Original Article

Effects of Selected Metal Oxide Nanoparticles on Multiple Biomarkers in *Carassius auratus*[*]

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Abstract

Objective To study the biological effects of nanoscale copper oxide (nCuO), zinc oxide (nZnO), cerium dioxide ($nCeO_2$) and their mixtures on *Carassius auratus*.

Methods Juvenile fish (*Carassius auratus*) were exposed to aqueous suspensions of nCuO, nZnO, and $nCeO_2$ (alone and in mixtures) at concentrations of 20, 40, 80, 160, and 320 mg/L. The biomarkers-acetylcholinesterase (AChE) in brain, sodium/potassium-activated ATPase (Na^+/K^+-ATPase) in gill, and superoxide dismutase (SOD) and catalase (CAT) in liver-were determined after 4 days of exposure. Integrated biomarker response (IBR) was calculated by combining multiple biomarkers into a single value.

Results AChE and SOD activities were significantly inhibited by all test metal oxide nanoparticles (NPs) at high concentrations (≥160 mg/L) with the exception of $nCeO_2$. Na^+/K^+-ATPase induction exhibited bell-shaped concentration-response curves. CAT activity was significantly inhibited at concentrations equal to or higher than 160 mg/L. The order of IBR values was $nCeO_2 \approx nZnO/nCeO_2 \approx nCuO/nCeO_2 < nCuO/nZnO/nCeO_2 < nZnO < nCuO < nCuO/nZnO$. The joint effect seemed to be synergistic for nCuO/nZnO mixtures, additive for the ternary mixture and less than additive or antagonistic for the binary mixtures containing $nCeO_2$.

Conclusion Concentration-dependent changes of enzymatic activities (AChE, Na^+/K^+-ATPase, SOD, and CAT) were observed in fish exposed to nanoscale metal oxides. IBR analysis allowed good discrimination between the different exposures and might be a useful tool for the quantification of integrated negative effects induced by NPs toward fish.

Key words: Metal oxide NPs; Coexposure; Biomarker; *Carassius auratus*

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INTRODUCTION

Metal oxide nanoparticles (NPs) are already manufactured in a large scale for both household and industrial use. Nanoscale copper oxide (nCuO) has been applied to gas sensors, catalysts, magnetic storage media, solar energy conversion, superconductors and ceramic pigments[1-2]. Nanoscale zinc oxide (nZnO) can enhance antimicrobial and ultraviolet

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filtering properties and has been used in products like paint, plastic, toothpaste, and sunscreen[3-4]. Nanoscale cerium dioxide ($nCeO_2$) is one of the most important rare-earth oxides that have been widely used in the automotive industry and electrolyte materials of solid oxide fuel cells[5]. NPs are of interest because of their novel properties such as small particle size, large surface-to-volume ratio and greater reactivity. However, the unique properties of these materials have raised questions concerning potential adverse effects on human and environmental health[6].

Metal oxide NPs may leak into natural bodies of water in their life cycles (production, storage, transportation, consumption, disposal, or reproduction), but relatively little is known about the magnitude of NPs released and exposed to organisms living in impacted aquatic environments, as well as the potential for toxic effects to aquatic species[7]. Therefore, there is an urgent need for information on the ecological risks of metal oxide NPs. Heinlaan et al.[8] found that both nCuO and nZnO were toxic to bacteria and crustaceans. García et al.[9] obtained a 48 h medican lethal concentration of 12 mg/L for $nCeO_2$ treated on *Daphnia magna*. Furthermore, 50 and 100 mg/L of nZnO killed zebrafish embryos after 96 h of exposure and caused malformation at concentrations ranging from 1 to 25 mg/L[10]. nCuO at 1.5 mg/L caused acute mortality on zebrafish (*Danio rerio*) after 48 h of exposure[11].

In an aquatic environment, the exposure of living organisms to NPs leads to interactions between the chemical and the biological system and may give rise to biochemical disturbances or/and adaptive responses[12-13]. The biological responses, termed biomarkers, can be used to assess the health status of organisms and to obtain the earliest signs of environmental disturbances[14]. However, current information is lacking on the biomarker responses of NPs on fish, especially the joint effects of coexposed NPs.

In order to investigate the possible negative effects of nCuO, nZnO, and $nCeO_2$ (alone and in mixtures) on fish, brain acetylcholinesterase (AChE) activity, gill sodium/potassium-activated ATPase (Na^+/K^+-ATPase), liver superoxide dismutase (SOD) and catalase (CAT) activities were measured in *Carassius auratus.* The integrated biomarker response (IBR) computed with biomarker measurements was used to assess the comprehensive effects of the different exposures of metal oxide NPs.

