Theriogenology 95 (2017) 89–95

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Consequences of uncontrolled cooling during sterlet (*Acipenser ruthenus*) sperm cryopreservation on post-thaw motility and fertilizing ability

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ARTICLE INFO

Article history:
Received 10 October 2016
Received in revised form
3 February 2017
Accepted 11 March 2017
Available online 15 March 2017

Keywords:
Sturgeon sperm
Cooling device
Freezing rate
Cryopreservation
Sperm motility
Fertilization

ABSTRACT

The significant influence of the number and position of fish sperm sample straws in uncontrolled cooling devices on post-thaw spermatozoa parameters, such as motility and fertilizing ability, is presented in this study. The two most popular uncontrolled cooling devices were used in this study: a Styrofoam box setup with a polystyrene floating raft on liquid nitrogen and the dry shipper setup with a straw holder. We tested the effect of different quantities of straws (6 or 60) placed on the polystyrene floating raft and the position of the straws in the holder (on the periphery or in the centre). Using these cooling methods, sperm of 10 male sterlets diluted with methanol containing cryoprotective medium was frozen. All temperature changes were recorded by a thermocouple inside the straw, and the thermogram intervals were analysed. Spermatozoa motility was evaluated by video microscopy with integrated computer-assisted sperm analysis software. Fertilization trials were conducted at a 10^5 spermatozoa/egg ratio. Post-thaw spermatozoa parameters, including the percent of motile spermatozoa, curvilinear velocity, velocity according to the smoothed path, linearity of track, beat-cross frequency and fertilization rate, were significantly decreased in the 60-straw floating raft setup in comparison to all of the other cooling methods. The freezing rate between −10 °C and −30 °C was significantly decreased by up to 18.6 ± 0.61 °C/min for the 60-straw floating raft setup in comparison to the other freezing conditions. Considering the above results, efforts to standardize cryopreservation protocols using uncontrolled cooling devices should take into account the amount of straws subjected to freezing.

1. Introduction

Sturgeons occupy a specific niche in the modern fish market worldwide. The demand for sturgeon aquaculture is increasing, especially for their caviar and boneless meat, which are valuable worldwide [1]. Current caviar production is estimated at 260 tons per year and could increase to 750 tons in the next 10 years [2]. However, due to the continuous decline of wild sturgeon populations and growing market demand for caviar, they have been included in biodiversity conservation programs for population restoration [3]. Currently, fish sperm cryobanking is considered to be a potentially powerful tool in aquaculture for fish hybridization, selective breeding programs for biodiversity, and also for the restoration of endangered species [4,5].

Many successful protocols for sturgeon sperm cryopreservation have been developed over the past decade [6–9]. Nevertheless, the success of fish sperm cryopreservation still depends on a broad range of factors, including initial sperm quality, extender and cryoprotectant type, dilution ratio, ability to be activated by the freezing/thawing process itself, and appropriate freezing and thawing rates [5,10,11]. Moreover, during freezing, spermatozoa can be injured by physical factors, such as aggregative transition in membranes, formation of ice crystals inside and outside the cell, and osmotic or oxidative stress [12]. It is currently accepted that many damaging effects of cryopreservation depend on the freezing rate, which in turn provokes osmotic stress during slow freezing or

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http://dx.doi.org/10.1016/j.theriogenology.2017.03.007
0093-691X/

intracellular ice formation during rapid freezing [13]. However, optimal freezing rates can minimize these damaging factors. Achieving optimal freezing rates is possible thorough commercial programmable freezers (Asymptote EF600, Asymptote Ltd.; Cryomed™, Thermo Scientific; PLANER Kryo 550-16, Planer PLC, and others) that deliver stable, controlled, and repeatable freezing rates [14]. However, despite the advantages of programmable cooling devices, many researchers studying fish sperm cryopreservation prefer devices with uncontrolled freezing rates, which are much simpler to use, less expensive, and easily transportable. The most popular uncontrolled freezer devices are Styrofoam boxes containing liquid nitrogen and equipped with a floating raft (which is designed to keep the set of freezing straws above the liquid nitrogen surface) or dry shippers into which straws are loaded directly in a liquid nitrogen-free cold interior space [6,15–18].

Regarding these devices, an exploration of publications in the "ScienceDirect" database (www.sciencedirect.com) using "fish sperm cryopreservation" as key word over the last 10 years shows that 62.5% of such studies used uncontrolled cooling devices and that the remaining 37.5% used programmable cooling devices.

Nevertheless, despite the higher usage rate of uncontrolled cooling devices, standardized protocols for freezing in these devices have not been published. Therefore, there is a risk of negatively modifying cryopreservation procedure by many factors, including the size and geometry of the device as well as different volumes and quantities of straws. All of these factors could influence the convective stream of nitrogen vapours and consequently change the freezing rate.

In the present study, we explore whether the number or position of straws frozen by application of uncontrolled cooling devices in fish sperm cryobanking influence the post-thaw sperm parameters, such as motility and fertilizing ability.

