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Effects of Individual and Co-exposure of Copper Oxide Nanoparticles and Copper Sulphate on Nile Tilapia *Oreochromis niloticus*: Nanoparticles Enhance Pesticide Biochemical Toxicity

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Abstract

Copper, like iron and zinc, is one of the most essential trace elements for organisms. Different forms of copper have distinctive and specific uses. For example, copper oxide nanoparticles (CuO-NP) are widely used in the world as a nanomaterial. Copper sulphate ($CuSO_4$) is worldwide used as a fungicide in agriculture and as an algaecide in aquaculture. Nowadays, the increasing use of these chemicals raises concerns regarding their potential effects on the health of aquatic organisms and ecological risks. Therefore, in the present research, toxic effects of $CuSO_4$ and CuO-NP, alone and in combination, were evaluated using biochemical markers (plasma biochemical and gill and liver oxidative stress) in freshwater fish, *Oreochromis niloticus*. The fish were exposed to 0.05 mg/L $CuSO_4$, CuO-NP, and $CuSO_4$+CuO-NP for 4 and 21 days. Especially at 21 days, $CuSO_4$ and CuO-NP, alone and combined, generally increased plasma alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, cortisol, glucose, creatinine, blood urea nitrogen, and tissue malondialdehyde while they decreased plasma total protein, and tissue superoxide dismutase, catalase, glutathione-S-transferase, glutathione reductase, and glutathione. Consequently, our results illustrate that $CuSO_4$ and CuO-NP have similar toxic effects in fish, however, co-exposure of CuO-NP and $CuSO_4$ is more toxic than effects of these chemicals alone.

Keywords: Fish; metal; nanoparticles; blood; biomarkers

1. Introduction

Most aquatic environments (e.g., seas and rivers) are contaminated by pollutants from natural and anthropogenic sources. These ecosystems are considered to be the ultimate receiving medium for pesticides, metals, and nanoparticles.[1] The entry of these dangerous substances into aquatic environments impairs the water quality to the extent that it is not suitable for aquatic organisms.

Copper (Cu) is one of the most essential trace elements for organisms like iron and zinc. The central role of copper in the cells is as a cofactor for many enzymes such as superoxide dismutase, monooxygenases, and cytochrome-c oxidase.[2] Different forms of copper have distinctive and specific uses. For example, copper oxide nanoparticles (CuO-NPs) are widely used in the world as a nanomaterial. Copper sulphate ($CuSO_4$), another form of copper, is worldwide used as an algaecide in aquaculture and as a fungicide in agriculture.[3] Nowadays, the increasing use of these chemicals raises concerns regarding their potential health problems on aquatic organisms and ecological risks.

Application, production, and use of nanoparticles (NPs) are increasing worldwide. While the global market for NPs reached $ 2.0 billion in 2017, it is estimated to reach approximately $ 7.0 billion by 2022.[4] CuO-NPs globally are one of the most widely used NPs and the fourth most commonly used metal oxide nanoparticle after titanium dioxide (TiO_2), silicon dioxide (SiO_2), and zinc oxide (ZnO). CuO-NPs are used in consumer products, medicine, and industrial applications. CuO-NPs are utilized in many different applications, including in gas sensors, cata-

lytic processes, solar cells, and lithium batteries, as well as in face masks, wound dressings, and socks.[5] These nanoparticles can also be toxic, which may be due to the particles themselves or the disintegration of ions from the particles.[6] In the aquatic environment, CuO-NPs are considered as a significant source of contamination due to their widespread applications in antifouling paints that used in boats and immersed structures, therefore, the potential toxicity CuO-NPs should not be ignored.[7]

$CuSO_4$ is used in aquaculture applications as a therapeutic agent for bacterial infections and various ectoparasitic and is reducing the incidence of fish parasites (trematodes, protozoa, and bacteria and external fungi, etc.).[8] Another application area of $CuSO_4$ is its usage as an effective fungicide in agriculture.

The blood indices, important biochemical indicators, provide valuable information to assess, monitor and quantify the health of the organisms e.g., fish. Therefore, they are used to explain and diagnose the toxicological effects of various stressors and chemicals. Plasma enzymes [alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH)] activities and metabolite [cortisol, glucose, cholesterol, total protein, creatinine, blood urea nitrogen (BUN), etc.)] levels are often measured as sensitive indicators of the harmful effects of pesticides, metals, and nanoparticles on fish vital tissues (e.g., liver and kidney).[9,10]

The main disturbances occur in biological systems of organisms and are caused by pollutants released in aquatic ecosystems.[11] Various aquatic pollutants, such as pesticides and nanoparticles induce reactive oxygen species (ROS), which may lead to oxidative stress, showing role of ROS in pesticide and nanoparticle toxicities.[1,12] The oxidative stress induces as a result of unbalance between oxidating and antioxidating compounds, which may be triggered by the predominance of ROS production, incapacity of defence or changes in antioxidant systems of organisms.[13] Enzymatic [catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GPX), glutathione reductase (GR)] and nonenzymatic [glutathione (GSH)] antioxidant defence systems play a vital role to neutralize the toxicity of oxidative stress on the biological functions/structures of the cells. Malondialdehyde (MDA) is widely used as a biomarker of toxic effects of pollutants on the cell membrane.

