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## SIMPLE AND INEXPENSIVE METHOD FOR CRYOPRESERVATION OF FISH SPERM COMBINING STRAW AND POWDERED DRY ICE

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### Abstract

Here, we propose a simple and inexpensive method for fish sperm cryopreservation. Sperm samples of the loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae) were diluted 7-fold by an extender containing 63.5mM NaCl, 114mM KCl, 20mM Tris and 10% methanol. The cryogenic straws were placed in three kinds of self-made tubes which diameter was changed by commercially available materials and then immersed into powdered dry ice for 2 min and plunged into liquid nitrogen. This procedure resulted in a cooling rate at  $-421.4 \pm 119.84$  (control),  $-55.8 \pm 4.32$  (tube 1),  $-40.2 \pm 3.43$  (tube 2) and  $-33.3 \pm 2.09^\circ\text{C}/\text{min}$  (tube 3). In the slowest cooling rate by the tube 3, total motility ( $72 \pm 3\%$ ), duration ( $146 \pm 12\text{s}$ ) and hatching rates ( $29 \pm 04\%$ ) were higher than those by other rates. Progressive motility ( $83 \pm 5\%$ ) did not differ significantly from fresh samples.

**Keywords:** cryopreservation, dry ice, fish, cooling rate, *Misgurnus*, sperm, straw

### INTRODUCTION

Freezing procedure during cryopreservation is a critical factor on sperm viability, because higher or lower rates are detrimental for the cells. Rapid cooling causes a shortage in the period of water efflux, resulting in excessive intracellular ice formation (IIF) and consequent cell death (26, 32). On the contrary, slow cooling often injures the cells due to mechanical and/or osmotic effects of external medium (36, 41). To determine the optimum cooling rates, the method using the programmable freezer is the most precise, but this device is expensive.

Vapour nitrogen is widely employed for cryopreservation and can be achieved in “dry-shippers” (7, 25) or placing the sperm package at different distances from the surface of liquid nitrogen, using a floating apparatus (1, 15, 20, 30, 31). It permits a simultaneous cooling of several samples that can be placed in different sperm packages. This method can also realize different cooling rates by changing the distance of sperm samples from liquid nitrogen. The cost for liquid nitrogen is a negative aspect and its manipulation may offer some risks for

users. Dry-shipper container is another useful procedure, but the cost is generally expensive. In addition, variation in model, position of the straw in the container and the influence of air temperature are other sources of variation during cooling (7).

Pellet-freezing on dry ice permits the mass cryopreservation of sperm and this method is interesting from the viewpoint of large-scale aquaculture. However, cells are exposed to different cooling rates because the localization is different among cells within the pellet at the moment of cooling. Precise volume and cooling rates inside the pellet are also difficult to evaluate. Furthermore, cooling rates vary according to the diluent. The placement of cryovials inside container for cooling into dry ice and nitrogen was also utilized successfully (12, 13, 29). Dry ice is inexpensive and a portable cooling medium, and is suited to field conditions or fish hatcheries.

In the present study, we propose simple and inexpensive procedure for the cryopreservation of sperm of the loach *Misgurnus anguillicaudatus*, in cryogenic straws.

## MATERIALS AND METHODS

### *Sperm sampling and motility assessment*

Four adult male loaches (*Misgurnus anguillicaudatus*) were anaesthetized with 2-phenoxyethanol (0.1%), and then induced maturation by hCG injection (100 I.U./male). Fishes were maintained at 27°C for 10 hours, and then sperm was collected using hematocrit glass tubes. Samples were immediately placed in a 1.5mL microtubes containing 1mL of immobilizing solution (128.4mM NaCl, 2.7mM KCl, 1.4mM CaCl<sub>2</sub>, 2.4mM NaHCO<sub>3</sub>) (18).

Sperm was activated by a 70-fold dilution with dechlorinated tap water and observed under the microscope. The motility analysis was conducted three-times (triplicates) and video recorded. Total motility (%), progressive motility (from percentage of motile cells) and its duration (s) were obtained from video sequences. Total motility denoted all types of movement and was expressed as percentage of all the cells observed in the same field. Progressive motility regards sperm movement along a linear track, and was expressed as percentage of the motile cells. The duration of motility regards to the elapsed time of progressive motility, and it was considered from the sperm activation until ~5% of motile cells.

