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Effects of Widely Used Heavy Metals on Embryological Development of Model Organism Zebrafish (Danio rerio)

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A B S T R A C T:

This study aimed to investigate the toxic effect of widely used heavy metals, which are KCN, $CuSO_4$, $K_3Fe(CN)_6$, and FeClOH on the embryological development of model organism zebrafish (*Danio rerio*). Zebrafish embryos were exposed to these heavy metals at a concentration was 0.5 mg/L for 96 hpf (hours post-fertilization). Mortality, hatching, and malformation rates were examined during the exposure period. The result showed that FeClOH and $K_3Fe(CN)_6$ were toxic to zebrafish embryos and caused an increased mortality rate and delayed hatching. Moreover, FeClOH and $K_3Fe(CN)_6$ caused a lot of types of abnormalities while KCN and $CuSO_4$ were more toxic to zebrafish embryos and caused a mortality rate, which was 100% at 24 hpf. In conclusion, this study showed that widely used heavy metals, such as KCN, $CuSO_4$, FeClOH, and $K_3Fe\ (CN)_6$ cause toxicity in embryos, delay hatching time and rate, and/or cause many different malformations.

آثار المعادن الثقيلة المستخدمة على نطاق واسع على التطور الجنيني لسمك Zebrafish (Danio rerio)

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الكلمات المفتاحية:

عيب الجنين،
التشوه ، السمية ،
وقت الفقس

الملخص:

تهدف هذه الدراسة إلى التحقيق في التأثير السام للمعادن الثقيلة المستخدمة على نطاق واسع، والتي هي KCN, $CuSO_4$, $K_3Fe(CN)_6$, and FeClOHعلى التطور الجنيني للكائن الحي zebrafish (Danio rerio). تعرضت أجنة Zebrafish لهذه المعادن الثقيلة بتركيز كان 0.5 ملغم / لتر لمدة 96 ساعة بعد الإخصاب. تم فحص معدلات الوفيات والفقس والتشوه خلال فترة التعرض. وأظهرت النتيجة FeClOH and $K_3Fe(CN)_6$ كانت سامة لأجنة Zebrafish، مما تسبب في زيادة معدل الوفيات وتأخر الفقس. وعلاوة على ذلك، تسبب FeClOH and $K_3Fe(CN)_6$ الكثير من أنواع التشوهات، في حين كان KCN and $CuSO_4$ أكثر سمية لأجنة Zebrafish وزيادة معدل الوفيات ، الذي كان 100 ٪ في 24 ساعة بعد الإخصاب. في الختام ، أظهرت هذه الدراسة أن المعادن الثقيلة المستخدمة على نطاق واسع، مثل KCN, $CuSO_4$, FeClOH, and $K_3Fe(CN)_6$ تسبب سمية في الأجنة، وتأخير وقت الفقس ومعدله ، و تسبب العديد من التشوهات المختلفة.

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Introduction

Environmental pollution is the presence of a pollutant in the environment; air, water, and soil which may be poisonous or toxic and will cause harm to living things in the polluted environment the pollutant emissions are increasing worldwide and bringing huge health and environmental problems, particularly in the aquatic milieu (Duruibe et al. 2007). Pollution is frequently composed of a mixture of heavy metals (Richetti et al. 2011). Heavy metals are individual metals and metal compounds that can impact human health and the environment. Potassium cyanide (KCN), copper sulfate ($CuSO_4$), potassium ferricyanide ($K_3Fe(CN)_6$), and ferrous hydroxyl chloride (FeClOH) are widely used heavy metals in a lot of industries, which are considered to be systemic toxic substances that can cause toxicity even at low exposure levels and are at the top of the dangerous substances.

KCN is a potent inhibitor of cellular respiration, acting on mitochondrial cytochrome c oxidase. This inhibition results in an inability to utilize oxygen for the generation of high-energy phosphates (adenosine triphosphate) through oxidative phosphorylation (Leavesley et al. 2008). KCN affects organ systems with the highest demand for energy such as the central nervous, cardiovascular, and pulmonary systems (Hawk et al. 2016).

$CuSO_4$ is the most common copper salt; it is used chief for agriculture purposes as a pesticide and in the leather industry, copper is an essential trace element that is widely distributed in tissues of animals and plants (Hashimyousif et al. 2019) excess copper is harmful to cells, leading to protein damage and reduced cell proliferation (Zhao et al. 2020). Liver toxicity was seen in doses high enough that result in death. Copper is currently categorized by the EPA (United States Environmental Protection Agency) as a group D carcinogen (Mahurpawar, 2015).

