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Oxidative stress and motility in tench *Tinca tinca* spermatozoa

V. Dzyuba[1,2], J. Cosson[1], B. Dzyuba[1], M. Rodina[1]

[1]South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Vodňany, Czech Republic

[2]Scientific-Research Institute of Biology, V.N. Karazin Kharkiv National University, Kharkiv, Ukraine

ABSTRACT: The attachment of the urinary bladder to the seminal duct near the anal aperture in tench constitutes a potential risk for urine contamination of sperm during collection, leading to spontaneous activation of sperm motility by urine hypotonicity. It was hypothesized that sperm hypotonic exposure can provoke oxidative stress which could be involved in sperm quality degradation. Our study aimed to describe spermatozoa motility parameters and levels of oxidative stress in activating media (AM) of differing osmolality. Tench sperm samples were collected from 6 males into Kurokura 180 immobilizing medium (IM) (180mM NaCl, 2.68mM KCl, 1.36mM $CaCl_2$ $2H_2O$, 2.38mM $NaHCO_3$, 340 mOsm/kg). Motility was recorded in AM of 0 mOsm/kg or 100 mOsm/kg using video microscopy combined with stroboscopic illumination. Video records were analyzed to calculate spermatozoa curvilinear velocity (VCL), motility rate, and motility duration. The level of thiobarbituric acid reactive substances (TBARS), measured by spectrophotometry, was used as an oxidative stress index. VCL and motility rate during the initial phase of motility (10 s post-activation) were not dependent on AM osmolality, while motility duration was significantly increased with 100 mOsm/kg AM. TBARS was significantly increased with reduction of AM osmolality. Increased TBARS was observed even at 5 s post-activation with AM of 0 mOsm/kg. These observations suggest that even a short period of sperm exposure to hypotonic conditions induces oxidative stress. Any contact of sperm with hypotonic urine during sperm collection should be avoided. The use of motility AM of moderate hypotonicity (≥ 100 mOsm/kg) is recommended for tench propagation.

Keywords: tench sperm; activating medium osmolality; motility reactivation; lipid peroxidation intensity

INTRODUCTION

The common tench, *Tinca tinca* (L.), is a freshwater cyprinid that has become important for pond aquaculture in many countries of Europe and Asia (Gela et al. 2006; Wang et al. 2006; Celada et al. 2009). Tench is utilized for food, sport fishing, and as an ornamental fish. Growing importance of tench in pond aquaculture has resulted in advances in reproduction techniques such as development of immobilizing medium (IM) for collection and preservation of spermatozoa, sperm cryopreservation, hormone induction of ovulation, and hybridization (Rodina et al. 2004, 2007; Mamcarz et al. 2006; Podhorec 2011; Targonska et al. 2012).

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Because of connection of the tench urinary bladder to the seminal duct near the anal aperture in the caudal abdominal cavity, there is a risk of sperm contamination by urine during collection (Linhart et al. 2003). The lower osmolality of urine compared to seminal fluid (Linhart et al. 2003) may lead to spontaneous activation, but this can be prevented by sperm collection into IM. For tench, the best IM has been reported to be modified Kurokura solution: Kurokura180 (Rodina et al. 2004). This IM was developed taking into account the fact that hypotonicity is the main activating factor for spermatozoa motility in cyprinids (Krasznai et al. 2000). Spermatozoa in the sperm ducts are immersed in the isotonic and high K^+ environment of the seminal plasma, in which they remain immotile. Spawning into hypotonic fresh water with low K^+ induces spermatozoa motility through a Ca^{2+}-dependent and cAMP-independent cell signalling cascade.

Physical properties of the spermatozoa cell membrane control the majority of signal transduction processes. The activation of spermatozoa may involve specific stretch-activated channels, sensitive to osmotic pressure. As demonstrated by Krasznai et al. (2003), hypoosmolality results in an increase in membrane fluidity. A stretch-activated channel blocker, gadolinium, was shown to block the initiation of carp sperm motility and significantly decrease the fluidity of the spermatozoa membrane.