MATERIALS AND METHODS

Chemicals

Aqueous dispersion of nCuO (stated particle size of 40 nm, surface area 80 m^2/g, purity 99.9% W/W), nZnO (stated particle size of 40 nm, surface area 90 m^2/g, purity 99.9% W/W) and $nCeO_2$ (stated particle size of 20 nm, surface area 95 m^2/g, purity 99.9% W/W) was obtained from Beijing Nachen S&T Ltd. (Beijing, China). Acetylthiocholine iodide, ouabain and ammonium molybdate were purchased from Sigma Chemical Company (St. Louis, MO, USA), and the stated purities were >99.9%. Coomassie brilliant blue G-250 (Ultra Pure Grade) and 5,5-dithiobis (2-nitrobenzoic acid) (99% purity) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Triton X-100 was purchased from Shanghai Lingfeng Chemistry Reagent Co., Ltd. (Shanghai, China), and the purity was >99%. Bovine serum albumin was purchased from Shanghai Huixing Biochemistry Reagent Co., Ltd. (Shanghai, China), and the purity was >98%. All other chemicals were of analytical grade and obtained from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China).

Nanoparticle Preparation

The stock solution was prepared by dispersing the NPs in ultrapure water (Millipore, Billerica, MA, USA) with ultrasonication (50-60 kHz) for 15 min before dosing every day. For the present study, the aqueous suspensions of nCuO, nZnO, and $nCeO_2$ (all at 80 mg/L) were characterized by transmission electron microscopy (TEM) in ultrapure water. The particle size distributions of NPs at 80 mg/L and pH 7.7 were determined by Malvern Mastersizer 2000.

Animals

Carassius auratus (Linnaeus, 1758) can be found in freshwaters throughout China. Approximately 400 juvenile fish of both sexes (weighing 25.23±4.12 g) were obtained from Nanjing Institute of Fishery Science (Nanjing, China). The fish were acclimatized for two weeks in dechlorinated municipal water prior to testing. Fish were fed with OSI freshwater aquarium pellet food (6% of body weight/day). Feces and uneaten food were removed every day by suction. Fish were not fed for 24 h prior to the experiments, and no food was provided during the test period.

Sublethal Toxicity Test

Three randomly assigned fish were kept in 30 L

glass tanks (40 cm × 25 cm × 30 cm) containing 20 L of various experimental solutions under constant aeration. According to the results of preliminary experiments, no mortality occurred when the concentrations of the selected metal oxide NPs were less than 320 mg/L. The tested animals were exposed to nominal nCuO, nZnO, and $nCeO_2$; binary mixtures of nCuO/nZnO, nCuO/$nCeO_2$, nZnO/$nCeO_2$; and a ternary mixture of nCuO/nZnO/$nCeO_2$ at concentrations of 20, 40, 80, 160, and 320 mg/L. The binary and ternary mixtures were tested at an equi-concentration ratio of 1:1 or 1:1:1 (W/V). A blank control was included in the experimental design. A semi-static test was conducted by replacing 10 L of experimental solutions every day. There were three replicate tanks per treatment. Water temperatures ranged from 16 to 18 °C, with pH at 7.0±0.2, dissolved oxygen at 5.8±0.2 mg/L and natural photoperiod at 4 d of exposure.

Biomarker Assays

All fish (9 fish in each treatment) were collected after 4 d of exposure and were killed by cervical transection. The brain, gill, and liver tissues were carefully dissected, washed in 0.15 mol/L of cold KCl, weighed and immediately frozen in liquid nitrogen. Each tissue sample of each fish was individually stored at -80 °C[15].

Brain samples were homogenized in 9 volumes of cold phosphate buffer (0.1 mol/L, pH 7.2, triton 1%) on ice and centrifuged for 20 min at 10 000 ×g at 4 °C. The supernatants were used as the enzyme extract for AChE activity determination. Forty volumes of cold buffer (0.04 mol/L imidazole, 0.25 mol/L saccharose, 0.005 mol/L EDTA, pH 7.0) with samples of gill were homogenized and then centrifuged for 25 min (9000 × g) at 4 °C. Liver samples were homogenized in nine volumes of cold buffer (0.15 mol/L KCl, 0.1 mol/L Tris-HCl, pH 7.4) and centrifuged for 30 min at 10 000 ×g.

