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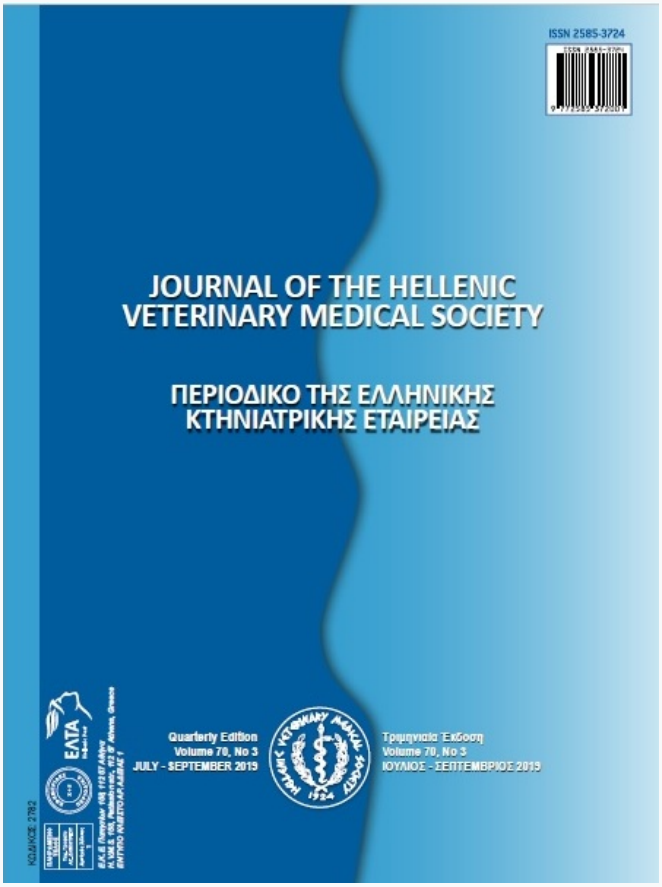

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Research article
Ερευνητικό άρθρο

The *in vitro* toxicity of atrazine on kinematics and DNA fragmentation in common carp (*Cyprinus carpio*) sperm cells

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ABSTRACT. This study investigated the in vitro effects of different concentrations of Atrazine (0.001, 0.01, 0.1, 0.5 mg/L) added to motile and immotile solutions on kinematics quality of sperm cells of common carp, *Cyprinus carpio*, which is a fish of economic significance. The kinematics of the sperm cells was analyzed by a computer-assisted sperm analysis system (CASA). As a result of the study, while there was a significant difference ($P < 0.05$) between the groups in terms of the VSL (µm/s) and VCL (µm/s) values after the Atrazine-added immotile solution's (IMS) and incubation for 3 hours, there was a significant difference ($P < 0.05$) in only the VSL values directly activated by the Atrazine-added motile solution (MS). DNA fragmentation was evident but not in higher numbers in the 0.1 mg/L atrazine group. Finally, it was determined the effective concentration (EC50) values of the VSL value of the motile and immotile solution as 0.34 mg/L and 0.03 mg/L, respectively.

***Keywords*:** *Cyprinus carpio, sperm cells kinematics, DNA fragmentation, atrazine, reproduction*

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INTRODUCTION

With increased pollution in the aquatic environment, in order to achieve environmental risk evaluation, it is needed to monitor the toxicity of pollutants, understand their mechanism and determine the areas they affect. Studies are limited on finding the ranges of concentration to determine the maximum levels of environmental pollutants that are not effective on reproduction. *In vitro* techniques allow application of important methods for comparing toxicity levels of pollutants, application of different concentrations and times of exposure, and determining the mechanisms and areas affected by pollutants (Hatef et al., 2013). As opposed to mammals, the sperm cells of teleost fish start to move right away with water and this motility lasts only for a few minutes. However, they may be preserved for 24 hours using some diluents and re-animated via water. For observing the sub-lethal effects of pollutants on reproductive systems, it is possible to test toxicity by both incubation in these diluents for 24 hours and adding pollutants during the last dilution (Kime et al., 1996).

