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Effectiveness of glucose–methanol extender for cryopreservation of *Huso huso* spermatozoa

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ABSTRACT

The present approach was designed to evaluate the methanol–glucose extender effects on sperm cryopreservation in beluga sturgeon, *Huso huso*. Sperm quality was examined by measuring post-thaw sperm motility and fertilizing rate at hatching stage. We first tested the effect of glucose concentration (0, 0.10, 0.15, 0.20 and 0.30 M) in a methanol extender on post-thaw sperm motility. The optimal cryopreservation conditions were found to be 0.2 M glucose in the extender. Then, motility and fertilization rates of sperm cryopreserved with 0.2 M glucose and 10% methanol (GM) were compared to Tris–sucrose–KCl in 10% methanol extender (TSKM). Additionally, sperm motility and fertilizing ability in relation to 15 and 30 min equilibration in GM extender before and after cryopreservation were measured. Higher post-thaw sperm motility duration and percentage as well as fertilization rate were obtained with the GM extender when compared to TSKM extender. Equilibration of sperm in extender did not affect the motility quality of either fresh-diluted or frozen/thawed sperm, while fertilization rate showed a significant decline alone after 30 min of post-thaw storage. Our results indicated that the use of a simple extender consisting of 0.2 M glucose in 10% methanol can be an alternative cryopreservation method to those previously described for sturgeons.

1. Introduction

Most endangered and commercially valuable fish species, such as sturgeons are in a great need of aquacultural restocking, which relies on artificial reproduction (Havelka et al., 2011). Cryopreservation is a promising way for preservation of rare and endangered species of sturgeon. It plays an important role in transporting genetic material between facilities, optimal use in aquaculture, reducing the risk of spreading infections, performing hybridization studies, protecting gene pool, conserving biodiversity, selective breeding activities, and protecting endangered species (Sarder et al., 2012; Kutluyer et al., 2014).

Early studies on sturgeon sperm cryopreservation were carried out in the 1960s (Burtsev and Serebryakova, 1969) with limited success. Since the 90s, methods resulting in sufficient sperm post-thaw motility have been described (Glogowski et al., 2002; Psenicka et al., 2008). The latter methods employed extenders consisting of potassium ions and sucrose buffered by Tris–HCl in methanol. Recently,

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simple extenders containing glucose and methanol have been used for cryopreservation of salmonid fish (Ciereszko et al., 2014; Dietrich et al., 2014; Nynca et al., 2014) and also Siberian sturgeon, *Acipenser baerii* (Judycka et al., 2015) sperm. Furthermore, some studies about the short and long-term storage of *Huso huso* sperm have been conducted (Aramli, 2014; Aramli et al., 2015). Despite these advances, many issues still need to be studied in order to identify efficient and simple protocols for cryopreservation of this species sperm.

Cryopreservation involves several parameters that need be stabilized to improve post-thaw survival. These factors include cryoprotectant type and concentration (Lahnsteiner et al., 1996), equilibration time (Babiak et al., 2001; Perez-Cerezales et al., 2010), and number of spermatozoa (Ciereszko et al., 2013). This study was conducted to test the effectiveness of the glucose–methanol extender for the cryopreservation of sperm of *H. huso*. In the first experiment we aimed to optimize glucose concentration in the extender. In the second experiment sperm motility parameters and fertilizing ability of sperm cryopreserved in 0.2 M glucose and 10% methanol (GM) were compared to Tris–sucrose–KCl–methanol extender (TSKM). Additionally, we examined sperm motility and fertilization rate in relation to 30 min equilibration in GM extender and 30 min of post-thaw storage.