Fishes are consequential sources of proteins and lipids and the health of them is very paramount for human beings.[14] *Oreochromis niloticus* (Nile tilapia) is an important aquaculture species amongst cultivated freshwater fish in the world.[10] These fishes are being the most farmed tropical fish species globally depending on their strong immune systems, high growth rates, and vigorous tolerance to a wide range of environmental conditions including aquatic pollutants.[15]

Some studies have documented the toxic effects of co-exposure of nanomaterials with classical pollutants (pesticides or heavy metals) on aquatic organisms. For example, deleterious effects of carbon nanotubes as nanomaterial, carbofuran as pesticide, and the co-exposure of both on *Astyanax ribeirae* (fish),[16] *O. niloticus* (Nile tilapia)[17] and *Palaemon pandaliformis* (shrimp)[18] were identified in detail. In other studies, it was reported that co-exposure of graphene oxide (carbon-based nanomaterial) with trace elements (Cd, Zn) impaired the routine metabolism of the freshwater fish *Geophagus iporangensis*[19] and *P. pandaliformis*.[20]

In recent years, nanotoxicological researches show that nanoparticles are also dangerous for living organisms, just like pesticides and metals, which are more conventional pollutants.[1,21,22] The increasing use of CuO-NPs and $CuSO_4$ inevitably results in increased concentrations of their discharges into the aquatic environment, which in turn may then pose a potential risk to aquatic organisms. The effect of pesticides or heavy metals on fish has been the focus of extensive research for many years, however, the combined effect of these pollutants and nanomaterials is still a new subject that needs to be studied.[23] In addition, the effects of $CuSO_4$ and CuO-NPs on fish were individually investigated, but no study was found on the combined effects of these chemical. Considering the constant exposure of fish to these chemicals in the natural water medium, the present investigation aimed to determine the acute and subchronic effects of CuO-NPs as a nanoparticle and $CuSO_4$ as a pesticide, alone and in combination, on plasma biochemical indicators (ALP, ALT, AST, LDH, glucose, cortisol, cholesterol, total protein, creatinine, BUN) and tissue oxidative stress parameters (CAT, SOD, GR, GPX, GST, GSH, MDA) in freshwater fish, *Oreochromis niloticus*. The hypothesis of the present investigation was that $CuSO_4$ and CuO-NPs interact synergistically on the *O. niloticus*, thus provoking alterations in biochemical indicators in its blood, gill, and liver tissues.

2. Materials and Methods

Copper sulphate ($CuSO_4 \cdot 5H_2O$) and CuO-NPs (form: nanopowder particle size: <50 nm; surface area: 29 m^2/L) were purchased from Sigma–Aldrich Co. (USA). The morphology and size of CuO-NPs dispersed in distilled water were determined by transmission electron microscopy (TEM) (Hitachi High-Tech HT7700, Tokyo, Japan). TEM measurements demonstrated that CuO-NPs were 55 ± 10 nm of average particle size and showed spherical and oval shapes (Figure 1). For measurements of zeta potential and hydrodynamic diameter of CuO-NPs' suspension, Zetasizer instrument (Malvern Zetasizer Nano ZSP, UK) was used. The zeta size (328 nm), polydispersity index (0.236), potential (22.7 mV), conductivity (0.00792 mS/cm), and mobility (1.8631 μmcm/Vs) of these nanoparticles were found. The stock dispersion (10 g/L) of CuO-NPs was prepared immediately in redistilled

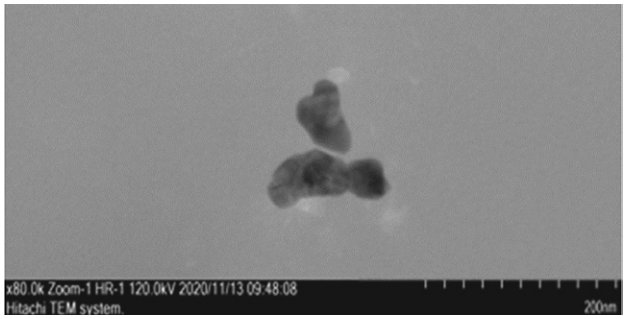

Figure 1. Transmission electron microscopy image of CuO-NPs.

water followed by sonication in an ultrasonic bath for 1 hour as previously described by Shahzad et al. (2018)[24]. 0.05 mg/L CuO-NPs (test concentration) was prepared daily by serial dilutions of this stock dispersion followed by sonication for 20 min to avoid aggregation before adding to the water of the experimental aquarium.

Male *O. niloticus* specimens, two years old, were used as research material in our study. *O. niloticus* (52.71 ± 0.63 g weight and 14.33 ± 0.28 cm total length, as mean ± SEM) were commercially obtained from the Aquaculture Unit of Fisheries Faculty of Cukurova University (CU), where they have been cultured for more than 30 years, and transferred to the Animal Ecophysiology Laboratory of the Science and Letters Faculty of the same university and kept in the glass aquariums containing clean tap water dechlorinated by intense aeration, static system for eight weeks to adapt to the ambient conditions (12-hour daylight /12-hour dark photoperiod, 25 ± 1 °C temperature, central ventilation system). The mean ± standard error of some physicochemical parameters of the waters was found as pH 7.98 ± 0.06, temperature 22.18 ± 0.42 °C, dissolved oxygen 7.65 ± 0.37 mg/L, and total hardness 318 ± 3.5 mg/L as $CaCO_3$. During the acclimatization and experimentation period, the fish were fed once daily at the same hour with commercial fish feed (Pinar Yem, Turkey), in an amount equivalent to 2% of their body weight.

