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SHORT COMMUNICATION

# Motility parameters, adenosine triphosphate content and oxidative stress indices of sterlet, *Acipenser ruthenus* sperm after 6 days of storage

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Short-term storage of fish gametes may be necessary when the availability of male and female gametes for *in vitro* fertilization is asynchronous (Lahnsteiner, Weismann & Patzner 1997) or the sources of gametes are separated spatially. This method may also be necessary during transport to a facility with the capacity to cryopreserve gametes for long-term storage when spermatozoa and oocytes have been removed from moribund or recently dead animals in the field. 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. Sperm 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, Garg & Aitken 2010). Oxidative stress 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 & Baker 2006). High concentrations of ROS can cause sperm pathology [adenosine triphosphate (ATP) depletion], leading to insufficient axonemal phosphorylation, lipid peroxidation (LPO) 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 signalling processes that ensure fertilization (Bansal & 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 effect of short-term storage on sperm quality of sturgeons have been studied, such as *Acipenser persicus* by Aramli, Kalbassi, Nazari and Aramli (2013); *Acipenser gueldenstaedtii* and *Acipenser baerii* by Shaliutina, Hulak, Gazo, Linhartova and Linhart (2013) and *Huso huso* by Aramli (2014). To the best of our knowledge, there are no available data in the literature on the effect of short-term storage on sperm quality in *A. ruthenus*. Hence, in this experiment, we evaluated the effect of short-term storage on the physiology of *A. ruthenus* sperm with respect to motility parameters, oxidative stress indices and ATP content.

Sperm of sterlet was obtained from five males (body mass: 0.5–3 kg; body length: 45 ± 8.3 cm) reared in Rajaee Sturgeon Propagation Center (Sari, Mazandaran, Iran; lat: 36°37′ N, long: 53°05′ E) in April 2012. Fish were kept in a 75.4 $m^3$ tank with a freshwater supply and water temperature varying between 14.5 and 16.5°C. Fish were feed using a commercial diet (no. 1.9; Biomars, Nersac, France; 50% crude protein, 18%

 

crude fat, 10% ash, 1.3% fibre) during the experimental period. Spermiation was stimulated by a single intramuscular injection of LHRH-$A_2$ (D-Ala6 GnRH Pro9-Net; Ningbo Renjian Pharmaceutical Group, Zhejiang, China) hormone at 5–10 μg $kg^{-1}$ (Nazari, Modanloo, Ghomi & Ovissipor 2010) body weight 18 h before sperm collection. Sperm samples ($n$ = 3 per each fish) were divided into 250 mL cell containers and kept under aerobic conditions at 4°C. From each sample, aliquots were removed at 24 (1 days), 48 (2 days), 72 (3 days) and 144 (6 days) h after collection to assess semen quality in terms of motility, oxidative stress indices and ATP content.

Tris-HCl buffer (10 mM, pH 8.0) containing 0.25% pluronic (a substance that prevents spermatozoa from sticking to slides) was used as an activating medium (AM). To trigger motility; sperm were diluted in AM with dilution rate 1:50 (Dzyuba, Cosson, Boryshpolets, Bondarenko, Dzyuba, Prokopchuk, Gazo, Rodina & Linhart 2014). Motility observations were made using a prefocused inverted microscope (400×, Olympus CK2; Olympus, Tokyo, Japan) at 10°C. 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 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.

Adenosine triphosphate content in the sperm was measured using the bioluminescence method described by Boryshpolets, Dzyuba, Rodina, Li, Hulak, Gela and Linhart (2009). Sperm samples were added to a boiling extraction medium consisting of 100 mM Tris-HCl, pH 7.75 and 4 mM ethylenediaminetetraacetic acid (EDTA). After boiling for 2 min at 100°C, samples of the sperm suspension were centrifuged (Heraeus, Sepatech, Berlin, Germany) at 12 000 $g$ for 20 min. ATP content in the supernatants was evaluated by bioluminescence using the Bioluminescence Assay Kit CLS II (Roche Diagnostics GmbH, Berlin, Germany). Luminescence was read using a SpectraFluor Plus plate reader (1-40; Tecan Group, Miyamachi, Japan), and data were expressed as nano-moles per $10^8$ cells.

