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Effects of cadmium on morphological structure of sperm in *Caspiomyzon wagneri* (Kessler, 1870) (Petromyzontiformes : Petromyzontidae)

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ABSTRACT

The present study aimed to investigate effects of the heavy metal Cd on the sperm morphological indices of *Caspimyzon wagneri*. The sperm were exposed to 0.01, 0.1, 1, 10, 100 and 1000 mg.L^{-1} Cd for three minutes, three and 36 hours. The sperms exposed to Cd showed an increase in the length, width and surface of the head, flagella degradation and slightly flagella breakage. With increase of Cd concentration or the exposure duration, the damages found in sperms increased. But when exposure to contaminant exceeds than certain period of time, damage effects caused changes in the sperm structure as head length reduction and head width increasing and thereby reducing the sperm's head surface, complete cutting of flagella at all samples and deformation of the sperm's head from oval to circular, which these changes became visible during 36 hours of exposure to cadmium. Therefore, the results revealed that the arrival of pollutants including cadmium to the natural reproduction ground of this species due to induction the harmful effects on sperm morphology factors will have adverse effects on sperm function and fertilization rate and reduce them, and as such will be considered a serious threat to the survival generation of this rare species.

Key words: Sperm, Morphological structure, Cadmium, *Caspimyzon wagneri*, Caspian Sea.

INTRODUCTION

Heavy metals get into aquatic ecosystems stemming from natural and anthropogenic sources becoming a serious threat to organisms (Eisler 1988). Heavy metals do not degrade and thus is led chronic exposure of fishes to contaminants. Due to the inability to excretion and detoxification, various physiological changes gradually decrease the growth and survival rates, and impair the continuity of the generations of fishes (Erickson *et al.* 2008). In addition, heavy metals are among the Endocrine Disrupting Chemicals (EDCs) that impact the reproductive system in fishes (Hatef *et al.* 2013), for example, they interfere the synthesis of sex hormones and delay the sexual maturation (Hela *et al.* 2005). In males, EDCs influence the sex hormones concentration, semen quality, spermatogenesis, number of spermatogeneic cells and sperm structure (Hela *et al.* 2005). In addition, EDCs accumulate in tissues of living organisms due to lack of biodegradation, transfer to trophic levels through food web and thus their risk becomes double creating ecological effects (Geyer *et al.* 2000).

Heavy metals are the common water pollutants (Kar *et al.* 2008) and among the heavy metals, Cd may have a greater risk as Cd salts dissolve easily in water, compared to salts of other elements (e.g. sulfides, carbonates, hydroxides, and fluoride) and thus, Cd is piled on the body of fishes (Kumar & Singh 2010). In addition, the biological half-life of Cd in animals' soft tissues

and bone is long, between 10 to 30 years (Kumar & Singh 2010).

Water pollution may have vast deleterious effects on the reproductive system of fishes (Hatef *et al.* 2013). For instance, accumulation of toxins in fish testis causes atrophy of leydig cells, a reduction in synthesis of sex hormones, size and number of germ cells and the diameter of the seminiferous ducts (Dutta & Arends 2003). *Caspiomyzon wagneri* is a native species, inhabited the northern, southern and western basins of the Caspian Sea (Sattari *et al.* 2002). Mature individuals migrate to upstream of rivers, where they spawn in shallow areas with sandy and rocky bed and die after spawning (Coad, 2016). In recent years, the stocks of this species have been reduced significantly due to the construction of dams, habitat degradation and contamination of rivers (Close *et al.* 2002). In this respect, there is a wide variety of human activities around the Caspian Sea including agriculture, industry and tourism that are possible source of contamination releasing materials into the Sea through rivers where anadromous fishes reproduce. Among the contaminants of the Caspian Sea, heavy metals have a special place and, in fact, there are numerous studies on heavy metals in the Sea (Watanabe *et al.* 2002; De Mora *et al.* 2004; Tabari *et al.* 2010). Hence, the present study aimed to evaluate effects of different levels of Cd on morphological parameters of sperm in *C. wagneri* sampled during its reproductive migration. Finding the impact of different levels of the metal in sperm morphology may help to better understanding of effects of pollutants on the spawning grounds and can thus be useful for conservational managers.