Fluidity of the plasma membrane is mainly determined by the presence of unsaturated fatty acids (Lenzi et al. 1996), double bonds of which are preferred targets for the attack of reactive oxygen species (ROS). High levels of unsaturated fatty acids in spermatozoa plasma membrane have been found in different animal species (Drokin 1993; Lenzi et al. 1996; Surai et al. 2001; Petcoff et al. 2008), making spermatozoa particularly vulnerable to the attack by ROS. In addition, it is known that lipid peroxidation resulting from the ROS effect leads to the lowering of membrane fluidity (Dobretsov et al. 1977).

The primary goal of the present study was determination of tench spermatozoa motility parameters and oxidative stress in AM of differing osmolalities. It was attempted to determine whether tench spermatozoa motility parameters are dependent on activating media osmolality and whether level of lipid peroxidation products changes during their motile phase.

MATERIAL AND METHODS

Sperm collection. Sperm samples were collected from six 4–6-year old tench during the natural reproductive season, mid-June 2012, at 24 h after injection with carp pituitary extract at 2 mg/kg. Sperm (0.2–0.5 ml) was collected from the genital papilla of each male into a 5 ml syringe containing 2 ml immobilizing media (IM) Kurokura 180 (K180: 180mM NaCl, 2.68mM KCl, 1.36mM $CaCl_2$, 2.38mM $NaHCO_3$, 340 mOsm/kg, pH 8.2) (Rodina et al. 2004).

Experimental design. Sperm in IM K180 was added to activating media (AM) of 100mOsm/kg (K180 diluted 1 : 2 with distilled water) or 0 mOsm/kg (distilled water), and motility was recorded. In addition, spermatozoa suspensions were diluted by the same AM and at the same dilution rate (1 : 30) and centrifuged at 10 000 *g* at 4°C for 10 min. The resulting supernatants were used for the evaluation of lipid peroxidation intensity by determination of thiobarbituric acid-reactive substance (TBARS) concentration.

The potential for tench spermatozoa motility to be reactivated was investigated using distilled water as AM. At 5 s post-activation, motility was arrested by the addition of KCl in an amount sufficient to create 300 mOsm/kg conditions. After 2 min of arrest, motility was re-activated by a second dilution (1 : 3) in distilled water. The content of TBARS after motility arrest was measured in the supernatants resulting from centrifugation as above.

Spermatozoa motility. Spermatozoa motibity was recorded in activating media of 100 mOsm/kg or 0 mOsm/kg using video microscopy (dark-field microscope (Olympus BX50F, lens UPlanF1 x20, dark field condenser, Olympus Optical Co. Ltd., Tokyo, Japan; CCD video camera, SSCDC50AP, Sony, Tokyo, Japan) combined with stroboscopic illumination (ExposureScope®, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Czech Republic) AM were containing 0.25% pluronic acid to prevent spermatozoa from adhering to microscope slides. Spermatozoa motility was recorded from the bottom part of drop, no cover slip was used. Video records were analyzed to estimate spermatozoa curvilinear velocity (VCL) at 10 and 40 s post-activation (μm/s), motility rate (% motile cells), and motility duration (s) using a micro-image analyzer (Olympus Micro Image software, Version 4.0.1., 1998, for MS Windows).

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Thiobarbituric acid-reactive substance content. As an index of oxidative stress resulting from lipid peroxidation, the level of TBARS was measured spectrophotometrically according to Asakawa and Matsushita (1980). Briefly, to 0.2–0.5 ml supernatant, 0.025 ml butylated hydroxytoluene solution (22 mg in 10 ml ethanol), 0.025 ml ferric chloride solution (27 mg of $FeCl_3.6H_2O$ in 10 ml water), 0.375 ml 0.2M glycine-hydrochloric acid buffer, pH 3.6, and 0.375 ml TBA reagent (0.5% TBA and 0.3% sodium dodecyl sulphate) were added. The tubes with mixture were capped and heated for 15 min in a boiling water bath. After cooling, 0.25 ml glacial acetic acid and 0.5 ml chloroform were added. The mixture was vigorously shaken and centrifuged at 1500 *g* for 10 min. The absorbance of samples was determined at 535 nm against a deionized water blank. A molar extinction coefficient of 1.56×10^5 $M^{-1} \cdot cm^{-1}$ was used for the calculation of TBARS content. The concentration of TBARS was expressed as nmol/ml of supernatant.