$K_3Fe(CN)_6$ is known to have relatively low toxicity but excessive potassium ferricyanide is harmful to the human body and the ecological impact has not been determined. Potassium ferricyanide is known to be highly soluble in water, in aqueous solutions and it can be produced the highly toxic gas hydrogen cyanide (Hantson et al. 1996). Hydrogen cyanide is the chemical responsible for tissue hypoxia (Xu et al. 2010). Chronic exposure to HCN may cause neurological, respiratory, cardiovascular, and thyroid defects. The onset of symptoms depends on the dose and duration of exposure (Dhas et al. 2011).

Iron is the most crucial element for the growth and survival of almost all living organisms (Valko et al 2005). Iron is the second most important cause of lung cancer (Jaishankar et al. 2014). It is said that

asbestos-associated cancer is linked to free radicals. Loose intracellular iron can also promote DNA damage and it can initiate cancer mainly by the process of oxidation of DNA molecules (Bhasin, 2002).

Zebrafish have become a vertebrate model organism in developmental biology, toxicology and genetic studies, and environmental sciences since it has a short generation time (3-4 months), they can lay hundreds of eggs weekly, the transparency of embryos. The embryo develops very fast; small size, easy maintenance, and external fertilization of eggs. Moreover, Zebrafish have a lot of physiological and genetic similarities with humans, including the brain, digestive tract, musculature, vasculature, and innate immune system (Laale, 1977; Nowik et al. 2015).

It is essential to identify the endpoints of toxicity and their dose-response relationships, elucidate the mechanisms of toxicity, and determine the toxicodynamics of the chemical (Hill et al 2005). Therefore, the toxicity of these widely used heavy metals was *in vivo* evaluated on zebrafish embryos in this study.

Materials and Methods

Experimental design

The study was carried out in Atatürk University, Fisheries Faculty, Fisheries Experimental Research Unit. The zebrafish (*Danio rerio*) and the eggs obtained from them were separated from the male and female fish, kept in the incubation unit and egg retrieval was carried out. Keeping the water temperature of the aquariums at 28 ºC, eggs were taken by standard photoperiod application. The eggs were carefully taken out of the aquarium, washed, and then transferred to petri dishes.

Heavy metals such as KCN, $CuSO_4$, $K_3Fe(CN)_6$, and FeClOH, which humans can always be exposed to in normal life, have been used. Zebrafish eggs were monitored and recorded for 96 hours on petri plates with a dose of 0.5 mg/L (Hisar et al. 2009; Scheerbaum and Noack, 2003) of KCN, $CuSO_4$, $K_3Fe(CN)_6$, and FeClOH, with 50 eggs in each group (Aksakal and Çiltaş, 2018). The incubation temperature was set as 28 ºC during the study period. Dead embryos or individuals with anomalies during observation were removed from the petri plates. Monitoring and imaging were performed with Zeiss brand Discovery V12 model stereo microscope and Axio Vision SE64 Rel 4.8 imaging software at 40x magnification using.

The rate of mortality, hatching, malformation was examined by one-way analysis of variance (ANOVA). Levels of significance were determined using Duncan's multiple comparison test, with critical limits being set at $P<0.05$. Values are expressed as means ± standard deviation (SD) for each measured variable.

Results

All of the eggs in the KCN and $CuSO_4$ treatment groups died at 24 hpf (hour post-fertilization) and the mortality rate was recorded as 100% (P<0.05). Therefore, no anomaly was recorded. Mortality rates of KCN and $CuSO_4$ treatment groups were given in Fig. 1 and 2.

