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Effects of in vitro exposure of mercury on sperm quality and fertility of tropical fish *Cyprinus carpio* L.

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ABSTRACT

Mercury is a widespread aquatic pollutant that adversely affects the reproductive system of male fish. Although the effects of mercuric chloride ($HgCl_2$) on the testicular structure, the sperm count and altered sperm morphology of fish have been investigated, the effects on sperm quality and fertilization are unknown. Therefore, the aim of this study was to investigate the effects of $HgCl_2$ exposure in different concentrations (0; 0.5; 1; 2.5; 5 ppm) on sperm parameters and fertility of male *Cyprinus carpio*. Sperm quality parameters, including mass and individual time of sperm motility (s), and viability (%) were measured using digital inverted microscopy. The fertilization (%) was measured by counting the number of fertilized eggs. Integrity or DNA fragmentation (%) was measured by Acridine orange test (AOT) using fluorescence microscopy. The change of sperm membrane surface was examined using scanning electron microscopy (SEM). The results showed that the in vitro exposure of mercury could significantly decrease some parameters of fish sperm qualities, i.e. decreased the time for mass and individual motility, viability, DNA fragmentation, and fertilization ability ($P < 0.05$) and significantly increase the malondialdehyde levels of sperm ($P < 0.05$). In addition, Hg exposure altered the morphological structure of the sperm head and interfered with the development of embryos in fish eggs.
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Introduction

River is an important ecosystem for human which provides water as a source for various activities such as agriculture, industry and domestic use (Moss, 2007). Water that comes from the spring has a good quality but in the process of water drainage, it will be affected by various pollutants. In recent years, river water quality in Indonesia has contaminated by many pollutants with different characteristics after passing through residential, industrial and agricultural areas. The river pollution in Indonesia has exceeded the quality standard and could negatively affect the life of aquatic biota including fish (Hayati et al., 2017a).

Mercury ($HgCl_2$) is one of the heavy metals found in highly polluting river water due to industrial activity. It is recognized as the most toxic heavy metal to organisms. High Hg concentrations in freshwater fish are from both aqueous and dietary containing mercury and decreased reproduction of adult fish (Pickhardt et al., 2006). Furthermore, the existence of mercury at high concentrations in the water could inhibit metabolic processes leading to fish death (Vangronsveld and Clijsters, 1994). Mercury also impaired the testes function of the tropical fish *Gymnotus carapo* (L.), including the decrease in sperm count and the alternation in sperm morphology (Vergilio et al., 2014). An in vivo study conducted by Lahnsteiner et al. (2004) showed that mercury had significantly decreased the percentage of sperm motility and velocity of *Clarias gariepinus* and *Lota lota.*

The major mechanisms behind metal toxicity including mercury have been attributed to oxidative stress. Mercury possesses the ability to form reactive oxygen species (ROS) that cause the oxidation of DNA and lipids, DNA fragmentation, and lipid peroxidation. Moreover, malondialdehyde (MDA) will be formed after exposure to ROS which is the secondary product of lipid peroxidation (Flora et al., 2008).

Cyprinus carpio is one of the tropical fish that cultivated for human consumption. It is known as a very sensitive fish to the environmental changes. The decline in fish reproduction is associated with the decreasing of the quality and fertilization ability of sperm. Fish sperm is also used as a bioindicator of toxicity parameters (Kime et al., 2001). Previous studies showed that cadmium

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may lead to a decrease in sperm quality, fertilization ability and affect the histology in some organs of *Oreochromis niloticus* and *Barbodes* sp. (Hayati et al., 2016, 2017a,b; Nursanti et al., 2017).

In addition, Mercury in the aquatic environment affects the reproductive health of fish. Accumulation of mercury in the fish brain has resulted in hypothalamic neuron degeneration, reduced gonadotropin-secreting cells at the pituitary, and reductions in gonad size, secretion steroids, gamete production, and spawning success (Crump and Trudeau, 2009). According to Webb et al. (2006), Mercury (methylmercury) has an effect on the reproduction of white sturgeon (*Acipenser transmonatus*) in the lower Columbia River. As well, this research showed that the mean of tissue mercury concentrations was higher in muscles compared to liver and gonads.