Data presentation and statistical analysis. Data distribution and homogeneity of dispersion were tested by Kolmogorov-Smirnov and Levene's test, respectively. Normally distributed data on VCL were analyzed by one-way ANOVA followed by Fisher's LSD test. Due to a low number of observations ($n = 6$), a nonparametric Kruskal-Wallis ANOVA followed by multiple comparisons of mean ranks for all groups were used for comparison of motility rate, motility duration, and concentration of TBARS. Data were presented as mean ± SD. Statistical significance was accepted at $P < 0.05$. All analyses were conducted using STATISTICA software (Version 9.1, 2013).

RESULTS AND DISCUSSION

Motility rate during the initial phase of tench spermatozoa motility (10 s post-activation) was not dependent on the AM osmolality (Figure 1). There was no significant change in this parameter at 40 s post-activation. Tench spermatozoa were able to be reactivated after motility arrest. At 10 s post-reactivation, the motility rate was not different from values at initial activation in either AM, but at 40 s post-reactivation, motility rate was significantly lower than that observed with 100 mOsm/kg AM.

Reduction in AM osmolality had no effect on the initial curvilinear velocity of tench spermatozoa (Figure 2). At 40 s post-activation, VCL was significantly decreased compared to its value at 10 s in both AM solutions, being lower at activation in distilled water (0 mOsm/kg). Reactivated spermatozoa were characterized by significantly lower initial VCL compared with spermatozoa activated in AM of either osmolality. As in the

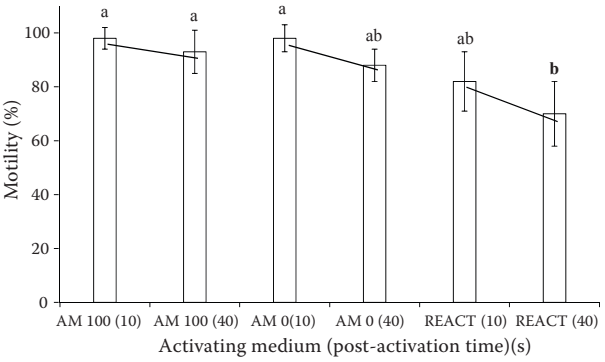

Figure 1. Motility rate (%) of tench spermatozoa at activation and reactivation

activating media (AM) 100 (10), AM 0 (10), REACT (10) = 10 s post-activation in 100 mOsm/kg AM, 0 mOsm/kg AM, and reactivation in 0 mOsm/kg AM, respectively; AM 100 (40), AM 0 (40), REACT (40) = 40 s post-activation in 100 mOsm/kg AM, 0 mOsm/kg AM, and reactivation in 0 mOsm/kg AM, respectively

[a,b]values with different letters are significantly different ($P < 0.05$, $n = 6$)

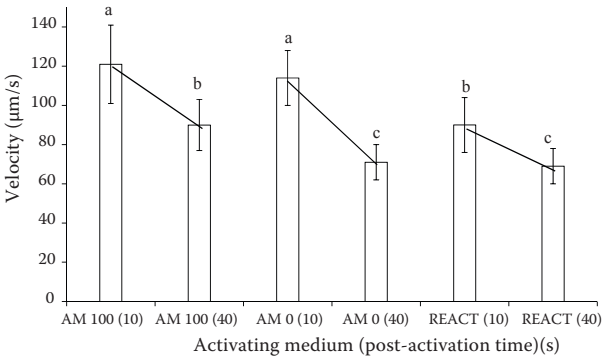

Figure 2. Curvilinear velocity (µm/s) of tench spermatozoa at activation and reactivation

activating media (AM) 100 (10), AM 0 (10), REACT (10) = 10 s post-activation in 100 mOsm/kg AM, 0 mOsm/kg AM, and reactivation in 0 mOsm/kg AM, respectively; AM 100 (40), AM 0 (40), REACT (40) = 40 s post-activation in 100 mOsm/kg AM, 0 mOsm/kg AM, and reactivation in 0 mOsm/kg AM, respectively