In most teleost fish with ability of external fertilization, sperm activity is short and has a tendency to decrease during the motility period. While the duration of motility is very short (20-25 seconds in the rainbow trout, *Oncorhynchus mykiss* and 1-2 minutes in the common carp, *Cyprinus carpio*), it rapidly decreases after activation (Alavi and Cosson, 2005). Computer-assisted sperm analysis (CASA) is very popular for fast and practical analysis of sperm motility in different species. It has replaced traditional methods and estimation methods based on the analysts' personal considerations (Kime et al., 2001).

The weeds in asparagus, maize, sorghum, sugarcane and pineapple have controlled by the triazine herbicide atrazine in worldwide and USA for over 40 years (Xing et al. 2014). Atrazine and its metabolites have found in surface and subsurface waters (around levels of 0.002 mg/L, under 0.0001 mg/L in general, probably higher in agricultural areas with intensive usage). Atrazine leads to acute toxicity in rats, while this toxicity is LD_{50}: 1870-3090 mg/kg body weight orally and LD_{50}>2000 mg/kg body weight on the skin. Various *in vitro* and *in vivo* test system studies have reported that immune system modulation takes place after treatment of Atrazine, it affects the neuroendocrine function and reduces development. Many studies also stated that Atrazine leads to prostate and ovary cancer and/or increased risks of such cancers in humans (Graham, 1999). Similarly, according to the United States Environmental Protection Agency (US EPA), 0.01-0.02 mg/L of Atrazine poses a significant environmental risk for aquatic life forms and communities. It was reported that it is moderately toxic for fish, while it is severely toxic for aquatic invertebrates (USEPA, 2006). It is stated that Atrazine is a herbicide with a moderate-severe toxic effect on aquatic life forms and especially fish. Its LC_{50} value was found as 76 mg/L in carps, 4.4 mg/L in Guppy, *Poecilia reticulata*, 4.5-8.8 mg/L in trout, and 98-154 mg/L in tilapia. It was observed that a low Atrazine concentration of 1 **µg**/L led to endocrine function changes in male Atlantic salmons (*Salmo salar)*. Recent studies reported that Atrazine harms especially the reproductive system, leads to abnormalities in sperm cells, ovaries and reproductive organs and reductions in sperm cells motility and numbers of sperm cells and ovaries (Wang et al., 2011).

Several and diverse studies have been conducted on acute, sub-acute and chronic toxicity of metals, nanomaterials, herbicides, pesticides and other toxic substances in fish and other aquatic life forms. In last decades, there have studied some researches for toxicity of atrazine such as general health, disease susceptibility and gene expression (Shelley et al., 2012b), behavioral responses (Shinn et al., 2015) and immunotoxic and cytotoxic effects (Shelley et al., 2012) in rainbow trout (*Oncorhynchus mykiss*), cytochromes of liver (Xing et al., 2014), hepatic damage and inflammation (Toughan et al., 2018) of common carp (*Cyprinus carpio*). However, *in vitro* studies on sperm toxicity are rather recent, and there is a dearth of research on the effects of Atrazine on fish sperm cells motility. Various studies were recently conducted on *in vitro* estimation of sperm toxicity via CASA systems regarding some toxic materials (Dietrich et al., 2010; Li et al., 2010b; Fabbrocini et al., 2012). No studies were found to investigate particularly the effects of Atrazine on the sperm cells of the common carp, *Cyprinus carpio* with *in vitro* methods.

This study aimed to investigate the *in vitro* effects of Atrazine on the sperm cells of the common carp. For this purpose, the study examined motility parameters after different levels of Atrazine intervention via a computer aided sperm analysis system (CASA), and analyzed kinematics such as the velocities values of VSL: straight line velocity (µm/s), VCL: curvilinear velocity (µm/s), VAP: angular path velocity (µm/s) and as the movement style values of LIN, linearity

(%,VSL/VCL), the ratio of net distance moved to total path distance, BCF: beat cross frequency (cross/second), ALH: amplitude of lateral displacement of the spermatozoa head (μm) and MAD: mean angular displacement (°), average change in direction of the sperm head from frame to frame which are used to quality-control sperm cells in fish (Kime et al., 2001; Fauvel et al., 2010).