MATERIALS AND METHODS

Fifteen male *C. wagneri* were caught using a hand-net from the downstream of the Shirud River (36°51'20"N, 50°47'57"E). Immediately after sampling, the abdomen of the specimens were dried using a clean towel and milt collected by pressing the abdominal area (Linhart *et al.* 1995). Working solutions of 0.01, 0.1, 1, 10, 100 and 1000 $mg.L^{-1}$ of Cd were prepared using $CdCl_2$ (Merck®).

The experiment of three minutes exposure was performed to simulate the effects of Cd on the sperm structure from the arrival time in the river through the time sperms reach the eggs.

The three and 36 hours experiments were performed to study the possible spermatozoids structure changes inducted by Cd accumulated in the gonad of fish. For this purpose, sperms were exposed to different concentrations of Cd for three minutes, three and 36 hours. Treatments at each exposure time were a control group and six concentrations of Cd, with three replicates. In this study, 21 sterile microtubes were prepared for each exposure time, each containing 1000 µL of various concentrations of Cd. Distilled water was used for the control group. Each microtube received 10 µL of sperm that were mixed slowly with Cd solution. At the end of the given exposure time, sperm were preserved at 2.5% glutaraldehyde solution and 0.1 M phosphate buffer and then kept at 4°C for 48 hours for later examination using a light microscopy and scanning electron microscopy (SEM) (Psenicka *et al.* 2007). Both normal and abnormal sperm were photographed using a SEM (model XL-30, Netherlands). Various sperm morphological characteristics and indicators were measured using ImageJ software, including the head length (L), head width (W), head surface (A), the index of head elongation (Elongation = 100 × (L-W)/(L+W)), the head ellipticity index (ellipticity = W/L), the flagellum length (Lt), and the percentages of the head, flagellum and total destructions (Brito 2007). The indicators of the head destruction were a large or small head, deformities in the head (the whip, needles and club-shaped), having two heads and the rupture of the head membrane. The indicators of flagellum destruction were flagellum breakage, shortening and twisting. The indicators of total destruction were the percentage of the head and flagellum destruction (Brito 2007). Data were analyzed using SPSS software.

The Kolmogorov-Smirnov and the Levene tests were used to examine normality of the data and homogeneity of variances. A One-Way

ANOVA was used to examine a significant difference between effects of various concentrations of Cd on sperm morphological characteristics, and in the case of a significant difference, the Duncan multiple range test was used to examine further the averages of the different concentrations of Cd. A Pearson correlation was used between the sperm characteristics and Cd concentration to examine a significant correlation between these variables.