AChE activity was determined at 405 nm using a microplate reader (Molecular Device VersaMax, USA) by the method of Guilhermino et al.[16]. AChE activity was expressed as nmol/mg protein/min. Na^+/K^+-ATPase activity in the gill was assayed according to the method of Agrahari and Gopal[17]. Na^+/K^+-ATPase was calculated from the difference of the activity of total ATPase and ouabain-insensitive ATPase and was expressed as μmol Pi liberated/mg protein/h. Liver SOD activity was determined at 420 nm by the method of Marklund and Marklund[18]. Using 300 μL Tris-HCl buffers in microplates warmed at 25 °C for 10 min, the auto-oxidation reaction was started by adding 10 μL of homogenate and 6 μL of preheated pyrogallol. SOD activities were expressed as U/mg protein. One unit (U) was defined as the enzyme that caused 50% inhibition of pyrogallol auto-oxidation. Liver CAT activity was measured according to the ammonium molybdate method[19]. CAT activity was expressed as μmol H_2O_2/min/mg protein. Protein concentrations of brain, gill, and liver were determined at 595 nm using a method developed by Bradford[20], with bovine serum albumin as the standard.

Calculation of the IBR

A method for integrating all the measured biomarker responses into one general "stress index", termed "Integrated Biomarker Response" (IBR)[21], was applied to evaluate an integrated impact of toxicants. The procedure for determining the IBR first involved calculating the mean and standard deviation of the biomarker response in each treatment. Then, data were standardized for each treatment according to the equation $F_i'=(F_i-\text{mean}F)/S$, where F_i' is the standardized value of the biomarker, F_i is the mean value of a biomarker from each treatment, mean F is the mean of the biomarker calculated for all the treatments and S is the standard deviation calculated for the treatment-specific values of each biomarker. Using standardized data, Z was computed as $+F_i'$ in the case of an activation and $-F_i'$ in the case of an inhibition, and then the minimum value for all treatments for each biomarker was obtained and added to Z. Finally, the score B was computed as $B=Z+|\min|$, where $B \geq 0$ and $|\min|$ is the absolute value of the minimum. The corresponding IBR value was: $\{[(B_1\times B_2)/2] + [(B_2\times B_3)/2] + \cdots [(B_{n-1}\times B_n)/2] + [(B_n\times B_1)/2]\}$[22].

Statistical Analysis

For each biomarker, the data were expressed as mean±standard deviation (SD, n=9). All the data from the different treatments were checked for normality and compared using one-way ANOVA. Statistically different treatments were identified by Dunnett's test. All differences were considered significant at P<0.05. Statistical analyses were performed using the SPSS statistical package (ver. 17.0, SPSS Company, Chicago, IL, USA).

RESULTS

Changes in various environmental parameters,

such as pH and ionic strength, can lead to the aggregation of NPs released in the environment. Both aggregates and individual nanoscale particles were present in nZnO, nCuO, and $nCeO_2$ suspensions. The TEM images of nCuO, nZnO, and $nCeO_2$ are presented in Figure 1. Average particle sizes of nCuO, nZnO, and $nCeO_2$ aqueous dispersion were 51.09±9.98, 48.16±13.56, and 32.24±10.12 nm, respectively. In addition, we measured the free ions released from metal oxide NPs, and the dissolution rates ranged from 4% to 10%. Nanoscale CuO was found to dissolve more free ions, and the dissolution rate decreased with increasing concentration.

Brain AChE activity, gill Na^+/K^+-ATPase activity, and liver CAT and SOD activities after 4 d of exposure to metal oxide NPs are presented in Figure 2. Exposures of nCuO, nZnO, $nCeO_2$ and their binary and ternary mixtures significantly altered brain AChE activity (Figure 2A). All the test metal oxide NPs significantly induced AChE activity at the concentration of 40 mg/L (P<0.05), with the exception of the nCuO/nZnO and nCuO/$nCeO_2$ mixtures. However, AChE activity was inhibited at higher concentrations of NPs, and the decreasing level of AChE activity correlated with increasing concentration. The highest concentrations of nCuO and nCuO/nZnO resulted in the most significant decrease of AChE activity, with inhibition rates of 47% and 53%, respectively. Similar AChE alterations were observed when exposed to mixtures containing $nCeO_2$ in all the test concentrations.

