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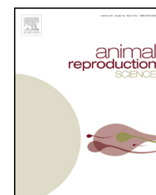

Effects of short-term storage on the motility, oxidative stress, and ATP content of Persian sturgeon (*Acipenser persicus*) sperm

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ABSTRACT

The effective short-term storage of semen is essential when processing multiple sperm samples and when semen must be transported from collection sites to hatcheries for the fertilization of ova, or to laboratories for cryopreservation. In the present study, the spermatozoa of Persian sturgeon (*Acipenser persicus*) were used to evaluate the effects of short-term storage on quality parameters (the percentage of motile cells and the total period of sperm motility), oxidative stress indices, and the ATP content. Spermatozoa cells exhibited >50% motility during 6 days of storage where the average total duration of sperm motility varied from 376.42 ± 80.86 s initially to 19.28 ± 10.96 s after 6 days. No motile spermatozoa were recorded after 9 days of storage. The levels of oxidative stress indices (TBARS and CP) and antioxidant activity (SOD) increased significantly with the storage time. The ATP content also decreased significantly after 2 days of storage. The results of this study may facilitate successful reproduction management and cryopreservation protocols for this endangered fish.

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1. Introduction

Various sperm storage methods are used frequently in aquaculture. Short-term or liquid storage is the most common technique. Non-diluted sperm storage (Park and Chapman, 2005), sperm storage after dilution in a simple ionic medium (Glogowski et al., 2002), or storage after dilution with multi-component media containing numerous additives such as antibiotics (Brown and Mims, 1995) or antioxidants (Stoss, 1983), are different methodological approaches. Storage methods are important tools on fish farms, such as when male broodstocks respond more rapidly to hormonal injections than females. In such cases, it is necessary to store the sperm in a quiescent state until the ova are released by females (Alavi et al., 2012). However, several factors can affect the quality and viability of the stored sperm. Individual male variability and the storage conditions are critical factors that determine the viability of sperm after short-term storage. Spermatozoa are also sensitive to stresses, because they possess limited endogenous antioxidant protection but abundant substrates for free radical attack in the form of unsaturated fatty acids (Koppers et al., 2010). Oxidative stress (OS) is the result of an imbalance between reactive oxygen species (ROS) and antioxidants in the body, which can lead to sperm damage, deformity, and eventually male infertility (Aitken and Baker, 2006). High concentrations of ROS can cause sperm pathology (ATP

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depletion), leading to insufficient axonemal phosphorylation, lipid peroxidation, and loss of motility and viability, but many studies have demonstrated that low and controlled concentrations of ROS play an important role in sperm physiological processes such as capacitation, acrosome reaction, and the signaling processes that ensure fertilization (Bansal and Bilaspuri, 2011). Thus, oxidative damage has important effects on sperm physiology and the study of these effects is of great importance in the field of gamete biology. The ATP content has often been used as a predictive factor of spermatozoa motility. Cell damage can be achieved without membrane rupture and the liberation of cell constituents, thereby compromising the internal structures of sperm and affecting essential metabolic pathways, such as ATP. It is known that ATP is required for the interaction between dinein and tubulin in the axonema, which subsequently generates flagellar movement (Perchec et al., 1995; Dorsey et al., 2011). However, studies in some species have shown that the ATP content is reduced during storage and decreases in the sperm ATP content are due principally to its hydrolysis by dynein ATPases when coupled with movement (Perchec et al., 1995).

Because of their life history characteristics, including slow growth and late age at maturity, sturgeons are particularly sensitive to low levels of exploitation and habitat destruction. At present, these species, including the Persian sturgeon (*Acipenser persicus*), are listed as endangered or critically endangered in the IUCN Red List (Rochard et al., 1990; Bemis and Kynard, 1997; Havelka et al., 2011). Therefore, knowledge of the reproduction of wild populations of sturgeon is essential for ensuring the perpetuation of this ancient group of animals (Haxton, 2006). In our experiment, undiluted sperm storage was conducted to determine the possible sources of sperm quality degradation during in vitro storage. We evaluated the effects of short-term storage on Persian sturgeon spermatozoa quality parameters, i.e., spermatozoa motility, oxidative stress, and the ATP content.

