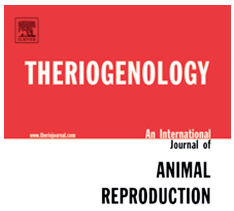

Effect of freezing rate for cryopreservation of Persian sturgeon (*Acipenser persicus*) spermatozoa

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Effect of freezing rate for cryopreservation of Persian sturgeon (*Acipenser persicus*) spermatozoa

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Abstract

This study examined the effect of freezing rate (–10°C, –15°C, –20°C, –30°C, and –40°C /min) on motility parameters, rates of fertilization and hatching, adenosine triphosphate (ATP) content, and indices of oxidative stress including thiobarbituric acid reactive substances (TBARS) and carbonyl derivatives of proteins (CP), in Persian sturgeon (*Acipenser persicus*) sperm. Following sampling, sperm was 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. For post-thaw sperm that were frozen at a rate of –40°C /min, sperm motile duration (134±27.01s), sperm motile percent (60 ± 4.1%), fertilizability (72±8.36% for fertilization rate and 65±7.58% for hatching rate) and ATP content (4.8 ± 0.57 nmol/10^8) were significantly higher than sperm frozen at any of the four slower rates (P<0.05). Moreover, sperm cryopreserved using the fastest freezing rate had significantly lower levels of TBARS (0.5±0.05 nmol/10^8) and CP (41.3±4.9 nmol/10^8) than all other freezing rates (P<0.05). In addition, there is a significant difference (P<0.05) between fresh sperm and the recovery of cryopreserved Persian sturgeon sperm using programmable freezing with –40°C /min being the optimal freezing rate among those tested.

Keywords: Motility, Fertility, ATP, Oxidative stress, Sperm, Cryopreservation, Persian sturgeon

1. Introduction

Sturgeons are commercially important fish that are valued for their meat, but primarily for their caviar [1]. Several sturgeon species, including the Persian sturgeon (*Acipenser persicus*), are listed as endangered or critically endangered on the IUCN Red List [2–4]. Restocking of the Persian sturgeon has occurred in Iran since 1969, but spawning populations have dramatically decreased due to many ecological disruptions as a result of human activities. The genetic resource banking of sperm along with restocking can maintain both the genetic variation and recover viable populations of *A. persicus* [5].

Cryopreservation as a method for the perpetuation of genetic resources of aquatic species offers many benefits to aquaculture and conservation biology. The advantages of cryopreservation include: (1) synchronization of gamete availability from sexes, (2) sperm economy, (3) simplification of broodstock management, (4) transportation of gametes between different fish farms, and (5) germplasm storage for genetic selection programs or conservation of endangered species [6]. However, despite the increasing success of sperm cryopreservation, this technique may causes extensive cell damage produced by the physical forces of ice crystal formation both within the cells and in the external medium, by osmotic stress, or by oxidative stress [7]. Thus, the conservation of cell structure and function will depend on the cryopreservation protocol. Sperm motility is crucial for the in vitro fertilisation of oocytes and depends on aspects of cell function including ATP production. The assessment of sperm quality using the available tools, such as viability tests, ATP content, and motility analysis, will be decisive for the selection of good sperm samples and for the standardization of the designed cryopreservation protocols [6]. Programmable freezing units are preferred for gamete cryopreservation as they ensure uniform freezing rates, thus providing more consistent results.

The freezing conditions described here offer consistency, precision, and accuracy. Furthermore, the whole procedure is rapid, as immediately after the ejaculate is obtained, the cells are processed, frozen, and ready to be stored under liquid nitrogen in less than 15 min [8].

Experiments have been conducted in an attempt to determine a protocol for the cryopreservation of semen from several sturgeon species including Atlantic sturgeon (*Acipenser sturio*) [9], Siberian sturgeon (*Acipenser baeri*) [10], Russian sturgeon (*Acipenser gueldenstaedtii*) [11], Persian sturgeon (*Acipenser persicus*) [12], and the sterlet (*Acipenser ruthenus*) [13]. To our knowledge, there are no data on the effects of freezing rate on the quality of *A. persicus* sperm. The present study was conducted to confirm the best freezing rate (from –10°C to –40°C/min) for *A. persicus* sperm. We also evaluated the effects of programmable freezing on sperm motility parameters, fertilizability, oxidative stress indices, and ATP content.