The responses of gill Na^+/K^+-ATPase activity exposed to different metal oxide NPs are presented in Figure 2B. Na^+/K^+-ATPase activity did not change much at the lowest and highest concentrations in most cases compared to the controls, whereas significant increase in activity was seen at the medial concentrations (P<0.05). Nanoscale CuO, ZnO, and CeO_2 (alone or in mixtures) exhibited bell-shaped concentration-response curves. The most significant inductions of Na^+/K^+-ATPase were found at 80 mg/L for all cases, with a maximum induction rate of 1.73-fold for the nCuO/nZnO mixture.

The responses of liver SOD activity exposed to metal oxide NPs are presented in Figure 2C. Exposures of nCuO, nZnO and nCuO/nZnO at 20 mg/L and $nCeO_2$ at 40 mg/L significantly increased SOD activity, while SOD activity was significantly inhibited at concentrations equal to or higher than 80 mg/L by all tested NPs with an exception of $nCeO_2$. SOD inhibition increase corresponded to the increase of nanoscale metal oxide concentrations. Similar concentration-response relationships were obtained for individual and mixed exposures in the present study, and there seemed to be an additive effect.

The responses of liver CAT activity exposed to metal oxide NPs are presented in Figure 2D. The response pattern of CAT was similar to that of SOD. Metal oxide NPs did not significantly alter liver CAT activity at the lowest concentrations compared to the controls. Exposures of nCuO, nCuO/nZnO (≥40 mg/L), and $nCeO_2$ (≥160 mg/L) significantly inhibited CAT activity, and the inhibition rates increased in a concentration-dependent manner. The inhibition of CAT activity in mixtures exhibited an additive effect when compared to that of the single compounds.

Standardization was carried out on brain AChE, gill Na^+/K^+-ATP and liver SOD and CAT activities obtained from each chemical and their mixtures, and the IBR index was calculated. The star plots of IBR after 4 days of exposure to metal oxide NPs at the concentration of 80, 160, and 320 mg/L are shown in Figure 3. In general, IBR values showed a large range of variation when exposed to different metal oxide NPs.

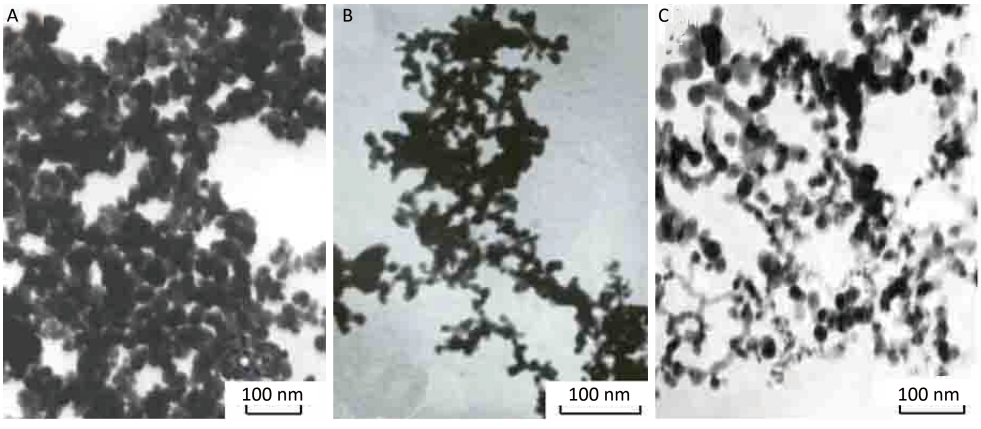

Figure 1. TEM images of nCuO (A), nZnO (B), and $nCeO_2$ (C).

