

## Determination of acute toxicity and the effects of sub-acute concentrations of CuO nanoparticles on blood parameters in *Rutilus rutilus*

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### ABSTRACT:

**Objective(s):** Copper oxide nanoparticles have different industrial applications so it is inevitable that nanoparticulate products finally find their way into aquatic ecosystems. Nevertheless there is little information available about their effects on some of edible fish. The present study aims to determine the acute toxicity and evaluate the effect of two sub-acute concentrations (50 and 70% 96 h LC50) of CuO-NPs on some hematological and biochemical parameters of *R. rutilus*.

**Materials and Methods:** 225 healthy specimen of *R. rutilus* (mean weight  $5.52 \pm 1.2$  g; mean length  $6.20 \pm 0.2$  cm) were transported to the laboratory. In order to prepare the stock solution, CuO-NPs was dispersed in pure water with ultrasonication (50-60 kHz) for 15 min every day before dosing. At first, *R. rutilus* was exposed to CuO-NPs to determine the lethal concentration (LC50) value. Following acute test, fish were treated with sub-acute concentrations of CuO-NPs (50 and 70% 96 h-LC50 at) with one control group (no CuO-NPs) for a week to determine the changes in the level of some plasma hematological and biochemical parameters.

**Results:** The 96 h-LC50 values of CuO-NPs was  $2.19 \pm 0.003$  mg/l. *R. rutilus* exhibited significantly lower RBC count, Hb and Hct values and a significant increase in the WBC numbers, MCH, MCHC and MCV indices ( $p < 0.05$ ). Low glucose and higher cortisol content in plasma were observed in the fish exposed to CuO nanoparticles than those in control group ( $p < 0.05$ ).

**Conclusion:** These alterations indicate *R. rutilus* sensitivity to CuO-NPs and changes in blood parameters would be a useful tool for measurement early exposure to CuO nanoparticles.

**Keywords:** Acute toxicity, CuO-NPs, Fish, Glucose, Hematological, *R. Rutilus*, Sub-acute toxicity

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### INTRODUCTION

As nanotechnology develops, its impacts on the environment and living organisms are becoming an important issue [1]. Nano-particles have many valuable properties and gained increasing attention because of their extensive surface area and tiny size, differed from those of the same materials in large scales [2]. Copper is

an essential trace element vital to the health of all living organisms as it is involved in several fundamental biological processes [3]. CuO-NPs have been applied in different industrial applications such as ceramic, glass [4]. In addition, they commonly used as bactericides because they are promising against microorganism *Escherichia coli* [5]. In recent years, it has been cleared that CuO-NPs in household application impose adverse effects on the health of living organisms [6]. Given various applications engineered nano-materials, it is inevitable that these products finally leak into the aquatic ecosystems in

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their life cycles, causing problems for non-target organisms [7]. Recently, CuO-NPs have been shown to have adverse effects on the survival and growth of living organisms [6]. So many authors have studied the effects of copper oxide nanoparticles on various organisms especially those in aqueous environments [8]. However there is little information available about their effects on fish as a model organism [9]. Therefore, there is an urgent need for studies on metal oxides like CuO-NPs impacts on different fish species because fish is considered as one of the main non-target aquatic organism affected by pollution [10]. Blood parameters could be measured easily and they are noteworthy indicators determining the condition of aquatic organisms [11]. For this, blood is a useful tool for prediction and diagnosis of chemicals toxicity [12]. Moreover, changes in stress indicators such as glucose and cortisol are widely used to detect physiological or environmental changes and can be considered as integrated measure of the physiological responses in organisms [13]. Glucose is a parameter having an important function in bioenergetics of animals [14]. Some authors have reported the levels of glucose changed under stress condition [15, 16, 17]. Cortisol level of plasma is typically used as a general indicator of stressful conditions in fish [18]. In this regard, Köprücü et al. [19], Ribeiro et al. [20], Hedayati and Jahanbakhshi [21], Hedayati et al. [22] and Shalvei et al. [23] studied the effects of some pollutants on hematological indices of various fish species. Whereas literature is full with several studies on the impact of pollutants on blood parameters of fish, there is no data published on the sub-acute toxicity of the CuO-NPs on hematological and biochemical parameters of *R. rutilus* as a suitable organism to evaluate the impact of pollution in fresh-water ecosystems (24). In the light of foregoing, the present study aims to determine the median lethal concentration (LC50) values (24, 48, 72 and 96 h) of CuO-NPs and the effects of the 50 and 70% of 96 h-LC50 of the nanoparticle on some hematological and biochemical parameters of the fish.

