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The *in vitro* effect of cypermethrin on quality and oxidative stress indices of rainbow trout *Oncorhynchus mykiss* spermatozoa

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ABSTRACT

There is limited information on the scientific literature about the effect of *in vitro* exposure of fish sperm to pesticides. *In vitro* effect of cypermethrin on sperm quality and oxidative stress has not yet been fully investigated. Therefore, the effects of cypermethrin, a type II pyrethroid insecticide, on quality and oxidative stress of spermatozoa were examined *in vitro*. To explore the potential *in vitro* toxicity of cypermethrin, fish spermatozoa were incubated with different concentrations of cypermethrin (1.025, 2.05 and 4.1 μg/l) for 2 h. The motility rate and duration of sperm were determined after exposure to cypermethrin. Reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT) and malondialdehyde (MDA) in spermatozoa were analyzed for determination of oxidant and antioxidant balance. Our results indicated that spermatozoa motility and duration significantly decreased with exposure to cypermethrin. Additionally, activity of GSH-Px ($P < 0.05$) and MDA and GSH levels increased in a concentration-dependent manner while CAT activity decreased ($P < 0.05$). Consequently, the oxidant and antioxidant status and sperm quality were affected by quantitative changes and different concentrations of cypermethrin.

1. Introduction

Synthetic pyrethroid pesticides have received great attention due to their widespread and indiscriminate usage in agriculture, forestry, horticulture and homes in the world [1–3]. Increase in the worldwide annual consumption rate of synthetic pyrethroid pesticides is owing to their low effect of toxicity on mammals and birds and limited soil persistence [4]. Cypermethrin, as a type II pyrethroid insecticide, is broadly used to control *Lepidoptera* and *Coleoptera* in citrus, moth pests of cotton, fruits, grapes and vegetable crops [3,5], and pest control in soybean culture [6,7]. It has also been adopted to prevent and treat ticks, lice and scab on sheep and as a treatment against infestation by the parasitic sea louse *Lepeophtheirus salmonis* in intensive salmonid aquaculture [8]. Cypermethrin is very highly toxic to fish and aquatic arthropods (in laboratory tests 96 h LC50 were generally within the range of 0.4–2.8 μg/l and aquatic invertebrates LC50 in the range of 0.01–5 μg/l) [3,7,9–11]. Residues of cypermethrin in water and sediment samples from streams and rivers draining major agricultural districts are affected to fish inhabiting these areas due to discharged into waters of the majority of pollutants [12–13].

Sperm quality is the most important due to its effect on fertilization success, hatching of embryos and survival of embryos, larvae and adults [14–16]. Sperm cells in most fish species are released into water and directly exposed to pollutants prior to fertilization [15,17]. Therefore, determination of the adverse effects of environmental pesticides on the sperm quality is essential in fish with external fertilization [13]. Reactive oxygen species (ROS) generate in consequence of expose of spermatozoa to contaminants due to contain highly polyunsaturated fatty acids (PUFA) in their membranes and a lack of protective cytoplasmic enzymes [15]. Production and increased levels of ROS in fish negatively affect sperm motion parameters and accelerate the process of germ cell apoptosis due to lipid peroxidation [13,15,18–21]. Due to these reasons, studies have recently focused on effects of toxicants on sperm quality as bio-indicator of aquatic pollution in different fish species (*Salmo trutta fario*, *Cyprinus carpio*, *Acipenser ruthenus*, *Heteropneustes fossilis*, *Rutilus frisii kutum*) [4,13,15,21,22–26]. To the best of our knowledge, the effect of toxicants on rainbow trout (*Oncorhynchus mykiss*) reproduction through ROS induction and sperm quality has not been documented thus far. In this context, the aim of this study was to investigate how spermatozoa physiology is affected by short-term (2 h) *in vitro* exposure of rainbow trout (*O. mykiss*) sperm to cypermethrin, by analyzing

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oxidative stress indices (lipid peroxidation–MDA), and antioxidant enzyme (Reduced glutathione-GSH, glutathione peroxidase-GSH-Px and catalase-CAT) activity of rainbow trout sperm, as well as spermatozoa motility and viability.

2. Material and methods

2.1. Broodstock handling and collection of gametes

Rainbow trout sperm was obtained from six males (2–3 years old; 36.6 ± 2.23 cm, 666.42 ± 129.15 g, as mean ± SD) reared in the fish farm BUTAŞ Trout Production Facility (Muğla, Turkey) between November and January. The males were anesthetized with 2-phenoxyethanol (0.6 ml l^{-1}) before stripping. The sperm was collected by a gentle abdominal massage, collected into glass vials and stored on ice until use. Caution was exercised to prevent contamination of the semen with urine, feces, blood, mucus or water. Sperm samples with a motility rate of ≤90% were excluded from the experiment and the percentage of motile sperm was checked using a light microscope with digital image processing software connected to the computer (Zeiss Axio Scope with AxioVision). Spermatozoa concentration of each male was estimated microscopically at 200× using a Burker cell hemocytometer.