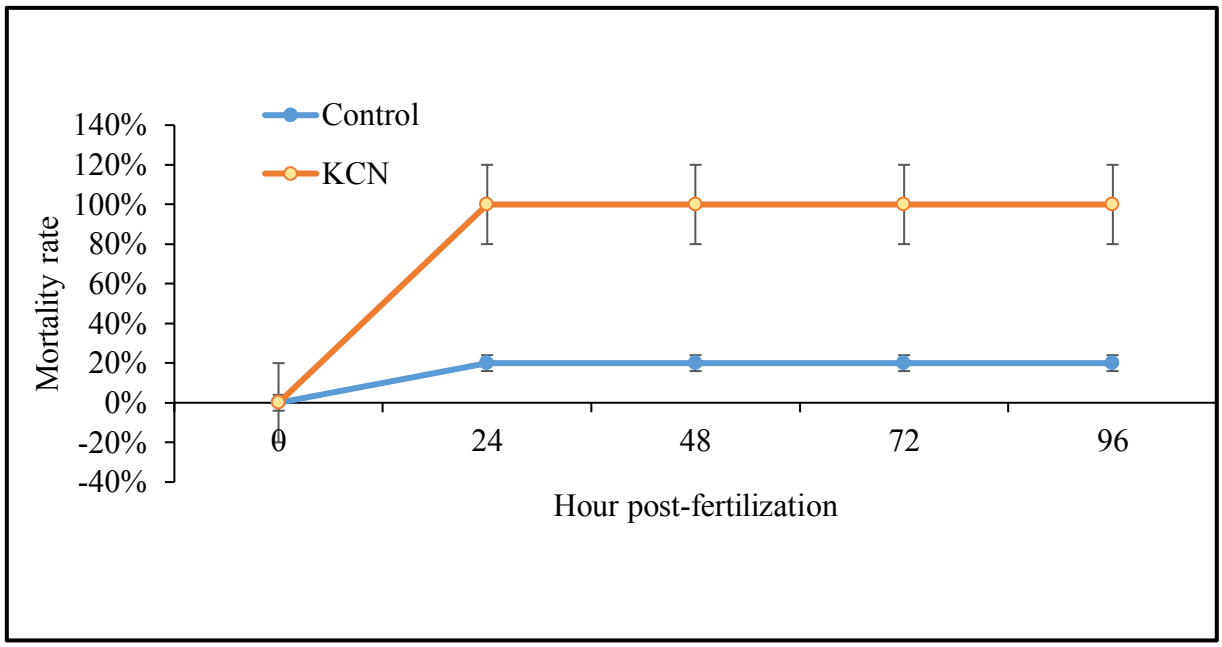


Figure 1. KCN mortality rate of eggs

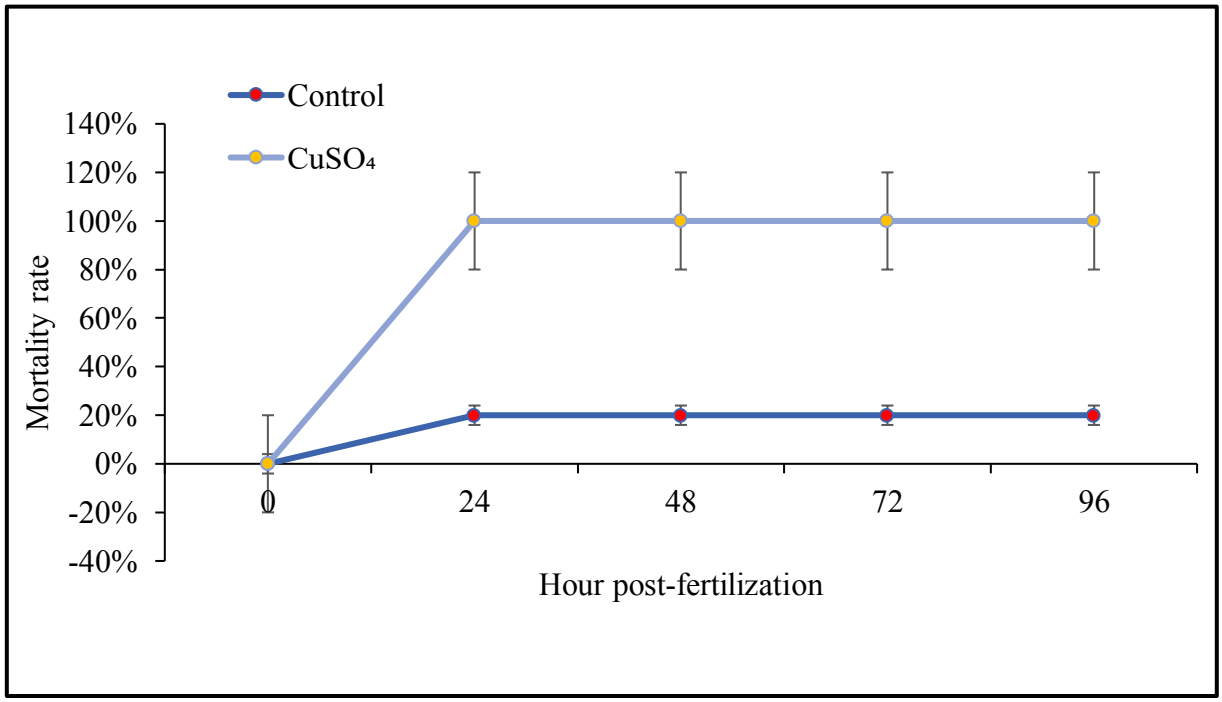


Figure 2. $CuSO_4$ mortality rate of eggs

The mortality rate was two times higher than the control group at the end of 24 hpf in the $K_3Fe(CN)_6$ treated group. Also, the mortality rate of the $K_3Fe(CN)_6$ treated group was 68% at 96 hpf and significantly higher than the control group ($P<0.05$) (Fig. 3).

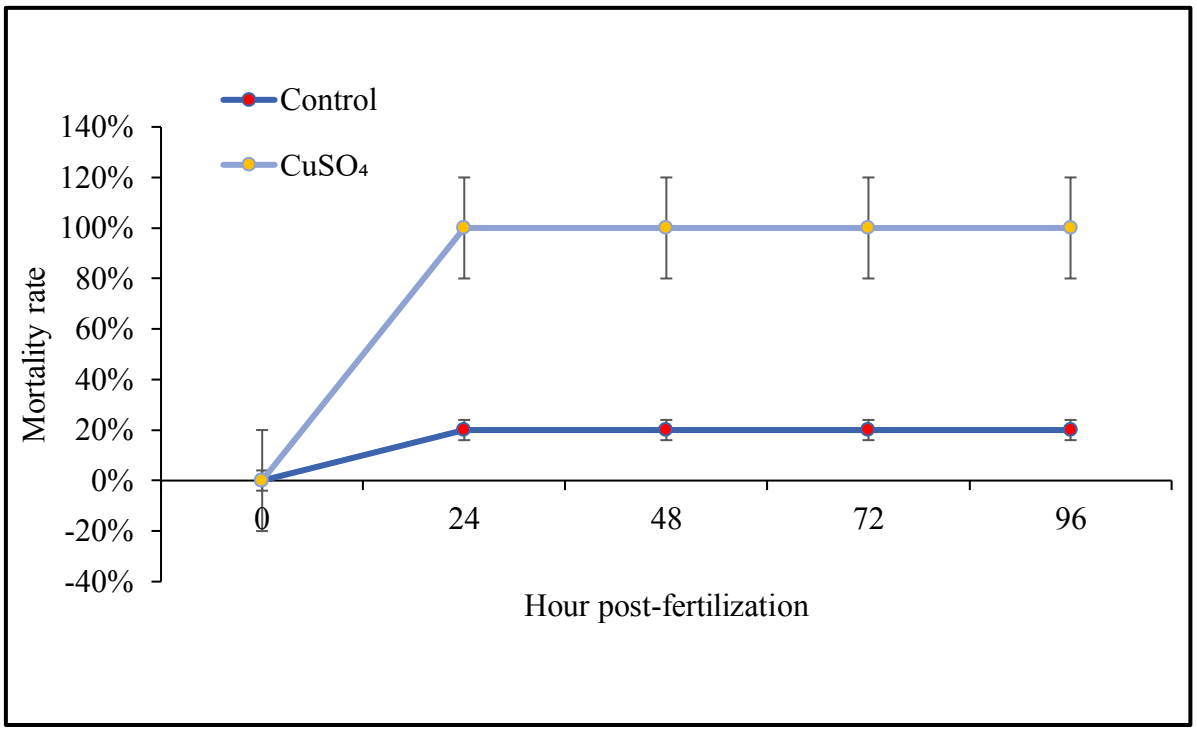
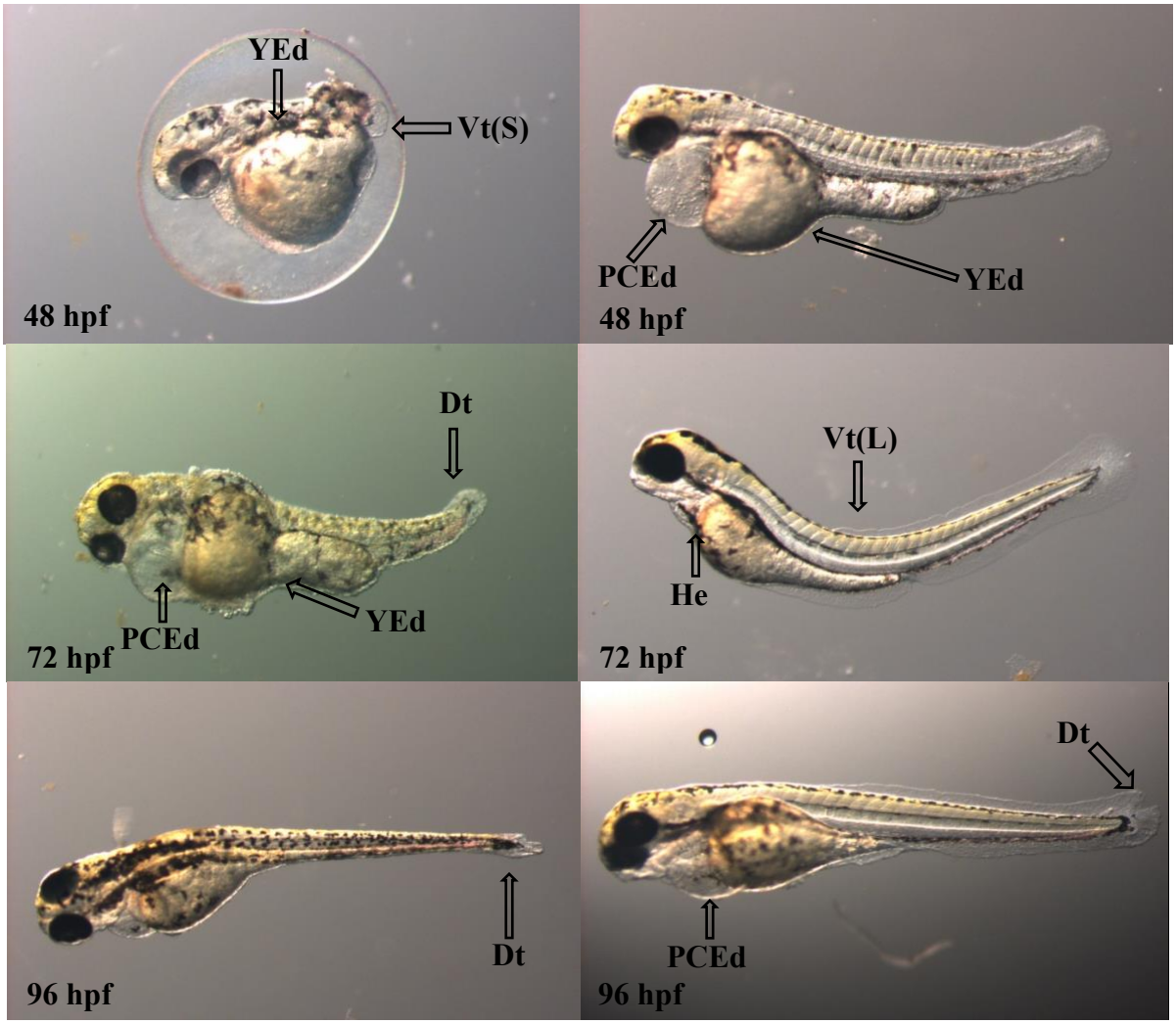


