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Effect of Sublethal Levels of Copper Sulfate on Some Hematological Parameters of *Rutilus frisii kutum* Fingerlings

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Abstract: As a consequence of human activity, various toxicants reach the aquatic ecosystems. Many fishes are exposed to a considerable concentration of pollutants such as copper sulphate ($CuSO_4$). The toxic effects of $CuSO_4$ on fish depend not only on water quality but also on its concentration in water. So this study was conducted to determine the effects of sublethal concentrations of $CuSO_4$ as a pollutant on some hematological parameters of *Rutilus frisii kutum* fingerling (mean weight ±SD: o.4±0.1 g and mean length ±SD: 3.9±0.3 cm). *Rutilus frisii kutum* fingerlings were exposed to three sublethal levels (0.4, 0.04 and 0.004 mg L^{-1} $CuSO_4$) for a period of 60 days. The obtained results revealed that there was significant difference in hematocrit, mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) among the control, 0.4, 0.04 and 0.004 treatments ($p<0.05$) that in the case hematocrit and MCV concentration, higher levels belong to control treatment. Also In the case of white blood cells (WBCs) count, significant difference was only detected among the control and 0.04, 0.004 mg L^{-1} treatments. Hemoglobin (Hb), red blood cells (RBCs) and mean corpuscular hemoglobin (MCH) concentration in fish exposed to sublethal concentration of $CuSO_4$ showed a significant decrease ($p<0.05$) and neutrophil concentration in fish exposed to 0.04 mg L^{-1} $CuSO_4$ showed increase than those in control group, but these differences was not significant.

Key words: *Rutilus frisii kutum* · Copper Sulphate · Hematological Parameters

INTRODUCTION

Kutum (*Rutilus frisii kutum* Kamensky, 1901) is one of the most important species in the southern Caspian Sea [1]. They are found in the Iran and Azerbaijan maritime borders [2], especially in the area between Astara and the Gorgan River [3, 4] and due to its high nutritional quality and excellent properties is preferred by the customers in the north of Iran [5].

The aquatic organisms' health is continuously being threatened with chemical contaminations present in aquatic environment [6-13].

Copper, an essential trace metal for cellular metabolism, may become extremely toxic for aquatic animals as its concentration in water increases [14, 15]. There are important natural sources of copper; multiple human activities (industries, agriculture, harbors) have considerably increased the input of this metal in estuarine and marine environments around the world [16-21]. At equilibrium, there are few free copper ions in natural waters since most copper is associated with inorganic ions or organic substances. However, $CuSO_4$ has been widely used to control algae and some pathogens in fish culture ponds, increasing copper concentration in water. $CuSO_4$ is highly toxic to fish, so the concentrations required to control algae or pathogen agents must be below the toxicity Threshold for fish. The effect of $CuSO_4$ on fish has been studied exhaustively and, as expected, some species have been found to be more susceptible to copper than others [14,15]. This implies previous knowledge of the susceptibility of a given cultured fish species before using $CuSO_4$. The experiments have shown that copper can accumulate in fish if it is present in excess amounts in the water [22, 23]. Hematological parameters are important for toxicological research, environmental monitoring and as indicators of disease and environmental stress [13, 24]. Many studies have demonstrated changes in blood variables as a result of environmental conditions and presence of contaminants. Fish exposed to environmental

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pollutants exhibit a variety of physiological responses, including blood balance disturbances, ion regulatory disturbances, oxygen uptake and transport inhibition [25] and study of hematological changes can be used as a diagnostic tool to assess health status, since blood is an indicator of the physiological condition of animals [17]. Therefore, hematological analysis and assessment of biochemical parameters of blood plasma are useful in monitoring the physiological status of fish and as indicators of the health of the aquatic environment, although they are not routinely used in fish disease diagnosis [26]. Blood parameters are often measured when clinical diagnosis of fish physiology is applied to determine the sublethal concentration effects of pollutants [27]. Large concentrations of $CuSO_4$ alter the blood parameters [3, 28-30] and immunosuppression can also be observed [3, 29], due to the fact that monocytes and neutrophils are sensitive to heavy metals [9]. Because fish are an important food resource and a major ecosystem component, it is important to assess the effect of Cu in fish [31]. This study aims to evaluate heavy metal toxicity stress symptoms in *Rutilus frisii kutum* fingerling blood during long-term exposure of sublethal concentration of $CuSO_4$.

