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The *in vitro* effect of Lambda-cyhalothrin on quality and antioxidant responses of rainbow trout *Oncorhynchus mykiss* spermatozoa

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ABSTRACT

There is little information in the scientific literature about effect of *in vitro* exposure of fish spermatozoa to pesticides. *In vitro* effect of Lambda-cyhalothrin (LCT) on sperm quality and oxidative stress has not been fully explored yet. The effects of LCT, which is a synthetic pyrethroid insecticide, on quality and oxidative stress of spermatozoa were investigated *in vitro* due to extensively use to control a wide range of insect pests in agriculture, public health, and homes and gardens. To explore the potential *in vitro* toxicity of LCT, fish spermatozoa were incubated with different concentrations of LCT (0.6, 1.2 and 2.4 μg/L) for 2 h. 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 the percentage and duration of sperm motility significantly decreased with exposure to LCT. Activity of GSH-Px and MDA ($P < 0.05$) and GSH levels ($P < 0.05$) increased in a concentration-dependent manner while CAT activity decreased ($P < 0.05$). In conclusion, the oxidant and antioxidant status and sperm quality were affected by increasing concentrations of LCT.

1. Introduction

Synthetic pyrethroids as major class of broad-spectrum organic insecticides are a widely used in agricultural, domestic, forestry, horticulture, and veterinary applications in the world owing to their the low toxic effect on mammals and birds and limited soil persistence. In addition, use of organochlorines and organophosphorus insecticides has been banned due to their highly toxic and environmental persistence (Bradberry et al., 2005; Singh and Singh, 2008; Shi et al., 2011; Ansari et al., 2011; Zeng et al., 2015). Lambda-cyhalothrin (LCT), as a type II α-cyano-pyrethroid insecticide and acaricide, is a non-systemic and extensively used to control a wide range of insect pests, including aphids, *Colorado* beetles, and butterfly larvae, ectoparasites, including cockroaches, lices, mosquitoes, in the cotton plantation and vegetable production (Velmurugan et al., 2007; Gökalp Muranli and Güner, 2011; Piner and Üner, 2012; Lofty et al., 2013; Ramadhas et al., 2014). It has also been adopted for structural pest management or in public health applications to control insects (Kidd and James, 1991; Gökalp Muranli and Güner, 2011). LCT is extremely toxic to aquatic animals, including fish, invertebrates, and amphibians (Velisek et al., 2006; Carriquiriborde et al., 2009; Ansari et al., 2011; Gökalp Muranli and Güner, 2011). LCT discharges into water through agricultural use, forest-spraying procedures and direct spraying of water bodies and accumulates in sediment (Campana et al., 1999). Due to these reasons, fish in aquatic habitat are extremely affected as non-target organisms by LCT (Marino and Ronco, 2005; Velmurugan et al., 2007; Gazo et al., 2013). In addition, pyrethroids have a high rate of gill absorption owing to their lipophilicity and cause the sensitivity of fish (Polat et al., 2002).

Spermatozoa in many aquatic animals with external fertilization are directly exposed to toxicants. Fertilization success, hatching success and survival of embryos, larvae and adults are affected by toxicants (Au et al., 2001; Li et al., 2010a,b; Kime and Nash, 1999; Rurangwa et al., 2002). Toxicants cause to generation of reactive oxygen substances (ROS) in spermatozoa due to highly susceptibility to lipid peroxidation (LPO) for including high concentrations of polyunsaturated fatty acids (PUFA) and a largely lack of protective cytoplasmic antioxidant enzymes activities [superoxide dismutase (SOD), glutathione peroxidases (GPX) and catalase (CAT)] in their membranes (Drevet, 2006; Li et al., 2010a). Sperm quality is negatively affected by increasing levels of ROS and germ

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cell apoptosis occur in consequence of lipid peroxidation (Ong et al., 2002; Agarwal et al., 2003; Baker and Aitken, 2004; Zhou et al., 2006; Li et al., 2010a; Gazo et al., 2013). For these reasons, studies have recently performed in different fish species (*Salmo trutta fario*, *Cyprinus carpio*, *Acipenser ruthenus*, *Heteropneustes fossilis*, *Rutilus frisii kutum*) about effects of toxicants on sperm quality as bio-indicator of aquatic pollution (Rurangwa et al., 2002; Zhou et al., 2006; Dietrich et al., 2007a,b; Singh and Singh, 2008; Singh et al., 2008; Li et al., 2010a; Fadakar Masouleh et al., 2011; Gazo et al., 2013; Linhartova et al., 2013).