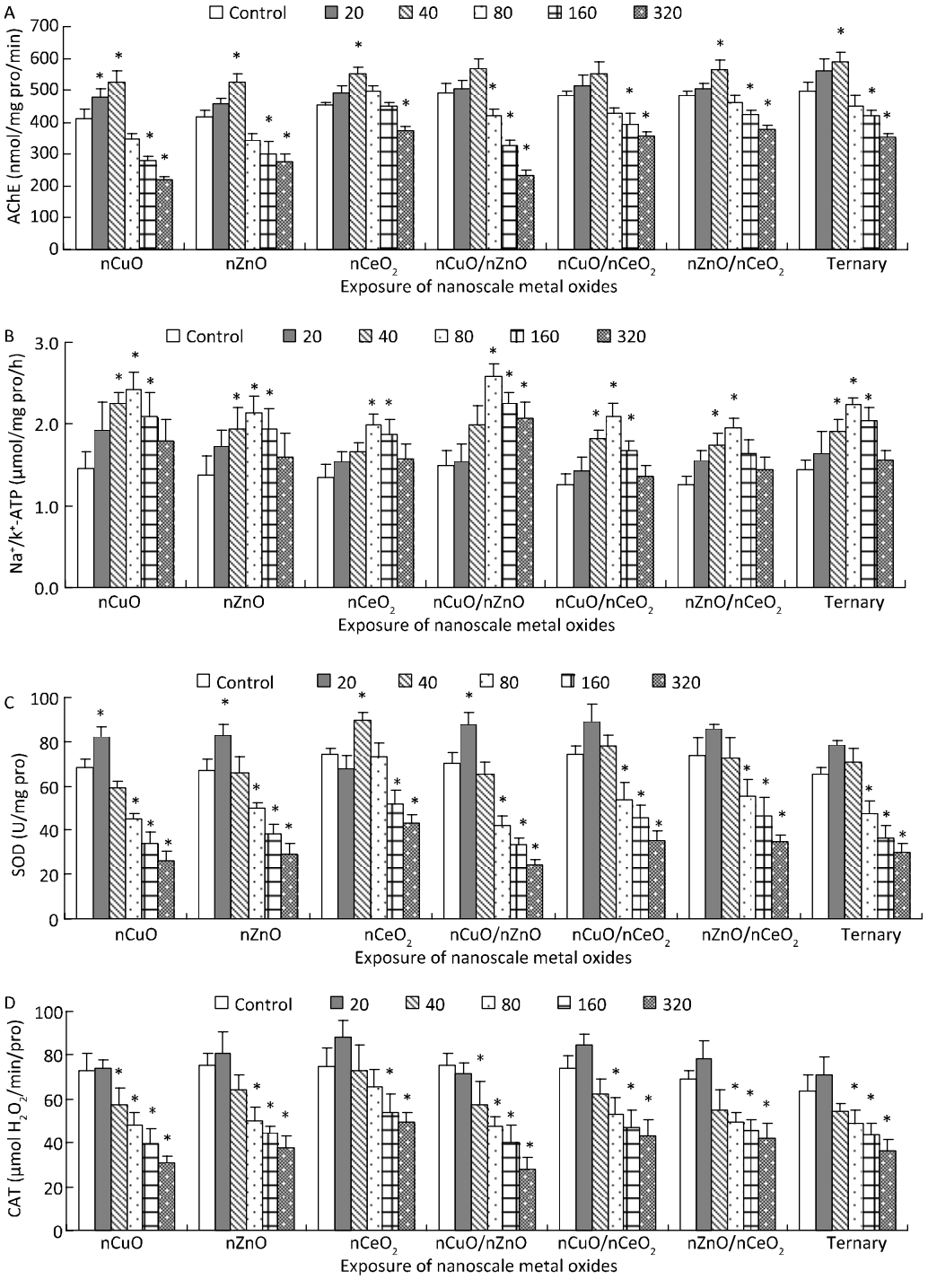

Figure 2. Biomarker response after 4 d of exposure to metal oxide NPs. Bars indicate standard error of the mean (n=9), and asterisks indicate values that are significantly different from control values (P<0.05).

In addition, a visual agreement can be observed among Figure 3A, B, and C. The IBR values elevated with increased concentrations of all NPs (alone and in mixtures), and showed the following order: $nCeO_2$ ≈ $nZnO/nCeO_2$ ≈ $nCuO/nCeO_2$ < $nCuO/nZnO/nCeO_2$ < nZnO < nCuO < nCuO/nZnO.

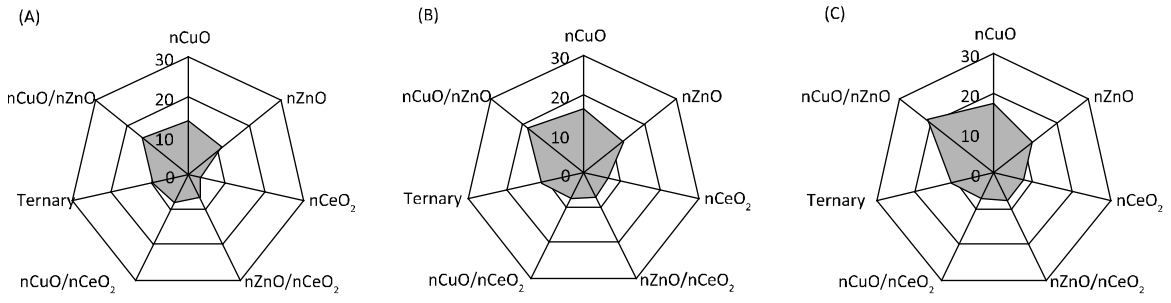

Figure 3. IBR variation after 4 days of exposure to NPs at 80 mg/L (A), 160 mg/L (B), and 320 mg/L (C).

