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The *in vitro* effect of temperature on motility and antioxidant response of common carp *Cyprinus carpio* spermatozoa

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ABSTRACT

The effect of temperature on *Cyprinus carpio* spermatozoa *in vitro* was investigated with spermatozoa activated at 4, 14, and 24 °C. At 30 s post-activation, motility rate was significantly higher at 4 °C compared to 14 and 24 °C, whereas highest swimming velocity was observed at 14 °C. The thiobarbituric acid-reactive substance (TBARS) content was significantly higher at 14 °C and 24 °C than at 4 °C in motile spermatozoa. No significant differences in catalase and superoxide dismutase activity relative to temperature were observed. This study provides new information regarding effect of temperature on lipid peroxidation intensity and spermatozoon motility parameters in carp. The elevation of TBARS seen at higher temperatures could be due to inadequate capacity of antioxidant enzymes to protect the cell against the detrimental effects of oxidative stress induced by higher temperatures.

1. Introduction

Spermatozoon motility is an important factor in fertilization capacity (Aas et al., 1991). Most fish exhibit external fertilization, so spermatozoon motility will be strongly influenced by environmental temperatures, and it has been demonstrated that temperature affects spermatozoon motility rate and swimming velocity (Lahnsteiner and Mansour, 2012; Beirão et al., 2014; Dadras et al., 2016). A trade-off between increased spermatozoon velocity and decreased motility duration at higher temperatures has been reported for common carp *Cyprinus carpio* (Perchec et al., 1995), grass carp *Ctenopharyngodon idella* (Jezierska and Witeska, 1999), hake *Merluccius merluccius* (Cosson et al., 2010), and burbot *Lota lota* (Lahnsteiner and Mansour, 2012).

The sperm activation process is accompanied by a series of cellular changes including a rise in respiration rate and rapid depletion of ATP (Lahnsteiner et al., 1999; Dzuba et al., 2001; Boryshpolets et al., 2009). This might be associated with increase in reactive oxygen species (ROS) level, since ROS are usually generated endogenously through cell respiration or through interaction with exogenous factors (Li et al., 2009).

Some changes in sperm viability might be related to ambient temperature (Nandre et al., 2013), and temperature-induced stress has been associated with enhanced generation of ROS and oxidative stress in spermatogenic cells (Nichi et al., 2006a). During times of environmental stress such as increased temperature, ROS levels can increase dramatically (Nichi et al., 2006a) which potentially leading to oxidative stress. Lipid peroxidation is an adverse effect of oxidative damage in fish spermatozoa associated with high poly-unsaturated fatty acid content resulting in susceptibility to ROS attack (Li et al., 2010; Cabrita et al., 2014; Gazo et al., 2015). In sperm, the measuring of TBARS content is currently used to evaluate lipid peroxidation level via quantification of malondialdehyde, which reacts with the thiobarbituric acid used as reagent (Mansour et al., 2006).

Antioxidant enzymes present in spermatozoa counteract oxidative damage. In fish sperm, the primary enzymes for detoxification of ROS are catalase (CAT) and superoxide dismutase (SOD) (Mansour et al., 2006; Lahnsteiner and Mansour, 2010; Cabrita et al., 2014); hence their activity will reveal oxidative status. Investigation of oxidative stress indicators during motility at different temperatures can provide valuable information about the effects of temperature on spermatozoon metabolism. The aim of this study was to evaluate the *in vitro* effect of temperature on carp sperm by analyzing lipid peroxidation (TBARS), the activity of CAT, SOD and motility characteristics.

2. Materials and methods

2.1. Semen collection

Cyprinus carpio reared at the fish farm of South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Vodnany, Czech Republic were used for the study. Six mature males of 3–4 kg body weight were injected with 0.2 mL of a

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suspension containing physiological solution and lyophilized carp pituitary powder at 5 mg kg^{-1} body weight. After 24 h, sperm was stripped by abdominal massage into dry 20 mL vials and stored on ice up to 2 h before experimentation.

2.2. Physiological saline solutions used for inhibition and activation of motility

The spermatozoon motility-inhibiting saline solution consisted of 103 mmol L^{-1} NaCl, 40 mmol L^{-1} KCl, 1 mmol L^{-1} $CaCl_2$, 0.8 mmol L^{-1} MgSO4, and 20 mmol L^{-1} HEPES (pH 7.8) (Lahnsteiner and Mansour, 2012). Activation medium (AM) containing 10 mM Tris–HCl buffer, pH 8.0, 1 mM $CaCl_2$, and 0.125% pluronic acid was used to trigger motility.

2.3. Motility assessment

Fresh sperm from each fish was added to a drop of AM (~50 μl) adjusted to 4, 14, or 24 °C on a microscope slide using the tip of a dissecting needle (~5 μl) with which the sperm suspension was thoroughly mixed for 2 s. Immediately after dilution, spermatozoon motility parameters at each temperature were recorded at 0, 10, 20, 30, 60, 120, 180, and 240 s post-activation using a CCD video camera (Sony, SSCDC50AP, Japan) mounted on an inverted microscope equipped with a cooling stage (Olympus IX83, United Kingdom) and a X20 NIC contrast objective lens. Motility records were analyzed to estimate spermatozoon curvilinear velocity (VCL, μm s^{-1}), percentage of motile spermatozoa after activation (motility rate), and motility duration in triplicate for each sperm sample. Temperature of the AM was adjusted by a thermoblock (HLC BO50/15, Germany) and monitored during the motility period by copper-constantan thermocouple (Omega, L-044T, Taiwan) via a data logger thermometer (Omega, HH127, Taiwan). Temperature fluctuation during motility analysis is shown in Fig. 1, with three replicate tests for each temperature.