2. Materials and methods

2.1. Broodfish handling and collection of semen

The study was performed at Rajaee Sturgeon Propagation Center (Sari, Mazandaran, Iran; 36°37′ N, 53°05′ E) during April 2011. Broodfish were selected from wild breeders that originated from the Caspian Sea. Seven male Persian sturgeon (average size and weight 110.2 cm and 16.3 kg, respectively) were kept in a 75.4 m^3 tank with a freshwater supply and the water temperature varied between 14 °C and 16 °C. Spermiation was stimulated by a single intramuscular injection of LHRH-A_2 (D-Ala6 GnRH Pro9-NEt) hormone at 5 μg kg^{-1} body weight at 18 h before sperm collection. The 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. The samples were stored on ice (0–4 °C) until the analyses.

2.2. Experimental protocol

Sperm samples from each specimen ($n = 7$ per fish) were divided into 250 mL cell containers and kept in aerobic conditions at 4 °C. From each sample, aliquots were removed at 24 (1 day), 48 (2 days), 72 (3 days), 144 (6 days), and 216 (9 days) h after collection to assess the functional parameters of sperm, including the sperm motility characteristics (total period of sperm motility and percentage of motile cells), ATP content, and oxidative stress indices.

2.3. Spermatozoa motility analyses

To evaluate the sperm motility, 10 μL of semen was pipetted onto a 1% (w/v) BSA-coated microscope slide. Activation was achieved by adding 25 μL of 0.3% NaCl solution. Motility observations were made using a prefocused inverted microscope (400×, Olympus CK2, Tokyo, Japan) at 10 °C. The percentage of 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.

2.4. ATP bioluminescence assay

The ATP contents of spermatozoa were determined using the bioluminescence method described by Boryshpolets et al. (2009). 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 12000 × *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.

2.5. Oxidative stress indices and antioxidant activity

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^{-1}, then homogenized in an ice bath using a 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 12000 × *g* for 30 min at 4 °C to obtain the post-mitochondrial supernatant to assay the activity of other antioxidant enzymes. The TBARS method described by Zhou et al. (2006) and Lushchak et al. (2005) 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^{-1}. The value of TBARS was

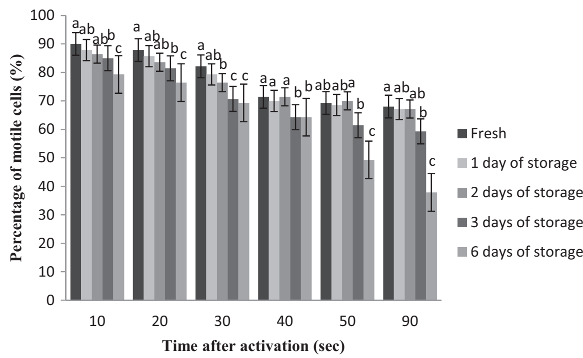

Fig. 1. Effects of short-term storage on the percentage of motile spermatozoa in *A. persicus*. The data represent means ± SD, $n = 7$ per specimen, and the time of storage. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

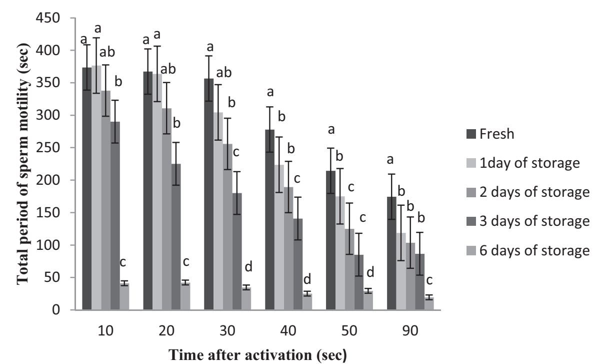

Fig. 2. Effects of short-term storage on the total period of spermatozoa motility in *A. persicus*. The data represent means ± SD, $n = 7$ per specimen, and the time of storage. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

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^{-1} and was expressed as nanomoles per 10^8 cells. The total superoxide dismutase (SOD) activity was determined using the method of Marklund and Marklund (1974), and was measured spectrophotometrically at 420 nm (expressed as milliunits per 10^8 cells).

2.6. Statistical analysis

First, the normality and the homogeneity of variance of all data were checked using the Kolmogorov test and the Bartlett test, respectively. The percentage motile cells and the total duration of spermatozoa movement were determined for each fish ($n = 7$ per specimen) at several time points following activation. The measurements were conducted in triplicate. Statistical comparisons of the percentage motile spermatozoa and spermatozoa movement values were performed using analysis of variance (factorial ANOVA) followed by Duncan's test. The values of TBARS, CP, SOD, and the ATP content were expressed as means ± SD. Differences in these values with various storage periods in the same specimens were analyzed with a one-way ANOVA, followed by Duncan's test. All of the analyses were performed at a significance level of $P < 0.05$ using SPSS statistical software (Version 11.5 for Windows 7).