RESULTS

When sperm exposed to Cd for three minutes, the head length of sperm increased with increasing in Cd concentration. There was a significant difference between the control and concentration of 1 $mg.L^{-1}$ in the head length of sperm. A Pearson correlation analysis showed a positive correlation ($r=0.185$, $P<0.01$) between different concentrations of Cd and the head length of the sperm at three-minute exposure (Fig. 1-A). There was no significant difference in head length between the control group and the group exposed to 0.01-1 $mg.L^{-1}$ in the three – hours experiment. However, a significant difference was found between the treatments exposed to Cd concentration > 1 $mg.L^{-1}$. There was a significant positive correlation between Cd concentration and the head length of the sperm over three hours of exposure ($r = 0.181$, $P<0.01$) (Fig. 1-A). The head length of the sperm exposed to different concentrations of Cd decreased compared to those of the control at the 36 - hour experiments, but the decrease was not statistically significant for the sperm exposed to 0.01 $mg.L^{-1}$. However, a significant difference was found in treatments exposed to the higher Cd concentrations ($P<0.05$). Again, no significant difference was found in the head length of sperms between the treatments exposed to Cd concentrations of 10 - 1000 $mg.L^{-1}$, and the control. The Pearson correlation analysis showed a negative significant relationship between different concentrations of Cd and the head length of the sperm over a 36-hour exposure ($r = 0.262$, $P<0.01$, Fig. 1-A). In the three-minute exposure experiment, there was no significant difference in the head width between the sperms exposed to different Cd concentrations and those in the control group. However, among different Cd concentrations, a significant difference was found between the head width of the sperms exposed to 0.01 and 1 mg L^{-1} ($P<0.05$). A Pearson correlation analysis indicated that there was no significant correlation between Cd concentration and the head width of the sperm at the three-minute exposure experiment ($r = 0.021$, $P>0.05$) (Fig. 1-B). Over the three-hour exposure, the head width of the sperms faced to 0.01 and 0.1 $mg.L^{-1}$ Cd, was similar to those of the control group ($P>0.05$). However, the head width of those exposed to 1 $mg.L^{-1}$ Cd decreased significantly compared to those of the control group ($P<0.05$). A decrease was found in the head width of the sperms exposed to 0.01-1000 $mg.L^{-1}$ Cd, but it was not significant. A Pearson correlation analysis between different concentrations of Cd and head width of the sperms showed a negative significant correlation at the three-hour exposure experiment ($r = 0.155$, $P<0.01$) (Fig. 1-B). Sixty six hours exposure to different concentrations of Cd did not result in a significant difference in the mean width of the sperm's head ($P>0.05$). A Pearson correlation analysis did not show a significant relationship ($r = 0.014$) between different concentrations of Cd and the width head of sperms within 36 hours of exposure time (Fig. 1-B).

During the three-minute exposure to Cd, the mean surface of the sperm's head increased with increasing in Cd concentration. There were significant differences in the mean surface of the sperm head between the sperms in the control group and those exposed to 1 $mg.L^{-1}$ and also between those exposed to 1000 $mg.L^{-1}$ and all other treatments. The Pearson correlation analysis showed a positive significant correlation ($r=0.135$, $P<0.01$) between different concentrations of Cd and the surface of the sperm head at three-minute exposure experiment (Fig. 1-C). During a three-hour exposure to Cd, almost none of the treatments had a significant difference with the

sperms in the control in the terms of the sperm head's surface. However, a significant difference was detected between the sperms exposed to 1 and 100 mg L^{-1} in this characters. The Pearson correlation analysis showed no significant correlation (r = 0.012) between different Cd concentrations and the head surface of sperm during the three-hour exposures (Fig. 1-C). During the 36-hour exposure, by increasing in Cd concentration, the surface of the sperm head decreased significantly, compared to those in the three-hour and three-minute exposures ($P<0.05$). Also, there was a significant difference in the surface of head between the sperms exposed to 0.1 $mg.L^{-1}$ Cd and those in the control group ($P<0.05$). However, no significant difference concerning to the surface of head was detected between the sperms exposed to 10 to 1000 $mg.L^{-1}$ and those in the control ($P>0.05$). A Pearson correlation analysis showed a significant negative correlation ($r=0.196$, $P<0.01$) between different Cd concentrations and the surface of the sperm head during the 36 hours of exposure (Fig. 1-C).

