Cryobiology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Effect of freezing rate on motility, adenosine triphosphate content and fertilizability in beluga sturgeon (*Huso huso*) spermatozoa ☆

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ARTICLE INFO

Article history:
Received 10 November 2014
Accepted 12 February 2015
Available online xxxx

Keywords:
Sperm motility
ATP
Fertilization
Freezing rate
Huso huso

ABSTRACT

Broodstock selection programs are currently underway for sturgeon species. To complement and further these selection programs we need to develop sperm cryopreservation procedures. In the present study, we describe the effects of freezing rate (−10 °C, −15 °C, −20 °C, −30 °C and −40 °C/min) on gamete quality characteristics (i.e., duration of motility (s), motility percentage (%), ATP content (nmol/10^8 cells), fertilization rate (%), and hatching rate (%)) in beluga sturgeon, *Huso huso*. After sampling, beluga sturgeon sperm were diluted in an extender composed of 23.4 mM sucrose, 0.25 mM KCl, and 30 mM Tris–HCl, pH 8.0 containing 10% methanol and subsequently frozen in a programmable freezer. Sperm frozen at −40 °C/min resulted in means for duration of motility (134 s), motility percentage (69%), ATP concentration (4.8 nmol/10^8 cells), fertilization rate (72%) and hatching rate (65%) that were higher ($P < 0.05$) than those for slower cooling rates. Based on our results, −40 °C/min was the best freezing rate (among those tested) for cryopreservation of beluga sturgeon sperm.

Introduction

In aquaculture, refrigerated storage is a simple and inexpensive procedure often needed to deal with logistics of large-scale hatchery operations. Cryopreservation is a powerful tool that allows sperm to be stored indefinitely. This method has been recognized as the most appropriate way for gene banking aimed to conserve specific genetic diversity [17]. There are numerous of factors affecting cryopreservation success including origin of the broodfish, initial quality of sperm, extender, cryoprotectant, equilibration time, dilution ratio, volume of straws, freezing rate, thawing rate, time between thawing and activation as well as physiological aspects of sperm that might be species-specific [46,12,36,16]. Therefore, determining the 'optimal' procedure for sperm cryopreservation from a given species is not a simple task.

Sturgeons are considered to be "living fossils" [10]. Their evolutionary history goes back to the early Jurassic period (approximately 100–200 million years ago). Caspian Sea is the habitat for the four commercial species of sturgeon and *Huso huso* is one of them [34], where their populations are declining due to excessive fishing for meat and caviar production, habitat destruction, and water pollution [13]. Now worldwide production of cultured sturgeon has increased from 2500 metric tons in 1999 to 25,600 metric tons in 2008 (FAO, 2008), and aquaculture caviar production increased from 1.69 metric tons in 2003 to 27.32 metric tons in 2007 [25].

Although sturgeon farming has a history of more than one hundred years, the basis for intensive artificial reproduction and methods of in vitro gamete manipulation were developed only in the second half of the 20th century [22]. The importance of semen storage to efficient husbandry was emphasized by Billard [14]. In addition, cryopreservation of sperm has been well developed in many fish species including sturgeons for resource conservation and aquaculture practices, such as Atlantic sturgeon *Acipenser sturio* by Kopeika et al. [35]; Siberian sturgeon *Acipenser baerii* by Glogowski et al. [29]; Russian sturgeon *Acipenser gueldenstaedtii* by Huang et al. [32]; Persian sturgeon *Acipenser persicus* by Aramli and Nazari [4]; Sterlet *Acipenser ruthenus* by Dzyuba et al. [25]; Common carp *Cyprinus carpio* by Wamecke et al. [49]; Brown trout *Salmo trutta* by Nynca et al. [41] and Rainbow trout *Oncorhynchus mykiss* by Ciereszko et al. [18].

Some experiments have been conducted in an attempt to determine a protocol for cryopreservation and short-term storage of beluga semen [5,1,2]. To our knowledge there are no data on the effect of freezing rate on the sperm quality of beluga sturgeon. Hence, in the completion of previous studies, the present study

☆ *Statement of funding:* The authors would like to acknowledge the Rajaee Sturgeon Propagation Center whose funding made this research possible.

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http://dx.doi.org/10.1016/j.cryobiol.2015.02.001
0011-2240/

was conducted to confirm the best freezing rate (from −10 °C to −40 °C/min) for beluga sperm and to quantify additional information on the effects of freezing rate on fertilizing ability, motility parameters, and energetics (ATP content).

Materials and methods

Fish and gamete collection

Males ($n = 12$; weight, 30–40 kg; total length, 1–2 m) and females ($n = 4$; weight, 40–45 kg; total length, 1.5–2 m) were captured using gillnets (length 18 m, width 5.4 m, mesh size 15 cm) from the southwestern part of the Caspian Sea and transported to the Rajaee Sturgeon Hatchery Center (Sari, Mazandaran, Iran) between March and April 2011. The fish were maintained in tanks with a water temperature of 15–16 °C, an oxygen content of >5 mg/L and a pH of 7.6–7.9. Spermiation was stimulated by a single intramuscular injection of the luteinizing hormone-releasing hormone agonist ($LHRHA_2$) at 5 μg/kg body weight at 18 h before sperm collection. Semen was collected from the urogenital papilla by aspiration through a plastic catheter (5–7 mm diameter) connected to a 50 mL syringe. Special care was taken to avoid contamination with mucus, feces, or water. Females were injected with the same hormone at 10 μg/kg body weight at 14 h before stripping. Fish were anesthetized and placed in lateral recumbency on a table. A finger was inserted into the gonopore to stretch the opening slightly. A scalpel (with a straight blade narrower than the gonopore) was inserted carefully into the gonopore opening, and a 1.5–3 cm incision was made through the ventral area of the oviductal (Mullerian duct) wall. The scalpel was withdrawn and the incision probed with one finger to ensure that the opening was not obstructed. The fish was inverted and slight pressure applied to the abdominal region by two individuals: the ova flowed through the incised opening in the oviduct and out of the gonopore [5,45]. The analysis in each trial was replicated three times.