All the experiments, including the controls, were set up in duplicate considering different exposure periods (4 and 21 days). In each repeat set the experiments were carried out in 4 glass aquariums sized 40 cm × 120 cm × 40 cm, each containing 120 L each of the experimental solutions and six fish. Solutions at the concentrations of 0.05 mg/L $CuSO_4$, CuO-NPs, and $CuSO_4$+CuO-NPs were added to the first three aquariums, respectively. The fourth aquarium contained only 120 L of free Cu-tap water and constituted the control. The range of 96-h LC_{50} for Nile tilapia was 5.03-14.27 mg Cu/L.[25] The 96 h LC_{50} value of CuO-NPs for *O. niloticus* was found as 100 mg/L.[26] The 0.05 mg/L concentration of CuO-NPs and $CuSO_4$ applied in the present investigation was therefore a sublethal concentration and eco-relevant considering the contamination levels of certain water resources.[5] The solutions of $CuSO_4$ and CuO-NPs in the treated groups were renewed every 24 hours.[27] The bottoms of aquaria were mixed very well with air at an interval of three times a day to minimize aggregation of NPs.[9] Test media were changed just after feeding, to prevent contamination of the environment with food remains. The control fish were maintained in the same manner. Fish were exposed to these chemicals for 4 and 21 days to determine their acute and subchronic exposures.

At the end of each duration six fish were removed from each aquarium and used as replicates for biochemical testing. After 4 and 21 days, the fish in the control and the treatment groups were individually caught and placed in the anaesthetic bath containing 75 mg/L tricaine methanesulfonate (MS222) for 1–2 min. Blood samples were taken from the caudal vein of each fish into tubes containing ethylene diamine tetra acetic acid (EDTA), anticlotting agent, and centrifuged at 3000 rpm over 10 min at 4 °C for the biochemical analyses of plasma. ALT, AST, ALP, LDH, cortisol, glucose, total protein, cholesterol, BUN and creatinine in the plasma samples were immediately determined using biochemical otoanalyzers (Beckman Coulter DXC 800 and Beckman Coulter DXI 800, USA). ALT, AST, and LDH activities were determined by UV test technique.[28,29] ALP activity was measured by use of the colorimetric assay.[30] Cortisol level was assayed using an electrochemiluminometric technique.[31] The enzymatic UV test was used for the determination of glucose level.[32] The levels of cholesterol,[33] total protein,[34] BUN,[35] and creatinine[36] were determined by colorimetric test. Following blood sampling, fish were dissected. The gill and liver tissues were homogenized in 0.05 M Na-P buffer (pH 7.4) containing 0.25 M sucrose with a ratio of 1/10 in using a steel homogenizer at 10000 rpm for 3 min. Thereafter, the homogenates were centrifuged at 10000 rpm for 30 min at +4 °C. The alteration in oxidative stress parameter in the gill and liver tissues determined using spectrophotometrically. The activity of CAT was evaluated following the method based on measuring the rate constant of hydrogen peroxide (H_2O_2) degradation by the enzyme.[37] The activity of SOD was determined by the inhibition of iodo-p-nitro tetrazolium violet reduction by superoxide anion radical generated by xanthine–xanthine oxidase.[38] The activity of GPX was measured according to Beutler (1984),[39] using t-Butyl hydroperoxide as the substrate. The activity was determined by calculating the difference in absorbance values during oxidation on nicotinamide adenine dinucleotide phosphate (NADPH) to $NADP^+$. The activity of GST was evaluated by the method of Habig et al. (1974)[40] who reported that activity of enzyme was calculated by monitoring the alterations in the absorbance at 340 nm. The GR activity was assayed by determination the oxidation of NADPH by oxidized glutathione at 340 nm.[41] MDA forms a pink complex with thiobarbutiric acid and this complex is measured at 535 nm in spectrophotometer.[42] Protein level was measured according to the method described by Lowry et al. (1951).[43] For statistical assessing, computer software pack-

age SPPS 22 was used. Before the statistical analysis, the data were analysed regarding normality distribution using Shapiro-Wilk's test, and Levene's test was used for homogeneity of variance (homoscedasticity). If the results were normal and homoscedastic, differences between means of experimental groups were evaluated using a variance analysis (one-way ANOVA) followed by Student-Newman-Keuls (SNK) multiple comparisons test. Significant differences were statistically considered at $p<0.05$. All procedures used in the animal experiment were carried out in accordance with the Animal Experiments Local Ethics Committee of the CU (Protocol 2/2018).

3. Results and Discussion

In the investigation, no death was observed in *O. niloticus* exposed to CuO-NPs and $CuSO_4$ and their combination. Similarly, CuO-NPs (0.02 mg/L) did not cause mortality in *O. niloticus*.[44] Aquatic ecosystems are the last ultimate receiving environment for almost all pollutants, and aquatic organisms are seriously threatened by toxic substances entering these environments. The ability of freshwater and marine fish to survive against both well-known pollutants such as metals and pesticides, and a new group of pollutants, nanoparticles, is primarily related to their adaptability and cellular defence mechanisms. It has been shown in many studies[1,10,45] that metals, pesticides and nanoparticles disrupt the internal balance in fish, cause serious toxic effects at the molecular, biochemical, and cellular levels, and even death. Similarly, in the present research significant biochemical and oxidative stress responses were observed in the *O. niloticus* following exposures of $CuSO_4$, CuO-NPs, and $CuSO_4$+CuO-NPs.