During the three-minute exposure to Cd, a significant difference was detected in the sperm head elongation indicators (i.e. elongation and ellipticity indexes) between the sperms exposed to concentrations >100 $mg.L^{-1}$ and the control group ($P<0.05$) (Fig. 2). The index of elongation increased with increasing in Cd concentration, whereas the index of ellipticity decreased. This represents a further increase in the head length rather than the head width over the three-minute exposure to Cd (Fig. 3). Over three hours of exposure, the elongation of the sperm head was more visible compared to the three-minute exposure, so that a significant difference was detected between the sperms exposed to 1 $mg.L^{-1}$ and those of the control ($P<0.05$). At the concentrations >100 $mg.L^{-1}$, there was a significant difference in elongation indices between the sperms exposed to 0.01 and 1 mg. L^{-1} and those in the control group. However, no such difference was detected between the sperms exposed to 100 and 1000 $mg.L^{-1}$ (Fig. 3). Over the 36-hour exposure to Cd, with increasing in Cd concentration, the length of the sperm's head decreased but the width did not, so that, the overall shape got closer to a circular one. No significant difference was found in the sperm elongation index between the sperm exposed to 0.01 $mg.L^{-1}$ Cd and the control group. However, by increasing in Cd concentration to 0.1 $mg.L^{-1}$, a significant difference was found ($P<0.05$). At 100 and 1000 $mg.L^{-1}$, the rate (%) of elongation was at the lowest and showed a significant difference with all other treatments with lower Cd concentrations ($P<0.05$) (Fig. 3). There was a significant correlation between different Cd concentrations and the ellipticity index in sperms at the three-minute ($r = 0.106$, $P<0.01$) and three-hour ($r = 0.228$, $P<0.01$) exposures. There was also a significant and positive correlation over the 36 - hour exposure ($r = 0.229$, $P<0.01$). The Pearson correlation analysis found a positive and significant correlation between different Cd concentrations and the rate (%) of elongation index at three-minute ($r = 0.106$, $P<0.01$) and three-hour ($r = 0.231$, $P<0.01$) exposures, while a negative and significant correlation over the 36-hours ($r = 0.234$, $P<0.01$).

During the three-minute exposure, tortuosity and shortening of flagella increased by increasing in Cd concentration. There was a significant difference between the sperms exposed to 10 $mg.L^{-1}$ and lower Cd concentration and those in the control group. Exposure to Cd for three minutes resulted in a high rate of damage to the sperm head rather than the flagella. There was a significant difference in the head degradation between the sperms exposed to 0.1 $mg.L^{-1}$ and those in the control group. There were no significant differences in the destruction of the sperm head from the concentrations of 0.1 through 100 $mg.L^{-1}$. However, by increasing to 1000 $mg.L^{-1}$, the destruction of the sperm head were significant compared to its all lower concentrations (Fig. 4-A).

The Pearson correlation analysis in the period of three minutes did not exhibit a significant correlation in the flagella destruction between

different Cd concentrations (r = 0.094, P>0.05), while showed a positive and significant correlation between its various concentrations and the rate (%) of the flagella degradation (r = 0.5090, P<0.05). It also showed a significant positive correlation between the various Cd concentrations and the rate (%) of sperm head degradation (r = 0.739, P<0.01) and as well as the rate (%) of the total destruction (r = 0.760, P<0.01).