Figure 3. $K_3Fe(CN)_6$ mortality rate of eggs

The eggs in the control group completed hatching at 72 hpf. During the same period, a significant difference was observed between the $K_3Fe(CN)_6$ group and the control group ($P<0.05$). It has been observed that the $K_3Fe(CN)_6$ has an inhibitory effect on hatching in zebrafish embryos. The first anomalies were observed in the early development stage at ≤48 hpf. Petechial cardiac edema, yolk sac edema, hemorrhage, tail malformations, vertebral defect (lordosis and scoliosis), hemorrhage, and pigmentation deficiency were observed in the $K_3Fe(CN)_6$ treated group (Fig. 4). At the end of 96 hpf, it was observed that the rate of healthy individuals was 26% in the $K_3Fe(CN)_6$ group.



Abbreviations: hpf, hour post fertilization; YEd, yolk sac edema; Vt(S), vertebra defect (scoliosis); PCEd, petechial cardiac edema; Dt, tail degeneration; He, hemorrhage; Vt(L), vertebra defect (lordosis)

Figure 4. Morphological abnormalities in zebrafish embryos exposed to $K_3Fe(CN)_6$

Similarly, with the $K_3Fe(CN)_6$ treatment group, the mortality rate was two times higher than the control group at the end of 24 hpf in the FeClOH treated group. At 96 hpf, a significant difference was found compared with the control group (P<0.05) and the mortality rate was observed as 50% at the end of the study (Fig. 5).