MATERIALS AND METHODS

Broodstocks and chemicals

The stocks of the species common carp, *Cyprinus carpio* were caught from the Karakaya reservoir located on the River Euphrates in end of March, 2018, and the fish were then transported to the fish breeding research unit of İnönü University, Sürgü Vocational School of Higher Education. After the period of adaptation there, the sperms were collected directly from 10 males (265±15 g, Mean±SD) without hormonal injection as they were in the reproduction period. All experiment procedures were approved with protocol no: 2016/A-113 by a committee on the ethical use of animals at İnönü University.

Atrazine [2-chloro-4-ethylamine-6-isopropylamino-l,3,5-triazine] was obtained from the Department of Analytical Chemistry, Faculty of Pharmacy, İnönü University. (FLUKA Atrazine, PESTANAL®, analytical standard, 250 mg, CAS No.:1912-24-9). It was dissolved in distilled water and added into the motile and immotile solutions.

Experimental design

In most species that have external fertilization, fish sperm cells remain immotile in the testis and in the seminal plasma. Then sperm cells become motile at spawning when released into the surrounding water (Kime et al., 2001). Therefore, the experimental design was done two basic *in vitro* settings were created to investigate the effects of Atrazine on the sperm samples:

Setting 1: Analysis after adding nominal concentrations of Atrazine (0.001, 0.01, 0.1, 0.5 mg/L) into the immotile solution (IMS) as artificial seminal plasma and incubation for 3 hours.

Setting 2: Analysis after adding nominal concentrations of Atrazine (0.001, 0.01, 0.1, 0.5 mg/L) into the motile solution (MS) as fertilization media and directly activating sperm cells.

For sperm samples preparation and motility analysis, the stock solutions were prepared to obtain the immotile solution (IMS) containing 200 mM KCl and 30 mM Tris-HCl with pH 8.0, and the motile solution (MS) containing 45 mM NaCl, 5 mM KCl and 30 mM Tris-HCl with pH 8.0, (Poupard et al., 1998). The sperm samples were firstly diluted in Eppendorf tubes with the IMS of 100 times their quantity, and Atrazine was then added into the solution, stirred gently, left at 4 ^{0}C for the duration of incubation. All sperm samples were kept over ice during the procedures. The sperm samples were analyzed under a microscope by activating them with the MS in proportion of 1:20. The final rate of dilution was 2000 times. The dilution rates of the sperm samples were set based on the 2-set procedure (Billard and Cosson, 1992). The microscope used in computer aided sperm analysis system (CASA) was an Olympus BX 53 phase contrast microscope with 20x1.25 magnification coupled with a Sony CCD VB600B camera in the BASA-Sperm Aqua module produced by Merk Biotechnology Co. from Turkey.

DNA fragmentation

For DNA fragmentation, the Sperm Chromatin Dispersion (SCD) test method previously described by Fernandez et al. (2005) was modified and the DNA fragmentation of sperm cells was determined by the present halos. In this method, firstly, 5 μL of sperm samples from control group and 0.1 mg/L dose of atrazine group in Setting 1 medium was diluted with 995 μL immotile solution. So, the concentration of sperm cells were about 16 x10^9 cells/mL. Then, the preparation method was modified with lysing solution (2 M NaCl, 0.5% SDS, 0.01 Triton X, 0,2 M Tris–HCl and 0.02 M EDTA, pH 7) for 5 minutes at room temperature. After dehydration, the sperm samples were stained with the Diff-Quick reagent for bright field microscopy. In staining, the samples immersed in Diff-Quick A solution for 10 seconds, Diff-Quick I solution for 5 seconds and Diff-Quick II solution for 5 seconds. Then slides was washed with water, dried air for 1 minute. The DNA fragmentation of sperm cells was determined to under 100x lens with immersion oil. The stained images were taken and converted to greyscale in Photoshop 8 version.