Cryopreservation protocol

Sperm was frozen using conventional freezing procedures: prior to freezing the samples were diluted 1:1 in an extender composed of 23.4 mM sucrose, 0.25 mM KCl, and 30 mM Tris–HCl, pH 8.0 [29] containing 10% methanol [27]. Diluted sperm were placed in 0.5 mL straws (CRYO-VET, France) and then placed into a programmable freezer (Planer Kryosave-Model KS30, Sunbury-on-Thames, Middlesex, UK) and frozen at −10 °C, −15 °C, −20 °C, −30 °C or −40 °C/min. The straws were removed from the freezer and immediately placed into dewars containing liquid nitrogen for storage. The initial freezing rate used was rotated for each day of each sampling week (to account for potential differences in the time samples were frozen relative to when they were collected). Each sample was removed from storage 30 days after it was frozen, thawed for 6 s in a 40 °C water bath, and re-evaluated for fertilizing ability, motility parameters and ATP.

Evaluation of sperm motility parameters and concentration

Tris–HCl buffer (10 mM, pH 8.0) containing 0.25% pluronic (a substance that prevents spermatozoa from sticking to slides) was used as activating medium (AM).To trigger motility, the post-thaw sperm and the fresh sperm were diluted in AM with dilution rates 1:500, and 1:1000, respectively [26,27,42]. Spermatozoa motility was recorded with a dark-filed microscopy (400×, Olympus CK2, Tokyo, Japan). The percentage motility was determined arbitrarily on a 0–10 point scale, where 0 denoted 0% motility and 10 denoted 100% motility. The duration of motility was determined by recording the time taken from activation to the complete cessation of activity by the last spermatozoa in a field. One person conducted all of the sperm motility observations to reduce the degree of variation. Sperm density was estimated using a Burker cell hemocytometer (Meopta, Czech Republic) at 200× magnification on an Olympus BX 50 phase contrast microscope (Olympus).

Fertilization assay

Eggs from the four females were pooled in equal parts 3 g eggs (approx. 200 eggs) and inseminated in a Petri dish with sperm stored from −10 °C to −40 °C/min. Based on the sperm concentration of the sample, the volume of sperm was adjusted to obtain a 10^5 sperm/egg ratio. To measure the fertilization rate, living and dead eggs were counted in each Petri dish during incubation and dead eggs were removed. Live embryos were counted after the second cleavage division at 4 h post-fertilization. Fertilization rate was expressed as the proportion of live embryos at corresponding post-fertilization times of the initial number of eggs incubated according to recommendations for sturgeon fishery practices according to Dettlaf et al. [22]. Hatching rate was determined by the proportion of yolk suck larvae from fertilized eggs.

ATP bioluminescence assay

The ATP contents of spermatozoa were determined using the bioluminescence method described by Boryshpolets et al. [15]. Sperm samples were added to a boiling extraction medium, which contained 100 mM Tris–HCl (pH 7.75) and 4 mM EDTA. After boiling for 2 min at 100 °C, samples of the sperm suspension were centrifuged at 12,000×*g* for 20 min. The ATP contents of the supernatants were evaluated using a Bioluminescence Assay Kit CLS II (Roche Diagnostics GmbH, Germany). The luminescence was read using a SpectraFluor Plus plate reader (Tecan Group, 1-40 Miyamachi, Japan) and the data were expressed as picomoles (pmol) of ATP per 10^8 sperm.

Statistical analyses

Data are presented as mean ± SD. All analyses were performed at a significance level of 0.05 using SPSS v 11.5 (Chicago, IL, USA). Normally distributed data were analyzed by ANOVA followed by Fisher's LSD test. For the statistical model, freezing rate was considered a fixed effect and week of semen sampling for each fish as a repeated measure. All variables were tested for rate by week interactions. A nonparametric Kruskal–Wallis test followed by the Mann–Whitney *U*-test with Bonferroni correction was used for comparison of motility parameters, fertilization rate and ATP content.

Results

Spermatozoa motility

For post-thaw sperm that were frozen at the −40 °C/min rate, total duration (s) and motile sperm (%) (134 ± 27.01 and 69 ± 4.18 respectively), were significantly higher than sperm frozen at any of the four slower rates. No significant differences observed among the four slower rates for both motion characteristics (Fig. 1a and b). In addition, total duration was 172 ± 13.5 s and percentage of motile cell was 88 ± 5.7 (%) for fresh samples.

ATP content

Fresh samples had an average ATP concentration of 7.2 ± 0.57 nmol/10^8 sperm. Sperm cryopreserved using the fastest

Please cite this article in press as: M.S. Aramli et al., Effect of freezing rate on motility, adenosine triphosphate content and fertilizability in beluga sturgeon (*Huso huso*) spermatozoa, Cryobiology (2015), http://dx.doi.org/10.1016/j.cryobiol.2015.02.001