2. Materials and methods

2.1. Collection of gamete and measurements of sperm density and seminal plasma osmolality

The study was performed at the Sturgeon Hatchery Center in Mazandaran, Iran. Broodfish (body weight 40–45 kg) were selected from wild breeders that originated from the Caspian Sea. Before stimulation, the fish (both male and female) were transferred from ponds to tanks with a water temperature of 15–16 °C, an oxygen content of >5 mg/L and a pH of 7.6–7.9. Next, the males were stimulated hormonally with an injection of 5 $\mu g\,kg^{-1}$ using a synthetic analog LHRH-A_2 (Ningbo Renjian Pharmaceutical Group Co., Ltd, China). Sperm was collected 24 h after hormonal stimulation using a syringe with an attached rigid tube inserted into the urogenital opening. Eggs were collected 24 h after stimulation with two doses of LHRH-A_2. The first dose contained 10% and the second 90% of hormone and the total injection was 10 $\mu g\,kg^{-1}$. Sperm density was estimated using a Burker cell hemocytometer (Meopta, Czech Republic) at 200× magnification on an Olympus BX 50 phase contrast microscope (Olympus). Freezing point depression (as an indication of osmolality) was measured with an osmometer (Osmomat 030-m, Berlin, Germany). Mean sperm denisty and osmolality of fresh undiluted semen were $2.4 \pm 1 \times 10^9$ sperm and 85 ±9.2 mOsm/kg, respectively.

2.2. Cryopreservation of sperm

Sperm samples from males were frozen individually. The cryopreservation was performed using a freezing unit for 20 straws which included an adjustable floating rack and Styrofoam box with an isolating Neopor block (MINITUB GmbH, Tiefenbach, Germany). Sperm suspensions were placed in 0.5 ml straws (CRYO-VET, France) and suspended 3 cm above liquid nitrogen in a Styrofoam box for 10 min and then plunged into the liquid nitrogen. The straws were then thawed by immersion in a water bath at 40 °C for 5 s. Cryopreservation trials were run in duplicates for each sample. Sperm motility was measured for fresh, diluted–equilibrated and frozen/thawed sperm.

2.3. Effect of glucose concentration in extender containing 10% methanol on sperm motility parameters of fresh and frozen/thawed sperm

The effect of glucose concentration (0, 0.10, 0.15, 0.20 and 0.30 M) in 10% methanol on the sperm motility of fresh-diluted and cryopreserved sperm was tested on three individual samples of sperm ($n = 3$). Cryopreservation was performed using a 1:1 sperm-to-extender dilution ratio.

2.4. Effects of extender composition on sperm motility and fertilization rate of fresh and frozen/thawed sperm

Two extenders were prepared: GM (0.2 M glucose in 10% methanol) and TSKM (30 mM Tris, 23.4 mM sucrose, 0.25 mM KCl in 10% methanol, pH 8.0) (Glogowski et al., 2002). The post-thaw sperm motility parameters and fertilizing ability of sperm cryopreserved in GM and TSKM extenders were tested on three individual samples of sperm ($n = 3$).

2.5. Effect of 15 and 30 min equilibration on sperm motility parameters of fresh sperm and sperm cryopreserved using GM extender

Sperm was cryopreserved with the use of an extender consisting of 0.2 M glucose and 10% methanol (sperm:extender 1:1). Straws were either cryopreserved immediately after dilution or after 15 and 30 min of incubation on ice at 4 °C. Sperm motility was measured for fresh-diluted and frozen-thawed sperm ($n = 6$).

2.6. Sperm motility parameters

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. The motility parameters of fresh sperm, fresh-diluted in extenders, sperm equilibrated for 15 and 30 min, and frozen/thawed sperm were recorded, in triplicates, with a video camera (AxioCamICc 5, Germany) mounted on a phase-contrast microscope (Olympus CK2, Tokyo, Japan) at 400× until the spermatozoa trajectories become tight concentric circles (Rurangwa et al., 2004). The video records were scanned to determine the percentages of progressive motility (%) and the durations of progressive motility (s). The sperm motility percentages were estimated as the percentage of cells that exhibited progressive forward movement (Billard and Cosson, 1992; Horvath et al., 2003), and the durations of motility were determined as the times until forward movement stopped and circular movement

began. The percentages of sperm motility were assessed using an arbitrary scale with 10% interval increments in which non-motility was recorded as 0% (modified from Borges et al., 2005).