2. Materials and methods

2.1. Fish

The fish species examined in this work is the Persian sturgeon, *A. persicus* (Borodin, 1897) of the Acipenseride family. Eight male (1–1.5 m total length and 15–20 kg mean body weight) and four female (1.5–2 m total length and 20–25 kg mean body weight) were captured using gillnets (length 18 m, width 5.4 m, mesh size 15 cm) in 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°C–16°C, an oxygen content of >5 mg/L, and a pH of 7.6–7.9. All fish manipulations were conducted in accordance with the guidelines on the care and use of animals for scientific purposes (National Health and Medical Research Council, Australia).

2.2. Collection of gametes

Males were injected with an analogue of releasing hormone LHRH-A_2 (D-Ala^6 GnRH Pro^9-Net, Ningbo Hormone Product Co., China) at 5 μg/kg body weight 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. After collection, fresh semen was divided into two parts; one to be used as control and the other for cryopreservation.

Females were injected with 10 μg LHRHA^2/kg body weight then after 14 h anesthetized and placed in lateral recumbency on a table for the stripping of semen. 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 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 incision in the oviduct and out of the gonopore [14, 15]. The analysis in each trial was replicated three times.

2.3. Sperm cryopreservation

Sperm (n=8) was frozen using conventional freezing procedures [16]. Prior to freezing, the samples were diluted 1:1 in a cryodiluent composed of 23.4 mM sucrose, 0.25 mM KCl, and 30 mM Tris–HCl, pH 8.0, containing 10% methanol (methanol concentration after semen dilution was 5%). The resulting sperm suspension was placed in 0.5 mL straws (CRYO-VET, France), 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 15 days after it was frozen, thawed for 6 s in a 40°C water bath, and re-evaluated for semen quality indices.

2.4. Sperm evaluation

2.4.1. Motion and density analysis

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 at dilutions of 1:500 and 1:1000, respectively [13, 17]. Spermatozoa motility was recorded under dark-field 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 using an Olympus BX 50 phase contrast microscope.

2.4.2. Fertilization assessment

Eggs from the four females were pooled in equal parts and 3 g of eggs (approx. 150 eggs) were inseminated in a Petri dish with sperm previously cryopreserved at rates of –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, all 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 the corresponding post-fertilization times compared to the initial number of eggs incubated according to recommendations for sturgeon fishery practices [18]. Hatching rate was determined as the proportion of yolk sack larvae obtained from fertilized eggs.

2.4.3. ATP bioluminescence assessment

The ATP content of spermatozoa was determined using the bioluminescence method described by Boryshpolets et al. [19]. 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 content of the supernatant was evaluated using a Bioluminescence Assay Kit CLS II (Roche Diagnostics GmbH, Germany). The luminescence was measured 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 cells.

2.4.4. Oxidative stress indices

Semen samples were centrifuged (Heraeus, Sepatech, Berlin, Germany) at 3000 × g for 10 min at 4°C. The supernatant was collected carefully and discarded. The pellet was diluted with potassium phosphate buffer (50 mM KPi-buffer, pH 7.0, 0.5 mM EDTA) to obtain a sperm density of 5×10^8 cells/mL and then homogenized in an ice bath using an Omni Ruptor 4000 Ultrasonic Homogenizer (Omni International, USA). The homogenate was divided into two portions: one to measure the thiobarbituric acid reactive substances (TBARS) and carbonyl derivatives of proteins (CP), and a second was centrifuged at 12,000 × *g* for 30 min at 4°C to obtain the post-mitochondrial supernatant for assays of other antioxidant enzyme activities. The TBARS method described by Zhou et al. [20] and Lushchak et al. [21] was used to evaluate

sperm lipid peroxidation (LPO). The TBARS concentration was calculated based on the absorption at 535 nm with a molar extinction coefficient of 156 mM/cm. The value of TBARS was recorded as nanomoles per 10^8 cells. The CP content was assessed spectrophotometrically at 370 nm using a molar extinction coefficient of 22 mM/cm and was expressed as nanomoles per 10^8 cells.