2. Materials and methods

2.1. Sperm collection

During the natural for sturgeon spawning season (April–May), ten sterlet males (3–4 years old, 0.6–2.0 kg body weight, BW) were transferred from fish farming ponds to 4-m^3 plastic tanks with a closed water recirculation system, and the temperature of the water was increased to 15 °C over the subsequent 24 h. Males were injected once with 4 mg/kg BW carp pituitary powder dissolved in a 0.9% (w/v) NaCl solution. Sperm was collected after 24 h using a syringe with an attached 4-mm plastic catheter inserted into the urogenital ducts. Prior to experimentation, sperm was stored at 4 °C on ice [15].

2.2. Sperm motility parameters assessment

Immediately after sperm collection, the motility parameters were evaluated. Motility of sperm samples was initiated (in a 1:100 dilution) in activation medium consisting of 10 mM Tris–HCl buffer and 0.25% Pluronic F-127 (catalogue number P2443, Sigma-Aldrich) at pH 8.0 [19]. Motility was observed using a negative phase-contrast microscope (UB 200i, PROISER, Spain) with an attached ISAS 782M digital camera (PROISER, Germany). As fish sperm tend to swim near the surface [20], motility was recorded on the bottom portion of the activation medium droplet. Video records were obtained at 10-s intervals from 10 to 90 s post-activation and were analysed using the Integrated System for Semen Analysis (ISAS) software (Proiser, Valencia, Spain). CASA analysis included the following parameters: percent of motile cells, VCL (curvilinear velocity over the actual path, in μm/s), VAP (velocity over the smoothed path, in μm/s), LIN (linearity of track, VSL/VCL × 100%) and BCF (beat-cross frequency, in Hz). The motility duration was measured as the time elapsed from activation to end of motility in approximately 95% of spermatozoa. The spermatozoa concentration was evaluated using a Burker cell hemocytometer (Meopta, Czech Republic) at 200× magnification on Olympus BX 50 phase-contrast microscope (Olympus, Japan) [19].

2.3. Cryopreservation: protocols, techniques and devices

Sperm samples were diluted to a 1:1 ratio (v:v) with a solution of the following composition: 30 mM Tris, 23.4 mM sucrose and 0.25 mM KCl supplemented with 10% methanol [6]. Following dilution, sperm samples were cryopreserved using uncontrolled cooling in either a Styrofoam box or dry shipper. The Styrofoam box (Fig. 1A) (dimensions: 52 × 33 × 30 cm) was filled to a depth of 10 cm with liquid nitrogen, and 6 or 60 straws (volume 0.5 mL) on a polystyrene raft (dimensions: 40 × 20 × 3 cm) were placed on the liquid nitrogen surface. After a 10-min exposure to liquid nitrogen vapour, straws were plunged directly into liquid nitrogen [19].

Before initiating experiments with usage of dry shipper, the device (Model CX 100, Taylor, Wharton, Alabama, USA) was cooled to −196 °C using liquid nitrogen one day before the experiments, and then, a straw holder containing 60 straws was placed inside the device (Fig. 1B). The cylindrical straw holder, which had 120-mm height and 65-mm radius, was purpose-made in our laboratory for freezing samples in a dry shipper. This design prevents the straws from contacting the wall of dry shipper or other straws and also maintains constant conditions for all experiments. After a 10-min cooling inside the dry shipper, straws were removed and

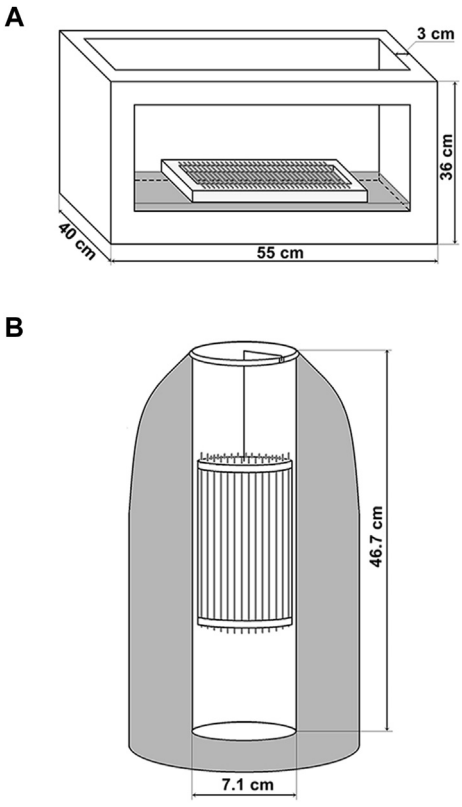

Fig. 1. Schematic illustration of uncontrolled cooling devices. (A) The Styrofoam box method consists in exposure of a 3-cm thick raft with straws to the liquid nitrogen surface (dark grey) inside a Styrofoam box. (B) The dry shipper method consists in insertion of a straw holder with straws in the middle of the interior space of a dry shipper.