first cleavage were scored and compared between the two conditions. The cleaved embryos were counted at approximately 50 min after insemination, when the increase of the number of cleaved embryos becomes saturated. This pairwise comparison was repeated 12 times (Fig. 1A). The success rates of fertilization were largely dependent on each pair, most probably on the male used. This is consistent with a previous observation that there are large differences in post-cryopreservation sperm quality among individual males, a phenomenon that has been recognized in many species (Holt, 2000).

The number of eggs fertilized with non-frozen sperm was 50/50 (100%, n = 10). An average of 25/50 and 14/50 eggs (asterisks) were fertilized under "on ice" and "room temperature" conditions, respectively. The slopes of most of the individual lines that were obtained from a single pair of male and female were negative (Fig. 1A), and a statistically significant difference was also found between these two sperm sampling conditions ($p < 0.05$, paired Wilcoxon signed-rank test, as the distribution of the data did not follow a normal probability distribution). Thus, we concluded that acclimation to DMSO on ice was better suited for cryopreservation.

Procedure for placing the samples in liquid nitrogen

After incubation with DMSO for 5 min on ice, the sample was transferred to a tank of liquid nitrogen. We then tested whether freezing the sperm suspensions rapidly or slowly was more suitable. For rapid freezing, we attached the cryotube containing the sample to a cane and plunged it directly into liquid nitrogen. In this procedure, the sperm suspension is completely frozen in 40 sec. For slow freezing, the sample was placed on a 3-cm-thick polystyrene plate floating on liquid nitrogen for 5 min. In this approach, the sperm suspension is completely frozen in 2 min. The cryotube was then picked up to confirm that it had frozen completely, fixed to a cane, and soaked in liquid nitrogen. Sperm suspensions from the same individual were used, and comparisons were performed between the two conditions. On average, 35/50 and 19/50 eggs were fertilized after "rapid" and "slow" freezing, respectively. Most of the individual lines obtained from a single male and female pair showed a negative slope (Fig. 1B), and a statistical comparison supported a significant difference ($p < 0.01$). Therefore, rapid freezing was shown to be more suitable.

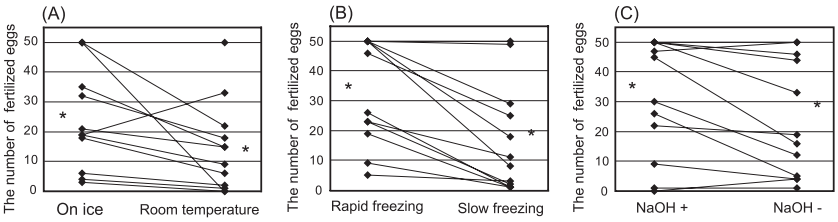

Fig. 1. Optimization of the conditions for freezing of *O. dioica* sperm. The number of fertilized eggs (as evidenced by the first cleavage) per 50 eggs is plotted on the ordinate (a value of 50 equals 100%). Results obtained using a single male and female pair are connected with a horizontal bar. Some points are on top of each other. Asterisks indicate the mean. **(A)** Acclimation to DMSO before freezing. The results for "incubation on ice with DMSO for 5 min before freezing" are shown on the left, and those for "incubation with DMSO at 20°C for 5 min before freezing" are indicated on the right. **(B)** Procedure for placing samples in liquid nitrogen. Left and right plots show the results obtained from the "rapid" and "slow" freezing experiments, respectively. **(C)** pH of artificial seawater (ASW) used for suspension of thawed sperm. Plots represent results obtained from suspension in ASW with NaOH (left) and without NaOH (right).

pH of ASW

To thaw the cryopreserved sperm, the sample tubes were briefly incubated at 35°C in a water bath for 40 seconds, and then the sperm were mixed with 200 μl of ASW. It is generally known that sperm motility can be activated by elevation of the pH level (e.g., Oda and Morisawa, 1993; Darszon et al., 2001). A small aliquot of NaOH solution has been added empirically to activate the sperm of the ascidians *Halocynthia roretzi* and *Ciona intestinalis*. To test whether this procedure can also activate the sperm of *O. dioica* recovered from cryopreservation, we compared two conditions in which 200 μl of ASW containing 0.4 mM NaOH, or ASW without NaOH, was added to 100 μl of the sperm suspension after thawing in a water bath (Fig. 1C). The pH of plain artificial seawater was 8.2. Addition of NaOH raised the pH of seawater containing DMSO from pH 8.7 to 9.4. We obtained a higher rate of fertilization with the NaOH-containing ASW, and statistical analysis supported a significant difference ($p < 0.05$). On average, 35/50 and 29/50 eggs were fertilized with and without the use of NaOH, respectively, indicating that elevation of the pH of ASW increases the rate of successful fertilization. Although every sperm is active in motility after natural spawning, after freeze and thaw, the ratio of swimming sperm was 3.1% on average (n = 6). Treatment with ASW containing NaOH did not improve the ratio (3.2%, n = 6). Therefore, higher pH diagnostically improves the fertilization rate; however, the reason for this is unknown.

Concentration of DMSO

All experiments described so far were performed using a mixture of 80 μl of 10% DMSO as a preservative, with 20 μl of sperm suspension (i.e., the final concentration of DMSO was 8%). Here, we examined the optimum concentration of DMSO for cryopreservation. We were normally able to obtain approximately six tubes containing 20 μl of sperm suspension from a single male. These samples were mixed with 80 μl of ASW containing different concentrations of DMSO (2.5% to 35%), and then cryopreserved. In this and following experiments, NaOH-containing ASW was added to the sperm suspension after thawing. In several series of experiments, each using a single male, the mean efficacies of fertilization were compared among various DMSO concentrations (Fig. 2A, Supplemental Table S1 online). The maximum efficiency of fertilization was obtained at 17.5%. When the DMSO concentration was below 5%, fertilization was rarely observed, and the fertilization rate gradually declined at concentrations over 17.5%.