Semen samples were centrifuged (Heraeus, Sepatech) at 3000 $g$ for 10 min at 4°C. The supernatant was collected carefully and discarded. The pellet was diluted with potassium phosphate buffer

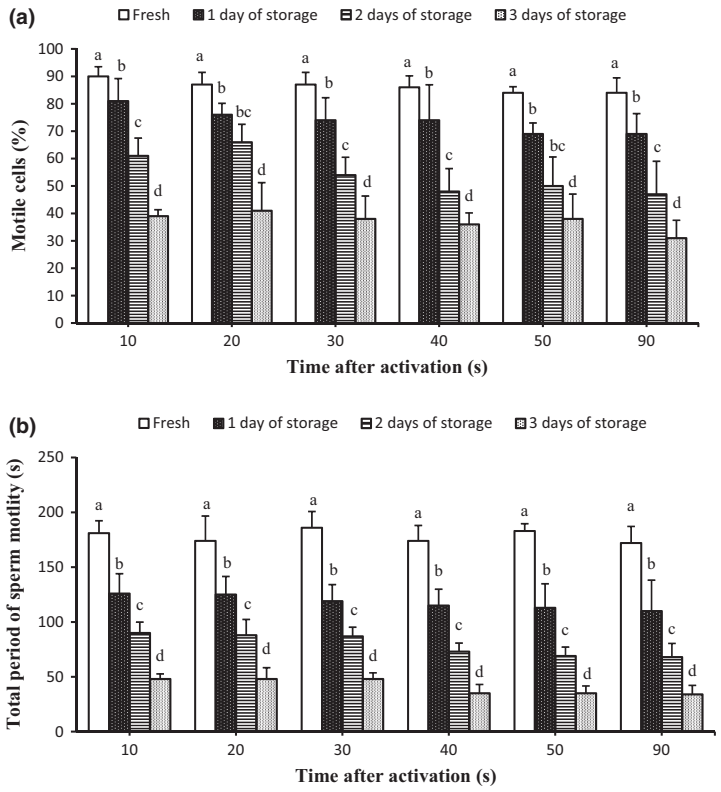


**Figure 1** Effect of short-term storage on the percentage of motile spermatozoa (a) and the total period of spermatozoa motility (b) in *Acipenser ruthenus*. The data represent as means ± SD, $n$ = 4. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

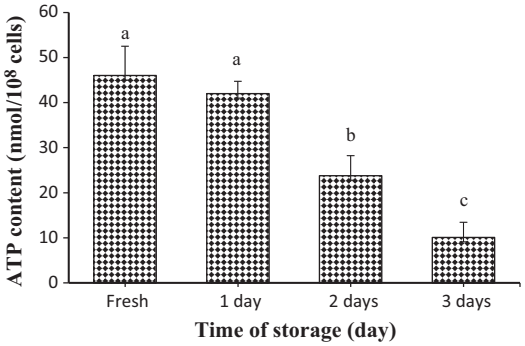


**Figure 2** Effect of short-term storage on the adenosine triphosphate (ATP) content of *Acipenser ruthenus* sperm. The data represent as means ± SD, $n = 4$. Different letters indicate significant differences among storage periods (ANOVA, $P < 0.05$).