Rainbow trout (Salmonidae) has been used as an alternative model organism in researches owing to its low rearing costs, an early life-stage ultrasensitive bioassay, sensitivity to many classes of carcinogen, a well-described tumor pathology, responsiveness to tumor promoters and inhibitors, and a mechanistically informative non-mammalian comparative status over the past 30 years (Hendricks et al., 1985, 1995; Kelly et al., 1992; Bailey et al., 1996; Walter et al., 2008; Williams, 2012; Kutluyer and Aksakal, 2013). In past decade, studies about using fish sperm cells in toxicity test have been conducted owing to its sensitive, like any other cells, and bio-indicator of aquatic pollution (Li et al., 2010a). To the best of our knowledge, no attempt has been made to evaluate the effect of LCT on rainbow trout (*Oncorhynchus mykiss*) reproduction through ROS induction and sperm quality thus far. In this framework, the aim of this study was to investigate effects of short-term (2 h) *in vitro* exposure of rainbow trout (*O. mykiss*) sperm to LCT, by analyzing 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.

2. Materials 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) 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 into glass vials by a gentle abdominal massage, 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 ≤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 of the six males was estimated microscopically at 200× using a Burker cell hemocytometer.

2.2. Sample preparation

Lambda-cyhalothrin (technical grade, α-cyano-3-phenoxybenzyl-3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethyl cyclopropanecarboxylate; empirical formula: $C_{23}H_{19}C_lF_3NO_3$), 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) (Lahnsteiner et al., 1998) to obtain a sperm density of 6×10^8 cells ml^{-1}. The sperm sub-samples ($n = 6$) were exposed for 2 h due to rapidly dissipate from water (He et al., 2008) and final concentrations of 0 (control), 0.6, 1.2 and 2.4 μg/L of LCT dissolved in ethanol. A control group was exposed to immobilization medium with 1% ethanol equal to the amount of ethanol in the experimental samples since this solvent was used to dissolve LCT (Hulak et al., 2013; Linhartova et al., 2013). Each experimental condition was triplicated.

2.3. Sperm motility assessment

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. Semen has high sperm density and high viscosity. 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) (Zhou et al., 2006; Ögretmen et al., 2014). Analysis of spermatozoa motility and duration was made in triplicate for each sample. The percent of motile spermatozoa and motility duration was immediately recorded for 1 min post-activation. Each sample was evaluated for the motility parameters using a light microscope with a digital image processing software connected to the computer (Zeiss Axio Scope with AxioVision) to evaluate the percentage of spermatozoa motility and duration. The obtained video records were scanned to determine the percentages of progressive motility (%) and the durations of progressive motility (s). 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. The percentage of sperm motility was assessed using an arbitrary scale with 10% interval increments in which non motile represents 0% (Ögretmen et al., 2014).

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 levels (as MDA) in the tissues were measured with the thiobarbituric acid reaction using methods described by Placer et al. (1966). The quantification of thiobarbituric acid reactive substances was determined by comparing the absorption to the standard curve of malondialdehyde equivalents generated by acid catalyzed hydrolysis of 1,1,3,3 tetraethoxypropane. The values of MDA were expressed as nmol g^{-1}/sperm cells. Glutathione peroxidase (GPx; EC 1.11.1.9) was assayed by the method of Matkovics et al. (1988) and expressed as unite per g of protein (U g^{-1} protein) per 10^8 cells. GSH-Px activity was determined by using cumene hydroperoxide 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 (1984) 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. (2005) 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. (1951).

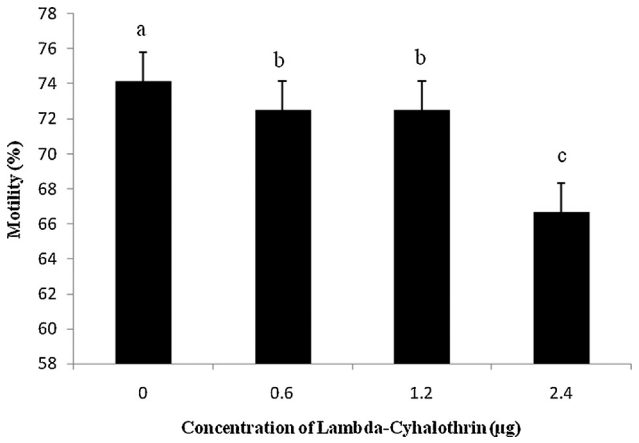

Fig. 1. *In vitro* effects of Lambda-cyhalothrin on spermatozoa motility rate (%) in *Oncorhynchus mykiss* ($n = 6$). Data are presented as means ± SD. Superscript letters indicate significant differences among samples at the same time post-activation ($P < 0.05$).