Samples with total motility higher than 90% were pooled. The final concentration of such pool was  $5.8 \times 10^8$  cells/mL.

### *Preparation of tubes for cryopreservation and adjustment of cooling rate*

We prepared three kinds of tubes for cryopreservation using plastic materials easily obtained from commercial sources (polyvinyl adhesive tape - Yamato, Japan, 1mL cryogenic straw - IMV, France; polyurethane pipe - Argyle, Japan; 1mL syringe barrel - Terumo, Japan). We used these in order to change the internal and external diameter (Fig. 1). For preparation of tube 1 we used polyurethane pipe, vinyl adhesive tape (single layer), 1mL cryogenic straw tube and vinyl adhesive tape (double layer). Tube 2 had the same composition of tube 1, but we increased diameter using a 1mL syringe barrel. Tube 3 was prepared from a 1mL syringe barrel.

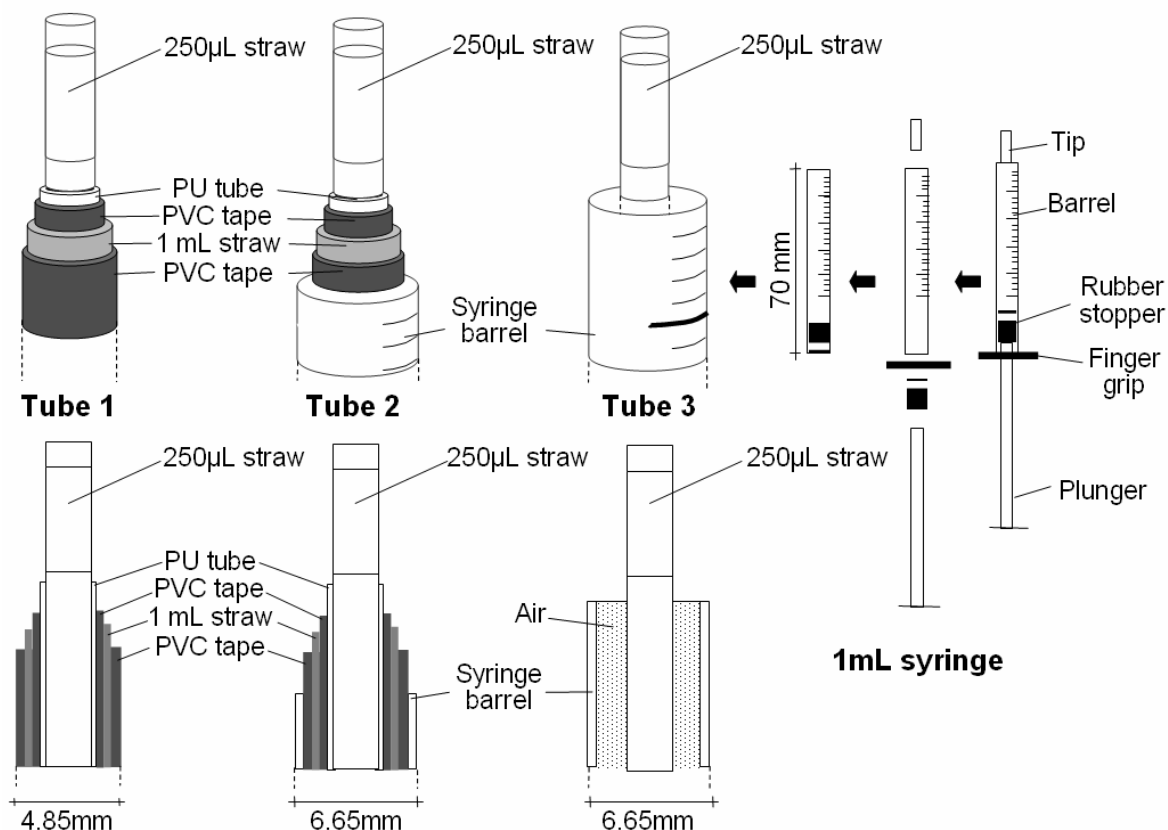


Figure 1. Structure and longitudinal section (lower) of the three different tubes used to control cooling rates for cryopreservation. Details of materials are the following: polyurethane tube (PU) - internal diameter: 2.04mm, external diameter: 3.23mm (Argyle, Japan); 1mL straw tube - internal diameter 3.91mm, external diameter 4.18mm (IMV, France); 1mL syringe tube - internal diameter: 4.66mm, external diameter: 6.6mm (Terumo, Japan) and polyvinyl adhesive tape (PVC). Schematic representation for preparation of tube 3 using 1mL syringe is detailed (right). Tip and finger grip are detached from the barrel and discharged. Rubber stopper is detached from the plunger, inverted and placed in the barrel extreme. A 250µL straw was cut to adequate to the barrel length (70mm) resulting in a 130µL final volume. All the tubes had the same length (70mm).

The cooling rate of each tube was measured using a thermo recorder (T&D Co., model TR-81, Japan). The probe was inserted into the tubes, placed in powdered dry-ice for 2 minutes and the reading was recorded at 1s intervals. In control group, cooling rate was recorded using the probe in powdered dry ice, without the tubes.

#### General procedure for cryopreservation and thawing

Sperm samples were mixed with the extender (63.5 mM NaCl, 114 mM KCl, 20 mM Tris and 10% methanol, pH 10.2) at a proportion of 6:1 (diluent:sperm). Methanol was used as a cryoprotectant based on previous results among cypriniforms (1, 5, 14). Diluted sperm samples were placed in 250µL cryogenic straw (IMV, France), cut to 130µL in order to adjust to tube's length (70mm). Straws were sealed using polyvinyl alcohol powder (PVA), inserted into different self-made tubes, placed into powdered dry ice (-79°C) and hand-compacted. An additional group was cryopreserved without the tubes (straw only). Commercial manual ice-crusher was employed for powdering the dry ice. After two minutes into dry ice, straws were