[a–c]values with different letters are significantly different ($P < 0.05$, $n = 6$)

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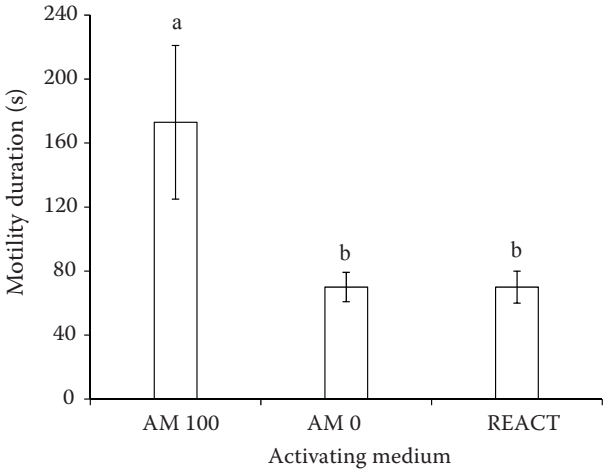

Figure 3. Motility duration (s) of tench spermatozoa at activation and reactivation

activating media (AM) 100, AM 0, REACT = spermatozoa activation in 100 mOsm/kg AM, 0 mOsm/kg AM, and reactivation in 0 mOsm/kg AM, respectively

[a,b]values with different letters are significantly different ($P < 0.05$, $n = 6$)

initial activation, VCL at 40 s post-reactivation was significantly lower than at 10 s.

Duration of tench spermatozoa motility in 100 mOsm/kg AM was more than two-fold that in AM of 0 mOsm/kg (Figure 3). There was no significant difference in this parameter in spermatozoa activated in 0 mOsm/kg AM from that reactivated in the same AM. Decrease in spermatozoa motility duration with the lowering of AM osmolality can be explained by the possibility that adenosine triphosphate (ATP) stock is needed not only to activate motility, but also to maintain numerous metabolic processes of sperm cells which could be directly involved in cell volume regulation. At reactivation (during the second motility period) spermatozoa swim slower (due to the partial use of ATP stock during the first period of motility), but for the same time period. It appears that sperm cells have developed regulatory systems which allow the duration of motility to be dependent not only on energy source.

Collection of sperm into Kurokura 180 IM preserves properties of tench spermatozoa that are typical of other cyprinids. Motility parameters were shown to depend on AM osmolality. Such dependence has been shown in the studies of other species (Morisawa et al. 1983; Perchec Poupard et al. 1997; Alavi et al. 2009). Common carp spermatozoa velocity significantly increased in AM from 60 to 140 mOsm/kg AM osmolality, with a progressive decrease when osmolality was increased to 300 mOsm/kg (Perchec Poupard et al. 1997). Motility duration of goldfish *Carassius auratus* spermatozoa at 100 mOsm/kg was significantly higher than at 0 mOsm/kg (Morisawa et al. 1983). Positive correlation of sperm motility and velocity with osmolality of AM was also reported for common barbell *Barbus barbus* (Alavi et al. 2009). In addition, as in other cyprinids, tench spermatozoa can be repeatedly activated. Common carp spermatozoa are able to be activated a second time following the transfer into a medium of high osmolality, interrupting motility, after the first phase of motility activation (Perchec et al. 1995; Linhart et al. 2008; Boryshpolets et al. 2009). The high variation in tench spermatozoa motility rate at reactivation observed in the present study was similar to that in carp spermatozoa shown by Boryshpolets et al. (2009).