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 were 90 ± 5.0% and 35 ± 7.0 s, respectively. Sperm motility parameters (motility and duration) are presented in Figs. 1 and 2. The percent of motile spermatozoa exposed to the different concentration of LCT (from 0.6 to 2.4 µg/L) was significantly different from the control ($P < 0.05$). Motility duration significantly reduced at the highest concentration (2.4 µg/L) ($P < 0.05$).

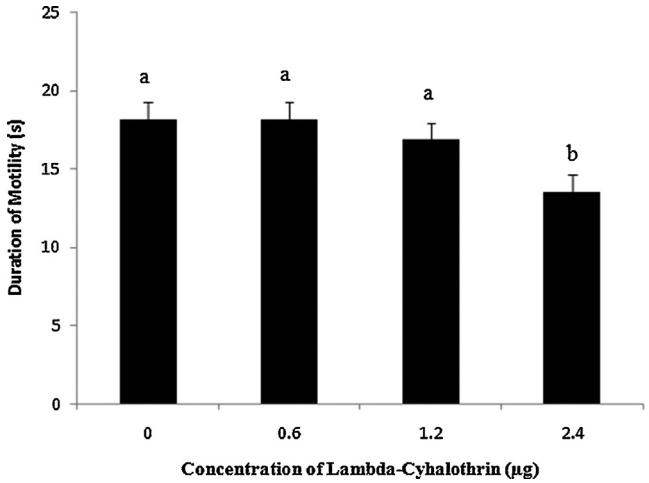

Fig. 2. *In vitro* effects of Lambda-cyhalothrin on spermatozoa motility duration (s) in *Oncorhynchus mykiss* ($n = 6$). Data are presented as means ± SD. Superscript letters indicate significant differences among samples at the same time post-activation ($P < 0.05$).

3.2. Antioxidant responses

Levels of malondialdehyde (MDA), GSH, GSH-Px and CAT are shown in Fig. 3. The results from the present study indicated that an increase in the concentration of LCT caused a significant increase in MDA and glutathione (GSH) levels of rainbow trout (*O. mykiss*) spermatozoa ($P < 0.05$). GSH-Px activity in spermatozoa significantly increased ($P < 0.05$). CAT activity significantly decreased with increasing concentration of LCT ($P < 0.05$).

4. Discussion

In this study, we examined a toxic effect of LCT on the motility parameters and oxidative stress indices in rainbow trout sperm. Overall, we demonstrated that motility rate and duration of spermatozoa decreased with increasing concentrations of LCT. Additionally, it is suggested that the cellular response of rainbow trout (*O. mykiss*) spermatozoa was affected by increasing LCT concentrations.

Sperm motility is the important functional parameter for successful fertilization and hatching in fish (Islam and Akhter, 2011). Additionally, fertilization success may depend on the capacity of sperm to move fast enough to find the egg in water after their releases (Zhou et al., 2006). Rapid loss of motility in rainbow trout (*O. mykiss*) spermatozoa occurs by 30 s after activation and motility lasts <1 min (Lahnsteiner et al., 1993). The results from the present study suggested that spermatozoa quality was impaired by LCT-mediated oxidative stress in comparison with fresh sperm. The percentage of sperm motility significantly decreased in all treatment groups compared to the control while motility duration significantly reduced at the highest concentration. The present results agree with previous studies about effects of toxicants on sperm quality of different fish species (*S. trutta fario*, *C. carpio*, *A. ruthenus*, *H. fossilis*, *R. frisii kutum*) (Rurangwa et al., 2002; Zhou et al., 2006; Dietrich et al., 2007a,b; Singh and Singh, 2008; Singh et al., 2008; Li et al., 2010a,b; Fadakar Masouleh et al., 2011; Gazo et al., 2013; Linhartova et al., 2013). In present study, decreasing in sperm motility and the motility period may be due to destroy the functional integrity of the axosome and mitochondria of the sperm cells of high doses of LCT or its toxic effect (Sikka, 2004; Ubilla and Valdebenito, 2011; Kledmanee et al., 2013). In addition, biological membranes readily absorb to pyrethroids because of the lipophilic nature (He et al., 2008). The plasma membrane plays an important role in the initiation of sperm motility through hypo-osmotic signals (Li et al., 2009, 2012). Hence, alterations of the plasma membrane can significantly change spermatozoa motion. Increased lipid peroxidation of unsaturated fatty acids in the sperm plasma membrane can reduce the fluidity of the membrane and lead to loss of cell function, thereby reducing sperm motility.