Table 1 shows the alterations in plasma enzyme activities of *O. niloticus* in response to the separate or combined effects of $CuSO_4$ and CuO-NPs. Changes in the plasma/serum biochemical parameters in response to environmental pollutants occur rapidly and therefore these parameters are attributed as biomarkers of the toxic effects of chemicals. Among these biochemical parameters, ALT, AST, ALP, and LDH are liver-originated enzymes. These enzymes are intracellular enzymes. Because ALT, AST, ALP, and LDH are sensitive to contaminants, they are recommended as key enzymes in the evaluation of hepatic cell damage and most liver diseases. These enzyme levels in blood plasma are low. However, due to the damage of hepatocyte cell membranes in the presence of toxicants that can cause cellular damage in the liver, their levels may increase by passing into the intercellular fluid and then into the blood. In the current work, all tested plasma enzyme activities of *O. niloticus* increased, especially at 21-d, under the effect of $CuSO_4$ and CuO-NPs and their combination compared that in the control, observing a statistically significant difference ($F = 60.289$, $p = 0.000$ for ALT; $F = 22{,}458$, $p = 0.000$ for AST; $F = 19.035$, $p = 0.001$ for ALP; $F = 13{,}233$, $p = 0.002$ for LDH). It is estimated that these increases in the plasma enzyme activities occur due to cellular damage caused by both copper forms in the fish liver. Similar elevation trends in the enzyme activities of fish blood serum were also found by Fırat et al. (2011)[46] for Nile tilapia *O. niloticus* after metals (copper and lead) and pesticide (cypermethrin) treatments. The researchers concluded that all tested pollutants induced significant increases in the serum ALT, AST, ALP, and LDH activities as a result of chemical toxicity on the liver. Also, it was reported that iron oxide nanoparticles and zinc nanoparticles increase serum ALT, AST, ALP, and LDH activities in *O. niloticus*.[21,47] In another investigation, it was observed that there was a significant elevation in serum ALT, AST, and ALP activities in CuONPs-exposed fish groups compared to the control group.[26]

Table 1. Effects of individual and co-exposure of $CuSO_4$ and CuO-NPs on plasma enzyme activity of *O. niloticus*

Group	4 days	21 days
	ALT activity (U/L)	
Control	18.21 ± 0.48 **a**	18.44 ± 0.77 **a**
0.05 mg/L CuSO4	20.49 ± 0.93 **a**	27.07 ± 0.68 **b**
0.05 mg/L CuO-NPs	31.15 ± 0.74 **b**	34.66 ± 0.56 **c**
0.05 mg/L Cu-Mix	34.28 ± 0.53 **b**	44.72 ± 0.39 **d**
	AST activity (U/L)	
Control	136 ± 4.5 **a**	128 ± 5.6 **a**
0.05 mg/L CuSO4	127 ± 6.1 **a**	169 ± 3.9 **b**
0.05 mg/L CuO-NPs	141 ± 5.4 **a**	197 ± 6.1 **c**
0.05 mg/L Cu-Mix	173 ± 3.3 **b**	213 ± 5.2 **c**
	ALP activity (U/L)	
Control	25.34 ± 0.51 **a**	24.79 ± 0.63 **a**
0.05 mg/L CuSO4	24.89 ± 0.47 **a**	33.21 ± 0.70 **b**
0.05 mg/L CuO-NPs	24.60 ± 0.39 **a**	34.59 ± 0.66 **b**
0.05 mg/L Cu-Mix	31.93 ± 0.41 **b**	36.05 ± 0.39 **b**
	LDH activity (U/L)	
Control	422 ± 12 **a**	429 ± 18 **a**
0.05 mg/L CuSO4	431 ± 22 **a**	558 ± 11 **b**
0.05 mg/L CuO-NPs	417 ± 27 **a**	573 ± 23 **b**
0.05 mg/L Cu-Mix	552 ± 19 **b**	581 ± 17 **b**

Data are expressed as mean ± standard error (n = 6). Small letters (a, b, c and d) are used to determine the differences between treatment groups at the same time. There is a statistical difference between data denoted by different letters ($p < 0.05$, Student-Newman-Keuls test). Cu-Mix: $CuSO_4$ + CuO-NPs

Energy may be urgently needed to cope with stressful situations that occur under the influence of toxic substances in the fish. Cortisol and glucose, important stress metabolites, play an active role in energy requirement processes in such cases. Under stress, the fish brain releases excessive amounts of catecholamines and corticosteroid hormones, which in turn increase the breakdown of liver glycogen, causing elevated blood glucose levels.[48] In our work, plasma cortisol and glucose levels of *O. niloticus* sig-

nificantly elevated in response to both alone- and co-exposure of $CuSO_4$ and CuO-NPs at 4 and 21 days (Table 2). Increases in the plasma metabolite levels of fish treated with 0.05 mg/L of $CuSO_4$, CuO-NPs, and $CuSO_4$+CuO-NPs at 4 days were found to be 47%, 51%, and 56% for cortisol (F = 26.100, p = 0.000), and 59%, 64%, and 86% for glucose (F = 20.916, p = 0.000), respectively. We concluded that the plasma cortisol and glucose levels increased depending on meet the increasing energy needs in stress situations caused by these chemicals. Similar to our study findings, it was observed that exposures to various toxicants such as metals (Zn, Cd, and Zn+Cd) and metal oxide nanoparticles (CuO-NPs) in *O. niloticus* caused significant elevations in serum glucose and cortisol levels.[47,49] The researchers emphasized in these studies that increases in glucose and cortisol levels might be important processes in dealing with stress caused by toxicants. In the study conducted by Soliman et al. (2021)[5] 15 mg/L $CuSO_4$ or CuO-NPs significantly increased blood glucose levels of *O. niloticus*.

