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The use of cryomicroscopy in guppy sperm freezing ☆

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ABSTRACT

The present study employed cryomicroscopy to derive an optimal sperm freezing protocol for guppy (*Poecilia reticulata*) sperm. Evaluation criteria during the freezing–thawing process were assessed for nucleation temperature (Tn), temperature when more than 50% of sperm display bending mid-piece (Tb), temperature when more than 80% of sperm stop moving (Tm), thawing temperature (Tt), and post-thaw motility. We compared four different cryoprotectants: 5% *N*-dimethyl formamide (DMF), 6% methanol (MEOH), 10% dimethyl sulfoxide (DMSO), and 14% glycerol, as well as glycerol at different concentrations of 7–50%; cooling and rewarming rates ranged from 5 to 100 °C/min. The protocol that yielded the highest post-thaw motility was samples suspended in 14% glycerol, cooled at 25 °C/min, and thawed at 100 °C/min, which was in complete agreement with our previous findings derived from a controlled-rate freezer. In addition, Tb and Tm were found to be negatively correlated with post-thaw motility, suggesting their possible role in predicting freezing success. The present study for the first time demonstrated the usefulness of cryomicroscopy in deriving an optimal sperm freezing protocol for aquatic species.

Introduction

Optimization of sperm freezing protocols has often been achieved through empirical trials using controlled-rate freezers or simple devices such as styrofoam boxes. The use of cryomicroscopy for evaluating various freezing or thawing parameters has been limited to species such as ram [7,8], mouse [14], boar [10,11,20], bull [15], human [12,13], marsupial [9], and okapi [16]. However, cryomicroscopy offers a significant advantage over the conventional cooling apparatus as it allows real time observation of the entire freezing and thawing process, thus events such as cell motility, membrane integrity, and ice formation status can be tracked throughout all temperature regions. Previous studies in boar sperm freezing have helped reveal significant observations in male-to-male variation [10,11]. Further exploration of its application on a large scale with multiple animal species or the use of this equipment itself has not been reported and this may be due to its prohibitive cost. In particular, its use in deriving an optimized freezing protocol for aquatic species has not been attempted.

In this study, we used cryomicroscopy for the development of an optimal freezing protocol for the guppy sperm. Guppies are an important aquatic model fish [19] and they represent an atypical reproductive mode of internal fertilization seen in teleost fishes. When compared with fish that employ external fertilization, guppy sperm are more similar to mammalian sperm in terms of motility activation, sperm morphology, and glycolytic activity [3]. Thus, findings in this study will not only be useful for other aquatic species, but may also be applicable to mammals.

Our objectives of this study were to (1) identify endpoints that are useful for predicting an optimal sperm freezing protocol in guppies; (2) evaluate the cold-shock sensitivity of guppy sperm, and (3) examine the effects of cryoprotectant type and concentration, cooling rate, and rewarming rate on various endpoints obtained through cryomicroscopy. We found that temperature at which more than 50% of sperm display mid-piece bending and the temperature when more than 80% of sperm ceased moving had significant correlations with post-thaw motility.

Materials and methods

Sperm collection and processing

Sexually mature male guppy fish were purchased from the pet market of Wenzhou, and held in a 20 l glass tank at 25 °C on a

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14:10 light/dark photoperiod for 2 weeks prior to experiment. Upon sperm collection, fish were anesthetized in 0.01% tricaine–methane sulfonate for 2 min, and their standard lengths (tip of snout to the crease of the caudal peduncle) and wet body masses were measured after blotting excess water with a paper towel. Testes were collected by the methods described previously [4–6], and were placed in 1.5-ml Eppendorf tube and weighed. Only mature testes (with a creamy white appearance) and samples with an initial motility above 75% for fresh sperm were used for experiments. Hanks' balanced salt solution (HBSS) was added before crushing of the testes to release sperm, and suspensions were filtered through a 40-μm cell strainer (BD Biosciences Discovery Labware, Bedford, MA) to get rid of tissue chunks. Dilutions with HBSS were based on the testis weight, and a ratio of testis to HBSS (mass:volume) of 1:20 generally yielded a sperm density of approximately 2–5×10^7 cells/ml. Sperm numbers were determined from the average of duplicate counts using a hemocytometer. Based on our preliminary experiments [17], HBSS at 300 mOsmol/kg was used for sperm suspension after collection.

General cryomicroscopy procedures

Cryomicroscopy was undertaken using a Linkam BCS 196 cryostage (Linkam Scientific, Surrey, UK) system mounted upon a microscope (Nikon ECLIPSE 80i, Kingston, UK) and a digital CCD camera (QImaging Retiga-2000R, USA). The BCS 196 cryostage had been previously validated to determine cooling rate consistency and repeatability of freezing and thawing protocols [15]. The specimen was situated on the silver block in the cryostage. The temperature of the cryostage was controlled via the TMS94 unit at rates between 0.01 and 130 °C/min. The TMS94 can be programmed with temperature profiles of up to 32 rates. Liquid nitrogen vapor was introduced directly into the block to produce stable and accurate temperature control. The block was surrounded by nitrogen gas after the cryostage chamber was sealed with its lid, ensuring that temperature conductance throughout the specimen was rapid and even. Prior to the experiment, the cryostage was cooled at 10 °C/min to −40 °C and warmed at the same rate back to 35 °C to remove any initial condensation in the stage chamber.