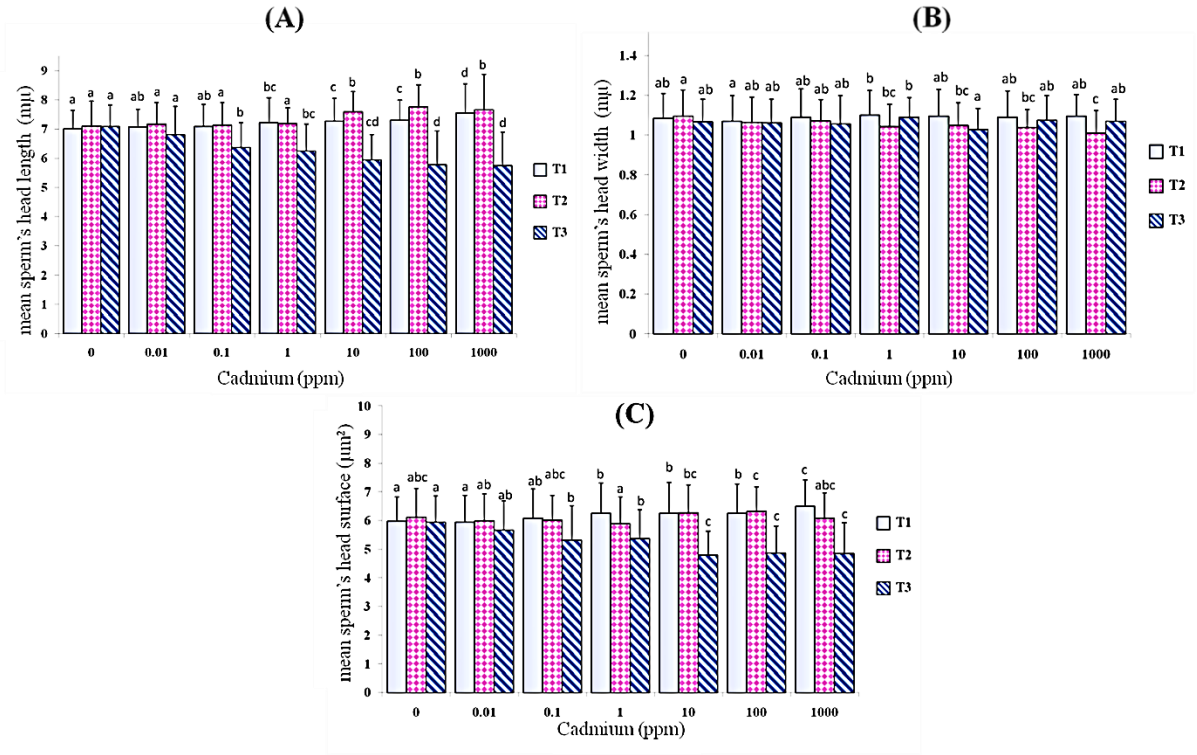

Fig. 1. (A) Sperm's head length, **(B)** sperm's head width and **(C**) sperm's head surface treated by different Cd concentrations (T1: 3 - min (n (number of observations)=1418), T2: 3 - hour (n=1369) and T3: 36 - hour (n=1442) exposure times).

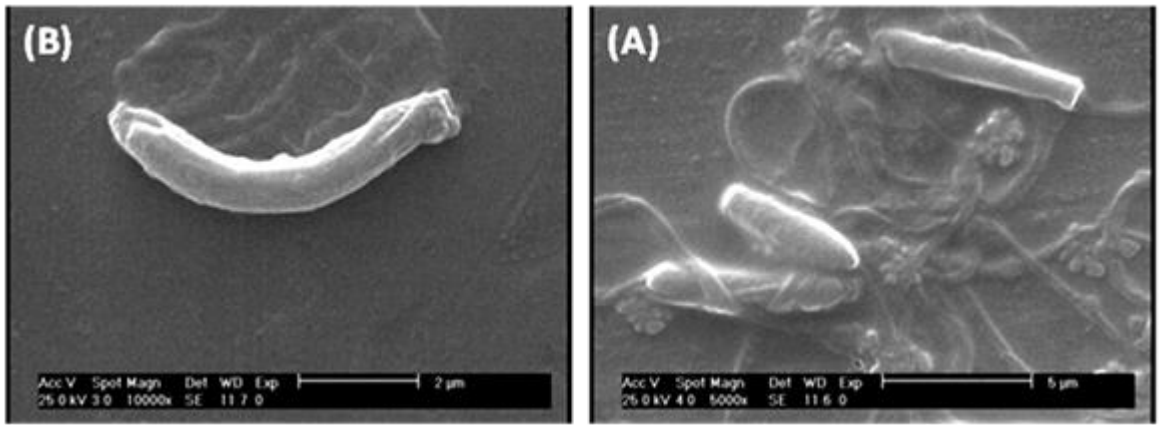

Fig. 2. (A) Sperm exposed to 1000 mg.L^{-1} Cd concentration for three hours (flagella twisting and sperm with increased and decreased head size was found; 5000X), and **(B)** sperm exposed to 10 mg.L^{-1} Cd for 36 hours (a decrease in the head length and curvature of the head and also the destruction of the cell wall and flagella were found; 1000X).