3. Results

3.1. Spermatozoa motility characteristics

After sperm activation, the majority of samples exhibited 90% spermatozoa motility within 10 s. During the first 3 days of sperm storage, the percentage of motile cells were as follows: (i) at 10–20 s post-activation, 80–90% of spermatozoa were motile (Fig. 1) and there were no significant differences ($P > 0.05$) between fresh and stored samples; (ii) at 30 s post-activation, 70–80% of spermatozoa were motile and significant differences ($P < 0.05$) were observed between fresh and stored samples (2 and 3 days after storage); (iii) at 40–50 s post-activation, 60–70% of the samples were motile and no significant differences ($P > 0.05$) were observed between fresh samples and the samples stored for up to 2 days. A significant decline in the percentage of motile cells ($P < 0.05$) was observed at 90 s after activation and also after 6 days of storage in all samples. The estimated total period of sperm motility had a normal distribution and the ANOVA showed that there were significant differences ($P < 0.05$) between fresh and stored samples: (i) after 3 days of sperm storage at 10–30 s post-activation; and (ii) after 1 day of sperm storage at 40–90 s post-activation. In addition, the period of motility was 7–8 min in Persian sturgeon. After 9 days of storage, no motile sperm were seen in any samples (Fig. 2).

3.2. Oxidative stress indices and antioxidant response

The levels of TBARS and CP increased significantly after 1 day of sperm storage (Fig. 3A and B). In addition, the levels of TBARS and CP varied from 0.21 ± 0.02 to 0.69 ± 0.11 and from 35.71 ± 5.12 to 58.92 ± 4.41 in sperm cells, respectively. The SOD activity, which is an indicator of antioxidant activity in fish spermatozoa, increased significantly with the storage time (Fig. 3C).

3.3. Evaluation of ATP content

To further investigate the potential mechanism that underlies the decrease in the sperm quality, the intracellular ATP content was analyzed in spermatozoa. The content of ATP in fresh spermatozoa was 6.57 nmol ATP/10^8 spermatozoa. A significant decline in ATP content ($P < 0.05$) was observed after 2 days of storage in all samples (Fig. 4).

4. Discussion

This study is the first to analyze the effects of short-term storage on the spermatozoa motility parameters, oxidative stress indices, and ATP content of Persian sturgeon spermatozoa. Our results showed that the spermatozoa of Persian sturgeon were fully capable of being activated immediately after transfer to swimming medium and 80–90% of spermatozoa became motile. During the first 2 days of storage, the percent of motile cells remained stable and there were no significant differences between the

Fig. 3. Effects of short-term storage on oxidative stress indices, i.e., TBARS (A), CP (B), and SOD (C), in *A. persicus* spermatozoa. The data represent means ± SD, $n = 7$ per specimen, and the time of storage. Different letters indicate significant differences among storage periods for the same specimen (ANOVA, $P < 0.05$).

fresh and stored samples. In addition, a significant decline in the spermatozoa motility parameters started after 2 days of storage in all samples. Similar results have been reported in Russian sturgeon (*Acipenser gueldenstaedtii*) and Siberian sturgeon (*Acipenser baerii*) by Shaliutina et al. (2013).

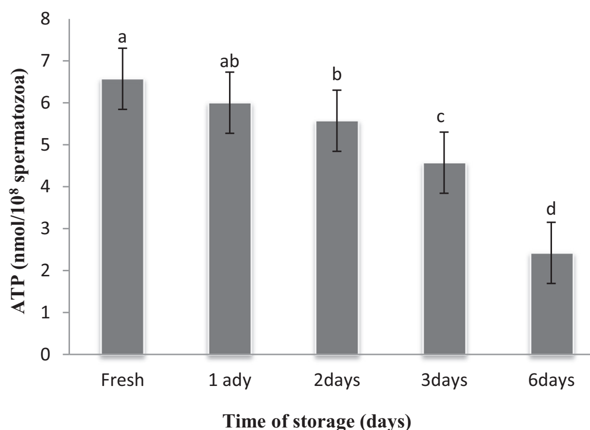

Fig. 4. Effects of short-term storage on the ATP content of *A. persicus* spermatozoa. The data represent means ± SD, $n = 7$ per specimen, and the time of storage. Different letters indicate significant differences among storage periods for the same specimen (ANOVA, $P < 0.05$).