For sample preparation, an aliquot of 10 μl sperm suspension was placed in the center of a circular glass coverslip (16 mm diameter), the circumference of which had previously been lightly coated with a layer of silicon grease. A second coverslip was gently pressed on top of it to form a seal, which prevents water evaporation from the sample. Samples were then transferred to the cryostage with a sample holder.

Assessment endpoints

Sperm motility and temperatures for sperm mid-piece bending, sperm stop moving, solution solidification (nucleation) and thawing were evaluated with each experimental trial. Sperm motility after rewarming was assessed at 35 °C and determined based on video images. Specifically, the number of motile and non-motile sperm was counted for each video clip (15 frames with 0.05 s per frame) and proportions of motile spermatozoa were calculated. Cells were considered motile if they displayed any form of active flagellar movement and a total of 200–350 sperm were counted per treatment. The typical morphological change observed during the cooling process was mid-piece bending (Fig. 2b), thus temperature changes associated with this phenomenon were recorded. To facilitate comparisons among different treatment groups, we used the temperature at which more than 50% of sperm exhibited mid-piece bending as an endpoint and referred hereafter as bending temperature (Tb). The temperature where sperm stop moving (Tm) was defined as more than 80% of sperm showing motionless flagellar activity. The temperature for complete solution solidification or spontaneous nucleation (Tn – when video clips changed from bright to completely dark), and thawing upon rewarming (Tt – when video clips changed from dark to bright) were also recorded.

Sperm motility before freezing was determined via a computer-assisted sperm analysis system (CASA, IVOS version 12.0, Hamilton Thorne Bioscience, Beverly, MA, USA). A 5-μl aliquot from each sample was filled to the 20-μm depth Leja 2-chamber slide (Leja Products B.V. Luzernestraat 10, 2153 GN Nieuw Vennep, The Netherlands). Five to six fields or at least 200 motile sperm were analyzed by the IVOS. Each of the sperm samples was tested in triplicate.

Cold-shock sensitivity

To test whether guppy sperm are sensitive to cold-shock, sperm samples were diluted with HBSS300 at 1:40 (v/v) without any cryoprotectants and the sperm–HBSS mixtures were placed on the cryostage at 35 °C and viewed continuously while being cooled at 10 °C/min to −8 °C and then rewarmed at the same rate back to 35 °C. Sperm samples pooled from two fish were used as one replicate and the experiment was repeated three times with a total of six fish.

Effect of cryoprotectants and concentrations

There were two trials in this experiment. In the first trial, sperm from ten males were used. Sperm samples pooled from every two fish (five replicates) were diluted with HBSS300 at a ratio of 1:20, and each was divided into four sub-samples. Each sub-sample was mixed with equal volumes of pre-cooled HBSS–cryoprotectant solution to yield the final treatments of 5% *N*-dimethyl formamide (DMF), 6% methanol (MEOH), 10% dimethyl sulfoxide (DMSO), and 14% glycerol (v/v). In the second trial, sperm from another 10 males was processed similarly for comparisons among 7%, 14%, 28%, and 50% glycerol (final concentration).

Effect of cooling rate

There were two trials in this experiment. In the first trial, sperm suspended in 14% glycerol was equilibrated for 10 min at 4 °C, cooled from 4 °C to −80 °C at 1, 25, and 100 °C/min, held for 2 min at −80 °C, rewarmed to 35 °C at a rate of 100 °C/min, and held for 1 min at 35 °C. In the second trial, cooling rates at a narrower interval of 5, 25, 45, and 65 °C/min were compared with all other parameters unchanged. Both trials were replicated three times each with three samples (a total of six fish).

Effect of rewarming rate

Sperm samples were suspended in 14% glycerol, cooled to −80 °C at 25 °C/min, held for 2 min at −80 °C, and then rewarmed to 35 °C at the rates of 5, 25, 45 or 100 °C/min. Sperm samples from individual fish were used as one replicate and this experiment was replicated three times with a total of three fish.

Validation with a controlled-rate freezer

The same batch of sperm samples suspended in 14% glycerol was divided into two equal portions for comparison between freezing with a controlled-rate freezer and the cryostage. A similar cooling program was used for both methods. For freezing with the controlled-rate freezer (Planer Products, Sunbury-on-Thames, UK), sperm suspensions were drawn into three 0.25-ml French straws (IMV International Corporation, Minneapolis, MN, USA) and held for 10 min at 4 °C. After equilibration, the straws were