Toxicity of copper pollution on sperm quality of *Cyprinus carpio*

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**Abstract.** Water pollution by heavy metal waste from industry, agriculture, and other human activities affect the reproductive health of aquatic biota, especially freshwater fish. This study aimed to evaluate the quality of *Cyprinus carpio* sperm contaminated with copper (Cu). The sample used was gonad from mature fish. The sperm and eggs were taken by stripping, six times of repetition. Cu with a concentration of 0, 10, 25, 50, and 75 ppm were used to examine the sperm quality (duration of motility and viability, DNA fragmentation, malondialdehyde level) and the ability of sperm to fertilize. The data was collected after sperm incubation with the variation of Cu concentration for five seconds. The sperm observation was done under inverted and fluorescence microscopes. The results showed that Cu exposure caused fertilization failure, thus reducing the number of fertilized eggs. The increase of Cu concentration from 10 ppm to 25 ppm caused a decrease in sperm quality and sperm fertilization. The increase in Cu concentration also raised the percentage of DNA fragmentation. In conclusion, in vitro exposure of Cu reduced sperm motility and viability as well as reduced the fertility in *Cyprinus carpio.*

1. **Introduction**

Water is one of the renewable and dynamic natural resources, that is essential for all living things on Earth. Some sources of water include lakes, groundwater, streams, and rivers. Rivers are one of the water sources which are beneficial for both humans and aquatic biota. The rapid increase in industrialization and humans activities near the stream areas cause many toxic contaminants to be discharged into the rivers result in water pollution [1]. One of the main water contaminants is heavy metals from industrial or agricultural activities. Heavy metal pollutants also affect the reproductive health and fertility of fish [2]; increase methalothionine concentrations [3]; and increases testicular and kidney damage in *Oreochromis niloticus* [4].

Although high concentrations of heavy metals can harm the aquatic organisms, the smaller quantities of heavy metals are important for physiological activity, such as Cu, Cr, Fe, Mg, Mn and Zn which are the important nutrients for various biochemical and physiological functions. These compounds play an important role in various oxidation-reduction reactions. They also serve as
important co-factors for several enzymes associated with oxidative stress including catalase, superoxide dismutase, peroxidase, cytochrome c oxidase, ferrooxidases, monoamine oxidase, and dopamine β-monoxygenase [5] [6]. Thus, heavy metals become important nutrients that are incorporated into several metalloenzymes involved in the formation of hemoglobin, carbohydrate metabolism, and catecholamine biosynthesis.

The heavy metals are known to affect cellular organelles like cell membranes, mitochondria, lysosomes, nuclei, and endoplasmic reticulum in systems of biology. Metal ions are capable of interacting with DNA and nuclear proteins, causing DNA damage and apoptosis [7]. Numerous studies demonstrated that reactive oxygen species (ROS) production and oxidative stress play an important role in the toxicity of the metal which is considered as systemic toxicants that could induce the damage of multiple organs, even at lower levels of exposure. Heavy metal Cu toxicity depends upon the absorbed dose, the duration, and route of exposure. This can lead to various disorders and can also result in excessive damage due to oxidative stress induced by free radical formation. One of the aquatic organisms that are prone to contamination is C. carpio fish. Good quality fish seeds are determined by the quality of fish sperms. The focus in this study is to evaluate the effect of Cu on sperms motility, sperms viability, DNA fragmentation, MDA level of sperms and fertility of C. carpio.

2. Materials and Methods
The materials used in this study were copper sulfate (CuSO4, Sigma-Aldrich) compounds (0, 10, 25, 50 and 75 ppm), 1% Eosin and 10% Nigrosin, 0.9% physiological NaCl solution, pH 7-7.2 carp (C. carpio), mature male’s and female’s gonad from the Freshwater Aquaculture Management Unit, Malang, East Java, Indonesia. The weight of male fish was around 1-1.2 kg and the age was around 1-2 years, while the weight and ages of female fish were around 1.5 kg and 1.5-3 years old respectively. The fish must be able to produce at least 2-3 ml of sperm and sires must be healthy, not deformed, or injured.