2.5. Data analyses

All analyses were performed at a significance level of 0.05 using SPSS version 11.5 (Chicago, IL, USA). Data are presented as mean ± SD. Normally distributed data were analyzed by ANOVA followed by Fisher's LSD test. A nonparametric Kruskal–Wallis test, followed by the Mann–Whitney U-test with Bonferroni correction, was used for comparison of motility parameters, fertilization rate, oxidative stress, and ATP content.

3. Results

The total duration of sperm motility of fresh samples was 140 ± 19.2 s and the percentage motility 90 ± 4.3%. For post-thaw sperm frozen at-40˚C/min., the duration (120 ± 21.15 s) and percentage (60 ± 4.1%, respectively) of sperm motility were significantly higher than those sperm frozen at any of the four slower rates. There were no significant differences among the four slower freezing rates for either motility characteristic (Fig. 1A, B).

Fresh sperm provided a fertilization rate of 80 ± 3.5% and a hatching rate of 75 ± 6.2%. Significantly higher rates of fertilization (Fig. 1C) and hatching (Fig. 1D) were observed for the sperm samples that were frozen at a rate of –40°C/min compared to those frozen at any of the slower rates. Similar to the data on sperm motility, no significant differences in fertilization or hatching were found between sperm frozen at the four slower rates.

Fresh sperm samples had an average ATP concentration of 6.1 ± 0.45 nmol/10^8 sperm cells. Sperm cryopreserved using the fastest freezing rate (–40°C/min) had significantly higher concentrations of ATP (4.5 ± 0.6 nmol/10^8 sperm) than all other freezing rates which were not significantly different from each other (Fig. 2).

Sperm cryopreserved using the fastest freezing rate (–40°C/min) had significantly lower levels of TBARS and CP than those at all other freezing rates which were not significantly different from each other. However, indices of oxidative stress were significantly higher in sperm from the fastest freezing rate than in fresh sperm, in which levels of TBARS and CP were approximately 0.3 nmol/10^8 cells and 28.3 nmol/10^8 cells, respectively (Fig. 3A, B).

Discussion

Semen cryopreservation is a practical method for banking germplasm from threatened fish species, and is especially useful when combined with conservation breeding or restocking programs. New approaches that can be easily used to cryopreserve samples and that process small volumes are urgently needed and the development of programmable freezing can satisfy this need [6].

Improvements in sperm cryopreservation techniques require an in-depth knowledge of gamete physiology and the biochemical processes occurring during sperm collection, processing, freezing, and thawing. These steps of the cryopreservation process are known to produce reactive oxygen species (ROS) in sperm [22]. During cryopreservation, sperm is exposed to cold shock and atmospheric oxygen, which increase the susceptibility to lipid peroxidation resulting from an elevated production of ROS [23].

In the present study, the best freezing rate was defined as the rate that produced the highest duration of motility, motility percentage, ATP content, fertilization rate, and hatching rate in

post-thaw Persian sturgeon sperm. Based on the results of the current study, motility parameters and fertilization success were significantly higher in samples frozen using the –40°C/min rate than at all other rates investigated. Similar observations were reported by Frankel et al. [24], who tested four different cooling rates (–10°C, –15°C, –20°C, and –40°C/min) for striped bass (*Morone saxatilis*) sperm and reported that the highest motility and fertility were observed at a cooling rate of –40°C/min. Trukshin [25] reported a deleterious effect on fertilization of a –10°C/min freezing rate (0% fertilization) compared to –40°C/min (22% fertilization) in *A. stellatus*. In data published by Liu et al. [26] on Chinese sturgeon (*Acipenser sinesis*) sperm, a freezing rate of –2°C/min to a temperature of –6°C produced the highest post-thaw motility.