Although the adverse effects of in vivo exposure on testicular structure, the sperm count and altered sperm morphology of fish have been investigated, the effects of in vitro exposure on sperm quality including mass and individual time of sperm motility, viability, integrity or DNA fragmentation, and fertilization are unknown. Therefore, it is essential to investigate the effects of the in vitro exposure of mercury on the sperm quality and fertility as well as to determine the concentration value of mercury that cause a decline in the fertility of *C. carpio*.

Materials and methods

Animals

Cyprinus carpio with adult gonads and healthy conditions were obtained from Freshwater Cultivation Management Unit, Batu City, Ministry of Fisheries, East Java Province, Indonesia. Twenty-five male fish, weighed 1–1.2 kg and aged 1–2 years were prepared for sperm collection. Five female fish, weighed 1.5 kg and aged 1.5–3 years were also prepared. Mature males and females were kept in a separate pool under temperature (23–25 °C) and photoperiod (12 h of light per day). Fish were fed with pellets twice a day. All procedures involving animal care were performed under the guidance of the cultivation unit.

Preparation of mercuric chloride ($HgCl_2$) solution

A stock solution of mercuric chloride (200 ppm) was prepared by diluting 0.02 g of mercuric chloride powder in 100 mL of aquadest. Then, solutions of mercuric chloride were prepared with 5 different concentrations (0; 0.5; 1; 2.5; 5 ppm).

Animal experiment

During the animal experiment, *C. carpio* were not anesthetized, but they remained quiet out of the water using cloth to cover their eyes while stripping. After rinsing with distilled water, the genital area was carefully dried. Sperm was collected in 5-ml syringes by gentle pressure. The sperm suspension was prepared by mixing fish sperm and 0.9% NaCl solution at a ratio 1:2 (v:v). Activation of fish sperm was performed by using water. Then, sperm suspension was dissolved in $HgCl_2$ solution with five different concentrations (0; 0.5; 1; 2.5; 5 ppm), five replications, and incubated for five seconds. $HgCl_2$ concentration was determined from LC50 assay conducted in a preliminary study. The result of LC50 assay was 0.214 ppm. Therefore, the concentrations used in this study were more than the LC50 result (0; 0.5; 1; 2.5; 5 ppm).

Sperm motility

Motility was objectively determined by mixing 1 drop of sperm suspension with 2 drops of tap water and observed under a digital inverted microscope (Olympus) at 100× magnification. The observed parameters were the mass duration and individual motility (seconds).

Sperm viability

Supravital staining with 1% aqueous Eosin Y and 10% aqueous Nigrosin solution (Sigma, USA) was used to examine sperm viability. A drop of fish sperm suspension was placed on a spot plate and mixed with one drop of Eosin solution. After 15 s, two drops of 10% Nigrosin solution was added and mixed thoroughly. A drop of this mixture was smeared in a glass slide and air-dried. The smears were examined under a light microscope with 100× magnification. Viable sperm cells appeared transparent and dead sperm appeared pink.

Gametes fertility

Fertilization was conducted by mixing the sperm and eggs in clean water with $HgCl_2$ in different concentrations and incubated for 5–10 s. After stirring using a chicken feather for 1 min, eggs were incubated for 5 min for fertilization process. The eggs were prepared in glass petri dish and placed in a 2 L aquarium. Water was constantly gently aerated at room temperature (20–23 °C). Each group consisted of 8 replications and each replication contained ± 200 eggs. Embryos were observed twice a day for three days and dead embryos (opaque eggs) were calculated.

DNA fragmentation

Dried smear was fixed in Carnoy's solution (Sigma Chemicals, St Louis, MO, USA) for at least 2 h and air-dried. Then, the smear was stained with Acridine Orange (AO) solution. After 5 min, the smear was washed with distilled water, covered with cover glass and mounted. Smears were examined using a fluorescence microscope (Olympus-FSX100, Japan) with the following filter combination: 450–490 nm excitation, 510 nm reflector and 520 nm barrier filter. The nuclei of 200–300 spermatozoa from each smear were examined and scored as green (normal) or red (DNA fragmentation).