2.1. Sperm analysis
Sperm and egg cells from fish were obtained by the stripping method, which was a way of massaging on the abdomen from the anterior direction towards the posterior direction. The sperm solution collected was first diluted with 0.9% NaCl (1:10), then used for analysis of sperm motility. Sperm motility analysis used single concave glass slides (depth 0.5 mm to 0.8 mm). Motion parameters of sperm (5x100 sperm) including duration of mass and individual motility (ies), and the velocity straight line of sperm (μm/s) were examined using a digital inverted microscope (Olympus-FSX100, Japan). Sperm viability analysis used a total of 20 μl of each aliquot sperm solution and mixed with 10 μl of 1% eosin Y and 10 μl of 10% nigrosin stain and then viewed by using a light microscope to determine the percentage of viable sperms. Live sperms remained white while dead sperms stained red, since the integrity of their plasma membranes had been compromised causing an increase in membrane permeability that led to an uptake of the dye.

2.2. DNA fragmentation
Dried smear was fixed in Carnoy's solution (Sigma Chemicals, St Louis, MO, USA) for at least 2 hours and air-dried. Then, the smear was stained with Acidine Orange (AO) solution (10 mL AO 1%, 40 ml of citric acid, 2.5 mL Na2HPO4.7H2O 0.3 M pH 2.5). After 5 mins, the smear was washed with distilled water, covered with cover glass and mounted on a microscope. Smear was 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). The result was expressed as the percentage of normal nucleus sperms.
2.3. MDA Assay
Lipid peroxidation was measured as the amount of malondialdehyde (MDA) determined by thiobarbituric acid (TBA). Briefly, 0.3 g of the sample was homogenized in 4 cm³ of 0.25% (w/v) thiobarbituric acid (TBA) in 10% (w/v) trichloroacetic acid (TCA) using mortar and pestle. The homogenate was heated at 95°C for 30 mins, quickly cooled in an ice bath and then centrifuged at 10000 rpm for 10 mins. The absorbance of the supernatant was recorded at 535 nm. The blank was 0.25% TBA in 10% TCA. The MDA concentration was calculated by subtracting the absorbance at 600 nm (nonspecific turbidity) and expressed in terms of μmol MDA/g protein.

2.4. Gametes fertility
Fertilization was conducted by mixing the sperms and eggs in clean water with HgCl₂ in different concentrations and incubated for 5-10 seconds. After stirring using chicken feathers for 1 min, eggs were incubated for 5 mins for the fertilization process to take place. The eggs were placed in petri dish glass and placed in a 2 L aquarium. Water was constantly gently aerated at room temperature (20-23°C). Each group consisted of 8 replications with each replication contained ± 200 eggs. Embryos were observed twice a day for three days and dead embryos (opaque egg) were calculated.

2.5. Data analysis
The data were analyzed using ANOVA followed by LSD test by Statistical Package for Social Studies (SPSS software version 21). A comparison was considered significantly different when p<0.05.

3. Results and Discussion
3.1. Sperm motility
The results showed that Cu exposure (10, 25, 50 and 75 ppm) affected sperm motility. The duration of mass motility in the control group (475 ± 32.1 seconds) was higher than in the other treatment groups. The exposure of 10 ppm of Cu caused a decrease in mass motility (443 ± 28.8 seconds), when the concentrations were increased (25, 50, and 75 ppm) it could decrease respectively as 247 ± 55.2; 133 ± 26.5; and 73 ± 12.7 seconds. The duration of individual motility in the control (421 ± 30.7 seconds) was higher than the other groups. Exposure to 10 ppm Cu decreased the duration of individual motility (295 ± 31.8 seconds). The lowest duration of individual motility (50 ± 7.6 seconds) occurred when C. carpio was exposed to 75 ppm Cu (Fig. 1).