## MATERIALS AND METHODS

For this experiment 225 healthy specimen of Roach (*Rutilus rutilus*) (mean weight  $5.52 \pm 1.2$  g; mean length  $6.20 \pm 0.2$  cm) were obtained from a fish farm. The animals were transported to aquaculture research center of Gorgan University of agricultural sciences and natural resources in many containers equipped with an oxygen capsule and were acclimatized for a period of seven days under laboratory conditions prior to the exposures commenced

in several 200-L glass aquaria supplied with dechlorinated aerated tap water. Some of water quality characteristics of aquaria during the test were recorded as follows; temperature ( $25.4 \pm 1^\circ\text{C}$ ), dissolved oxygen ( $6.8 \text{ mg/L} \pm 0.9$ ), pH ( $7.6 \pm 0.13$ ) (mean+SD) and photoperiod was a 12:12 light-dark cycle. During the acclimation period fish were fed twice a day.

### *CuO-NPs suspension preparation*

CuO nanoparticles (particle size in 20-30 nm with a purity > 98.0%) were purchased from NanoNasb Company, Tehran, Iran as uncoated nanoparticles. In order to prepare the stock solution, according to Zhao et al. (25), CuO-NPs were dispersed in pure water with ultrasonication (50-60 kHz) for 15 min every day before dosing.

### *Acute toxicity test*

Groups of 21 (each concentration was composed of three replicates aquaria and each aquarium contained 7 fish) fish were exposed to 1, 10, 100, 1000, 2000 and 4000 mg/l CuO NPs for 96h. Mortalities rate was measured at 24, 48, 72 and 96 h, and dead fish were immediately removed by dip net to avoid possible deterioration of the water quality. Test water was not changed during the 96-h time period and exposed fish were not fed. The LC50 values for 24, 48, 72 and 96h were calculated by Probit analysis and spss18 software (26).

### *Sub-acute toxicity test*

Following the toxicity test, in order to investigate the effect of CuO-NPs on the hematological parameters, two concentrations (50% and 70% of 96 h- LC50) were considered [27]. In this phase, 120 randomly *R. rutilus* were selected from acclimation aquaria and were randomly graded into several experimental 70-L aquaria exposed to concentrations of 50% and 70% of 96 h- LC50 for a period of seven days to hematological and plasma glucose analysis [27, 28]. For sub-acute toxicity assay, the exposure water in the aquaria was changed daily and freshly prepared solution was added to maintain the concentration of CuO-NPs at a constant level. Moreover, during the experiment, water was continuously monitored. The mean values for test water qualities were as follows (temperature of water was kept at about  $25.4 \pm 1^\circ\text{C}$ ) for each exposure. The pH in exposure water was 7–7.2, the dissolved oxygen was 6.5–7.8 mg/l, and total hardness in water was 300 mg/l (as CaO). A control test without CuO-NPs was conducted under the same conditions.

### **Blood collection**

At the end of the experiment (7 days), 21 fish per treatment were removed for hematological and biochemical studies. Fish were immediately anesthetized into a 200 ppm solution of clove powder [21]. Blood collected from each fish by cutting the caudal peduncle was decanted into heparinized tubes and placed immediately on ice for the estimation of red blood cells count, white blood cell count, hemoglobin percentage, hematocrit, MCV, MCH and MCHC [20, 13]. The rest of the samples were kept for plasma measurements.