2.7. Fertilization

Before fertilization the straws were thawed in a water bath at a temperature of 40 °C for 5 s. After thawing, an adequate volume of frozen/thawed milt (10^5 sperm/egg) was added to about 100 eggs, diluted with an activation solution (described above) and left for 5 min. Next, the samples were washed with hatchery water and then with a tannic acid solution. Egg samples (approx. 100 eggs per treatment) were stocked into separate baskets in an incubation apparatus. Undiluted fresh sperm was used at the beginning and at the end of the fertilization trial to test the quality of the eggs. Fertilization rates were calculated as the percentage of hatched larvae after 7 days of incubation at 16 °C (Dietrich et al., 2012). All fertilization trials were done in duplicate.

2.8. Statistical analysis

All values were expressed as mean values ± SD. The percentage data were subjected to normalization by arcsine transformation. Data were tested for normal distribution and equal variances (Bartlett's test). Data then were subjected to two-way analysis of variance (ANOVA) followed by Sidak's post hoc test using the statistical package GraphPad Prism (San Diego, CA, USA). One-way ANOVA followed by Tukey's test and the Kruskal Wallis test followed by Dunn's test were used to evaluate the effects of concentrated extenders on post-thaw sperm motility. The hatching success was compared using *t*-test. All analyses were performed at a significance level of 0.05.

3. Results

3.1. Effect of glucose concentration in extender containing 10% methanol on sperm motility parameters of fresh and frozen/thawed sperm

The results of comparing different concentrations of glucose on fresh and post-thaw beluga sperm parameters are presented in Fig. 1(A) and (B). Glucose concentration in the extender did not have any effect on the fresh-diluted sperm (~ 85%) but significantly affected post-thaw sperm motility parameters. The highest post-thaw sperm duration (48 s) and percentage (50%) was observed at 0.2 M glucose concentration. Therefore, this concentration was selected to be used in subsequent experiments.

3.2. Effects of extender composition on sperm motility and fertilization rate of fresh and frozen/thawed sperm

The results of comparing GM and TSKM extenders on fresh and post-thaw beluga sperm parameters and fertilization rate are presented in Fig. 2(A)–(C). No differences in the motility duration and percentage of fresh sperm diluted in both extenders were observed. Application of

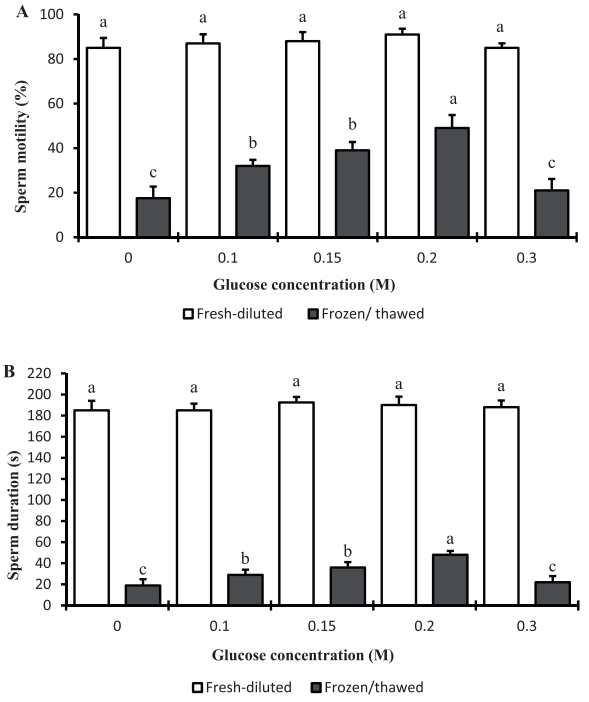

Fig. 1. The effect of glucose concentrations in extender on fresh-diluted and frozen/thawed sperm motility percentage (A) and duration (B) of beluga sturgeon ($n = 3$). ^{a,b,c} Values (mean ± standard deviation) with different superscripts are significantly different ($P < 0.05$).

GM extender showed a higher duration and percentage of motile spermatozoa after freezing/thawing compared to TSKM extender (Fig. 2(A) and (B)). Fertilization rate measured at hatching stage of fresh-diluted sperm were high (70–85%) and did not differ statistically in both extenders. While, GM extender for post-thaw sperm provided a better result in terms of fertilization rate when compared to TSKM extender (Fig. 2(C)).