![Figure 1](image.png)

**Figure 1.** Duration of mass and individual sperm motility of C. carpio after Cu exposure in various concentrations

3.2. Sperm viability and DNA fragmentation
Fish sperm viability after exposure to Cu decreased compared to controls. The control group showed the highest percentage of sperm viability 88 ± 2% compared to the other groups exposed with 10,25,50 and
75 ppm of Cu that have shown sperm viabilities of 83 ± 2; 46 ± 3; 30 ± 2; and 17 ± 6% respectively. There was no significant difference between the control with 10 ppm Cu treatment (P > 0.05), but there was a significant decrease (P < 0.05) in increasing Cu concentrations from 25, 50, and 75 ppm (Figure 2). Conversely, the results of the calculation of the percentage of sperm DNA fragmentation showed that the smallest percentage (4%) of sperm which had DNA damage (fragmentation) was the control group, and the highest was in the group exposed by 75 ppm of Cu. There was a significant increase in the percentage of DNA fragmentation between control and other groups (P < 0.05) (Figure 2).

![Percentage of viability and DNA fragmentation of sperm after Cu in vitro in various concentration](image1.png)

**Figure 2.** Percentage of viability and DNA fragmentation of sperm after Cu in vitro in various concentration

### 3.3. MDA level of sperm

The end product of lipid peroxidation was measured as the levels of MDA by the thiobarbituric acid reaction, and the end products were colored. The results revealed that MDA concentrations in control and treatment groups showed that exposure to Cu 10, 25, 50, and 75 ppm were not significant (P > 0.05) in the level of MDA. This showed that exposure of Cu was not induced lipid peroxidation (Figure 3)

![Sperm MDA levels (µmol/g) after Cu in vitro exposure in various concentration](image2.png)

**Figure 3.** Sperm MDA levels (µmol/g) after Cu in vitro exposure in various concentration

### 3.4. Fertility

Fertility is the successful fusion of the sperm with the nucleus of the egg. The results showed that Cu exposure decreased the percentage of successful fertilization. The control group showed the highest
percentage of fertilization $85 \pm 4\%$ followed by the fish group exposed by 10ppm of Cu $82\pm 3\%$ while the group exposed with the highest concentration of Cu showed the lowest percentage of successful fertilization. Compared to the control group, the percentage of fertilization was showed a significant decrease in all treatment groups $P<0.05$ except in the group exposed to 10 ppm of Cu (Figure 4).

![Figure 4](image)

**Figure 4.** Percentage of the sperm fertilized the eggs after exposure to Cu in various concentrations

Some elements of heavy metals like Se, Fe, Cu, and Zn are a very essential nutrient for animals [8], but they can be toxic in fish at high concentrations [4]. The elements of heavy metals are abundant and occur as natural minerals with widespread use. Cu contamination exists through extensive use of pesticides, agricultural chemicals, and discharge of industrial wastes [9]. Furthermore, CuSO4 is also used as an algaecide in commercial and recreational fish ponds to control the growth of phytoplankton and filamentous algae as well as to control the certain fish disease [10] [11]. Fish can accumulate Cu through diet or ambient exposure [12]. Even at low concentrations, Cu shows a distinct affinity to accumulate in the fish gonad and reduce fertility [13].

The results of the study showed a higher dose of Cu could reduce the motility and viability of sperm. Cu played a big role in the activation of transcription of various genes, cellular respiration activity, catecholamine production, connective tissue biosynthesis, and superoxide dismutation. But in high levels, Cu caused structural and functional disruption of cells. Excess Cu caused oxidative damage by catalyzing reactions that produce hydroxyl and other oxoyradicals [14]. Cu ions were well suited to facilitate the formation of reactive oxygen species that causes DNA damage (fragmentation) [15]. Sperm mitochondria are arranged at the base of the tail (middle piece), but the head does not equip with acrosomes like in mammalian sperm [16]. Sperm mitochondria are wrapped around the middle piece of sperm to provide energy that is accessed by the tail microfilament to move and facilitate efficient thrust to reach the egg, and to penetrate the pellucid zone in external fertilization.