Effects of DMSO on subsequent embryogenesis

From the results described above, the optimum concentration of DMSO for fertilization was found to be 17.5%. However, when we observed subsequent development of the eggs inseminated with sperm preserved in 17.5% DMSO, we noticed that this treatment was associated with frequent abnormalities in the hatched larvae, including individuals with a kinky tail, no tail, or failure to hatch. Therefore, we next examined the optimal concentration of DMSO to support normal embryogenesis until hatching. When we only examined eggs fertilized after insemination with frozen sperm, the proportion of normal hatched larvae gradually

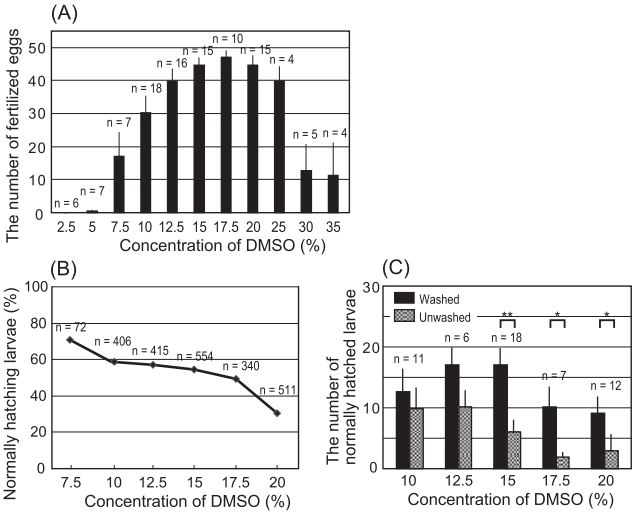

Fig. 2. Optimization of the concentration of DMSO. **(A)** Fertilization rate obtained using sperm frozen with various concentrations of DMSO. Twenty-microliter sperm suspensions were mixed with 80 μl of ASW containing different concentrations of DMSO (2.5% to 35%). The number of fertilized eggs (as evidenced by the first cleavage) per 50 eggs is plotted on the ordinate (a value of 50 equals 100%). Vertical bars indicate the standard error of the mean. Number of repeated experiments is indicated above each column. A table of the data is presented in Supplemental Table S1 online. **(B)** The proportion of normal hatched larvae after exposure to different concentrations of DMSO. The proportions of normal hatched larvae are plotted, where the denominator represents the sum of eggs successfully fertilized after insemination with frozen sperm in several experiments, excluding unfertilized eggs. The numerator represents the sum of normally developed larvae in the same experiments. The number above each plot indicates the number of specimens. **(C)** Effect of removal of DMSO after fertilization on normal development. The vertical and horizontal axes indicate the average number of normally hatched larvae among 50 inseminated eggs and the DMSO concentration, respectively. Dark columns indicate the results of removal of DMSO after fertilization, and the gray columns those obtained without DMSO removal. The error bar indicates the amplitude of the standard error of the mean. Removal improved the normal hatching ratio. Double asterisks indicate a *p*-value of less than 0.01, and a single asterisk indicates a *p*-value of less than 0.05. Number of repeated experiments is indicated above each column.

decreased as the DMSO concentration was increased (Fig. 2B), suggesting that DMSO introduced together with frozen sperm was harmful to embryogenesis.

Therefore, we examined the effects of removal of DMSO after insemination. In this experiment, sperm samples from single individuals were divided into two aliquots and frozen, and 50 eggs were inseminated with one aliquot of sperm and transferred to another dish containing fresh ASW at 20 min after insemination to wash out the DMSO. Observation of synchrony of the first cleavage in the previous experiments indicate that most eggs appeared to be fertilized within 20 min after insemination. The results of washing were compared with those obtained without washing (Fig. 2C, Supplemental Table S2 online). The results suggested that removal of DMSO increased the eventual number of normally hatched larvae. Considering the results presented in Fig. 2A and 2C, it seems that the optimal concentration of

DMSO for fertilization and subsequent normal development is approximately 15%.

Simple procedure for cryopreservation of *O. dioica* sperm

We confirmed that active sperm could be recovered after preservation in liquid nitrogen for more than one year. In addition, mature animals and even the next generations were obtained from eggs fertilized by cryopreserved sperm prepared using this protocol and the ordinary culture system for this animal (Spada et al., 2001; Nishida, 2008; Bouquet et al., 2009). We were then able to propose optimal conditions for cryopreservation of *O. dioica* sperm (Fig. 3). Of course, the evaluated parameters are not comprehensive, and it may be possible to further improve this procedure. However, the present procedure seems to be simple and sufficiently practical for the maintenance of lines that may be established in future. According to our recent communication with laboratories at the Sars International Center for Marine Molecular Biology in Norway, an independent study of cryopreservation of *Oikopleura* sperm has been conducted, and details will be published elsewhere (J-M. Bouquet, et al., personal communication). Their and our protocols for preserving the sperm of *O. dioica* appear promising as a basic method for use in future genetic studies of this organism.

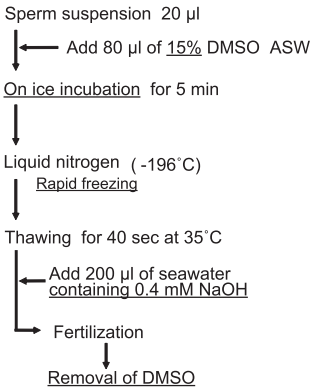

Fig. 3. The optimized procedure for cryopreservation of *O. dioica* sperm. The conditions examined and optimized in the present study are underlined.

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