Sperm morphology

Fish sperm was fixed in neutral buffered formalin (NBF) solution, dehydrated in ethanol series and air-dried. The dried cells were attached to stubs with carbon double-stick tape and teased apart with needles. The sample was sputter-coated with carbon and gold and was observed via FEI type Inspect S50 scanning electron microscope.

Malondialdehyde (MDA) assay

Around 150 µL of fish sperm was added into 300 µL of ice-cold phosphate buffered saline (PBS) and homogenized by brief sonication on ice (20 s). Malondialdehyde concentration was analyzed by QuantiChromTM TBARS Assay Kit (DTBA-100) (BioAssay Systems, USA) according to the manufacturer's protocol. The absorbance was measured at 535 nm.

Data analysis

The data were analyzed using ANOVA followed by LSD test by Statistical Package for Social Studies (SPSS software version 21).

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The results were presented as mean ± SD. P-values less than 0.05 were considered statistically significant.

Results

Effects of in vitro exposure of mercury on sperm motility

Mercury exposure in high concentrations affects sperm motility of fish. The measurement results of sperm mass duration parameters showed that the control group had significantly ($P < 0.05$) the longest duration (498 ± 15.46 s) compared to the other treatments (1; 2.5; and 5 ppm). In line with the duration of mass motility, the duration of individual motility decreased when mercury exposure increased. All treatment groups showed a significant decrease compared to the control group ($P < 0.05$), except for 0.5 ppm exposure that did not differ significantly ($P > 0.05$). (Fig. 1).

Effect of mercury on the viability of sperm

The results of the collected sperm showed that the control group contained viable sperm but not all of this sperm would continue to live. The percentage of live sperm was determined by identifying the sperm with intact cell membranes. The living cell will be clear, but the non-vital cell will absorb the color.

Exposure to mercury decreases the membrane integrity so that dyes can enter sperm. The viability of sperm in the control group was 97 ± 0.71%. Variations in exposure concentration could significantly reduce the sperm viability compared to the control group ($P < 0.05$). The results showed that the highest value of sperm viability was 91 ± 0.74% in the 0.5 ppm exposure and the lowest was 57 ± 1.19% after 5 ppm exposure (Fig. 2).

Effect of mercury on the fertility of sperm

The results showed that the control group had 96 ± 2.07% of eggs that successfully fertilized by sperm. However, exposure of mercury at 0.5 ppm or more had significantly decreased the ability of egg fertilization ($P < 0.05$). The number of unfertilized eggs increased due to the damage in structure and physiology of sperm and egg cells. Embryonic development was impaired because of the mercury contamination (Fig. 3).

The growth and development of embryos in eggs were normal in the control group. Successful eggs were appeared milky white, but those that fail to be fertilized would be dark. Summary of 72 h of *Cyprinus carpio* embryonic development after mercury exposure with different concentrations is presented in Table 1. This result proved that higher concentrations of mercury inhibited the development of fish embryo.

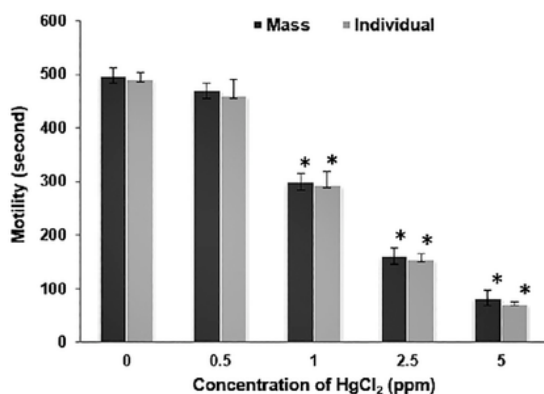

Fig. 1. The duration of mass and individual sperm after exposure to different concentrations of mercury $HgCl_2$. Each bar represents mean ± SD ($n = 5$). $^*P < 0.05$ compared to the control group (0 ppm).

Effect of mercury on DNA fragmentation and morphology of sperm

DNA fragmentation of sperm in the treatment groups was significantly different from the control group ($P < 0.05$). When the mercury concentration increased, DNA fragmentation of sperm was also increased (Fig. 2). Sperm that has normal DNA was appeared green and sperm with DNA fragmentation was appeared red (Fig. 4).