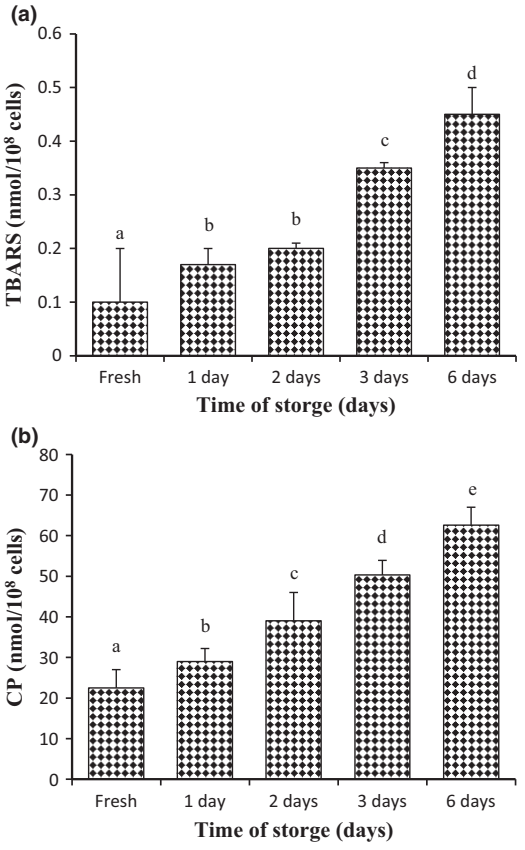


**Figure 3** Effect of short-term storage on oxidative stress indices expressed by thiobarbituric acid reactive substances (TBARS; a) and carbonyl derivatives of proteins (CP; b) in *Acipenser ruthenus* sperm. The data represent as means ± SD, $n = 4$. Different letters indicate significant differences among storage periods (ANOVA, $P < 0.05$).

(50 mM KPi-buffer, pH 7.0, 0.5 mM EDTA) to obtain a sperm density of $5 \times 10^8$ cells $mL^{-1}$, then homogenized in an ice bath using a Omni Ruptor 4000 Ultrasonic Homogenizer (Omni International, Kennesaw, GA, 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 to assay the activity of other antioxidant enzymes. The TBARS method described by Zhou, Liu, Siu, Desmond, Lam and Wu (2006) and Lushchak, Bagnyukova, Lushchak, Storey and Storey (2005) was used to evaluate sperm 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 recorded as nano-moles 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 nano-moles per $10^8$ cells.

All analyses were conducted using SPSS statistical software (version 11.5 for Windows 7; Chicago, IL, USA), and the significance level was set at 0.05. Normality and the homogeneity of variance of all data were first assessed using the Kolmogorov test and the Bartlett test respectively. Sperm motility, oxidative stress indices and ATP content were expressed as means ± SD. Differences in these values among times of storage were analysed by one-way ANOVA followed by Duncan's test.

After sperm activation, the majority of samples exhibited 90% spermatozoa motility within 10 s. During day 1 of sperm storage, 75–80% of spermatozoa were motile at 10–90 s post-activation (Fig. 1a), and there were significant differences ($P > 0.05$) between fresh and stored samples. After 2 days of sperm storage, 55–65% of spermatozoa were motile at 10–90 s post-activation and after 3 days of sperm storage 30–40% of spermatozoa were motile at 10–90 post activation. Significant differences ($P > 0.05$) were observed between fresh samples and the samples stored for up to 1 day. The estimated total period of sperm motility had a normal distribution, and ANOVA showed that there were significant differences in motility ($P < 0.05$) between fresh and stored samples after 1 day of sperm storage at 10–90 s post activation. In addition, the period of sperm motility was 2–3 min for this species. After 6 days of storage, no motile sperm were seen in any samples (Fig. 1b).

To investigate the potential mechanism underlying the decrease in sperm quality, the intracellular ATP content in spermatozoa was analysed. The intracellular ATP content in fresh sperm was detected at 46 nmol per $10^8$ cells (Fig. 2). After 2 days of storage, the ATP content significantly ($P < 0.05$) declined.

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 $0.9 \pm 0.01$ to $0.48 \pm 0.2$ nmol per $10^8$ cells and from $21.5 \pm 3.5$ to $65.92 \pm 5.02$ nmol per $10^8$ cells respectively.