According to Dettlaff et al. (1993), the fertilization period of stored Sterlet (*Acipenser ruthenus*) spermatozoa was 5 days with refrigerated storage. In the present study, no motility was detected after 9 days of storage. The ATP contents and oxidative stress are assumed to be the main causes of the decline in these functional characteristics (Perchec et al., 1995; Aitken and Baker, 2006). Disequilibrium between ROS and the spermatozoa antioxidant system can cause metabolic or functional disorders, thereby reducing spermatozoa motility and increasing LPO and CP (Li et al., 2009). LPO is particularly important for aquatic species because they normally contain larger amounts of highly unsaturated fatty acids (HUFA) than other species. HUFA has been reported to be a major contributor to the loss of cell function under oxidative stress and is usually indicated by TBARS in fish (Storey, 1996; Oakes and Van der Kraak, 2003). In our study, the level of TBARS increased significantly after 1 day of sperm storage. Similarly, Shaliutina et al. (2013) also found that an increased level of TBARS was associated with short-term storage of Russian sturgeon and Siberian sturgeon spermatozoa after 6 days of storage. In the present study, we observed that the CP level increased significantly in Persian sturgeon sperm after 1day of storage. Subsequently, the level of CP significantly increased with the storage time. Our results are in good agreement with data reported by Shaliutina

et al. (2013) in Russian sturgeon and Siberian sturgeon. We hypothesize that the loss of sperm motility parameters in Persian sturgeon during short-term storage was caused by oxidative stress, which significantly interrupted cellular metabolism in spermatozoa, thereby leading to a subsequent decline in motility indices. SOD is the main protective enzyme, which acts as a scavenging agent that removes ROS after they form. Fish sperm possess an antioxidant system, including enzymatic (e.g., glutathione peroxidase) and non-enzymatic (e.g., α-tocopherol) components, which is capable of counteracting the damaging effects of ROS to protect the cell structure (Li et al., 2009). However, few reports are available on the mode of action of antioxidants in fish spermatozoa (Lahnsteiner et al., 2010). In the present study the antioxidant activity expressed as the total SOD activity increased significantly after 2 days storage. Our results were contrary to results from cryopreservation where no increase were reported in the LPO (Lahnsteiner et al., 2011) and SOD (Li et al., 2010) values after freezing/thawing procedure. However, of the effect of each antioxidant is species-specific, improving different sperm quality parameters will depend on the type of antioxidant and concentration used (Catriona et al., 2011).

We observed a decreasing trend in the intracellular ATP concentration throughout the storage period. 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, which is catalyzed by dynein ATPase and is coupled to the sliding of adjacent microtubules, thereby leading to the generation of flagellar movement (Perchec-Poupard et al., 1998; Rurangwa et al., 2002). In mammalian spermatozoa, there is evidence that the intracellular ATP concentration decreases after exposure to oxidative stress (Cummins et al., 1994). In teleosts, the ATP level in sperm is likely to improve cryoresistance (Labbe et al., 1998). In *A. baerii*, studies have detected high intermale variability in the ATP content of sperm during the freezing procedure (Billard et al., 2004). In general, ATP depletion by ROS has been explained based on the inactivation of mitochondrial ATP synthase, or by the activation of nuclear enzyme poly (ADP-ribose) polymerase-1.

In conclusion, the results of the present study provide new insights into Persian sturgeon spermatozoa quality with respect to its short-term storage. Our results indicate that the decline in spermatozoa quality may be related to oxidative stress and the accumulation of LPO and CP in sperm cells, as well as declines in the ATP content. This study suggests that the application of antioxidants during the short-term and long-term storage of fish spermatozoa could prevent cellular injuries caused by oxidative stress. Moreover, with respect to the addition of antibiotics, we cannot exclude the possibility that bacteria had roles in elevating the levels of free radicals in the samples. Furthermore, we cannot ignore the role of pH, which can lead to membrane damage, enzyme deactivation, and ATP depletion. We suggest that future investigations of the effects of short-term storage on sturgeon spermatozoa should focus on the application of antioxidants to prevent the deleterious effects of storage on qualitative sperm parameters during short-term storage or cryopreservation.

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