The plasma/serum BUN and creatinine levels are measured frequently to assess the kidney dysfunction and damage caused by chemicals. In toxicological researches, these parameters have been used as biochemical indicators to provide valuable information about renal functions. In our investigation, the creatinine and BUN were significantly elevated by all tested chemicals at 21 days (Table 2). Significant increases in levels of the creatinine (F = 12.576, p = 0.002) and BUN (F = 19.109, p = 0.001) were found with the treatments of $CuSO_4$ (64% and 52%) and CuO-NPs (65% and 93%), while marginally significant elevations in these parameters were noted in fish exposed to $CuSO_4$+CuO-NPs (148% and 171%). The increased plasma creatinine and BUN levels may demonstrate the significant pathological alterations of fish kidneys associated with toxicity of all tested copper compounds. In agreement with our results, Canli et al. (2018)[9] reported that *O. niloticus* after exposure to 1, 5, 25 mg/L of metal oxide nanoparticles (Al_2O_3, CuO, and TiO_2) for 14-d showed striking elevations in the serum creatinine and BUN levels, as their levels elevated nearly 10 folds. The researchers noted increased creatinine and blood urea nitrogen may reflect kidney failure as a result of nanoparticle toxicities. Also, a significant dose-dependent increase in BUN and creatinine levels was reported in *O. niloticus* exposed to 10, 20 and 50 mg/L CuO-NPs for 25 days.[26]

The levels of plasma proteins are closely related to liver function as most of these proteins are synthesized in this tissue.[50] Various chemicals can cause significant changes in plasma total protein levels, which may indicate their effects on protein metabolism in the liver. Cholesterol, another biochemical parameter, is an important component of cell membranes. Compared with the control, the individual and combined effects of $CuSO_4$ and CuO-NPs declined total protein levels (F = 14.261, p = 0.000) after 21 days whereas they did not cause a significant change in cholesterol levels during both exposure periods (F = 0.426, p = 0.742) (Table 2). Declined total protein levels may be the result of increased protein degradation or reduced protein synthesis in the fish liver caused by these chemicals. These findings are in agreement with the results of Fırat et al. (2011)[46] who noted *O. niloticus* exposed to lead and cypermethrin for 21 days showed significant decreases in the serum total protein levels. The exposures of CuO-NPs and CuO-bulks declined serum total protein levels of *O. niloticus*.[47] Also, 21-d exposure of 0.5 and 1.0 mg/L silver-NP (Ag-NP) declined serum total protein levels of *Cyprinus carpio* (common carp).[51] In another study, significant changes in the serum cholesterol levels of *O. niloticus* were not observed following exposures of Al_2O_3-, CuO-, and TiO_2-NPs.[9]

Pollutants such as metals, pesticides, and metal-based nanoparticles that enter aquatic ecosystems from

Table 2. Effects of individual and co-exposure of $CuSO_4$ and CuO-NPs on plasma metabolite level of *O. niloticus*

Group	4 days	21 days
	Cortisol level (ng/dL)	
Control	4.67 ± 0.17 **a**	4.78 ± 0.11 **a**
0.05 mg/L $CuSO_4$	6.86 ± 0.13 **b**	6.16 ± 0.22 **b**
0.05 mg/L CuO-NPs	7.04 ± 0.21 **b**	6.20 ± 0.19 **b**
0.05 mg/L Cu-Mix	7.29 ± 0.16 **b**	6.77 ± 0.34 **b**
	Glucose level (mg/dL)	
Control	51.44 ± 0.63 **a**	53.61 ± 0.71 **a**
0.05 mg/L $CuSO_4$	81.88 ± 0.74 **b**	75.18 ± 0.46 **b**
0.05 mg/L CuO-NPs	84.25 ± 0.52 **b**	76.09 ± 0.84 **b**
0.05 mg/L Cu-Mix	95.73 ± 0.81 **b**	98.57 ± 0.84 **c**
	Cholesterol level (mg/dL)	
Control	211 ± 3.51 **a**	205 ± 4.63 **a**
0.05 mg/L $CuSO_4$	217 ± 2.12 **a**	221 ± 5.27 **a**
0.05 mg/L CuO-NPs	208 ± 3.05 **a**	214 ± 2.71 **a**
0.05 mg/L Cu-Mix	223 ± 2.42 **a**	230 ± 4.30 **a**
	Total Protein level (g/dL)	
Control	4.30 ± 0.11 **a**	4.33 ± 0.08 **a**
0.05 mg/L $CuSO_4$	4.28 ± 0.13 **a**	3.40 ± 0.06 **b**
0.05 mg/L CuO-NPs	4.31 ± 0.07 **a**	3.28 ± 0.15 **b**
0.05 mg/L Cu-Mix	4.34 ± 0.08 **a**	3.17 ± 0.10 **b**
	BUN level (mg/dL)	
Control	0.015 ± 0.002 **a**	0.014 ± 0.002 **a**
0.05 mg/L $CuSO_4$	0.015 ± 0.001 **a**	0.023 ± 0.003 **b**
0.05 mg/L CuO-NPs	0.016 ± 0.002 **a**	0.027 ± 0.003 **b**
0.05 mg/L Cu-Mix	0.017 ± 0.003 **a**	0.038 ± 0.004 **c**
	Creatinine level (mg/dL)	
Control	0.022 ± 0.003 **a**	0.023 ± 0.002 **a**
0.05 mg/L $CuSO_4$	0.022 ± 0.002 **a**	0.035 ± 0.002 **b**
0.05 mg/L CuO-NPs	0.024 ± 0.002 **a**	0.038 ± 0.003 **b**
0.05 mg/L Cu-Mix	0.025 ± 0.003 **a**	0.057 ± 0.002 **c**