### **Hematological analyses**

Examination of red blood cell (RBC) and total white blood cell (WBC) were carried out according to the hemocytometer method under the light microscope [29]. The micro-hematocrit method of Hesser [30] was used to estimate the hematocrit (Hct). Hemoglobin values (Hb, milligrams per liter) were immediately assessed calorimetrically according to Lee et al. [31] by determining the formation of cyanomethemoglobin. Red cell indices include mean corpuscular hemoglobin (MCH: pg/cell), mean corpuscular volume (MCV:  $\mu^3$ /cell) and mean corpuscular hemoglobin concentration (MCHC in g/dl) were calculated from red blood cell count, hemoglobin and hematocrit according to formula were suggested by Lee et al. [31].

### **Glucose and cortisol measurement**

In order to glucose assessment after assessing hematological parameters, the rest of the blood samples were allowed to clot at approximately 22°C (room temperature) for half an hour before centrifugation. The blood was centrifuged at 3500 rpm over 5 minutes for the collection of plasma. The supernatants were stored at -80°C until analysis. The plasma obtained was assayed for determine glucose and cortisol values. The plasma glucose was quantified using spectrophotometry method as described by Shaluei et al. [26]. Cortisol level of plasma was determined using ELISAKit (DRG Diagnostics, Mountainside, NJ, USA) as described by Shaluei et al. [26].

### **Statistical analysis**

LC50 values and confidence intervals were calculated using EPA Probit Analysis Program V. 1.5 for each group, separately. All data were accepted if calculated chi-square for heterogeneity was lower than the tabular value at the

0.05 level. After determining LC50 values, statistical analysis was used to compare the significant difference between treatments. In order to estimate the mean values of hematological indices and biochemical parameters was used of all replicates. Each parameter was statistically analyzed for normality and homogeneity. Analysis of variance (One-way ANOVA) with Duncan post hoc test was applied to detect the significance of CuO-NPs levels on hematological and biochemical parameters. Values were expressed as means  $\pm$  standard deviation ( $\bar{X} \pm SD$ ). Differences were considered to be significant at  $p < 0.05$

## **RESULTS**

### **Acute test**

The number of dead fish was increased significantly with increasing concentration and time during the acute toxicity test (24-96 h). Therefore the highest mortality was recorded in the fish exposed to highest amount of CuO nanoparticles for 96h. The 96 h-LC50 of the material were found to be  $2.19 \pm 0.003$  mg/l. Obtained results for LC1-99 values tests are shown in Table 1.

### **Hematological parameters**

Results of hematological parameters (RBC, Hb, Hct, WBC, MCH, MCHC and MCV) of the test and control *R. rutilus* exposed to 50 and 70% of LC50 are shown in Figures 1-7. At seventh day, *R. rutilus* specimens exhibited significantly lower RBC count, Hb and Hct values ( $p < 0.05$ ). While, a significant increase in the WBC numbers, MCH, MCHC and MCV indices was found after exposure to 50 and 70% LC50 of CuO-NPs for seven days ( $p < 0.05$ ).

### **Biochemical parameters**

Changes in glucose and cortisol levels of plasma in control and treated fish are shown in figures 8-9. There was a significant differences between cortisol levels in the treated fish and control group ( $p < 0.05$ ). Fish exhibited significantly ( $p < 0.05$ ) higher cortisol level in plasma during exposure to CuO-NPs. On the other hand, values of cortisol increased with increasing concentration. The average plasma glucose content in the unexposed control group of *R. rutilus* was  $65.33 \pm 2.08$  mg/dl. As it is obvious from Figure 8, there was a significant decrease in the glucose levels of the treated groups when compared with their respective controls ( $p < 0.05$ ). In fact, the value of glucose was decreased with increasing the concentration.