MATERIALS AND METHODS

Test Animals and Acclimatization: The sublethal bioassay was conducted using kutum fingerlings (mean weight ± SD: o.4±0.1 g and mean length ±SD: 3.9±0.3 cm) prepared from Bony Fish Propagation and Rearing Center of Sijeval (Bandar Torkaman, Gorgan, Iran). Fish were transferred to Aquaculture Research Center of Gorgan University of Agricultural Sciences and Natural Resources. Fish were allowed to acclimate to laboratory conditions for 2 weeks to the start of experimentation. A total of 180 kutum fingerlings were randomly distributed on 12 glass aquaria (30×40×60 cm) filled with 60 L water. In each aquarium, fifteen kutum fingerlings were introduced. The aquaria were provided with continuous aeration from an air blower. After acclimation period, the aquaria were assigned as 4 treatments (three replications per treatment). During the trials, fish were hand fed twice daily with pellet feed to satiation. The physicochemical parameters such as temperature, dissolved oxygen and pH of water were measured daily. Total hardness and alkalinity levels were measured once every week following standard procedures [8]. During the trials, no mortality was recorded in any of the aquarium. The test concentrations and the control were all conducted with three replicates each. The duration of the experiment was 60 days.

Blood Parameters Measurements after Sublethal Exposure to $CuSO_4$: Kutum fingerlings were anaesthetized by immersion in Tricaine Methanesulphonate (MS-222) at 200 mg L^{-1}. Approximately 1.5-2.0 ml blood was taken from the caudal vein of kutum fingerlings using heparinized capillary tubes. Due to small amounts of blood sample from each fish, blood samples from 15 specimens, belonging to the same treatment and with similar conditions were used as a single pooled sample. Hematological parameters including RBC count, WBC count, Hb, hematocrit, MCV, MCH and MCHC concentration, white cells differential count such as neutrophils and lymphocyte were determined [32].

An aliquot of blood was diluted 1:200 with 0.4% formaldehyde and 3% trisodium citrate, to determine the number of RBC in a Neubauer counting chamber (hemocytometer). The hematocrit value was determined by centrifuging the blood in a capillary or micro-hematocrit tube at 12,000 rpm for 5 min. The hemoglobin concentration was obtained using the cyanmethaemoglobin method.

- MCV, MCH and MCHC concentration were calculated:
- MCV (μ^3) = PCV (%)/RBC (cell L^{-1})
- MCH (pg) = Hb (g dL^{-1})/RBC (cell L^{-1})
- MCHC (g dL^{-1}) = Hb (g L^{-1})/PCV (L L^{-1}).

Test Solution: Required test concentrations were prepared by dissolving 1 g of $CuSO_4$ in 1 L of water to prepare 1000 mg L^{-1} stock solution of $CuSO_4$. The water used in the toxicity tests was the same as the acclimation period and no copper was added to the aquaria containing the control fish. Wastes were removed daily by siphoning and water was exchanged 50% on alternate days. Three sublethal levels of $CuSO_4$ (0.001, 0.01, 0.1 of the 96 h LC_{50} value; i.e. 0.004, 0.04, 0.4 mg L^{-1}) and one control were selected for experimentation. The 96 h LC_{50} values for same size groups previously obtained by Gholami [33] were used for the present study (96 h LC_{50} for kutum fingerling was 4.02 mg L^{-1}).

Table 1: Changes in some blood parameters of *Rutilus frisii kutum* fingerling exposed to sublethal concentrations of $CuSO_4$