taken from the tubes and immediately plunged into liquid nitrogen (-196°C). Three hours afterwards all the samples were thawed in a water bath at 25°C for 10 seconds and evaluated the sperm parameters as mentioned above.

Fertilization

Sperm was cryopreserved using the cooling method that provided the best post-thawed motility. In this trial, we added 0.5% glycine to the diluent. Batches of 280 eggs were pipetted to 50mm diameter *Petri* dishes and inseminated with cryopreserved or fresh sperm at the minimum spermatozoa:egg ratio (300 spermatozoa/egg). Sperm was activated by adding 380µL of dechlorinated tap water. Hatching rates were measured 25-50 hours post-fertilization, at room temperature.

Statistics

Results are presented as mean ± SD. Comparisons were performed using analysis of variance, followed when appropriate by comparisons using the Student-Newman-Keuls test ( $P<0.05$ ).

RESULTS

The cooling characteristics were changed using different types of hand-made tubes and powdered dry ice. The final temperatures and cooling rates for particular phases of cooling curves are shown in Table 1. After placing the tubes into powdered dry ice for 2 minutes, we observed differences in all phases of cooling and the final temperatures.

Table 1. Temperature characteristics of the different tubes used to control cooling rates (n=3).

| Parameters                                | Control (probe) | Tube 1       | Tube 2       | Tube 3       |
|-------------------------------------------|-----------------|--------------|--------------|--------------|
| Final temperature (°C)                    | -79.0 ± 0.00    | -69.8 ± 1.07 | -64.0 ± 2.46 | -57.9 ± 1.57 |
| Total cooling rate (°C/min)               | -182.0 ± 54.82  | -36.9 ± 0.53 | -34.0 ± 1.23 | -30.9 ± 0.79 |
| Cooling rate from 4 °C to 0 °C (°C/min)   | -86.7 ± 27.32   | -23.8 ± 9.18 | -14.4 ± 0.96 | -18.0 ± 0.76 |
| Cooling rate from 0 °C to -50 °C (°C/min) | -421.4 ± 119.84 | -55.8 ± 4.32 | -40.2 ± 3.43 | -33.3 ± 2.09 |
| Time from 4 °C to 0 °C (s)                | 3.0 ± 0.89      | 11.0 ± 3.61  | 16.7 ± 1.15  | 13.33 ± 0.58 |

\*Total cooling rate was the temperature from 4°C until the final temperature.