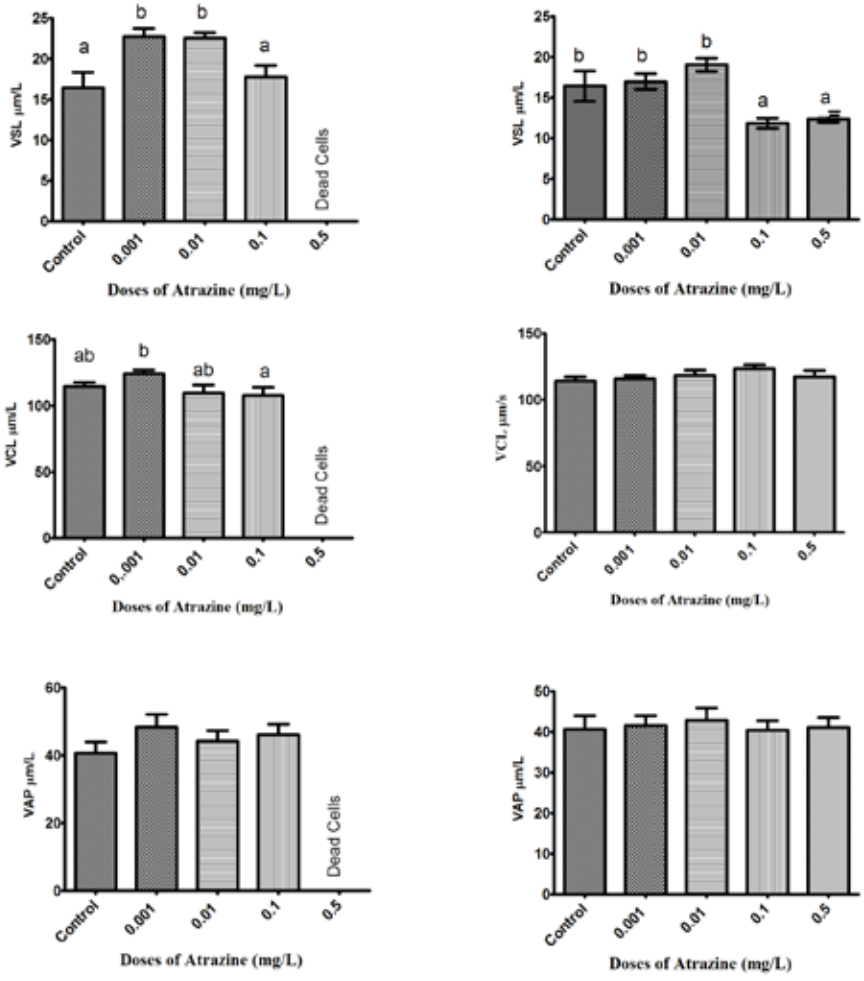
Statistics

The SPSS 17 software was used for statistical analysis. All results were expressed as mean ± standard deviation (Mean±SD) and the statistically significant

level was accepted as $P < 0.05$. The homogeneity of the data in all variables in the groups was tested using the Test of Homogeneity of Variances, and one-way ANOVA and Duncan Post-Hoc tests were used to make comparisons among the groups. Graph Pad Prism 5 was used to form the graphs.