Data are expressed as mean ± standard error (n = 6). Small letters (a, b and c) are used to determine the differences between treatment groups at the same time. There is a statistical difference between data denoted by different letters ($p < 0.05$, Student-Newman-Keuls test). Cu-Mix: $CuSO_4$ + CuO-NPs

natural or anthropogenic sources can cause oxidative stress in fish by producing ROS. It is well known ROS containing highly dangerous radicals such as hydroxyl and superoxide anion cause serious damage to cells. To cope with oxidative stress, there are mechanisms in cells that prevent ROS formation and/or repair cellular damage caused by them. One of the most important of these mechanisms is antioxidant defence systems. This system consists of enzymatic antioxidants such as CAT, SOD, GPX, GR and GST, or non-enzymatic antioxidants such as GSH. It has been emphasized by many researchers that cellular antioxidant defence systems can be used as biomarkers of oxidative damage caused by metal-based nanoparticles and metals.[1,44,52]

CAT and SOD constitute the cell's first line of defence against ROS and play important biological roles in protecting cells from oxidative stress.[53] In the current study, CAT and SOD activities indicated a significant decrease at the end of 21 days in both liver (F = 15.707, p = 0.001; F = 38.458, p = 0.000, respectively) and gill (F = 17.750, p = 0.001; F = 14.149, p = 0.001, respectively) of fish exposed to individually or in a mixture of $CuSO_4$ and CuO-NPs (Table 3). When compared to the control group, these declines in the fish liver in the treatment groups of $CuSO_4$, CuO-NPs, and $CuSO_4$+CuO-NPs were found to be 38%, 46%, and 48% for CAT, and 41%, 42%, and 51% for SOD, respectively. Considering the biological roles of these enzymes in antioxidant defence, the decreases in SOD and CAT activities under the effect of both copper forms may cause a decrease in the defence abilities of cells against the toxic effects of superoxide and hydroxyl radicals. Similar results to our study were also observed in the research conducted by Tunçsoy et al. (2017)[44]. They reported that the SOD and CAT activities reduced in the liver and gill tissues of *O. niloticus* exposed to 20 µg/L CuO-NPs. Also, it was found that the gill tissue SOD and CAT activities of *O. niloticus*, which was exposed to 1.0 and 5.0 mg / L TiO_2-NP for 4 and 14 days, decreased significantly at the end of the first exposure period.[1] These researchers noted that depending on reduced SOD and CAT activities the cells may remain vulnerable to the toxicity of radicals and suffer from oxidative stress. Ag NP and bulk Ag particle exposure caused consistent decreases in both SOD and CAT activities in estuarine ragworm (*Nereis diversicolor*).[54]

GPX protects the cell against damage induced by hydrogen peroxide. Therefore, this enzyme, like CAT, plays significant roles in cellular defence against ROS. Changes in GPX activity affect the defence abilities of cells against toxicants. In our study, liver GPX activity of *O. niloticus* decreased after 4 days in $CuSO_4$ (29%), CuO-NPs (39%), and $CuSO_4$+CuO-NPs (43%) (F = 10.937, p = 0.003) (Table 3). Declined GPX activity may cause the accumulation of H_2O_2 in the cell. Due to the decreasing activities of both CAT and GPX enzymes under the effect of both copper forms, the insufficient removal of H_2O_2 may induce this ROS to turn into hydroxyl radical and thus cause damage to cell components. Consistent with our results, in *C. carpio* exposed to different concentrations of ZnO-NPs for 14 days, 50 mg/L nanoparticle concentration declined the liver, gill, intestine and brain GPX activities.[55]

GR, like CAT and SOD, protects cells against oxidative stress as an antioxidant that forms the primary line of defence against oxidative damage. It also plays an important role in GSH metabolism. GST, another antioxidant enzyme, has very effective and important roles in detoxification processes in cells. This enzyme catalyses the GSH conjugation to xenobiotics, protecting cells and their components from the harmful effects of these chemicals. Our research showed that in response to the tested all copper forms, GR and GST activities increased in both tissues at 4 days and decreased in the liver at 21 days (F = 8.382, p = 0.008; F = 20.878, p = 0.000, respectively) (Table 3). The induction of GR and GST activities may be an adaptation response to the toxic effects of $CuSO_4$ and CuO-NPs. Similarly, it was reported that the gill GR and GST activities of *O. niloticus* increased after TiO_2-NPs exposure as a rapid adaptation response to neutralize the toxicity of this nanoparticle.[1] The inhibition of GST activity may be related to decreased intracellular GSH levels in the effect of these chemicals, as determined in our study. In parallel with the results in our study, a similar decrease in GST activity was found in the tissues of freshwater fish, *Labeo rohita* (Indian major carp), treated with Ag-NP for 28 days.[22]

GSH, a cysteine-rich and low molecular weight tripeptide, acts in the cell as a protective agent against many toxic compounds.[56] Therefore, maintaining intracellular levels of GSH is crucial in both normal cell function and neutralization of toxic stress. Under the single and combined effect of $CuSO_4$ and CuO-NPs, the liver and gill GSH levels of *O. niloticus* increased at 4 days whereas they decreased at 21 days (F = 31.336, p = 0.000; F = 12.103, p = 0.002, respectively) (Table 3). Increases in GSH levels are may be important in neutralizing the toxic effects of both copper forms on the cells. However, the decrease in its levels with increasing time of exposure may be the result of the toxic effect of the chemicals on the synthesis of GSH or the increased cellular utilization of this tripeptide under oxidative stress. Similar to our study results, it was noted that the GSH level of the gill and liver tissues of *C. carpio* significantly increased in the treatment group of 0.5 mg/L ZnO-NP at 14 days.[55] GSH levels increased in the initial periods of defence responses against aquatic pollutants.[57] In another investigation, the effect of ZnO and ZnO-NP caused a decrease in the liver GSH levels of *Danio rerio* (zebrafish).[58]