The destruction of head and flagella showed a significant increase at the three-hour exposure. During this period, flagella destruction at the Cd concentrations >100 mg. L^{-1} showed a significant difference with those of the control. The highest destruction of flagella was found in the concentration of 1000 mg.L^{-1}. During the three-hour Cd exposure, flagella twisted and shortened significantly compared to exposure for three minutes.

The mean head destruction in sperms exposed to 0.01 mg.L^{-1} (12.6%) Cd had a significant difference with those of the control group. However, no significant difference was found between 0.01 and 1 mg.L^{-1}.

There was a significant difference in the head destruction between the sperms exposed to 10 mg.L^{-1} (27.3%) and 1 mg.L^{-1}. However, no significant difference was found between the sperms exposed to 10-1000 mg.L^{-1}. By increasing in Cd concentration, the number of sperms with only head destruction increased. Therefore, the difference between the total damage chart and the flagella destruction plot increased (Fig. 4-B).

The Pearson correlation analysis showed a positive significant correlation between different Cd concentrations and the rate (%) of flagella destruction ($r = 0.622$, $P<0.01$), as well as the head destruction ($r = 0.500$, $P<0.05$) over the three-minute exposure.

During the 36 hours of exposure to Cd, sperms were damaged severely, even at concentration as low as 0.01 mg.L^{-1} causing the head destruction, flagella breakage, flagella destruction and total destruction with a mean of 87.6%, 41%, 94.6% and 97.1%, respectively (Fig. 4-C). The flagella destruction showed a significant difference with increasing in Cd concentration to 1000 mg.L^{-1} compared to 0.01, 0.1 and 10 mg.L^{-1} ($P<0.05$). A significant difference in the sperm head degradation was found between exposure to 1000 mg.L^{-1} Cd and 0.01 mg.L^{-1} ($P<0.05$).

The Pearson correlation analysis during the 36 - hour exposure displayed only a positive and significant relationship between different Cd concentrations and flagella destruction ($r = 0.476$, $P<0.05$).

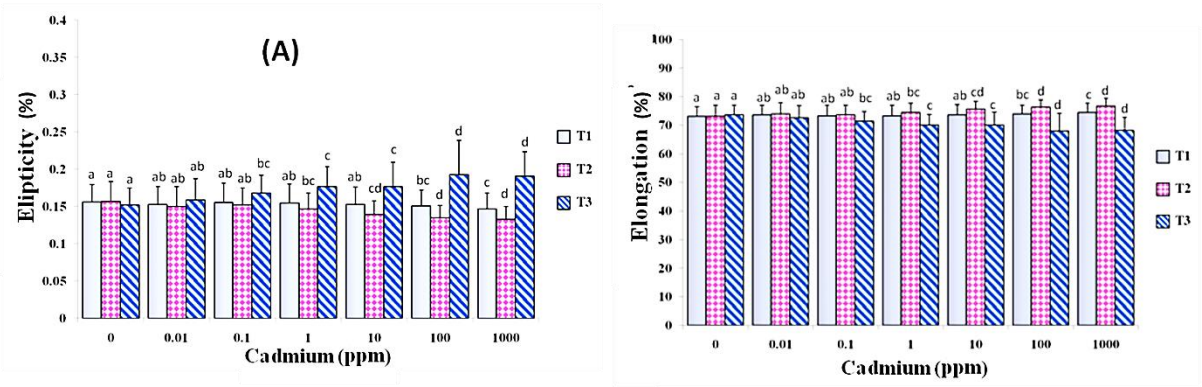

Fig. 3. (A) The sperm head ellipticity index and **(B)** elongation index under the effect of different Cd concentrations (T1: 3 min (n=1418), T2: 3 - hour (n= 1369), and T3: 36 - hour (n= 1442) exposure times).