Table 1. Lethal Concentrations (LC1-99) of CuO-NPs (mean ± Standard Error) depending on time (24-96h) in *R. rutilus*

Roach point	Concentration (mg/l) (95 % of confidence limits)			
	24h	48h	72h	96h
LC1	3.07±0.003	2.72±0.002	1.73±0.16	1.36±0.003
LC10	3.84±0.003	3.34±0.002	2.06±0.16	1.73±0.003
LC30	4.40±0.003	3.79±0.002	3.02±0.16	2.01±0.003
LC50	4.78±0.003	4.10±0.002	3.69±0.16	2.19±0.003
LC70	5.17±0.003	4.41±0.002	4.35±0.16	2.38±0.003
LC90	5.72±0.003	4.86±0.002	5.31±0.16	2.65±0.003
LC99	6.49±0.003	5.48±0.002	6.64±0.16	3.03±0.003

**DISCUSSION**

Release of metal oxide nanoparticles into the environment as a consequence of increasing production and exploitation, make it necessary to assess the environmental and health hazards that these compounds could exert [32, 33]. In toxicity studies, the LC50 values of new materials should be determined in the first stage [34]. LC50 is the most widely accepted basis for acute toxicity test kills 50% of the test organisms after a particular period of exposure, usually 96 h [23]. The individual variability in acute toxicity even within a species and with the same toxicant depends on the size, age, and condition of the tested organism as well as on experimental factors [35]. To the best of our knowledge, this study is one of the first reports detailing the effects of CuO-NPs on *R. rutilus*. The results obtained from acute toxicity test showed that the 96 h-LC50 value was 2.19±0.003 mg/l. Griffith et al [36].

Studied the acute toxicity Cu-NPs to adult zebrafish (*Danio rerio*) and reported 48 h-LC50 value as 1.56 mg/l, and 7.20 mg/l in larvae. Das and Das [37] recorded 1.40 mg/l Cu as 96 h-LC50 to fry of common carp (*Cyprinus carpio*), explanation, the LC50 values indicated that Cu was highly toxic to the organisms. Moreover, the acute toxicity of copper to some types of fish was reported by Richey and Roseboom [38]. They illustrated that the 48 h-LC50 of Cu to rainbow trout (*Oncorhynchus mykiss*) in size of a yearling and alkalinity of 250, was 0.75 mg/l. In addition, the 96 h-LC50 value of Ag-NPs (Nanocid) in Caspian roach (*Rutilus rutilus caspicus*) was found to be 0.028 mg/l [39]. As described by Kalbassi et al. [40], CuO NPs in the present survey can be classified as moderately toxic for *R. rutilus* (96 h-LC 50 in the of range 1 to 10 ppm).

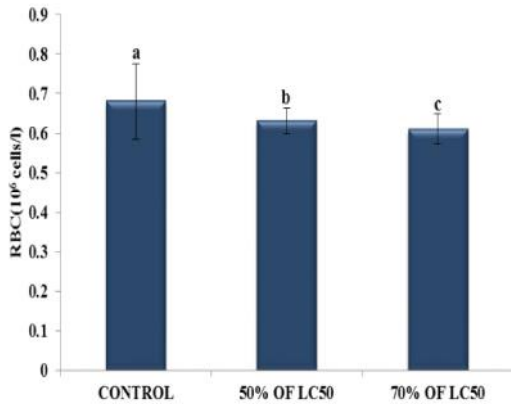


Fig. 1. Changes in number of red blood cells (10<sup>6</sup> cell /L) in *R. rutilus* after exposure to 50-70% 96 h-LC50 of CuO-NPs after 7 days. Statistical significance was determined using a one-way analysis of variance (ANOVA) and a Duncan post hoc test ( = 0.05). Values are means ± SD (n = 21). Different letters show significant differences among exposure concentrations