3.3. Effect of 30 min equilibration on sperm motility parameters of fresh sperm and sperm cryopreserved using GM extender

Equilibration did not cause significant changes in the percentage and duration of motility of fresh and frozen/thawed sperm (Fig. 3(A) and (B)). The percentage of fertilization rate for fresh sperm did not differ from frozen/thawed sperm stored for 15 min, however, a significant decline was observed after 30 min of post-thaw storage (Fig. 3(C)).

4. Discussion

External cryoprotectants that do not permeate sperm cells often are used to protect sperm from damage during the freezing and thawing process. In fish sperm, the effects of egg yolk (Babiak et al., 2001), low density lipoproteins (Perez-Cerezales et al., 2010), soybean proteins (Cabrita et al., 2001), and bovine serum albumin (Stoss and Holtz, 1983) have been examined. Although these non-permeating cryoprotectants usually have a

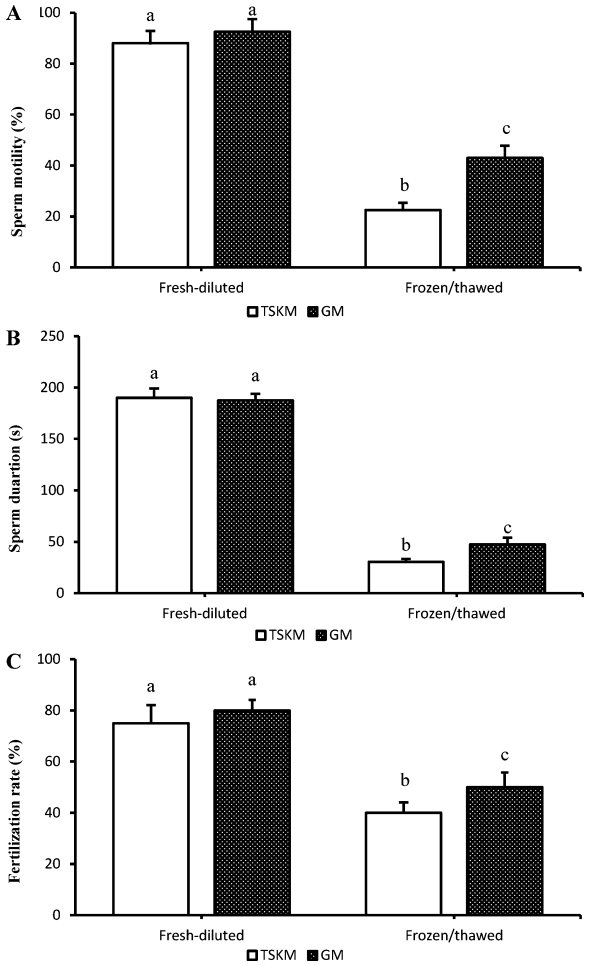

Fig. 2. Sperm motility percentage (A), duration (B) and fertilization rate (C) of fresh-diluted and frozen/thawed Beluga sturgeon sperm cryopreserved in TSKM and GM extenders ($n = 3$). [a,b,c] Values (mean ± standard deviation) with different superscripts are significantly different ($P < 0.05$).