In addition, external investigations were conducted on each fish sperm before and after the experiment. Control sperm had no change in morphological structure (head and flagella). Meanwhile, mercury treatment group showed a change in the shape of sperm head (Fig. 5).

Effect of mercury in malondialdehyde level of fish sperm

Malondialdehyde (MDA) concentration in sperm, in relation to mercury concentration was presented in Fig. 6. The figure revealed that mercury exposure increased the level of MDA. Exposure of 0.5 ppm or more had significantly increased the MDA levels of fish sperm compared to the control group ($P < 0.05$).

Discussion

In aquatic organisms, the male reproductive system was susceptible to the side effects of heavy metals. Heavy metals that accumulated in fish tissue and cells can decrease the quality of gametes especially the sperm motility (Moss, 2007; Govind and Madhuri, 2014). Mercury is one of the abundant metals in the earth which has toxic effects. High concentrations of mercury induce free radical-mediated cytotoxicity and could be toxic for the male reproductive system (Jaishankar et al., 2014). It has been shown that exposure to mercury could decrease the sperm motility and viability due to its ability to produce ROS (Martinez et al., 2014).

Reactive oxygen species are highly reactive oxidizing oxygen derivative compound that can cause cell and tissue damage. Many of researches showed that free radicals which affect the quality of sperm could cause lipid peroxidation. Lipid peroxidation occurs in cell membranes, especially unsaturated fatty acids which are an important component of cell membrane constituents (Powers and Jackson, 2008). When free radicals are produced in vivo or in vitro inside the cells beyond the normal defense mechanism, there will be various metabolic and cellular disorders. Free radicals can damage cells by destroying the cell membrane using some mechanisms including (a) binding covalently with enzymes and/or receptors located in the cell membrane, altering the activity of the components present in the cell membrane; (b) binding covalently to cell membrane components, altering the membrane structure and resulting in changes in membrane function and/or altering the membrane character to be antigenic; (c) interfering with the transport system of cell membranes through covalent bonding, oxidize thiol groups, or by changing polyunsaturated fatty acids; and (d) initiating lipid peroxidation directly to polyunsaturated cell wall fatty acids. Free radicals will cause cell membrane lipid peroxidation. Lipid peroxides will form longer chains and may damage the organization of cell membranes (Sikka et al., 1995).

The success of fertilization was determined by the quality of sperm. In this study, the examination of sperm motility was divided into two parameters, the duration of the mass motility and individual motility (s). Motility of sperm occurs because of

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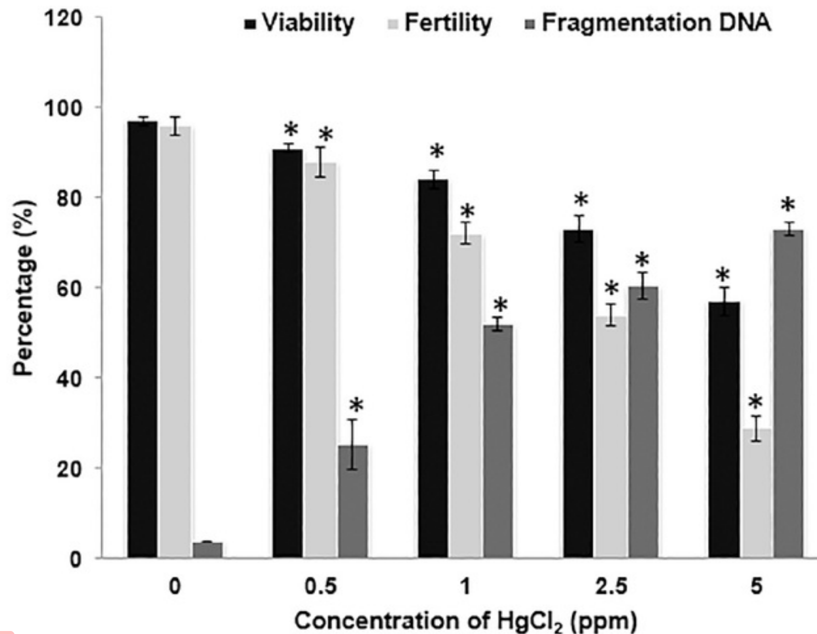

Fig. 2. Viability, fertility and DNA fragmentation of sperm after exposure to different concentrations of mercury ($HgCl_2$). Each bar represents mean ± SD ($n = 5$). $^*P < 0.05$ compared to the control group (0 ppm).