The mitochondrion is the main source of cell oxidative energy by producing ATP through glycolysis, the Kreb cycle, and the electron transport chain. However, one of the sperm organelles that are more sensitive to oxidants in the mitochondria. Mitochondria depend on various antioxidants and anti-oxidation systems to defend against oxidative stress. The presence of excess Cu endangers the function of the mitochondria and further interferes the sperm motility. Sperm motility is a functional parameter that can be directly influenced by mitochondrial function [17]. Higher doses of Cu reduce the duration of sperm motility of *C. carpio*. The structure of fish sperm is almost identical to mammalian sperm although there is a slight difference in the acrosome. Sperm of fish does not equip with acrosomes like mammalian sperm. During fertilization, fish sperm penetrates the egg cell membrane through a small pore, microple. Microple is a gap in the egg membrane where sperm enter to fertilize the egg. The structure of the sperm tail of all animals contains mitochondria in the middle
piece. Sperm motility is a functional parameter that may be directly affected by sperm structure and function and depends on mitochondrial function [17]. The effect on the mitochondria decreases the energy produced by the mitochondria hence affect the sperm motility. Exposure of *C. caprio* with various doses of Cu in this study decreased both mass and individual sperm motility, this may be a result of damaged mitochondria due to Cu.

In this study, heavy metal Cu was able to induce oxidation of fish sperm DNA and cause DNA damage or fragmentation. These results indicate that each heavy metal has a unique effect that shows results that vary depending on the type and concentration of the particular metal being tested [18]. DNA is a genetic material whose structure and pattern can be damaged. Genotoxic substances are the main reason behind the destruction of this genetic material. Cu as a metal is the best example, when it reacts with DNA causes oxidation damage and DNA fragmentation [8]. The reproductive effect recorded at low Cu levels reduced the success of fertilization of the egg. The reduced ability of sperm and egg cell fertility could be explained by a decrease in sperm quality due to exposure to heavy metals, thereby stimulating oxidative stress [19]. Sperm DNA oxidation caused DNA damage, thus inhibiting the secretion and enzymatic function in sperm cell metabolism. Besides, oxidation also occurs, thereby reducing the permeability of the sperm membrane therefore many molecules cross the sperm plasma membrane. Thus, the motility and viability decrease and sperm DNA damage increases.

One of the most damaging oxidative effects of heavy metals is membrane lipid peroxidation. Malondialdehyde (MDA) is one of the end products of lipid membrane peroxidation and is an important indicator of injury of the cellular membrane. The Cu toxicity reported by other studies differs from this study, the reason may be due to the difference in concentrations of Cu used and depending on the membrane permeability mechanism. In this study, Cu exposure did not have a significant effect because the concentration was low to be able to damage the sperm cell membrane permeability. This can be attributed to the higher activity of cellular antioxidant enzymes to protect the biomembrane from oxidative damage by lipid peroxidation. Besides, Cu plays an essential function in a variety of metabolic processes. But in high concentrations, Cu causes oxidative damage which has the most damaging effect of the cellular membrane through lipid peroxidation.

The function of sperm is to fertilize the egg. Health sperm can fertilize an egg to form a zygote that develops into larvae then grow into adult fish. This study shows that Cu exposure caused oxidative damage. Cu caused lipid peroxidation thereby reduces membrane permeability. The oxidized DNA caused impaired sperm motility and viability which fails sperm to successful fertilize the egg.

4. Conclusion
Based on this study, we can conclude that Copper pollution in waters can directly increase DNA damage and reduce the quality and function of fish sperm.

5. References
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