2.2. Sample preparation

Cypermethrin (technical grade, a-cyano-3 phenoxybenzyl-(IR,IS) cis,trans-3 2, 2-dichlorovinyl)-2,2-dimethylcyclopropanecarcoxylate; empirical formula: $C_{22}H_{19}C_{l2}NO_3$), was obtained from HEKTAŞ Insecticide Limited, Gebze, Kocaeli, Turkey and was dissolved in 96% ethanol and diluted with distilled water to obtain a stock solution of 0.1 g/l. Stock solutions were prepared daily. Analytical grade chemicals were obtained from Sigma Chemicals Co. (USA). Fish sperm from six individual males was pooled. The pooled sample was then diluted with an immobilization medium (NaCl, 103 mmol/l; KCl, 40 mmol/l; $CaCl_2$, 1 mmol/l; $MgSO_4$, 0.8 mmol/l; hepes, 20 mmol/l; pH 7.8) to obtain a sperm density of 6×10^8 cells ml^{-1}. The sperm sub-samples (n = 6) were then exposed for 2 h to final concentrations of 0 (control), 1.025, 2.05 and 4.1 μg/l of cypermethrin dissolved in ethanol. Each experimental condition was triplicated.

2.3. Sperm motility

After the sperm was *in vitro* exposed to the toxicant in the immobilization medium, sperm motility and duration was assessed following a two-step dilution. This was immediately followed by a second five-fold dilution in an activation medium (45 mM NaCl, 5 mM KCl, 30 mM Tris–HCl, pH 8.2) [27]. The percent of motile spermatozoa and motility duration was immediately recorded for 1 min post-activation using a CCD video camera mounted on a phase-contrast microscope (Zeiss Axio Scope with AxioVision) at room temperature (20 °C). The percentage of sperm motility was estimated as the cell performing progressive forward movement, while the duration of motility was determined as the time until forward movement stops. Determining the percentage of sperm motility was assessed using an arbitrary scale with 10% interval increments in which non motile represents 0%.

2.4. Oxidative stress and antioxidant indices analyses

The sub-sperm sample was centrifuged at 3000 × *g* at 4 °C for 10 min and the pellet in an ice bath was homogenized using a Sonopuls HD 2070 ultrasonicator (Bandelin Electronic, Berlin, Germany) with the immobilization buffer. Lipid peroxidation in spermatozoa was measured by the thiobarbituric acid reacting substance (TBARS) method [28], and was expressed in terms of the MDA content, which served as standard of 1,1,3,3-tetraethoxypropane. The TBARS concentration was calculated by the absorption at 535 nm. The content of TBARS was expressed as nanomoles per 10^8 cells. Values were expressed as MDA equivalents in nmol/sperm cells. Glutathione peroxidase (GPx; EC 1.11.1.9) was assayed by the method of Matkovics et al. [29] and expressed as unit per g of protein (U.g^{-1} protein) per 10^8 cells. GSH-Px activity was determined by using cumenehydroperoxide and reduced glutathione (GSH) as co-substrates and the loss of GSH following enzymic reaction at 37 °C was measured spectrophotometrically with Ellman's reagent at 412 nm. Spermatozoa CAT activity was determined according to the method of Aebi [30] and expressed as kat.g^{-1} protein per 10^8 cells. The decomposition of H_2O_2 can be directly followed by the decrease of absorbance at 240 nm. The difference in absorbance at 240 nm per time unit allows determining the CAT activity. Glutathione reductase (GR; EC 1.6.4.2) was assayed by the method of Chavan et al. [31] and expressed as μmol.g^{-1} protein. The method is based on the capacity of sulfhydryl groups present in whole blood to react with 5, 5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) and form a yellow dye with maximum absorbance at 412 nm. The protein content in spermatozoa was measured by method of Lowry et al. [32].

2.5. Statistical assays

All values were expressed as mean ± SD and analyzed by SPSS for Win 14.0 software. One-way ANOVA with Duncan test was used to determine whether results of treatments were significantly different from the control group ($P < 0.05$).

3. Results

3.1. Spermatozoa motility

In fresh semen, the percentage and duration of motile spermatozoa was 90 ± 5.0% and 35 ± 7.0 s, respectively. Sperm motility parameters (motility and duration) were determined after 2 h exposure to cypermethrin *in vitro* (Figs. 1 and 2). Motility and duration were significantly lower than in the control compared to other concentrations of cypermethrin from 1.025 to 4 μg/l ($P < 0.05$), but did not significantly affect the sperm motility duration ($P > 0.05$).See Fig. 3

3.2. Antioxidant responses

Levels of MDA, GSH, GSH-Px and CAT are shown in Fig. 1. The results from the present study indicated that an increase in the concentration of cypermethrin caused a slightly increase in Malondialdehyde (MDA) and

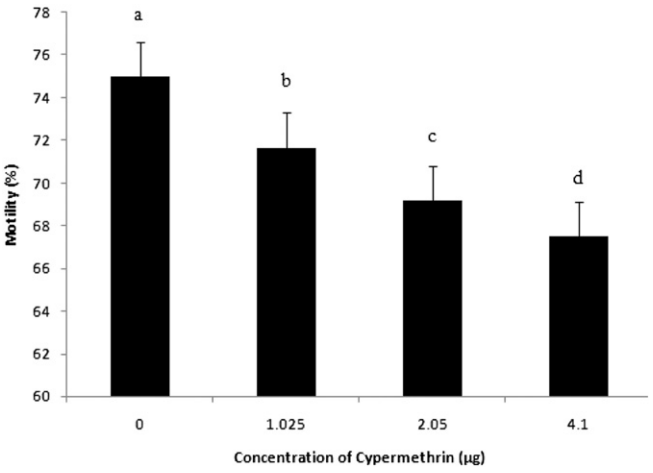

Fig. 1. *In vitro* effects of cypermethrin on spermatozoa motility rate (%) in *Oncorhynchus mykiss*. Data are presented as means ± SD. Superscript letters indicate significant differences among samples at the same time post-activation (ANOVA, $P < 0.05$).