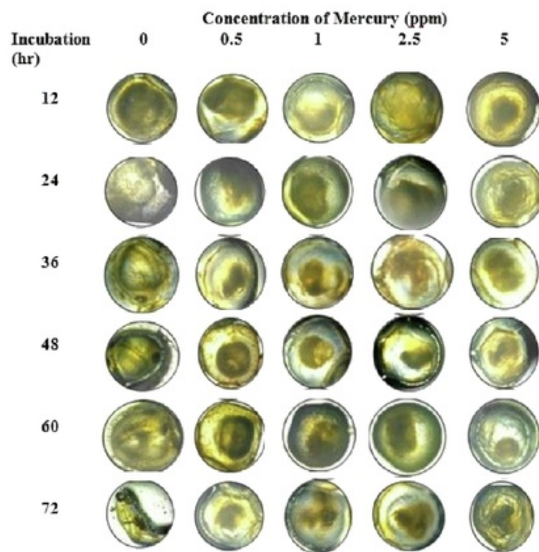

Fig. 3. Development of fish embryo after fertilization on mercury media with different concentrations.

the mitochondrial sheath in the middle of the sperm tail. Sperm tail movements depend on the ATP production by mitochondria with a source of metabolic energy derived from the fructose contained in the diluents (Ansari and Ansari, 2012).

The results showed that the higher concentration of mercury decreased the duration of mass motility and individual motility. Treatment group of 0.5 ppm mercury exposure showed a significant difference although the mercury concentration was small. Mercury was very toxic so that even in small concentrations, it could affect the quality of sperm. Sperm motility was decreased because sperm membranes that were rich in unsaturated fatty acids were particularly vulnerable to ROS attacks. Increase of mercury concentration caused a rapid formation of ROS. High levels of ROS would affect the integrity of sperm DNA and sperm membrane flexibility by oxidizing the membrane and decreasing sperm motility. The integrity of cell membranes was crucial to the metabolic processes that depend on ATP energy supply. Damaged membranes inhibited the ATP production by disruption of outflow and electron transport required in cell metabolism, so cells could not metabolize properly (Jaishankar et al., 2014; Martinez et al., 2014).

The ability of ROS to decrease the sperm motility through membrane lipid peroxidation and production of MDA, resulted in a decreasing of sperm tails flexibility and movement. Furthermore, mercury could bind to sperm tail proteins that affect sperm movement and beat-cross frequency or could bind to enzymes affecting sperm cell metabolism that could lead to a decrease in the duration of motility of mass and individual sperm (Dietrich et al., 2010).

Viability was one of the sperm quality parameters that is required to examine the fertilization function of sperm. It is the ability to survive and to allow sufficient time for sperm to reach the place of fertilization. Based on observations and data analysis, the higher the concentration of mercury, the more sperm will die. Higher mercury concentration could increase the level of toxicity that caused lack of nutrients for sperm to survive. Mercury was also able to produce ROS that directly damages the DNA of sperm by attacking purine and pyrimidine bases. Reactive oxygen species could initiate the occurrence of apoptosis in sperm, causing the activation of caspase enzymes to degrade sperm DNA that can decrease the survival ability of sperm (Tremallen, 2008).

Fertility was the ability of a fish sperm to fertilize an egg. In the process of fertilization, merging the sperm core with the egg core occurs in the cytoplasm to form a zygote. Not all of the fertilized eggs had developed and hatched into larvae, this was included in the type of eggs with poor quality and the pollution of heavy metals (Nica et al., 2012; Mojer, 2015). The present results showed that

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Table 1
Summary of 72 h of *Cyprinus carpio* embryonic development after mercury exposure with different concentrations.