RESULTS

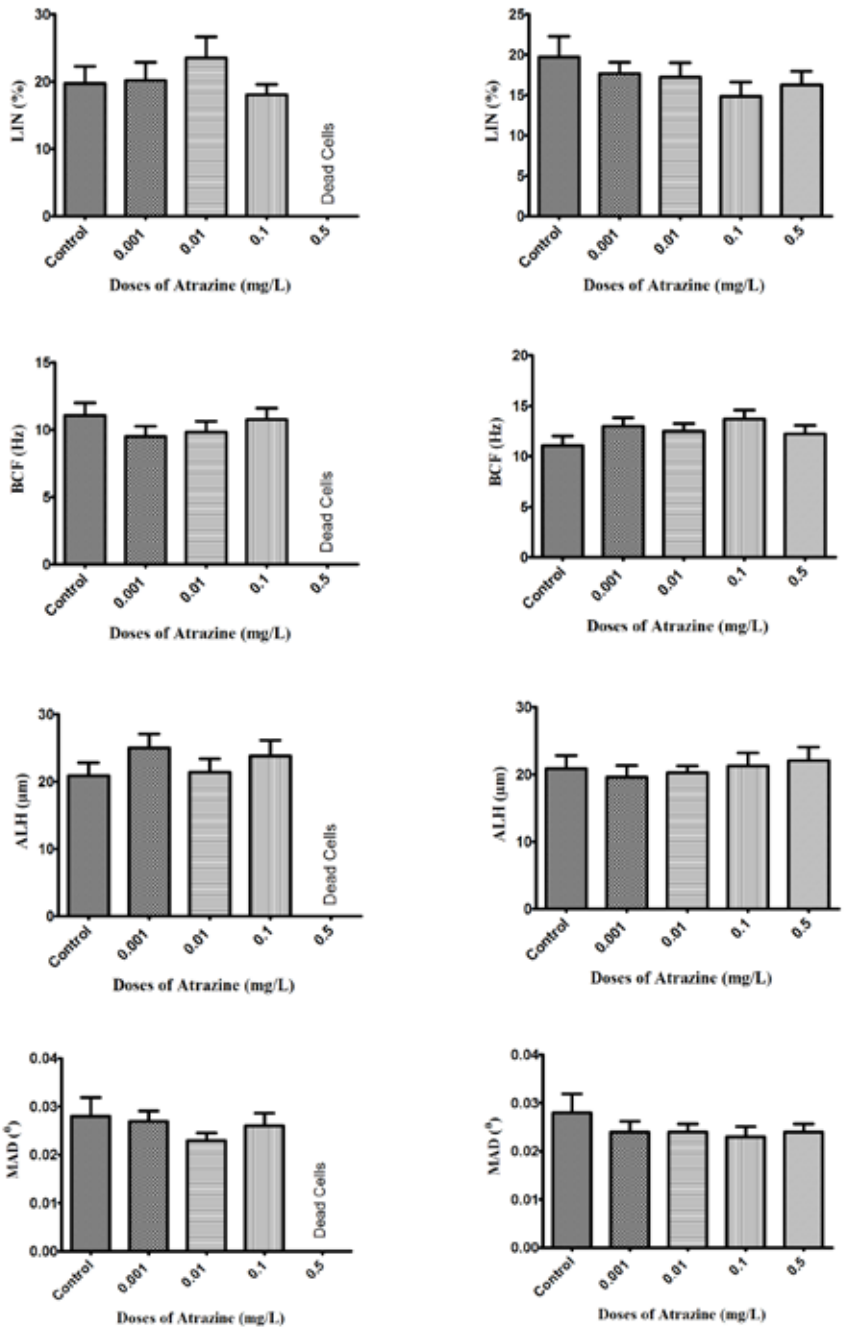
In comparison to the control group, the Atrazine-added samples had gradual reductions in Setting 2 and decreased-increased velocities in Setting 1 at VSL (Straight line velocity) values. While the mean VSL value of the control group was found as 16.44±5.91 μm/s, it was seen that all sperm cells died in the samples with the 0.5 mg/L dose of Atrazine. The changes in the mean VSL values due to Atrazine addition were found to be statistically significant ($P < 0.05$) in Setting 1. Similar statistical results for VSL were observed in Setting 2. The highest reduction was seen in the group that was exposed to 0.1 mg/L of Atrazine in Setting 2 and the mean value was determined as 11.84±1.99 μm/s. This reduction in the mean VSL values due to mg/L of Atrazine in Setting 2 was statistically significant ($P < 0.05$) (Figure 1 a, b). The control group's mean VCL (Curvilinear velocity) value was found as 114.70±10.01 μm/s. While the velocity of the sperm cells suddenly increased with the minimum dosage of Atrazine (0.001 mg/L) but then it started to decrease in Setting 1. The highest decrease in velocity was seen in the sample with the dose of 0.1 mg/L in both Setting 1 and Setting 2. While it was found that the differences between VCL mean values were statistically significant ($P < 0.05$) between 0.001 mg/L and 0.1 mg/L of Atrazine doses in setting 1, they were insignificant ($P > 0.05$) for all groups in setting 2 (Figure 1 a, b).