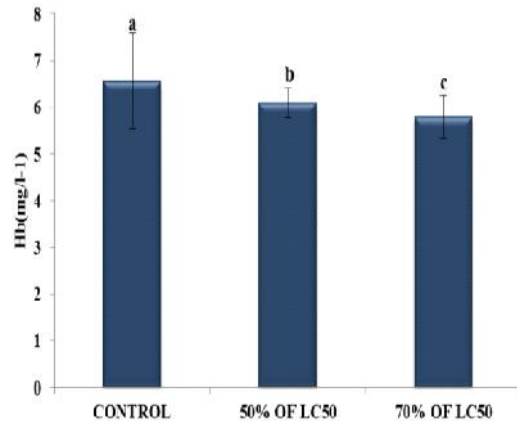


Fig. 2. Changes in values of hemoglobin (mg /L) in *R. rutilus* after exposure to 50-70% 96 h-LC50 of CuO-NPs after 7 days. Statistical significance was determined using a one-way analysis of variance (ANOVA) and a Duncan post hoc test ( = 0.05). Values are means ± SD (n = 21). Different letters show significant differences among exposure concentrations

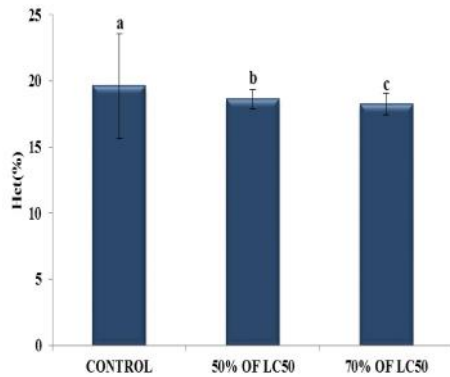


Fig. 3. Changes in values of hematocrit (%) in *R. rutilus* after exposure to 50-70% 96 h-LC50 of CuO-NPs after 7 days. Statistical significance was determined using a one-way analysis of variance (ANOVA) and a Duncan post hoc test ( $\alpha = 0.05$ ). Values are means  $\pm$  SD (n = 21). Different letters show significant differences among exposure concentrations

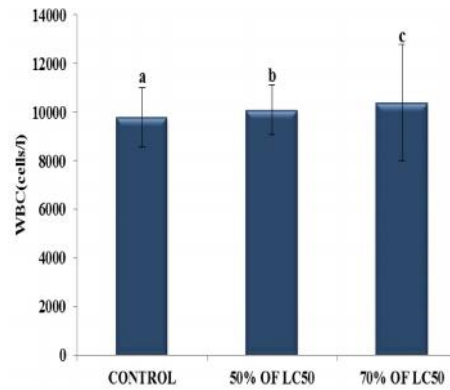


Fig. 4. Changes in number of white blood cells (cell /L) in *R. rutilus* after exposure to 50-70% 96 h-LC50 of CuO-NPs after 7 days. Statistical significance was determined using a one-way analysis of variance (ANOVA) and a Duncan post hoc test ( $\alpha = 0.05$ ). Values are means  $\pm$  SD (n = 21). Different letters show significant differences among exposure concentrations

#### Sub-acute toxicity test

#### The effects of CuO-NPs on hematological and biochemical parameters

Blood is a very good indicator of toxic stress and analysis of hematological parameters in fish is greatly used to estimate toxic stress and practical status of the animals' health [41]. Hematological data showed that CuO-NPs exerted a certain influence on afore-mentioned blood indices in this study. Condition of the specimens during a long period could be reflected by erythrocytes [42]. Decreased RBC, Hb and Hct content in *R. rutilus* were observed in our study. Similar results were also reported in fish exposed to metals [43], pesticides [44] and other toxicant [45]. A reduction in the number of RBC of trout (*Salmo gairdneri*) was observed after exposure to 0.301 mg/l of copper for a day [46]. Moreover, the analysis of hematological indices of rainbow trout (*Oncorhynchus mykiss*) treated with CuO-NPs for a period of 96 h showed that CuO-NPs stimulated red and white blood cells, hematocrit, MCH, MCHC and MCV, and did not have any effects on hemoglobin content [47]. The decrease in RBC count, Hb and Hct levels observed in this study may be due to anemia or erythropoiesis disorder [23]. As mentioned by Thomas and Egee [48], the transport of carbon dioxide and oxygen within the blood is related to the electrolytes and the acid-base status of the red blood cells (RBCs). Therefore respiratory disorders may occur following exposure to CuO-NPs which in turn led to changes in the number of RBCs [47]. Hemolysis also can be expressed as a reason for reduction observed in RBC count in the affected fish [49]. Significant reduction in