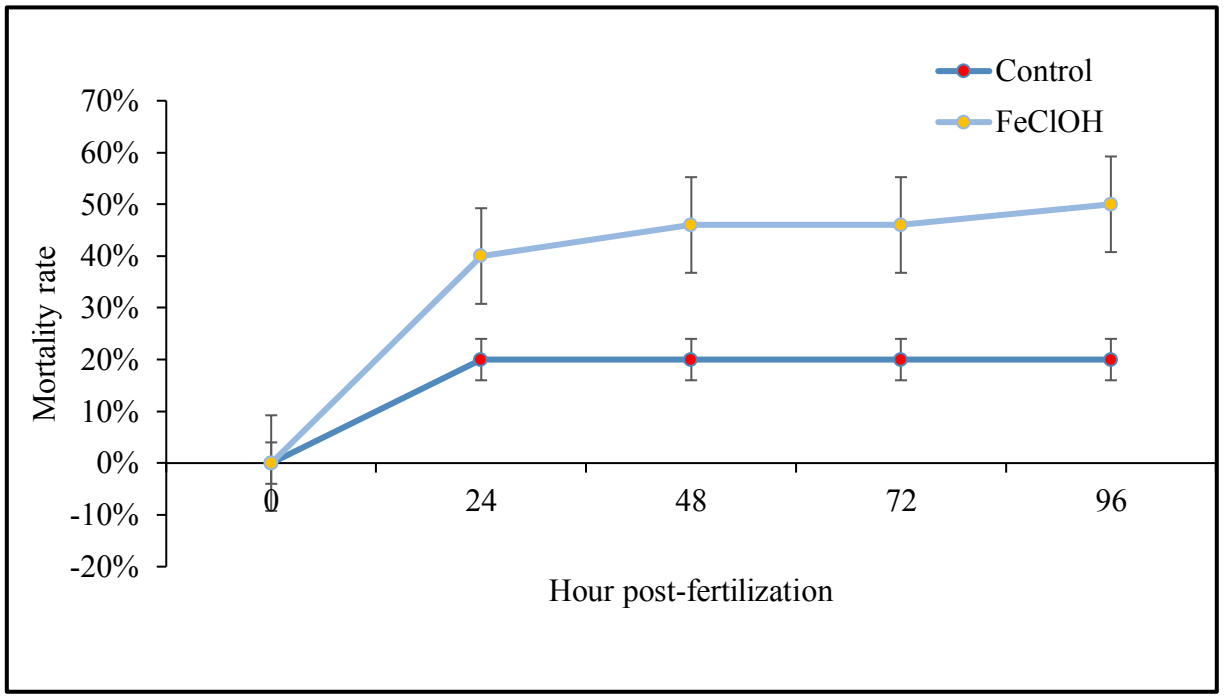
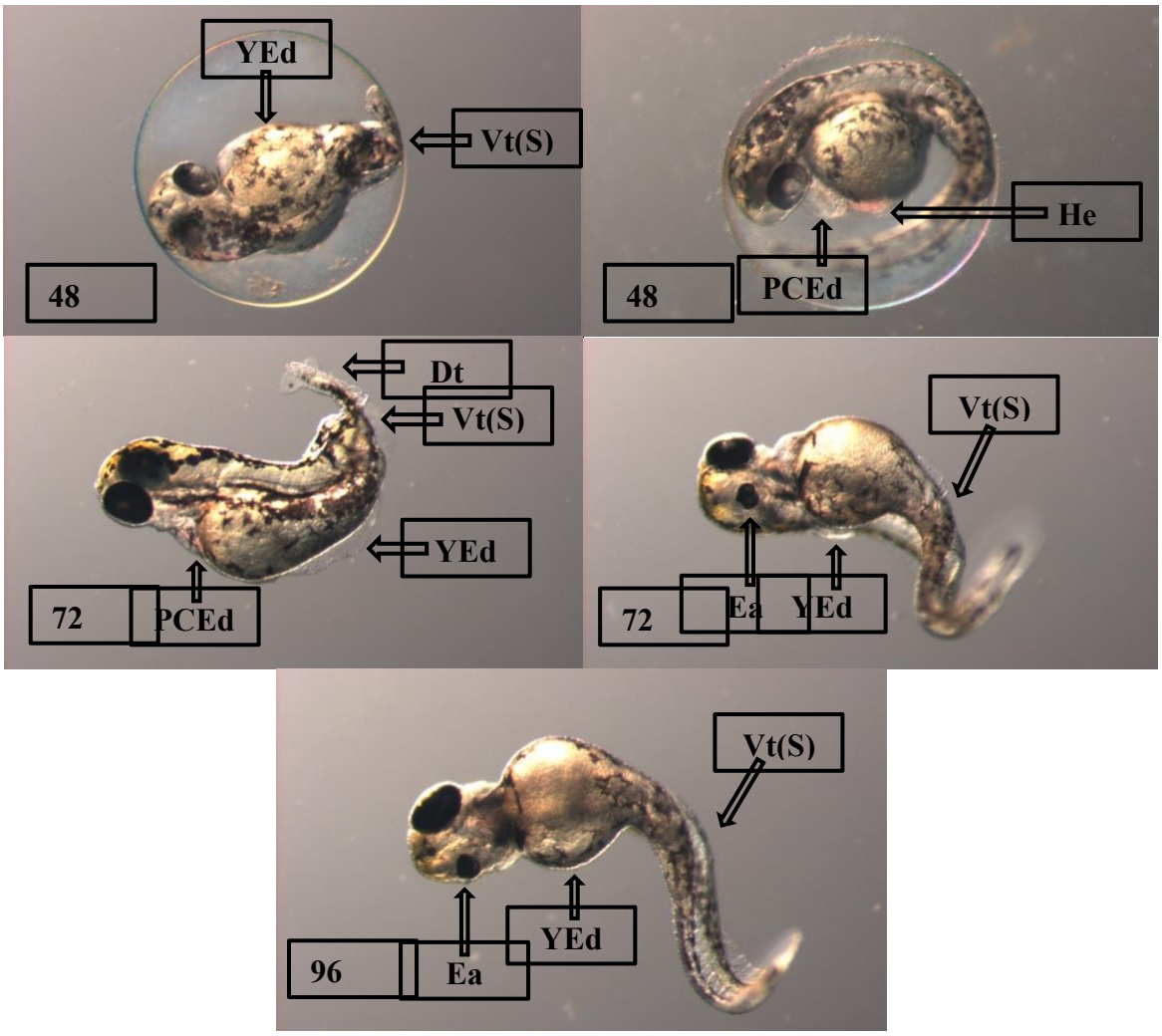