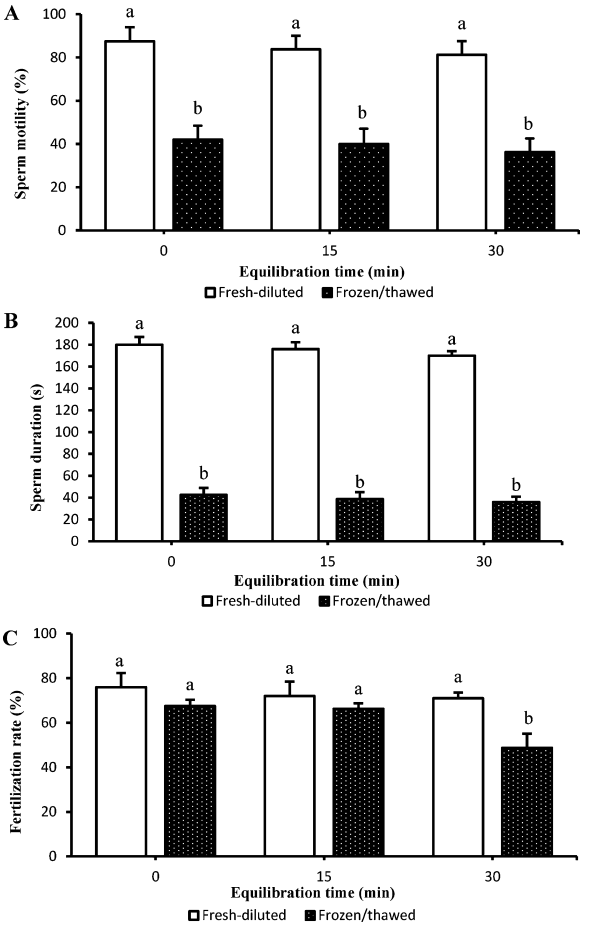

Fig. 3. The post-thaw sperm motility percentage (A), duration (B) and fertilization rate (C) of beluga sturgeon sperm ($n = 6$). [a,b] Values (mean ± standard deviation) with different superscripts are significantly different ($P < 0.05$).

positive effect, high variability in the results often is observed, probably due to significant interactions related to their effects (Babiak et al., 2001; Tekin et al., 2007). Furthermore, during the last decade, the most extenders used for the cryopreservation of sturgeon sperm usually consists of Tris–sucrose–KCl, Tris–sucrose and Tris–NaCl combined with methanol or dimethyl sulfoxide cryoprotectants (Glogowski et al., 2002; Boryshpolets et al., 2011). To our knowledge, few studies on the effects of sugar concentrations for cryopreservation success of sturgeons sperm have been conducted. Recently cryopreservation procedures with the use of 0.1 M glucose in 15% methanol were successfully implemented for Siberian sturgeon, *A. baerii* sperm (Judycka et al., 2015). In the current study, we have developed this procedure for the cryopreservation of beluga sturgeon sperm. Our results indicated that beluga sturgeon sperm are sensitive to external damage and that the cryoprotective effect of glucose is effective within a narrow range of concentrations. However, the mechanism for the cryoprotective action of glucose and its modulation by glucose concentration need to be elucidated in further studies.

An equilibration is rarely used for the cryopreservation of fish sperm due to a lack of improvement of post-thaw fertility or even a decrease in the fertilizing ability of cryopreserved sperm (Lahnsteiner et al., 1996). However, studies reported that equilibration times up to 15 min did not affect the post-thaw fertility of fish semen, and even equilibration times of 30, 45 and 60 min have been used (Bozkurt et al., 2005; Cabrita et al., 1998). Similar to our results, Aramli and Nazari (2014) and Judycka et al. (2015) reported that equilibration times up to 30 min did not affect the post-thaw motility and fertility of Persian sturgeon and Siberian sturgeon sperm, respectively. In contrast, Babiak et al. (2001) and Perez-Cerezales et al. (2010) used a 10 min equilibration time for rainbow trout sperm and found that equilibration led to decreased fertilization rates of semen cryopreserved with DMSO- and ethylene glycol- but not DMA-based extenders. In addition, interaction between equilibration time and extender constituents may explain conflicting information about the use of equilibration in cryopreservation of fish sperm.

The post-thaw motility of fish sperm is usually quite low to comparison to fresh sperm, which indicates that severe cryogenic injuries occur during the freezing and thawing process (Babiak et al., 2002; Glogowski et al., 1996). In the current study, however, we recorded more than 45% post-thaw sperm motility. High percentage of post-thaw motility of sperm was also observed recently for Persian sturgeon (Aramli and Nazari, 2014). Our results confirm the remarkable usefulness of the glucose–methanol extender for securing high sperm motility upon thawing, and suggest that this method can be universal for the freezing of sturgeon sperm.

In conclusion, our results demonstrated that beluga sturgeon sperm can be successfully cryopreserved with the application of a simple glucose–methanol extender. The concentration of glucose is important for cryopreservation efficiency. The use of an equilibration period and the possibility of post-thaw sperm storage can improve the organization of hatchery work and help with the logistics of large-scale hatchery operations. This producer extender after scaling up can be recommended for routine hatchery practice.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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