hematocrit and hemoglobin has been recorded in various toxicant-treated fish by some authors. Decreased Hb and hematocrit content may be attributed to the stress induced by innutrition during the test, collapse of erythrocytes because of toxicant stress [23, 50] and or lysing of RBC owing to stressor [51]. Therefore, decreased RBC count and Hb content seen in the current survey may be as a result of disarranging action of the used material on the erythro-poietic tissue [19].

Average volume of red blood cells by dividing the hematocrit is expressed as MCV. Since The MCV, MCH and MCHC values are exactly calculated based on hemoglobin and hematocrit content and RBC number, changes in these parameters will lead to changes in MCV, MCH and MCHC values [52]. In this regard, a marked increase in MCV value might be resulted from decrease in RBC number induced by hypoxic condition [53]. According to Barton et al. [54], alternations in WBC numbers can be used as a susceptible indicator of stress in fish. The increase in white blood cells of *R. rutilus* is in accordance with those of Oliveira Riberio et al. [20] who noted disorders of hematological tissues (spleen and kidney) and Remyła et al. [13] who stated increase in WBC numbers may be occur in order to overcome stressful condition. Major reduction in plasma glucose levels after exposure to stressors has reported by few authors. noticeable reduction in glucose content of pesticide treated *Channa punctatus* [55], *Catla catla* affected by acute of arsenate [56] and rainbow trout (*Oncorhynchus mykiss*) subjected to waterborne copper nanoparticles [57] are examples of these studies. In addition, the marked

decline in glucose content of plasma during the study may be associated to excessive consumption of stored carbohydrates in the body followed by hypoxic condition caused by nanomaterials [56], which in turn may be a reason for hypoglycemic condition [55].

Cortisol is the major corticosteroid produced by teleostean fish. Different stressors activate the hypothalamus–pituitary–inter-renal (HPI) axis, resulting in a cortisol release, which causes secondary stress responses [23]. Cortisol maintenance the homeostasis through mobilizing some factors such as fatty acids and glucose and so cortisol has an important role in exerts direct and indirect effects on intermediary metabolism, particularly in response to stress [58]. Increased cortisol of plasma can be considered as the reaction of the species to recognize the presence of a lethal or potential harmful substance in the environment [59].

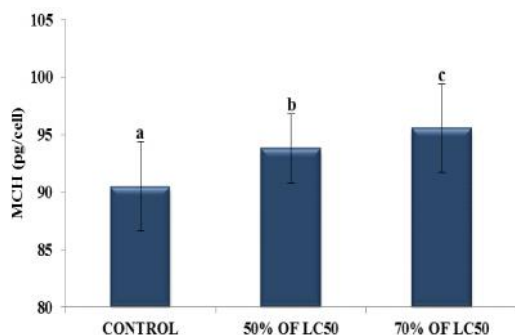


Fig.5. Changes in values of MCH (pg/cell) in *R. rutilus* after exposure to 50-70% 96h-LC50 of CuO-NPs after 7 days. Statistical significance was determined using a one-way analysis of variance (ANOVA) and a Duncan post hoc test ( $\alpha = 0.05$ ). Values are means  $\pm$  SD (n = 21). Different letters show significant differences among exposure concentrations