Fish are affected by residues of toxicants in water and sediment samples due to discharge into waters of the majority of pollutants. Sperm cells in most fish species release into water and directly expose to pollutants prior to fertilization (Gazo et al., 2013). Sperm cells are highly susceptible to oxidative damage and poorly equipped to fight against free radical-mediated attacks because of being transcriptionally inactive and largely devoid of cytoplasm. Additionally, the high poly-unsaturated fatty acids (PUFA) content of the spermatozoa plasma membrane causes being susceptible of sperm to ROS attacks. Lipid peroxidation of sperm cell membranes, damage of midpiece, axonemal structure, and DNA, malfunctions of capacitation and acrosomal reaction, loss of motility, and infertility may carry out (Sikka, 2004; Ubilla and Valdebenito, 2011) when there is a high production of reactive oxygen species (ROS) in gametes (Saleh and Agarwal, 2002; Membrillo et al., 2003; Lahnsteiner et al., 2011). Therefore, balanced ROS levels are

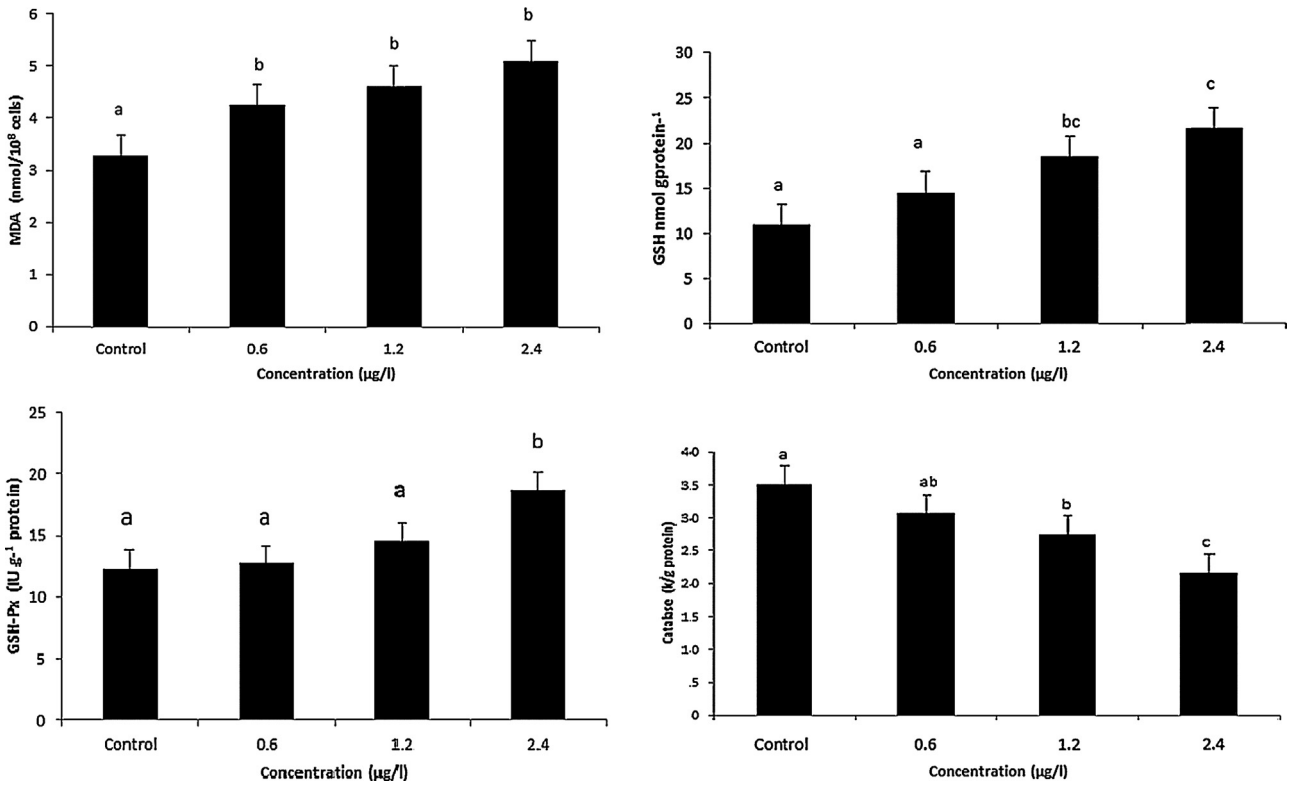

Fig. 3. Effect of Lambda-cyhalothrin on (a) MDA, (b) GSH, (c) GSH-Px and (d) CAT of spermatozoa in *Oncorhynchus mykiss* ($n = 6$). Data are presented as means ± SD. Superscript letters indicate significant differences among samples at the same time post-activation ($P < 0.05$).