Blood parameters	Control	0.4 mg L^{-1}	0.04 mg L^{-1}	0.004 mg L^{-1}
RBC ($\times 10^6$/mm^3)	1.49 ± 36055.51 [a]	1.46 ± 37546.11 [a]	1.45 ± 9451.63 [a]	1.48 ± 23630.77 [a]
WBC ($\times 10^3$/mm^3)	7.03 ± 152.75 [b]	7.73 ± 404.14 [b]	8.23 ± 642.91 [a]	8.3 ± 721.11 [a]
Hemoglobin (g/dl)	6.66 ± 0.32 [a]	6.40 ± 0.26 [a]	6.43 ± 0.057 [a]	6.47 ± 0.21 [a]
Hematocrit (%)	20.70 ± 0.20 [a]	18.56 ± 0.41 [b]	18.80 ± 0.10 [b]	18.73 ± 0.20 [b]
Neutrophil (%)	9.66 ± 0.57 [a]	9 ± 1 [a]	9.66 ± 0.57 [a]	9.33 ± 0.57 [a]
Lymphocyte (%)	90.3 ± 0.57 [a]	90.3 ± 0.57 [a]	90.3 ± 0.57 [a]	90 ± 1 [a]
MCV (μ^3)	1.38 ± 2.11 [a]	1.26 ± 0.49 [b]	1.28 ± 1.50 [b]	1.26 ± 0.62 [b]
MCH (Pg)	44.72 ± 1.10 [a]	43.74 ± 0.69 [a]	44.14 ± 0.67 [a]	43.74 ± 0.79 [a]
MCHC (%)	32.19 ± 1.27 [b]	34.46 ± 0.66 [a]	34.21 ± 0.17 [a]	34.57 ± 0.77 [a]

Means with the same superscript letters at the same row are not significantly different (p>0.05).

Statistical Analysis: Data were subjected to one-way ANOVA and significant difference between the treatments was determined by Duncan's test. Data are presented as treatment mean±SD. The values of P<0.05 were considered significantly different. All analyses were performed using statistical software SPSS v. 16.

RESULTS

Hematological parameters in *Rutilus frisii kutum* fingerling affected by sublethal concentration of $CuSO_4$ are presented in Table 1. The mean quality parameters of water used for experiment were: temperature: 23.1±1°C; pH: 8.5±0.4; dissolved oxygen: 7.9 mg L^{-1}; total hardness: 300±0.2 mg L^{-1} as $CaCO_3$; Alkalinity: 275±0.00 mg L^{-1} as $CaCO_3$; PO_4: 0.35±0.09.

In the present study, number of RBC were 1/49× 0^6 ± 36055.51 per mm^3, number of WBC were 7.03 ×10^3 ± 152.75 per mm^3, Hb concentration was 6.66±0.32 g/dl and hematocrit concentration was 20.70±0.20%. Differential count of WBC showed that 90.3±0.57% belonged to lymphocyte and 9.66±0.57% to neutrophil. Also blood indicators were included of MCV: 1.38 ± 2.11 μ^3, MCH: 44.72 ± 1.10 Pg, MCHC: 32.19 ± 1.27 %.

Hematological parameters analysis showed that RBC, Hb and MCH concentration and also the number of neutrophil and lymphocyte did not differ among control and fish exposed to sublethal concentrations of $CuSO_4$ (0.4, 0.04 and 0.004 mg L^{-1}). There was significant difference in hematocrit, MCV and MCHC concentration among the control, 0.4, 0.04 and 0.004 treatments (p<0.05) that in the case hematocrit and MCV concentration, higher levels belong to control treatment. In the case of WBC concentration, significant difference was only detected between the control and 0.04, 0.004 mg L^{-1} treatments in which control treatment showed significantly lower level (p<0.05).

Nevertheless, fish exposed to 0.4, 0.04 and 0.004 mg L^{-1} $CuSO_4$ showed a significant decrease (p<0.05) in hematocrit and MCV concentration when compared to those exposed to control treatment. Also, Hb, RBC and MCH concentration in fish exposed to 0.4, 0.04 and 0.004 mg L^{-1} of $CuSO_4$ showed decrease (p<0.05) than those of control treatment, but these differences was not significant. Fish exposed to 0.4, 0.04 and 0.004 mg L^{-1} of $CuSO_4$ showed a significant increase (p<0.05) in MCHC concentration when compared to control treatment. Lymphocyte concentration increased in fish exposed to 0.4, 0.04 mg L^{-1} $CuSO_4$ when compared to those exposed to 0.004 mg L^{-1} and also increased neutrophil concentration in fish exposed to 0.04 mg L^{-1} $CuSO_4$ was observed when compared with the groups exposed to 0.4 and 0.004 mg L^{-1}, but these differences was not significant.

DISCUSSION

Fish exposed to environmental pollutants exhibit a variety of physiological responses, including blood balance disturbances [25]. The evaluation of hematological characteristics in fish has become an important means of understanding normal and pathological processes and toxicological impacts [34, 35].