Spermatozoa activation is an extremely complex and precisely orchestrated process. In general, upon reception of external activating stimuli, specific signalling pathways are switched on allowing the transduction of received signals and, ultimately, triggering motion through the activation of axonemal dyneins (Dzyuba and Cosson 2014). Initial signal transduction processes depend upon the physico-chemical properties of the cytoplasmic membrane. Similar physical changes of spermatozoa plasma membrane (e.g. changes in fluidity and membrane potential) may be also involved in physiological processes such as epididymal maturation, hyperactivation, and capacitation (de Lamirande et al. 1997; Nolan and Hammerstedt 1997).

As it appears that the loss of spermatozoa membrane fluidity would be the consequence of intensification of the processes of lipid peroxidation, the content of free radical lipid peroxidation products in spermatozoa supernatants before motility activation, at the motility activation step in media of differing osmolalities, and at motility arrest was quantitatively estimated. The concentration of TBARS during spermatozoa motility activation in 100 mOsm/kg AM was similar to that in non-activated spermatozoa (Figure 4). With reduction of AM osmolality, the TBARS content significantly increased. The level of lipid peroxidation products observed at 5 s post-activation and that following motility arrest did not differ. This may indicate that the process of lipid peroxidation was imme-

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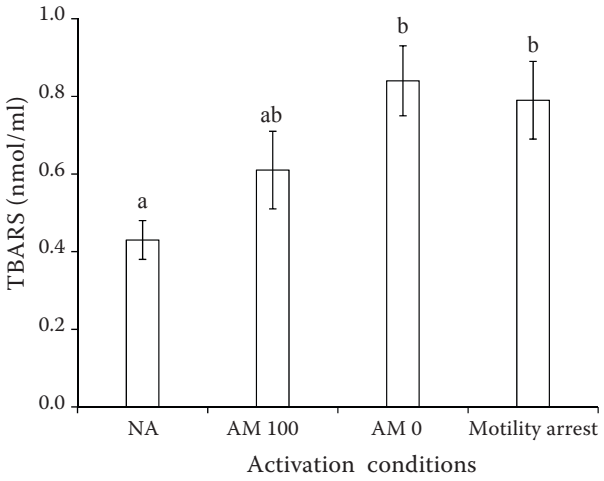

Figure 4. Concentration of thiobarbituric acid-reactive substances (nmol/ml) at activation and motility arrest of tench spermatozoa

NA = before activation, AM 100 and AM 0 = spermatozoa activation in 100 mOsm/kg activating media (AM) and 0 mOsm/kg AM, motility arrest = motility stopped by the addition of KCl up to osmolality of 300 mOsm/kg at 5 s post-activation

[a,b]values with different letters are significantly different ($P < 0.05$, $n = 6$)

diately enhanced upon activation in AM of low osmolality. The high concentration of TBARS seen at activation in medium with low osmolality and immediately following motility arrest may be the source of decreased motility duration at activation and reactivation of tench spermatozoa.

Data on decreased spermatozoa motility duration with activation and reactivation in distilled water, decreased VCL at reactivation, and increased TBARS content at 5 s post-activation in distilled water and after motility arrest are evidence that even a short exposure of tench sperm to hypotonic conditions can provoke oxidative stress.

CONCLUSION

The results indicate that correctly collected tench spermatozoa, implying the use of immobilizing media, possess the properties typical of cyprinid sperm, including dependence of motility parameters on AM osmolality and potential for reactivation after the initial motile phase.

During tench artificial propagation, the application of AM of moderate hypotonicity (≥ 100 mOsm/kg) is preferable. During sperm collection even a minimal contact of spermatozoa with hypotonic urine should be avoided in order to prevent oxidative stress. It can be assumed that the addition of antioxidants to immobilizing media may be useful for tench sperm preservation. The latter assumption and the endogenous antioxidant system in tench sperm are topics for further research.

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Corresponding Author

Viktoriya Dzyuba, Ph.D., University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25 Vodňany, Czech Republic
Phone: +420 387 774 614, e-mail: vdzyuba@frov.jcu.cz