a) Setting 1 **b)** Setting 2

Figure 1. The velocities values as VSL, VCL and VAP in different Atrazine doses. [a,b,] Different letters show differences between groups and plots show Mean±SD ($P < 0.05$)

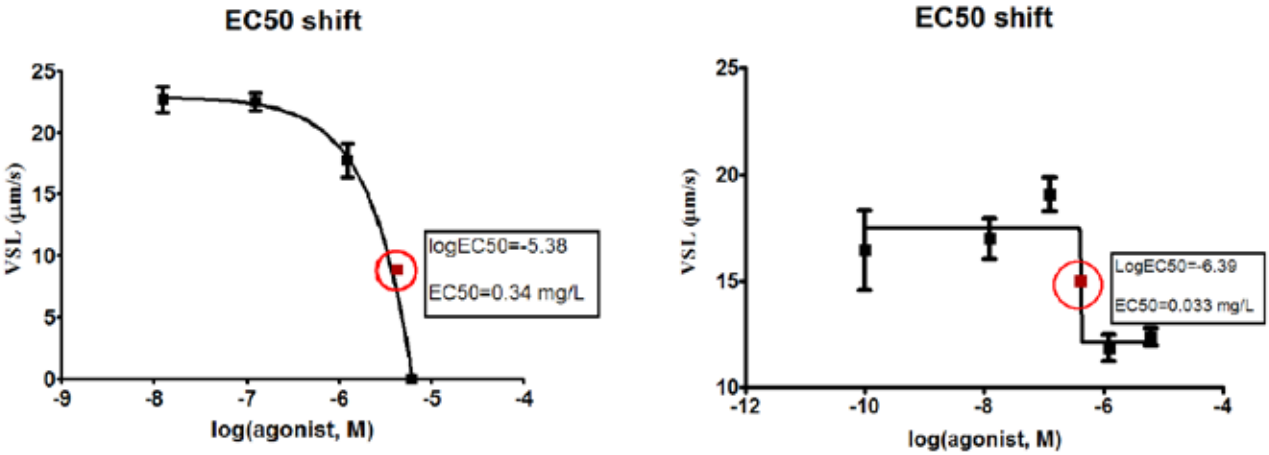
The VAP (Angular part velocity) values of the sperm samples exposed to different rates of Atrazine increased as the dosage was increased, but these increased values were not significant (P > 0.05). While the mean VAP value of the control group was 40.65±10.73 μm/s after incubation, the highest increase was found in the group that was given the dosage of 0.001 mg/L by 48.49±11.50 μm/s in Setting 1. Although the similar statistical results were observed for VAP in Setting 2, but the VAP values increased slightly by increased doses. In the sperm samples in both Setting 1 and Setting 2 after exposure, it was found that the differences were insignificant (P > 0.05) regarding the control values of LIN (19.74±8.17), BCF (11.07±2.98), ALH (20.91±6.13) and MAD (0.03±0.01) (Figure 2).