The quantity of stored ATP has been implicated as the primary source of immediate energy that supports spermatozoa motility. Indeed, motility is initiated and maintained by the hydrolysis of ATP catalyzed by dynein ATPase, which is coupled to the sliding of adjacent microtubules, thereby leading to the generation of flagellar movement [27, 28].The intracellular concentration of ATP is decreased or lost and it value is usually affected by the osmotic stress caused by the cryopreservation procedure [29]. In the present study, sperm from male *A. persicus* cryopreserved using the–40°C/min rate had significantly greater concentrations of ATP after thawing. This result obtained from the present study is in agreement with observations Aramli et al [8] on Beluga (*Huso huso*) and Frankel et al. [24] on *M. saxatilis.* Moreover, decrease in cell ATP content have been reported in the cryopreserved sperm of several species like gilthead sea bream (*Sparus aurata*), common carp (*Crprinus carpio*) or rainbow trout (*Oncorhynchus mykiss*), causing a reduction in motility duration and sperm velocity after cryopreservation[29, 30, 31].

Investigations of cryopreserved sperm from many mammalian and fish species have demonstrated that the production of ROS is increased during the freeze–thaw process [22, 32]. The mechanisms of ROS-induced spermatozoa damage includes an oxidative attack on membrane lipids leading to initiation of an LPO cascade [33]. Lipid peroxidation not only disrupts sperm motility, but also impairs sperm functions which are dependent upon the integrity of the plasma membrane, including sperm–oocyte fusion and ability to undergo acrosomal exocytosis [24]. Concentrations of carbonyl derivatives of proteins (CP) also indicate the extent of oxidative stress in the sperm and is the most frequently used marker of protein oxidation [34]. The current study has demonstrated dramatic increases in indices of oxidative stress in thawed sperm at all tested freezing rates, as compared to fresh sperm. Similar to the present findings, Li et al. [22] detected an increase in concentration of CP associated with the thawing process after cryopreservation of *C. carpio* sperm, indicating that the ROS generation was primarily related to the freezing/thawing procedure. In addition, excessive production of ROS during cryopreservation has been associated with reduced post-thaw motility, viability, membrane integrity, antioxidant status, fertility, and other sperm functions. Thus, we hypothesize that the loss of sperm motility parameters in Persian sturgeon during cryopreservation was caused by oxidative stress, which significantly disrupted cellular metabolism in spermatozoa, thereby leading to a subsequent decline in motility indices.

5. Conclusion

This study has revealed that Persian sturgeon sperm cryopreserved at a freezing rate of –40°C/min have significantly higher post-thaw motility than those frozen at slower rates (–10°C, –15°C, –20°C, and –30°C/min). When used in fertilization trials, sperm cryopreserved at –40°C/min resulted in rates of fertilization and hatching that were close to those observed with

fresh semen. An examination of faster freezing rates in the present study was not possible as the programmable freezer used was only able to consistently and repeatedly freeze at rates up to –40°C/min. Thus, future studies should examine the effect of controlled freezing rates faster than –40°C/min, and also the effects of novel cryoprotectants, on the post-thaw quality of Persian sturgeon sperm. Furthermore, to increase the efficiency of artificial reproduction using stored or cryopreserved sperm, further studies should aim to optimize the composition of sperm activation medium and to determine optimal sperm/egg ratios.

Competing Interests

None of the authors have any conflicts of interest to declare.