Lipid peroxidation disrupts the selective permeability of cell membranes and can initiate processes that cause serious damage to cells. Lipid peroxidation has been attributed as one of the most important markers of oxidative damage caused by toxicants such as metals, pesticides,

and nano-metals in aquatic organisms. MDA is one of the lipid peroxidation products and increases in its levels provide critical information about the oxidative stress of toxicants and the severity of this stress. In our research, $CuSO_4$ and CuO-NPs exposures, either separately or in combination, after 21 days caused significant increases in MDA levels of liver (F = 10.855, p = 0.003) and gill (F = 6.747, p = 0.014) (Table 3). The levels of MDA elevate as a result of lipid peroxidation that occurs due to copper-induced ROS. These increases in MDA levels most likely demonstrate that these chemicals induce oxidative stress in fish tissues. In agreement with the current investigation, it was reported a similar elevation in the levels of tissue MDA, clearly indicating the lipid peroxidation in 5 and 50 mg/L ZnO-NP treated the fish, *C. carpio*, for 10 and 14 days.[55] Also, $CuSO_4$ and Cu-NPs increased lipid peroxidation in the gill tissue of *Oncorhynchus mykiss* (rainbow trout).[59] In another study, an elevation in MDA levels was observed in rat liver following aluminium chloride administration.[60] In a study investigating the comparative toxicity of copper oxide bulk and nanoparticles on fish, it was found that CuO-NPs have a more toxic ef-

Table 3. Effects of individual and co-exposure of $CuSO_4$ and CuO-NPs on tissue oxidative stress parameters of *O. niloticus*

	Liver		Gill	
Group	**4 days**	**21 days**	**4 days**	**21 days**
		CAT activity (U/mg)		
Control	470 ± 13 **a**	461 ± 15 **a**	165 ± 6.8 **a**	172 ± 3.8 **a**
0.05 mg/L $CuSO_4$	481 ± 16 **a**	285 ± 20 **b**	171 ± 5.5 **a**	129 ± 4.4 **b**
0.05 mg/L CuO-NPs	493 ± 21 **a**	247 ± 16 **b**	166 ± 4.7 **a**	122 ± 2.9 **b**
0.05 mg/L Cu-Mix	497 ± 18 **a**	241 ± 21 **b**	164 ± 2.3 **a**	98 ± 1.7 **c**
		SOD activity (U/mg)		
Control	27.40 ± 0.62 **a**	27.98 ± 0.43 **a**	21.70 ± 0.51 **a**	21.95 ± 0.44 **a**
0.05 mg/L $CuSO_4$	27.89 ± 0.54 **a**	16.65 ± 0.34 **b**	20.97 ± 0.34 **a**	14.13 ± 0.26 **b**
0.05 mg/L CuO-NPs	26.71 ± 0.78 **a**	16.24 ± 0.59 **b**	22.06 ± 0.65 **a**	13.60 ± 0.51 **b**
0.05 mg/L Cu-Mix	28.22 ± 0.83 **a**	13.83 ± 0.27 **c**	21.14 ± 0.49 **a**	13.19 ± 0.74 **b**
		GPX activity (U/mg)		
Control	0.51 ± 0.02 **a**	0.52 ± 0.04 **a**	0.31 ± 0.03 **a**	0.30 ± 0.02 **a**
0.05 mg/L $CuSO_4$	0.36 ± 0.04 **b**	0.50 ± 0.04 **a**	0.30 ± 0.03 **a**	0.34 ± 0.04 **a**
0.05 mg/L CuO-NPs	0.31 ± 0.03 **b**	0.48 ± 0.05 **a**	0.33 ± 0.02 **a**	0.31 ± 0.02 **a**
0.05 mg/L Cu-Mix	0.29 ± 0.04 **b**	0.47 ± 0.03 **a**	0.31 ± 0.03 **a**	0.35 ± 0.04 **a**
		GR activity (U/mg)		
Control	0.081 ± 0.003 **a**	0.085 ± 0.004 **a**	0.035 ± 0.002 **a**	0.034 ± 0.003 **a**
0.05 mg/L $CuSO_4$	0.104 ± 0.004 **b**	0.064 ± 0.005 **b**	0.045 ± 0.003 **b**	0.033 ± 0.002 **a**
0.05 mg/L CuO-NPs	0.108 ± 0.003 **b**	0.063 ± 0.003 **b**	0.047 ± 0.002 **b**	0.030 ± 0.005 **a**
0.05 mg/L Cu-Mix	0.133 ± 0.002 **c**	0.058 ± 0.004 **b**	0.051 ± 0.004 **b**	0.029 ± 0.003 **a**
		GST activity (U/mg)		
Control	29.18 ± 0.84 **a**	31.41 ± 0.64 **a**	14.76 ± 0.57 **a**	15.28 ± 0.63 **a**
0.05 mg/L $CuSO_4$	37.14 ± 0.69 **b**	24.49 ± 0.33 **b**	18.61 ± 0.73 **b**	14.91 ± 0.49 **a**
0.05 mg/L CuO-NPs	44.85 ± 0.51 **c**	23.55 ± 0.48 **b**	18.89 ± 0.89 **b**	15.13 ± 0.54 **a**
0.05 mg/L Cu-Mix	47.29 ± 0.77 **c**	17.91 ± 0.21 **c**	23.04 ± 0.61 **c**	14.77 ± 0.42 **a**
		GSH level (μmol/mg)		
Control	2.61 ± 0.14 **a**	2.72 ± 0.18 **a**	1.49 ± 0.05 **a**	1.54 ± 0.04 **a**
0.05 mg/L $CuSO_4$	3.40 ± 0.23 **b**	2.08 ± 0.15 **b**	1.85 ± 0.04 **b**	1.23 ± 0.03 **b**
0.05 mg/L CuO-NPs	3.52 ± 0.19 **b**	1.65 ± 0.22 **c**	1.96 ± 0.05 **b**	1.22 ± 0.03 **b**
0.05 mg/L Cu-Mix	4.16 ± 0.17 **c**	1.51 ± 0.13 **c**	1.99 ± 0.06 **b**	1.17 ± 0.02 **c**
		MDA level (nmol/mg)		
Control	2.11 ± 0.03 **a**	2.04 ± 0.03 **a**	1.73 ± 0.02 **a**	1.74 ± 0.03 **a**
0.05 mg/L $CuSO_4$	2.06 ± 0.02 **a**	2.89 ± 0.04 **b**	1.75 ± 0.03 **a**	2.13 ± 0.02 **b**
0.05 mg/L CuO-NPs	2.07 ± 0.04 **a**	2.97 ± 0.03 **b**	1.72 ± 0.02 **a**	2.22 ± 0.04 **b**
0.05 mg/L Cu-Mix	2.05 ± 0.03 **a**	3.58 ± 0.02 **c**	1.71 ± 0.03 **a**	2.32 ± 0.03 **b**