As first step towards understanding how short-term storage might alter sperm physiology of sterlet sperm, spermatozoa motility parameters, oxidative stress and ATP content were evaluated. Our results showed that beluga spermatozoa were fully capable of being activated immediately after being transferred to swimming medium and that ~90% of sperm became motile. However, a significant decline in the sperm motility parameters began after 1 day of storage in all samples. Similar studies have been handled in Russian sturgeon and Siberian sturgeon by Shaliutina *et al.* (2013) and also Persian sturgeon and beluga sturgeon by Aramli *et al.* (2013) and Aramli (2014) respectively. According to results obtained by researchers, the period of stored sperm in these species was 6 days and no motile spermatozoa were recorded after 9 days of storage (see Table 1). The ATP contents and oxidative stress are assumed to be the main causes of the decline in these functional characteristics (Perchec-Poupard, Jeulin, Cosson, André & Billard 1995; Aitken & Baker 2006). We observed a decreasing trend in the intracellular ATP concentration throughout the storage period. The intracellular ATP content in fresh sperm was detected at 46 nmol per $10^8$ cells (Fig. 2). After 2 days of storage, the ATP content significantly ($P < 0.05$) declined. This result is in good agreement with our previous data published in the Persian sturgeon and beluga sturgeon (Aramli *et al.* 2013; Aramli 2014). 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 catalysed by dynein ATPase and is coupled to the sliding of adjacent microtubules, thereby leading to the generation of flagellar movement (Perchec-Poupard, Paxion, Cosson, Jeulin, Fierville & Billard 1998; Rurangwa, Biegniewska, Slominska, Skorkowski & Ollevier 2002). In teleosts, the ATP level in sperm is likely to improve cryoresistance (Labbe, Maisse & Billard 1998). In *A. baerii*, studies have detected high intermale variability in the ATP content of sperm during the freezing procedure (Billard, Cosson, Noveiri & Pourkazemi 2004).

Disequilibrium between ROS and the spermatozoa antioxidant system can cause metabolic or

**Table 1** Motility parameters, ATP contact, oxidative stress and antioxidant activity during short-term storage of sperm in some sturgeon species according to the literature

| | Parameters | | | | | | |
|---|---|---|---|---|---|---|---|
| Species | Motility duration (s) or velocity (μm $s^{-1}$) | Motility (%) | ATP (nmol per $10^8$cells) | TBARS (nmol per $10^8$cells) | CP (nmol per $10^8$cells) | SOD (mU per $10^8$ cells) | References |
| *Acipenser gueldenstaedtii* | 190–90 (μm $s^{-1}$)* | >50* | – | 0.33–0.75† | 40–74† | 2.2–7.5† | Shaliutina *et al.* (2013) |
| *Acipenser baerii* | 185–87 (μm $s^{-1}$)* | >50* | – | 0.31–0.75† | 20–76† | 3–8† | Shaliutina *et al.* (2013) |
| *Acipenser persicus* | 376–19 (s)* | >50* | 6.57–2.42‡ | 0.21–0.69† | 35–69† | 4.04–7.41† | Aramli *et al.* (2013) |
| *Huso Huso* | 287–13 (s)§ | >50§ | 8.83–3‡ | 0.53–1.16† | 18–66† | 5.8–9.3† | Aramli (2014) |
| *Acipenser ruthenus* | 180–30 (s)‡ | >50¶ | 45–10** | 0.9–0.48‡ | 21–65‡ | – | Present study |

*Motility during 6 days of storage.
†Ranged from fresh semen to 9 days after storage.
‡Ranged from fresh semen to 6 days after storage.
§Motility during 3 days of storage.
¶Motility during 2 days of storage.
**Ranged from fresh semen to 3 days after storage.
ATP, adenosine triphosphate; TBARS, thiobarbituric acid reactive substances; CP, carbonyl derivatives of proteins; SOD, Total superoxide dismutase.

  

functional disorders, thereby reducing spermatozoa motility and increasing LPO and CP (Li, Hulak & Linhart 2009). LPO is particularly important for aquatic species because they normally contain larger amounts of highly unsaturated fatty acids than other species. Highly unsaturated fatty acids have been reported to be a major contributor to the loss of cell function under oxidative stress and are usually indicated by TBARS in fish (Storey 1996; Oakes & Van der Kraak 2003). In this study, the levels of TBARS and CP increased significantly after 1 day of sperm storage. Shaliutina *et al.* (2013) also found an increasing level of TBARS and CP associated with short-term storage of sperm in Russian sturgeon and Siberian sturgeon, and Aramli *et al.* (2013) reported similar findings for Persian sturgeon. We hypothesize that the loss of sperm motility during short-term storage of beluga spermatozoa was due to oxidative stress that significantly interrupted cellular metabolism, leading to the subsequent decline of motility indices.

In conclusion, the results of this study provide new insights into sterlet sperm 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.

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**Keywords:** sperm motility, sperm storage, oxidative stress, ATP, sterlet

  