2.4. Quantification of TBARS content

In this experiment, two media were used to prepare samples for quantification of TBARS level. Samples (150 μl) of fresh sperm were incubated in 1350 μl of AM to suppress motility at 4, 14, and 24 °C. A solution containing 0.9% NaCl was used as non-activation medium (NAM) at 4, 14, and 24 °C. Subsequently, samples were centrifuged at 1000 × *g* for 5 min at 4 °C. The TBARS content of the supernatant was measured spectrophotometrically according to Asakawa and Matsushita (1980): Briefly, 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 of 0.2 M glycine-hydrochloric acid buffer, pH 3.6, and 0.375 mL TBA reagent (0.5% TBA and 0.3% sodium dodecyl sulfate) were added to 0.08–0.25 mL supernatant. The tubes 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 for 10 min at 1500 × *g*. The absorbance of samples was determined at 535 nm against a blank with deionized water substituted for the biological sample. A molar extinction coefficient of 1.56 10^5 M^{-1} cm^{-1} was used for calculation of TBARS content. The concentration of TBARS was expressed as nmol mL^{-1} supernatant. Measurements were taken in triplicate for each sample, and the average of the three measurements was used for the results.

2.5. Extraction and evaluation of antioxidant enzymes

Before extraction of enzymes, 200 μl of fresh sperm was diluted in 1 mL of motility-inhibiting solution and centrifuged 1000 × *g* for 10 min at 4 °C. The sperm pellet was re-diluted in motility-inhibiting solution to remove the seminal plasma. Thereafter, the sperm suspension was centrifuged once more under similar conditions, and the sperm pellet was diluted in 1 mL of 0.1 mol L^{-1} Tris buffer (pH 8.0) containing 0.01% Triton-X-100 and 0.5% glycerol. Samples were homogenized and the suspensions were pooled, placed in an ice bath, and sonicated (Bandelin Electronic UW 2070, Germany) for 15 s Insoluble particles were removed by centrifugation at 3000 × *g* for 10 min at 4 °C. Before analysis, extracted samples were stored at −80 °C until analysis.

The activity of CAT and SOD were determined at 4, 14, and 24 °C. The activity of SOD (EC 1.15.1.1) was measured by a spectrophotometer equipped with a thermo-regulated cuvette holder at 420 nm according to the procedure of Marklund and Marklund (1974). The inhibition of pyrogallol autoxidation by the sample was used to determine SOD activity. The autoxidation of 0.2 mM pyrogallol in air-equilibrated 50 mM Tris–HCl buffer, containing 1 mM EDTA, pH 8.2, was inhibited by the addition of the assayed sample. One unit of the enzyme is generally defined as the amount of enzyme that inhibits the reaction (in this case, pyrogallol autoxidation) by 50%. The specific activity of SOD was expressed as unit mg^{-1} of protein.

The activity of CAT (EC 1.11.1.6) was measured by a spectrophotometer equipped with a thermo-regulated cuvette holder at 240 nm. The reaction medium contained 10 mM K^+-phosphate buffer with 0.1 mM EDTA, pH 7.4, and 15 mM H_2O_2, according to the method of Marklund et al. (1981). The CAT activity was calculated from the H_2O_2 decomposition rate using the molar extinction coefficient 39.4 M^{-1} cm^{-1}, and the specific activity was expressed as micromole min^{-1} mg^{-1} of protein. Measurements were carried out in triplicate for each sample.

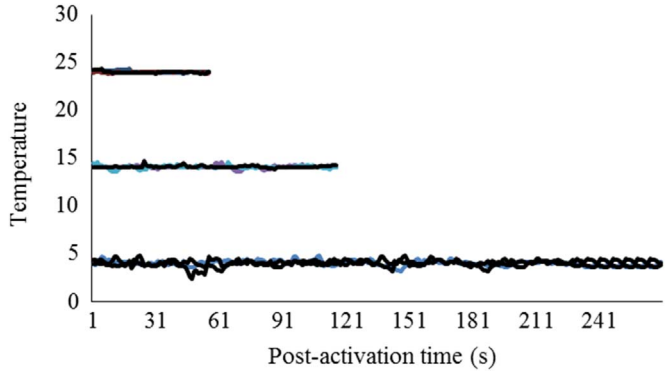

Fig. 1. Supplementary data about temperature fluctuation during spermatozoon motility analysis in Cyprinus carpio.

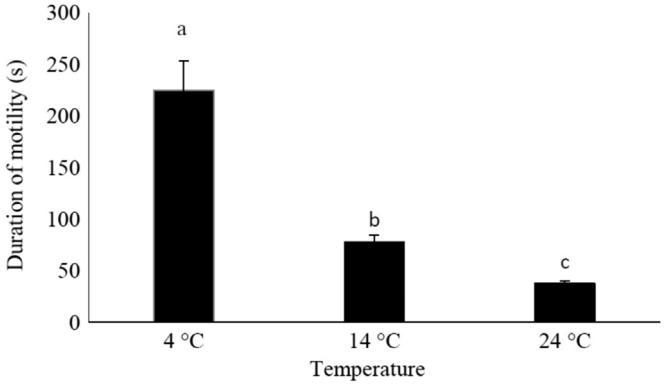

Fig. 2. Effect of temperature on *C. carpio* spermatozoon motility duration. Data are mean ± SD. Different lowercase letters indicate significant difference ($P < 0.05$).