Incubation (h)	Concentration of mercury (ppm)				
	0	0.5	1	2.5	5
12	Gastrula period. Early differentiation indicates development of the head and tail	Showed the mass of clustered eggs	The eggs were damaged	The eggs were damaged	The eggs were damaged
24	The segmentation period. Eggs turned whitish. the tail and head were slighty seen	The eggs were damaged	The eggs were damaged	Dead embryo	Dead embryo
36	The end of segmentation period. The tail and head were seen, eyes were clearly seen	Dead embryo	Dead embryo	Dead embryo	Dead embryo
48	The beginning of the pharyngula period. Head, eye and tail region were clearly seen	Dead embryo	Dead embryo	Dead embryo	Dead embryo
60	The end of pharyngula stage. Head, eye and tail were more elongated than before	Dead embryo	Dead embryo	Dead embryo	Dead embryo
72	The larvae, pectoral fins were slightly seen	Dead embryo	Dead embryo	Dead embryo	Dead embryo

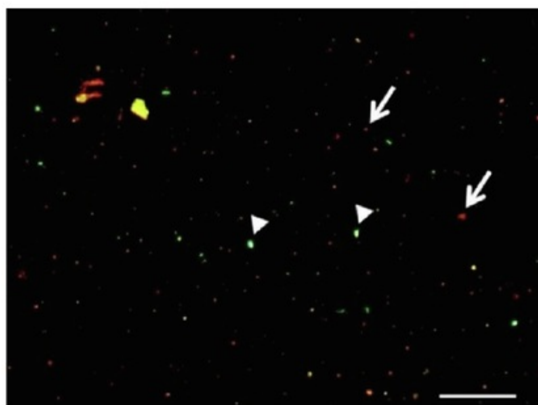

Fig. 4. Fragmentation of sperm DNA after $HgCl_2$ exposure (200×). Sperm that has DNA fragmentation (◄), normal sperm (↓). Scale bar = 100 μm.

0.5 ppm mercury had significantly decreased the success of sperm fertilization. This result happened because of the very toxic nature of mercury and the increase of ROS, which may affect the fertility and the development of fish embryos. In the treatment group with the highest concentration (5 ppm mercury), it showed a very low fertility percentage. A study conducted by El-Greisy and El-Gamal (2015) also reported that heavy metal pollution specifically cadmium could affect the development of the embryos of common carp. The decrease in fertility percentage occurred because of the mercury contamination of the egg that affected the quality of sperm, so it could inhibit the occurrence of fertilization in fish eggs (Fürböck et al., 2009; Martinez et al., 2014; Ansari and Ansari, 2015).

In the control group, fertilized egg appeared to have a complete embryonic development. Embryo development was influenced by the stability of the DNA of sperm which was organized to keep chromosomes stable. The organization of this DNA not only allows a very tight packed genetic information to be transferred to the eggs but also ensures that the DNA is delivered in a physical and chemical form that enables the embryo to develop for easy access to genetic information. Moreover, abnormalities in sperm chromatin and DNA fragmentation of fish were influenced by environmental factors. The fertile sperm has a stable DNA, capable of decongestion at just the right time in the fertilization process and transmits the DNA completely. The genetic material in the sperm was damaged when the chromosome was in condensation or during maturation of the sperm nucleus, or aneuploid chromosome. The main factors causing sperm DNA damage include several mechanisms: abnormal chromatin packing, ROS, and apoptosis. Detection of damage or fragmentation of oxidative DNA in sperm was related to sperm morphology structure, function, and infertility.

Oxidative stress which changes the DNA integrity of sperm is one of the failure causes of sperm fertilization. This change caused abnormality in sperm function, especially affecting motility, morphology, and sperm viability (Zini et al., 2008). Changes in DNA integrity have a risk of increasing DNA fragmentation, that was associated with both in vivo and in vitro fertility potential. There was an increase in DNA fragmentation in sperm (≤ 40%). On the other hand, from the results of this study, there was a positive correlation between motility and sperm viability, which indicated that the sperm motility was depended on the sperm viability. Meanwhile, there was a negative correlation found between sperm