a) Setting 1 **b)** Setting 2

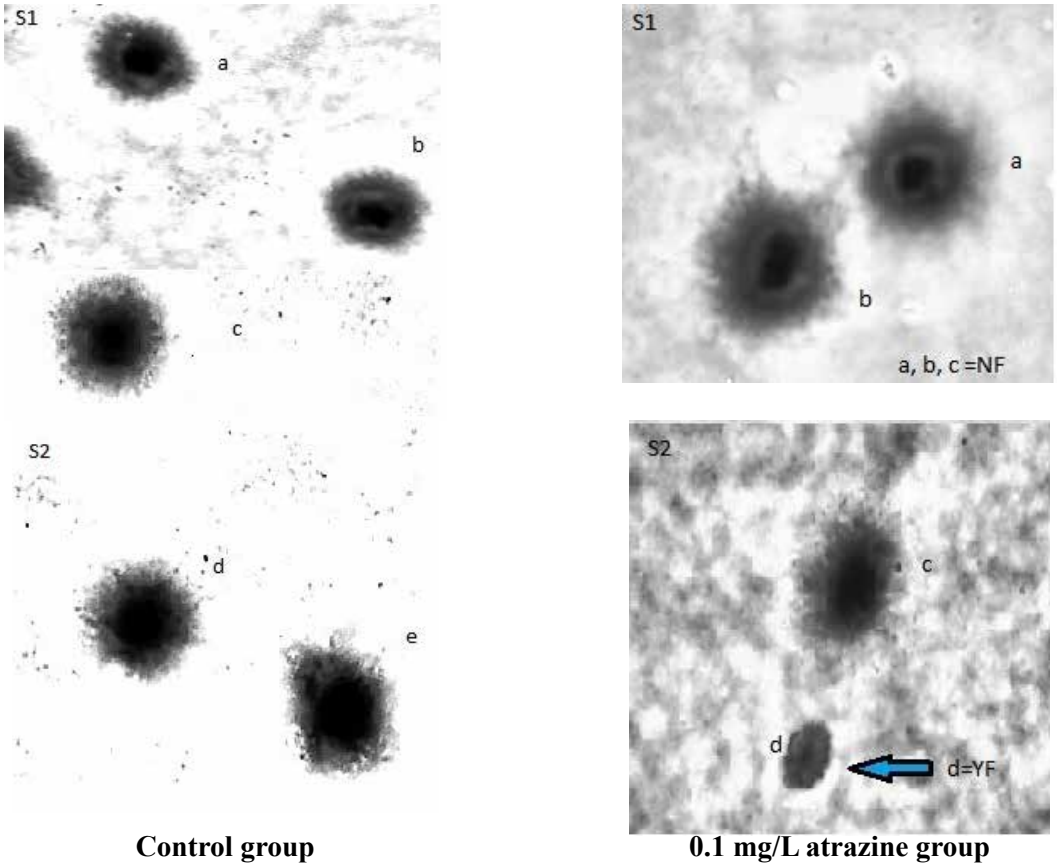
Figure 2. The movement style values as LIN, BCF, ALH and MAD in different Atrazine doses. [a,b,] Different letters show differences between groups and plots show Mean±SD (P < 0.05)

Additionally, on the basis of these experiments, the EC50 values of the VSL parameters after exposure to different doses of Atrazine were calculated as 0.34 mg/L in the immotile solution (Figure 3a) and as 0.033 mg/L in the motile solution (Figure 3b). In the SCD test, although we determined no fragmentation in DNA of sperm cell at control group, there was fragmentation in DNA of sperm cells at 0.1 mg/L Atrazine group but the DNA fragmentation was not increased after 3 hours incubation at +4 °C (Figure 4).



a) Setting 1 **b)** Setting 2

Figure 3. EC50 shift values for VSL in different Atrazine doses



Control group **0.1 mg/L atrazine group**

Figure 4. The Sperm Chromatin Dispersion (SCD) test processed sperm cells showing DNA dispersion halo in Control group (a, b, c, d) and 0.1 mg/L Atrazine group (a, b, c); no showing DNA dispersion halo in 0.1 mg/L Atrazine group (d) with arrow; YF= Yes fragmentation in DNA

DISCUSSION

While *in vitro* sperm toxicity studies are very recent, no studies that were particularly conducted with a herbicide like Atrazine were found. On the other hand, some studies were conducted to estimate *in vitro* sperm toxicity of some toxic substances using CASA systems.

A study investigated the effects of mercury on sperm cells of goldfish, *Carassius auratus,* and considered the sperm motility parameter VCL and sperm morphology (Van Look and Kime, 2003). In their study, they found that 1-10 mg/L concentrations of mercury chloride decreased VCL values dramatically, and 100 mg/L concentration of mercury chloride shortened the length of the flagellum in the sperm cells. In another study (Abascal et al., 2017), 0.01, 0.1, 1, 10 and 100 mg/L concentrations of lead chloride ($PbCl_2$), copper chloride ($CuCl_2 \cdot 2H_2O$) and mercury chloride ($HgCl_2$) were added onto the activation solution on the sperm cells of sea bass, *Dicentrarchus labrax*, and their effects were studied. In the study, a reduction in all sperm motility parameters after 20 seconds was recorded in this fish species, whose sperm motility has a very short duration (<50s). Additionally, it was found that 100 mg/L of copper and lead added to the activation solution did not affect the sperm cells motility parameters. On the other hand, it was found that 0.4-1 mg/L (1:39 dilution rate) of mercury chloride changed the final swimming morphologies of the sperm cells, and dosages lower than 0.1 mg/L (1:2500 dilution rate) affected the motility parameters with a tendency for a complete halt (Abascal et al., 2007).