important for sperm cells (Drevet, 2006). Seminal plasma provides the major defence against ROS due to the low content of cytoplasm in spermatozoa (Shiva et al., 2011). In fish sperm, the main responsible enzymes for detoxification of ROS are catalase, superoxide dismutase (SOD), glutathione reductase (GSR) and peroxidase (GPX) (Lahnsteiner and Mansour, 2010; Mansour et al., 2006; Cabrita et al., 2014). For these reasons, assessing of these enzymes' activities provides understanding the effects of LCT on oxidative status of spermatozoa. To the best of our knowledge, this is apparently the first report about effect of LCT on rainbow trout (*O. mykiss*) sperm oxidative status, although studies have been conducted about effect of LCT on oxidative status as bio-indicator of aquatic pollution in different tissues of fish species (*Oreochromis niloticus*, *O. mykiss*) (Piner and Üner, 2012; Alak et al., 2013). In studies about effect of LCT on oxidative status in tissues, it was determined that LCT exposure caused oxidative stress. Piner and Üner (2012) determined that LCT caused increase in tGSH, GSH, TBARS contents, and GST activity in the liver of juvenile (*O. niloticus*). Alak et al. (2013) demonstrated that the activity of antioxidant enzymes in liver tissue of rainbow trout was significantly increased by LCT exposure. Nevertheless, only a few studies have been examined the effect of pesticides on oxidative status of spermatozoa in different fish species (*C. carpio*, *A. ruthenus*) (Zhou et al., 2006; Gazo et al., 2013; Linhartova et al., 2013). In these studies, it was determined that ROS production in fish sperm exposed to pesticides caused oxidative damage and reproductive impairment. In a study on oxidative stress through exposure of spermatozoa to duroquinone in common carp (*C. carpio*), Zhou et al. (2006) stated an increase in ROS production as a result of be metabolized by cytochrome P450 reductase, which use a variety of small and large molecules as substrates in enzymatic reactions, or the mitochondrial electron transfer chain in carp sperm. Linhartova et al. (2013) determined an increase in lipid peroxidation, content of carbonyl proteins (CP) and SOD activity with increasing concentrations of duroquinone when sterlet (*A. ruthenus*) spermatozoa exposed to duroquinone. Gazo et al. (2013) found that TBARS levels, SOD activity and carbonyl proteins (CP) were greater in sterlet (*A. ruthenus*) spermatozoa exposed Vinclozolin. In agreement with the studies noted above our results indicated that malondialdehyde (MDA), which is one of the oxidative damage products of lipid peroxidation, significantly increased. LCT may be caused to generation of reactive oxygen substances (ROS) in spermatozoa due to highly susceptibility to lipid peroxidation (LPO) for including high concentrations of polyunsaturated fatty acids (PUFA) and a largely lack of protective cytoplasmic antioxidant enzymes activities [superoxide dismutase (SOD), glutathione peroxidases (GPX) and catalase (CAT)] in their membranes (Drevet, 2006; Li et al., 2010a). This might be associated with increased GSH and decreased CAT activities in spermatozoa after LCT exposure. The decrease in catalase activity could be due to flux of superoxide radicals, which have been reported to inhibit CAT activity (Pandey et al., 2001). Reduced GSH is one of the most important antioxidant agents, protects cell membranes from lipid peroxidation and the main non-protein thiol and one of the main reductant found in cells. The increase in GSH may be due to protective role against oxidative stress-induced toxicity (Parvez and Raisuddin, 2006). This oxidative damage in spermatozoa may be due to susceptibility of PUFAs in spermatozoa and, lipid peroxidation and cell dysfunction as a result of exposure LCT (Sanocka and Kurpisz, 2004).

In conclusion, based on the data obtained within the context of this study, rainbow trout spermatozoa are highly sensitive to the presence of LCT. The antioxidant system possesses a protective response to increased lipid oxidation and prevents cell damage. In addition, different doses of LCT for rainbow trout significantly affected sperm motility and the motility period. From our data, it is recommended that the use of fish sperm for *in vivo* or *in vitro* assays may be practical, quick and cost effective for routine monitoring and toxicological assessment of chemicals compared to another

non-invasive method, such as the use of blood cells, tissues. Further studies would be needed to evaluation the precise mechanisms involved and the relationship between motility and oxidative stress in rainbow trout sperm as well as its consequences on fertilization.

Conflict of interest statement

None.

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