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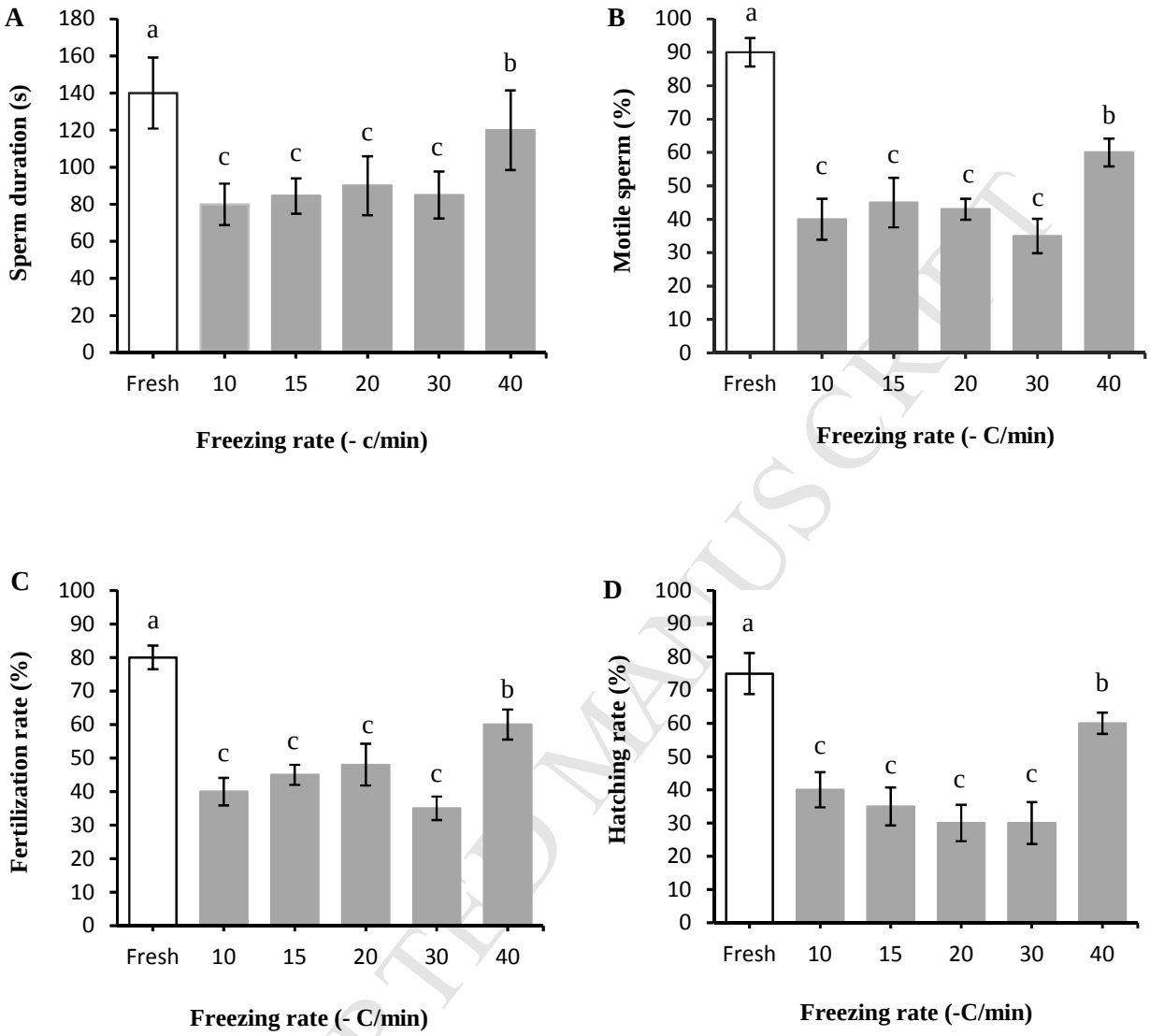


Figure 1. Mean (± SD) total duration of sperm movement (A), percentage of motile cells (B), fertilization rate (C) and hatching rate (D) for fresh and post-thaw male Persian sturgeon sperm (n= 8) when cryopreserved at various freezing rates. The same superscript in each chart indicates no significant difference ($P > 0.05$) and different superscripts indicate significant differences ($P < 0.05$).