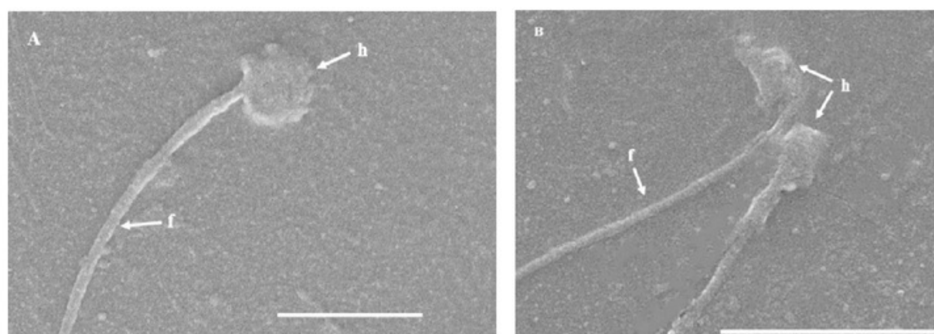

Fig. 5. Sperm morphology was observed with scanning electron microscopes (SEM). A, normal sperm; B, abnormal sperm (abnormal head morphology); h, head; f, flagellum; (15,000×). Scale bar = 3 μm (normal sperm) and 5 μm (abnormal sperm).

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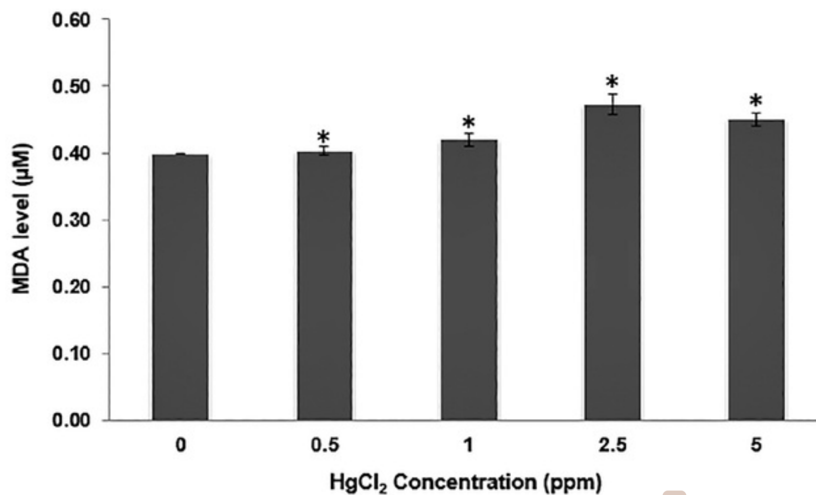

Fig. 6. Malondialdehyde levels of fish sperm after exposure to different concentrations of $HgCl_2$. Each bar represents mean ± SD ($n = 5$). $^*P < 0.05$ compared to the control group (0 ppm).

motility with DNA fragmentation and MDA level. Oxidative stress could increase lipid peroxidation, resulting in an increase of MDA level and also indicate the infertility (Hosen et al., 2015). In particular, semen with a high percentage of damaged spermatozoa (e.g. denatured DNA) has a very low potential for natural fertility. In external aquatic fertilization animals, DNA fragmentation of sperm was probably occurred because of internal factors, such as apoptosis in seminiferous tubular epithelium, defects in chromatin remodeling during spermatogenesis and free radical production during sperm migration from seminiferous tubules to other (urogenital) male reproductive tracts. The external factors that affect DNA fragmentation of sperm are changes in the aquatic environment such as pollution and changes in water temperature, acidity, and dissolved oxygen level (Hayati et al., 2017c). The success of sperm fertilization in egg cells is also influenced by the integrity of DNA in sperm including the process of fertilization and the development of embryos in eggs. The result of this study showed a high success rate of fertilization (96%) with low DNA fragmentation (4%) in the control group.

To conclude, It was found that mercury ($HgCl_2$) exposure at 0.5 ppm and above could lower the sperm motility (mass and individual duration), viability, gamete fertilization, and could increase the DNA fragmentation as well as the MDA levels of *C. carpio* sperm. Exposure to mercury was also found to change the shape of sperm head and it could impair the embryonic development of *C. carpio*.

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