Dietrich et al. (2010) treated the sperm cells of the rainbow trout, *Oncorhynchus mykiss* with 1-10 mg/L of mercury and 10 mg/L cadmium, and observed changes in the motility of the sperm cells after 4 hours of incubation (Dietrich et al., 2010). Li et al. (2010a) conducted studies on the motility parameters of sterlet sturgeon (*Acipenser ruthenus*) sperms and their antioxidant responses against environmentally risky heavy metals. They studied the effects of heavy metals Cd, Cr and Cd+Cr after 2 hours of incubation, and found that the dosages of 5 mg/L of Cr, 0.05 mg/L of Cd and 5 mg/L of Cr + 0.05 mg/L of Cd affected sperm motility values negatively (Li et al., 2010a). Moreover, the ecotoxicological effects of cadmium on storing the sperm cells of the gilt-head sea bream, *Sparus aurata* were also investigated (Fabbrocini et al., 2012)**.** The authors found that 50 mg/L of cadmium affected sperm motility values insignificantly in comparison to the control group. Nevertheless, in teleost fish, sperm cells generally move on a straight or slightly curved route right after activation. Under these conditions, Linearity (LIN, the ratio of net distance moved to total path distance (VSL/VCL)) may become a very important determining parameter in finding the curvature of the route. Fertilization may be based on both the number of motile sperm cells, and their velocity (Rurangwa et al., 2004; Bozkurt and Yavaş, 2017).

In our experimental design, the sperm cells incubated in Atrazine added artificial seminal plasma in Setting 1, while they did exposure directly with Atrazine in Setting 2. The value of VSL in Setting 1 did not more decrease than the values of VSL in Setting 2. Other hands, the values of VCL decreased at 0.1 mg/L dose of Atrazine in Setting 1. This results may be explained by protective role of artificial seminal plasma which inhibited to all acts of sperm cells in incubation time (Alavi and Cosson, 2006; Dietrich et al., 2010). Additionally, the decreasing of velocities in sperm cells may take place because of especially ATP consumption after activation (Dzyuba and Cosson 2014)spermatozoa must access, bind, and penetrate an egg, processes for which activation of spermatozoa motility is a prerequisite. Fish spermatozoa are stored in seminal plasma where they are immotile during transit through the genital tract of most externally fertilizing teleosts and chondrosteans. Under natural conditions, motility is induced immediately following release of spermatozoa from the male genital tract into the aqueous environment. The nature of an external trigger for the initiation of motility is highly dependent on the aquatic environment (fresh or salt water in Setting 2. However, our results on the VSL and VCL values were in parallel to those in studies conducted with other species (Van Look and Kime, 2003; Dietrich et al., 2010; Li et al., 2010a)**,** and are also in agreement with the results reported by Wang et al (2011).

Finally, it may be stated that Atrazine on the level of 0.5 mg/L showed lethal effects in all samples and 0.1 mg/L Atrazine level had negative effects on DNA fragmentations of sperm cells after *in vitro* incubation for 3 hours. However, it was shown that the motility parameters of sperm cells in *Cyprinus carpio* were affected negatively in direct exposures of the motile solution to Atrazine. Consequently, while the data obtained here showed that fish sperm cells are significant indicators in ecotoxicological studies, this study

may be seen as a contribution in determining the toxic dosages of Atrazine. Moreover, it is also expected that it will help understand ecotoxicology of herbicides in general.

CONFLICT OF INTERESTS

None of the authors has any conflict of interests to declare.

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