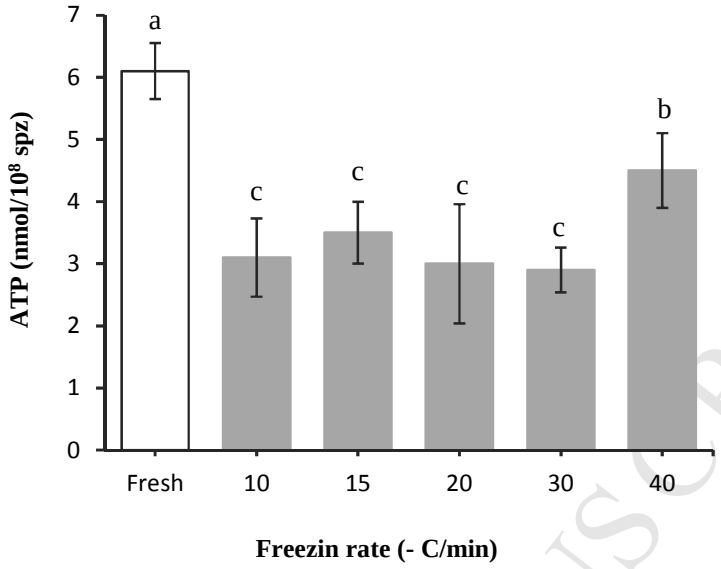
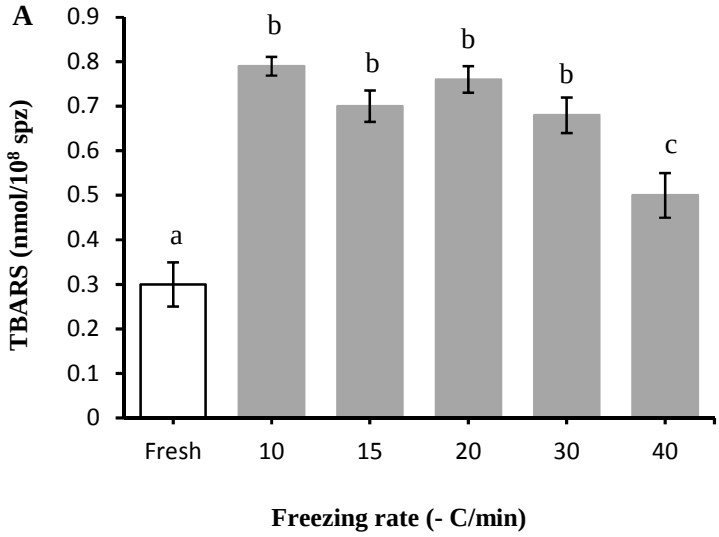


Figure 2. Mean (± SD) adenosine triphosphate (ATP) content for fresh and post-thaw male Persian sturgeon sperm (n= 8) when cryopreserved at various freezing rates. The same superscript in each chart indicates no significant difference ($P > 0.05$) and different superscripts indicate significant differences ($P < 0.05$).



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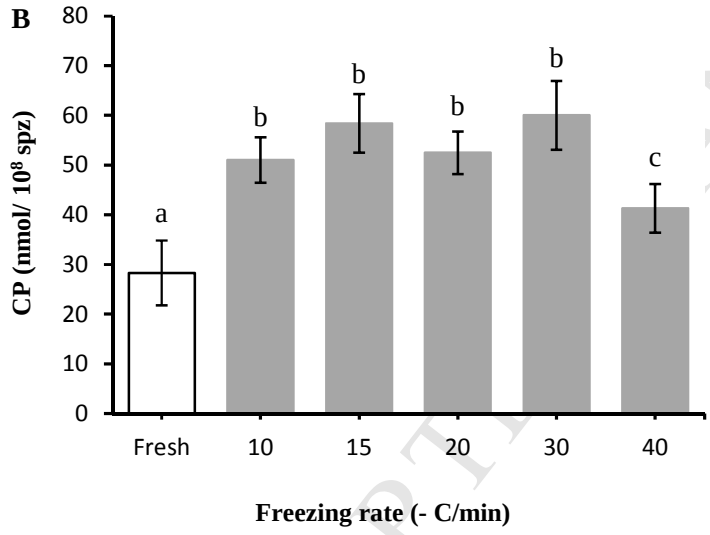

392

393 **Figure 3.** Mean (± SD) thiobarbituric acid reactive substances (TBARS; A), and carbonyl
394 derivatives of proteins (CP; B) for fresh and post-thaw male Persian sturgeon sperm (n= 8) when
395 cryopreserved at various freezing rates. The same superscript in each chart indicates no
396 significant difference ($P > 0.05$) and different superscripts indicate significant differences ($P <$
397 0.05).

398

399

Highlight

1. *This technique is useful for the controlled freezing of sturgeons sperm.*
2. *–40˚C/min is the best freezing rate for cryopreservation of Persian sturgeon sperm.*
3. *Semen quality indices at –40˚C/min were close to those observed with fresh semen.*