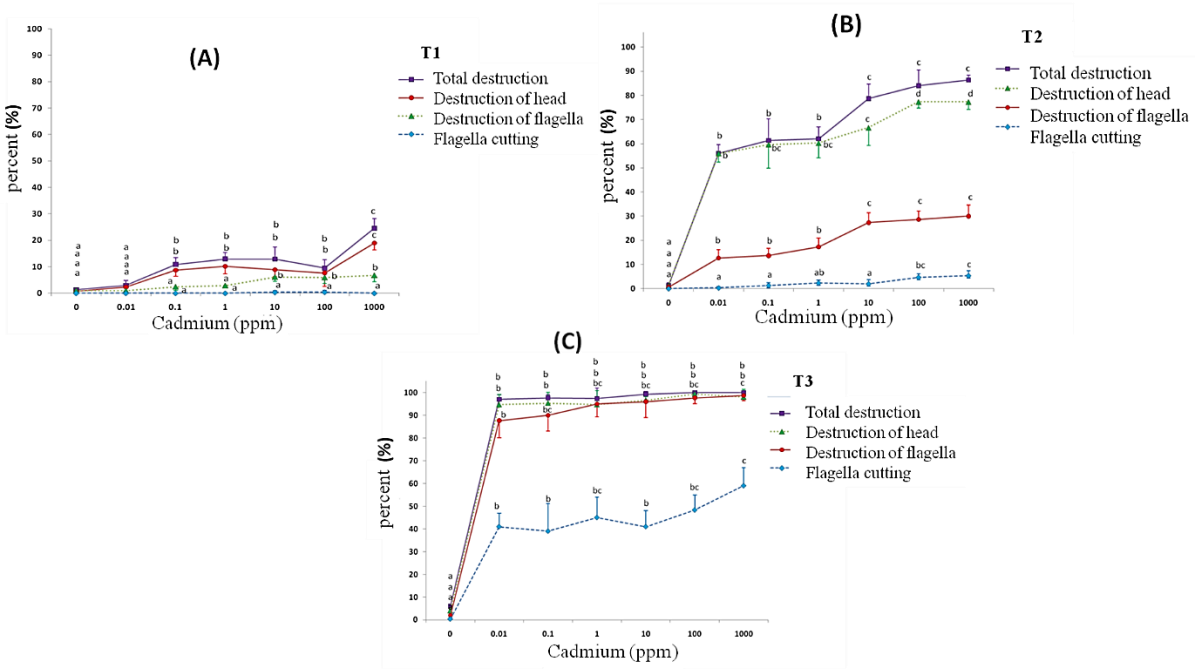

Fig. 4. (A) Sperm destruction under effect of different Cd concentrations at 3 min (n: 0 = 236, 0.01 = 334, 0.1 = 375, 1 = 283, 10 = 269, 100 = 312, and 1000 = 278), **(B)** 3 - hour (n: 0 = 312, 0.01 = 226, 0.1 = 352, 1 = 384, 10 = 268, 100 =219 and 1000 = 324), and **(C)** 36 - hour (n: 0 = 218, 0.01 = 348, 0.1 = 264, 1 = 231, 10 = 325, 100 = 275, and 1000 = 304) exposure times.

DISCUSSION

Sexual reproduction in fish is associated with the production of high-quality sperms. Any damages to the structure of sperm due to chemicals and pollutants can lead a sperm with big or small head, shortening or flagella cutting, head sperm twisted or rupture of the membrane which affect the ability of the sperm motility and fertilization (Van Look & Kime 2003).

During the three-minute exposure of the sperms to Cd, an increase was found in the sperms' head length and width. Van Look and Kim (2003) exposed the goldfish sperm (*Carassius auratus*) to different Hg concentrations and reported that the sperm's head had no significant change but the flagella was shorter compared to normal sperms. Heavy metals such as Cu, Cd and Zn bind to proteins or enzymes, degenerating the proteins, influencing the metabolism and structure of sperm flagella, and cease the sperm motility (Dietricha *et al*. 2010).