The cooling rates from 0°C to -50°C also differed when using the tubes. This range of cooling is related to intracellular ice formation. Curves of cooling rates are plotted on Figure 2.

Total motility, progressive motility and its duration for fresh and cryopreserved sperm are shown on Table 2. All cryopreserved samples showed significant decrease in sperm motility.

The reduction of cooling rates significantly improved the post-thawed sperm parameters, and our best result was observed in tube 3. In this treatment the highest progressive motility was also observed, without any statistical difference from the fresh sperm. Regarding the duration of motility, all the treatments significantly differed from fresh samples. Sperm cooled without the tubes yielded the poorest result. However, significant differences were not observed among the three tubes.

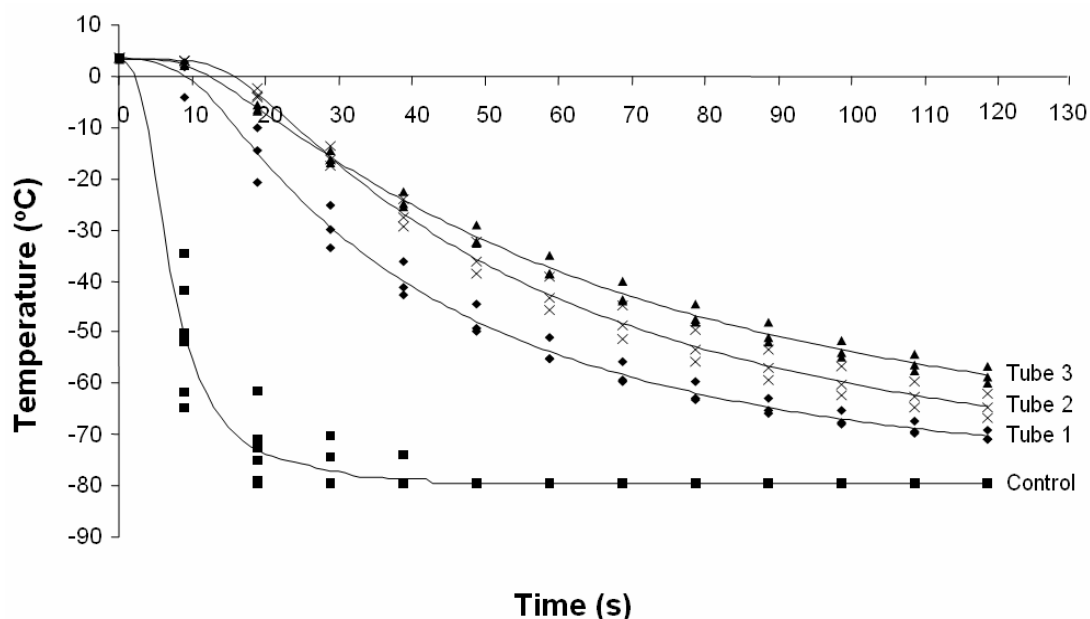


Figure 2. Cooling curves of the different tubes in powdered dry-ice. Temperature was evaluated each 1 s from 4°C until 120s (n = 3). Tubes containing only air were used for measuring the cooling rates.

Table 2. Sperm parameters of fresh and cryopreserved samples frozen at different cooling rates using self-made tubes (n=3).

| Sperm sample         | Motility (%)         | Progressive motility (%) | Duration (s)          |
|----------------------|----------------------|--------------------------|-----------------------|
| Fresh sperm          | >90% <sup>a</sup>    | >90% <sup>a</sup>        | 247 ± 16 <sup>a</sup> |
| No tube (straw only) | 20 ± 05 <sup>b</sup> | 58 ± 08 <sup>b</sup>     | 102 ± 12 <sup>c</sup> |
| Tube 1               | 32 ± 03 <sup>b</sup> | 62 ± 03 <sup>b</sup>     | 147 ± 14 <sup>b</sup> |
| Tube 2               | 53 ± 15 <sup>c</sup> | 65 ± 05 <sup>b</sup>     | 141 ± 20 <sup>b</sup> |
| Tube 3               | 72 ± 03 <sup>d</sup> | 83 ± 06 <sup>a</sup>     | 146 ± 12 <sup>b</sup> |

Identical superscript letters within a column denote no significant difference ( $P < 0.05$ )

The hatching rates of the eggs were significantly different between fresh sperm and cryopreserved using tube 3 ( $P < 0.05$ ).