Data are expressed as mean±standard error (n = 6). Small letters (a, b, c and d) are used to determine the differences between treatment groups at the same time. There is a statistical difference between data denoted by different letters ($p<0.05$, Student-Newman-Keuls test). Cu-Mix: $CuSO_4$ + CuO-NPs

fect than CuO-bulks in liver and gill tissues of *O. niloticus* in most oxidative stress parameters.[47]

Similar to our study results, it was determined in other studies that the combined effect of chemicals had more toxic effects. The combined toxic effects of silica nanoparticles (SiNPs) and methylmercury (MeHg) on zebrafish *D. rerio*, a good model organism for toxicological researches, had more severe toxicity than the single exposure alone.[61] Concomitant (iron oxide nanoparticles+mercury) exposure displayed a synergistic response to that of individual responses of either iron oxide nanoparticles or mercury which was evident by significant increases in GST and lipid peroxidation of the gills of *Anguilla Anguilla* (European eel).[62] In an investigation determining impact of co-exposure of aldrin, a pesticide, and titanium dioxide nanoparticles at biochemical and molecular levels in Zebrafish (*D. rerio*), it was observed that the combined effect of chemicals on oxidative stress parameters was generally higher than the effect alone.[63] Similarly, the combined effect of carbon nanotubes as nanomaterial and carbofuran as pesticide on *A. ribeirae* (fish) was found to be higher than the effect of these chemicals alone.[16]

4. Conclusions

The current investigation demonstrated that almost all biochemical and oxidative stress parameters examined were negatively affected by $CuSO_4$ and CuO-NPs, alone or in combination and that these chemicals caused cytotoxic and oxidative damage in *O. niloticus*. Also, our results illustrate that $CuSO_4$ and CuO-NPs have similar toxic effects in the fish; however, the combined effects of these two chemicals were higher than on the individual exposure regarding the biochemical changes and the oxidative stress observed in *O. niloticus*.

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Conflict of interest

The authors declare that they have no conflict of interest

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Povzetek

Baker je, tako kot železo in cink, eden najpomembnejših elementov v sledovih za organizme. Različne oblike bakra imajo značilno in specifično uporabo. Nanodelci bakrovega oksida (CuO-NP) se npr. v svetu pogosto uporabljajo kot nanomaterial. Bakrov sulfat ($CuSO_4$) se po vsem svetu uporablja kot fungicid v kmetijstvu in kot algicid v ribogojništvu. Danes vse večja uporaba teh kemikalij vzbuja zaskrbljenost zaradi njihovih možnih učinkov na zdravje vodnih organizmov in ekoloških tveganj. Zato so bili v pričujoči raziskavi ovrednoteni toksični učinki $CuSO_4$ in CuO-NP, samostojno in v kombinaciji, z uporabo biokemijskih markerjev (plazemsko-biokemijski ter škržni in jetrni oksidativni stres) pri sladkovodnih ribah *Oreochromis niloticus*. Ribe so bile izpostavljene 0,05 mg/L $CuSO_4$, CuO-NP in $CuSO_4$ + CuO-NP 4 in 21 dni. Predvsem po 21 dneh sta $CuSO_4$ in CuO-NP, samostojno in v kombinaciji, na splošno povečala nivo plazemske alkalne fosfataze, aspartat aminotransferaze, alanin aminotransferaze, laktatne dehidrogenaze, kortizola, glukoze, kreatinina, dušika iz sečnine v krvi in tkivnih proteinov, medtem ko sta zmanjšala nivo skupnega malondialdehida v tkivih, tkivne superoksidne dismutaze, katalaze, glutation-S-transferaze, glutation reduktaze in glutationa. Posledično naši rezultati kažejo, da imata $CuSO_4$ in CuO-NP podobne toksične učinke pri ribah, vendar je sočasna izpostavljenost CuO-NP in $CuSO_4$ bolj strupena kot učinki posameznih kemikalij.