During the three-hour exposure, by increasing in Cd concentration, the sperm length increased. In the present study, increasing in the size of the sperm due to exposure to Cd may be related to loss of its consistency because of uncoiling DNA that leaves contents of the nucleus through disconnecting the sulfur bridges (Leno *et al*. 1996). In addition, the flagellum twisting is probably due to axial compression and tension in microtubules and thereby separating them, which eventually led to the break of flagella (Au *et al*. 2001). During the three-hour exposure, the total destruction was mainly affected by the flagella destruction. In addition to physiological changes, such as disturbance in motility factors and respiratory system, the SEM exhibited morphological changes, verifying role of Cd on structural and anatomical defects (Bradl 2005; Ebrahimi 2005). When sperm were exposed to Cd for 36 hours, a decrease in the length of the sperms was found in all concentrations, so that, the sperms in many cases lost their cylindrical form and changed to spherical. Furthermore, the head width and surface decreased by up to 35.5% compared to control group. This result may suggests that the severity of sperm destruction increases up a specific value, then the sperm head size starts to decrease. Therefore, a high Cd concentration decreases the size of the

sperm head. In a study on goldfish (*C. auratus*), effects of Hg on the sperms over a 24-hour exposure resulted in an increase in the length, width and surface of the head, while exposures to 10 and 100 $mg.L^{-1}$, lead to decrease in these morphological structures (Van Look & Kime 2003).

The present study indicated that Cd can affect the reproductive success of *C. wagneri* in the contaminated environments through morphological defects in the structure of the sperm during the simulation of Cd accumulation in the testis, exposing sperms to Cd and incubating for three and 36 hours. The damage to the sperms structure increased so that after 36 hours, almost all sperms were injured severely being quite different than those of normal shape. Any contact with a contaminant at a stage of life especially in the reproductive season, even in low concentrations may affect negatively reproductive success and thus survival of fish species.

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اثرات کادمیوم بر ساختار ریختی اسپرم ماهی دهان‌گرد خزری *Caspiomyzon wagneri* (Petromyzontiformes: Petromyzontidae)

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چکیده

مطالعه حاضر با هدف بررسی اثرات فلز سنگین کادمیوم بر شاخص‌های ریختی اسپرم ماهی دهان‌گرد خزری (*Caspiomyzon wagneri*) به اجرا درآمد. برای این منظور، اسپرم در معرض غلظت‌های ۰/۰۱، ۰/۱، ۱، ۱۰، ۱۰۰ و ۱۰۰۰ میلی‌گرم در لیتر کادمیوم به مدت ۳ دقیقه، ۳ و ۳۶ ساعت قرار گرفت. اسپرم‌های در معرض کادمیوم افزایش طول، عرض و مساحت سر، تخریب تاژک، و تعدادی شکستگی تاژک نشان دادند. با افزایش غلظت کادمیوم و یا دوره در معرض قرارگیری، میزان آسیب‌ها نیز زیاد شدند. اما هنگامی که دوره در معرض قرارگیری از حد معینی بیشتر شد، اثرات آسیبی سبب تغییر ساختار اسپرم از جمله کاهش طول سر و افزایش عرض سر شد و به دنبال آن، کاهش مساحت سر، قطع کامل تاژک و تغییر شکل سر از حالت بیضی به دایره‌ای به وقوع پیوست که به وضوح در تیمارهایی که به مدت ۳۶ ساعت در معرض کادمیوم بودند، قابل مشاهده بود. بنابراین نتایج نشان داد که ورود آلاینده‌ها از قبیل کادمیوم می تواند به جایگاه‌های تخم‌ریزی این گونه، به واسطه القای اثرات زیانبار بر ساختار ریختی اسپرم، به عملکرد و نرخ لقاح آن آسیب رسانده و سبب کاهش آنها شود. از این‌رو، چنین روندی باید به‌عنوان یک تهدید جدی بر بقای نسل این گونه نادر مد نظر قرار گیرد.

*مولف مسئول