## DISCUSSION

The control of cooling rates by using different kinds of tubes and dry ice is effective for better cryopreservation. It was possible to adjust cooling rates in all periods of cooling by changing the internal and external diameter of the tubes.

The cooling temperatures were recorded inside the tubes without fluid (diluent) around the probe. This procedure makes the tubes versatile for utilization in other cryopreservation protocols, since the cooling characteristics may vary according to each diluent. The measurement at dry conditions did not result in exotherms during the cooling phase.

In this study, we divided the cooling rates in two periods (4 to 0°C and 0 to -50°C). During the first period of cooling, chilling injury (cool injury in absence of ice) may occur in a wide range of sperm cells due to exposure to low temperatures and/or high rates of cooling

(cold shock) (28, 38, 42). Fish as ectothermic organisms presents a wide range of physiological temperatures and consequent intrinsic resistance to temperature and its variations. In cryopreservation or cool storage, fish sperm is commonly pre-cooled at around 0 to 4°C presenting low chilling sensitivity (8, 10, 24, 37), but injury caused by supra-optimal cooling rates may occur. Moreover, as we have limited the cooling period for 2 minutes, the prolongation of this initial period (0 to 4°C) may interfere the cooling characteristics and final temperatures of the following stages.

We focused the cooling rates from 0 to -50°C because critical events for cell survival such as ice nucleation and subsequent intracellular ice formation occur in this range of temperature for most of animal cells (27).

The optimum cooling rate has been determined in other fish species (16, 17, 21, 23, 24, 33). For cryogenic straws, cooling methods are standardized by the description of cooling procedures (9, 6, 15, 24, 35) and/or measurement of cooling rate inside the straw (21, 22, 34). However, cooling characteristics must vary according to the straw volume, diluents and sperm:diluent ratio. In the present study, we determined the cooling rates inside the tubes and it standardizes cooling rates for application in other protocols.

In the pellet-freezing on dry ice, equilibration periods ranging from 1 to 20 min prior to plunging into liquid nitrogen have been utilized in fish (2-4, 19, 40). Since dry ice has a constant cooling temperature (-79°C), the cooling rate may be changed by the exposure time or sperm package (pellet volume, straw size, cooling tube). These changes must be performed to prevent cell injuries and optimize the post-thawed sperm motility.

In our study, sperm samples were cooled by powdered dry ice for two minutes. This period was established based on our previous observations, since the prolongation of cooling period decreased the post-thawed sperm motility (data not shown). Our best results of post-thawed sperm motility were observed at the slowest cooling rate (and consequent lowest final temperature), indicating that the critical range of temperature was also attained for other treatments. So, our cooling period seems to be adequate for cryopreservation in powdered dry ice.

Cryopreserved sperm showed a decrease in hatching rates. Lower fertilization success using cryopreserved sperm is also reported in other fish species (4, 11, 39). Powdered dry ice becomes a portable cooling medium for cryopreservation, which is interesting for field conditions or fish hatcheries. Moreover, it is inexpensive and commonly available in the market, which is interesting in some areas where liquid nitrogen is not easily available. Since dry ice is a solid medium and utilizes higher temperatures (-79°C) when compared to liquid nitrogen (-196°C), it increases safety during manipulation. In addition, according to our experience, powdered dry ice is able to be stored for several weeks in the deep freezer at -80 to -83°C.

In this study we cut 250µL straws to adjust it to the tube's length (70mm). In the case of higher volumes of diluted sperm, it is recommendable to connect syringe barrels in order to increase length. Although a more detailed experiment should be conducted to optimize such performance, it was demonstrated that this technique is an inexpensive and simple method for controlling cooling rates during cryopreservation.

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