Our results showed that $CuSO_4$ at various concentrations exerted a certain influence on some of the blood indices studied. In this study, the main hematological response of *Rutilus frisii kutum* fingerling to the sublethal concentration of $CuSO_4$ was a significantly lower hematocrit and MCV concentration and also decrease in the RBC, Hb, MCH and neutrophil concentration than to the control group after exposure to 0.4, 0.04 and 0.004 mg L^{-1} $CuSO_4$ for 60 days.

Unlike mammals, the hematopoietic system of fish is mainly located in the interstitium of the kidney. So, a reduction in the hematological parameters may be

attributed to the malfunctioning of the hematopoietic system caused by morphological alterations in renal interstitium [36]. Additional, the hematological parameters changes can be interpreted as a compensatory response that improves the O_2 carrying capacity to maintain the gas transfer and a change in water blood barrier for gas exchange in gill lamellae, which were also reported in previous results [37]. Similar results were found in *Channa punctatus* that exposure to sublethal concentration of $CuSO_4$ and reduced the RBC, Hb and hematocrit concentration [38].

Also similar results have been reported with significant reduction of RBC and Hb concentration in fishes exposed to different heavy metals [39] and Goel and Sharma [40]. In *C. macropomum*, it was observed that the number of RBC decreased after treatment with various concentrations $CuSO_4$ [41].

According to Pamila *et al.* [42], the reduction in Hb concentration in fish exposed to toxicant could also be due to the inhibitory effect of the toxic substance on the enzyme system responsible for synthesis of Hb. Joshi *et al.* [43] suggested that heavy metal exposure also decreased the RBC, Hb and hematocrit concentration due to impaired intestinal absorption of iron. The anemia might have led to a fall in the RBC, Hb concentration and hematocrit volume. Anemia, under copper induced stress, may also be due to blood cell injury and disrupted Hb synthesis [44, 45]. The anemic condition in fish results from an unusually low number of RBC or too little hemoglobin in the red blood cells. Anemia is an early manifestation of acute and chronic intoxication of heavy metals. Significance of these changes may be understood in terms of reduced oxygen consumption in fish resulting in death due to heavy metal pollution [46].

In other hand, Increased hemoglobin, higher hematocrit levels and elevated numbers of erythrocytes have been observed in brook trout (*Salvelinus fontinalis*) after 21 days of $CuSO_4$ [44].

Also In common carp *Cyprinus carpio* [47] and *Prochilodus lineatus* [30]. The exposure to copper induces blood alterations, characterized by an increase on the hematocrit, Hb and RBC concentration. These negative results can be also due to the different concentrations and times used in the treatments, as wells as to the physicochemical parameters of water and the monogenean species sensibility to $CuSO_4$ [3]. Moreover, in this study, significantly higher levels of MCHC concentration were observed in fish exposed to 0.4, 0.04 and 0.004 mg L^{-1} $CuSO_4$ than to control groups and significant increase of WBC concentration in fish submitted to 0.04 and 0.004 mg L^{-1} $CuSO_4$ than to control groups was found. One of the most elementary ways to assess the immune system is to explore changes in the WBC count and its types [48].

High WBC counts indicate damage due to infection of body tissues, severe physical stress and as well leukemia. In most cases, abnormal red cell morphology is noted [49]. Singh *et al.* [49] and Carvalho and Fernandes [30] showed that various concentrations of $CuSO_4$ increased the number of WBC [30, 49]. Similar findings were also documented significantly higher in fish exposed to increased copper concentration [29, 50]. Lymphocyte concentration increased in fish exposed to 0.4, 0.04 mg L^{-1} $CuSo_4$ when compared to those exposed to 0.004 mg L^{-1}. Increasing in the number of lymphocytes (lymphocytosis) combined with decreases in the number of neutrophils (neutropenia), are indicative of infections that set in after the exposure to metals [51] that This finding is agreement with our results. The exposure of *Colisa fusciatus* to manganese evoked leucocytosis resulting from an increase in the number of small lymphocytes (lymphocytosis) [52].

The $CuSO_4$ toxicity may also vary significantly among fish species due to other factors such as fish size, exposure dose and time, species unique mechanisms for the metabolism of copper ion [53] and physiological conditions of the individuals and also water physicochemical parameters [3,54,55]. Thus, it is concluded that the hematological parameters are the most sensitive parameters in monitoring the toxicity of copper especially at sublethal concentrations and changes in hematological variables in response to copper exposure indicate ionoregulatory or respiratory disturbances that imply an increase in energy consumption to restore homeostasis instead of other physiological functions and weight gain and growth.

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