Figure 5. FeClOH mortality rate of eggs

Although the hatching of all embryos in the control group was completed at 72 hpf, it was observed that FeClOH embryos had an inhibitory effect on the hatching time of the chorion. The anomalies observed in the early development stages at 48 and 72 hpf of the groups are given in Fig. 6. Various defects such as petechial cardiac edema, yolk sac edema, hemorrhage, eye anomalies, and spinal malformations (scoliosis) were observed in the FeClOH treated group. At the end of 96 hpf, it was observed that the rate of healthy individuals in the group treated with FeClOH was 34%.



Abbreviations: hpf, hour post fertilization; YEd, yolk sac edema, Vt(S), vertebra defect (scoliosis); PCEd, petechial cardiac edema; He, hemorrhage; Dt, tail degeneration; Ea, eye abnormality

Figure 6. Morphological abnormalities in zebrafish embryos exposed to FeClOH

Discussion

Embryonic development of the zebrafish (*Danio rerio*) has been described in several studies. These studies have described seven broad periods of embryogenesis as the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods in the embryological developmental stage. A normally developing embryo completes all stages in four days. Zebrafish is a genetic model organism in the embryotoxicity test and several lethal, sub-lethal, and teratogenicity endpoints were observed (Kimmel et al. 1995). The fish embryo stage is highly sensitive to chemicals and pollutants. Since embryos are covered by semi-permeable protective membrane chorion, it does not fully protect the embryo against all chemical penetration (Abdelkader et al. 2012).