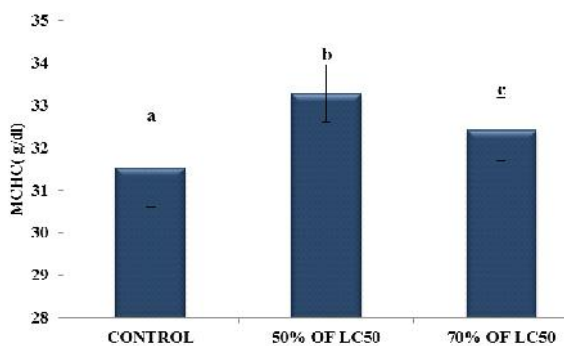


Fig. 7. Changes in values of MCHC (g/dl) in *R. rutilus* after exposure to 50-70% 96 h-LC50 of CuO-NPs after 7 days. Statistical significance was determined using a one-way analysis of variance (ANOVA) and a Duncan post hoc test ( $\alpha = 0.05$ ). Values are means  $\pm$  SD (n = 21). Different letters show significant differences among exposure concentrations

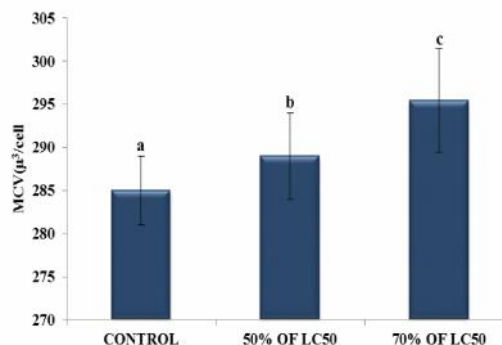


Fig. 6. Changes in values of MCV ( $\mu^3$ /cell) in *R. rutilus* after exposure to 50-70% 96h-LC50 of CuO-NPs after 7 days. Statistical significance was determined using a one-way analysis of variance (ANOVA) and a Duncan post hoc test ( $\alpha = 0.05$ ). Values are means  $\pm$  SD (n = 21). Different letters show significant differences among exposure concentrations

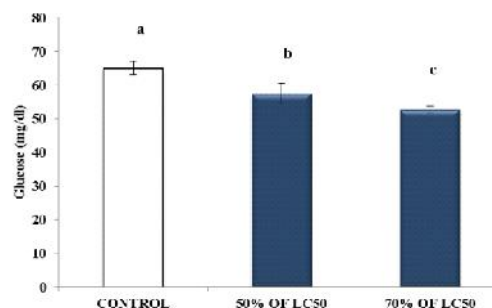


Fig. 8. Changes in glucose levels (mg/dl) in *R. rutilus* after exposure to 50 and 70% of LC50 after 7 days. Statistical significance was determined using a one-way analysis of variance (ANOVA) and a Duncan post hoc test ( $\alpha = 0.05$ ). Values are means  $\pm$  SD (n = 21). Different letters show significant differences among exposure concentrations

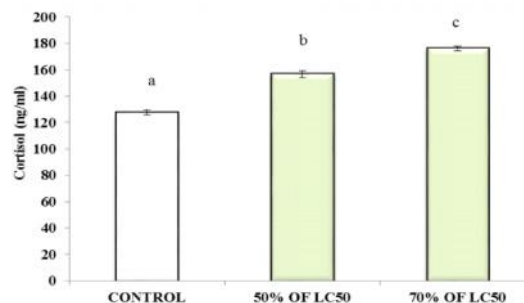


Fig. 9. Changes in cortisol levels (ng/ml) in *R. rutilus* after exposure to 50 and 70% of LC50 after 7 days. Statistical significance was determined using a one-way analysis of variance (ANOVA) and a Duncan post hoc test ( $\alpha = 0.05$ ). Values are means  $\pm$  SD (n = 21). Different letters show significant differences among exposure concentrations

## CONCLUSION

Though little surveys have focused on the effects of copper oxide nanoparticles on blood parameters of various fish species, the current investigation proves that exposure to lower concentrations than LC50 of copper oxide nanoparticles leads to change in hematological and biochemical parameters of *R. rutilus*. Since the species is among edible species, infection in turn affects human health. Furthermore, the results show that alternation in blood parameters would be a useful tool for measurement early exposure to CuO nanoparticles.

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