The present study demonstrated that short-term exposure to heavy metals at concentrations commonly found in the

aquatic ecosystems brings about significant changes in zebrafish embryos. Similarly, study results were supported that very early life stages in which the chorion has not yet hardened are particularly susceptible to waterborne contaminants (Herrmann, 1993). The toxicity of KCN, $CuSO_4$, $K_3Fe(CN)_6$, and FeClOH were evaluated by using the toxicological endpoint on zebrafish embryos. Fe exposure adversely affected the physiological conditions of zebrafish larvae, including a decrease in hatching rate between 2 and 3 dpf (day post-fertilization). The reduced hatching rate was possibly a result of the binding of Fe onto the chorion (Hassan, 2020). The application of FeCIOH on zebrafish increased mortality and anomalies and decreased hatching rate and also it reduced the percentage of hatching in a time-dependent manner in this study. Interference with or inhibition of the secretion or activity of the hatching enzyme, chorionase, which degrades the zona interna of the chorion (Yamamoto and Yamagami, 1975) leaving the softer zona externa to be broken down by osmotic and mechanical processes (Schoots et al. 1982). Yolk sac edema in embryonic and larval fish, following metal exposure, has commonly been identified by changes in the shape of the yolk sac and the space between the yolk and yolk sac itself (Cheng et al. 2002). In the present study, according to the test result, the concentration of 0.5 mg\L FeCIOH caused some anomalies. At the 48 hpf, different anomalies were observed such as yolk sac edema, petechial cardiac edema, hemorrhage, and vertebra defect. Differently, eye abnormality was observed in both 72 hpf and 96 hpf (Fig. 6). Some embryos showed all anomalies while some only showed 1 or 2 anomalies. These types of anomalies were reported in other studies using iron oxide nanoparticles on zebrafish (Oliveira et al 2020). Fe is an essential trace metal for all vertebrates including fish, but in excess, it can be deleterious. The absorption and homeostasis of Fe are regulated by a suite of Fe-transport and Fe-storage proteins, including divalent metal transporter-1 (DMT1), Fe-regulated transporter-1 (IREG1), and ferritin (Hassan and Kwong, 2020). Anomalies observed in this study result can be attributed to inhibition DNA synthesis causing inhibition of protein and enzyme as a result of an excessive level of Fe. In another study, long-term exposure of zebrafish to heavy metals caused DNA damage in all tissues (Marins et al 2019). In the present study, when exposed zebrafish embryos to $K_3Fe(CN)_6$ caused increased mortality and anomalies and decreased hatching rate at 0.5 mg\L. Hatching is often the result of a combination of biochemical (enzymatic), biophysical (mechanical), and osmotic mechanisms (Yamagami, 1981) and so the

observed inhibition may result from the action of $K_3Fe(CN)_6$ on more than one or all of these mechanisms. Alternatively delayed hatching may be attributed to a slower developmental rate. The decreases in the hatching rate of zebrafish embryos after exposure to $K_3Fe(CN)_6$ may also be associated with anomalies. However, exposure to K3Fe(CN)6 caused various anomalies such as yolk sac edema, vertebra defect (lordosis and scoliosis), petechial cardiac edema, tail degeneration, and hemorrhage at all monitored hpf (Fig. 4). Besides, the rate of development abnormalities increased in a time-dependent manner. On the other hand, exposed zebrafish embryos to KCN at 0.5 mg/L all of the embryos died, at 24 hpf mortality rate was 100% this means, KCN was very toxic on zebrafish embryos. The effects of cyanide administration in zebrafish embryos are very similar to the effects observed during anoxia (Mendelsohn et al. 2008). Copper significantly increased the mortality of zebrafish, beyond 24 hpf, above a threshold concentration between 50 and 100 ug/L. The majority of mortalities occurred during the gastrulation and segmentation periods occurring between 5 and 24 hpf (Schilling, 2002), which have previously been termed "critical windows" in the development of fish (Chow and Cheng, 2003). In the present study, zebrafish embryos were exposed to $CuSO_4$ at 0.5 mg/L all of the embryos died at 24 hpf, and the mortality rate was 100%, which means $CuSO_4$ was very toxic on zebrafish embryos. In the other study, the toxicity of CuO NPs 0.5, 1, and 1.5 on zebrafish embryos was evaluated by using toxicological endpoints. The application of CuO NPs on zebrafish embryos increased the mortality and malformation rate and decreased the hatching and heartbeat rate. This situation indicated that CuO NPs lead to developmental toxicity on the zebrafish embryos/larvae (Aksakal and Çiltaş, 2019). In addition, Kondera (2016) was found the minimum concentration significantly reducing egg swelling, embryo survival, and hatching success at 0.1 mg/dm^3 dose of Cu in 24 hpf.

In conclusion, the results of this current study indicated that KCN and $CuSO_4$ were very toxic on zebrafish embryos. Also, FeCIOH and $K_3Fe(CN)_6$ compounds during the embryological development of zebrafish; it has been observed to cause toxicity in embryos, delayed hatching time and rate, and/or cause many different malformations.

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