DISCUSSION

AChE can hydrolyze the neurotransmitter acetylcholine in cholinergic synapses and is one of the most important enzymes for many physiological functions in higher organisms, such as locomotion, orientation, feeding and predator evasion[23]. Alterations of AChE activity can influence the process of cholinergic neurotransmission and promote undesirable effects, which have been observed in several neurological disorders[24-25]. AChE inhibition was observed in an *in vitro* study of electric eels exposed to copper NPs, multi-walled carbon nanotubes and single-walled carbon nanotubes (SWCNT)[26]. However, nCuO, nZnO, and $nCeO_2$ (alone and in binary and ternary mixtures) enhanced brain AChE activity at the lower concentrations in the present study. Perhaps this contradictory result is a response to an injurious toxicological situation, and fishes could be compensating stress as demonstrated by enhanced AChE activity[25]. A slight inductive effect of NPs on AChE activity observed in this study can be related to a generally favorable biological response to low exposures of toxicants and other stressors (hormesis effect)[27]. Moraes et al.[28] reported that herbicide containing imazethapyr and imazapic (98.5 and 20.9 μg/L, respectively) increased brain AChE activity in carp (*Cyprinus carpio*). Xuereb et al.[29] found that chlorpyrifos and methomyl significantly altered feeding rate and locomotor behavior when the AChE inhibition in *Gammarus fossarum* was higher than 50% during short-term exposure (96 h). NPs significantly decreased AChE activity in the current study at the higher concentrations, although the highest inhibition rates in most cases were less than 50%. Similarly, 100 mg/L of nCuO significantly inhibited brain AChE activity in juvenile carp after 10 d of exposure with an inhibition rate of about 40%[30].

The inhibitory rates of nCuO/nZnO mixtures on AChE activity were slightly higher than those of the corresponding individual exposures at higher concentrations (80, 160, and 320 mg/L), suggesting that nCuO combined with nZnO may induce more than just additive effects on fish neurotoxicity. In addition, the inhibition rates of $nCuO/nCeO_2$ and $nZnO/nCeO_2$ were higher than that of $nCeO_2$ but lower than that of nCuO and nZnO, and there may be additive effects. Additive effects on AChE inhibition were also observed at higher concentrations of pesticide coexposure by Laetz et al.[31].

Gill Na^+/K^+-ATPase is a membrane-bound enzyme that catalyzes the active Na^+ and K^+ transport in animals, providing a driving force in the gill epithelium[32]. In the present study, the tested metal oxide NPs caused elevated Na^+/K^+-ATPase activity in gills compared to the controls. A similar result was reported in rainbow trout (*Oncorhynchus mykiss*) exposed to SWCNT for 10 days[33]. In contrast, Federici et al. reported that $nTiO_2$ (0.5 and 1.0 mg/L) statistically significantly decreased gill Na^+/K^+-ATPase activity in rainbow trout after 14 d of exposure[34]. Gill ATPases are intimately involved in osmoregulation, acid-base regulation and respiration of fish[35]. The marked alteration of Na^+/K^+-ATPase activity may disrupt the processes and produce adverse effects in organisms[17].

The induction effects of binary and ternary mixtures on Na^+/K^+-ATPase activity did not significantly differ from those in individual exposures. However, Na^+/K^+-ATPase activity induced by nCuO/nZnO was significantly higher than that of the corresponding individual exposures at the three highest concentrations ($P<0.05$), suggesting that there might be a synergistic effect.

Cellular oxidative stress occurs when pro-oxidant forces overwhelm antioxidant defenses. These antioxidant defenses comprise enzymatic and non-enzymatic mechanisms[36]. SOD is important in the disproportionation of superoxide anions into

hydrogen peroxide (H_2O_2) and dioxygen[37]. The SOD-CAT system provides the first line of defense against oxygen toxicity and is usually used as a biomarker of reactive oxygen species (ROS) production[38]. Results of the present investigation indicated that individual chemicals and the nCuO/nZnO mixture significantly induced SOD activity at the lowest concentration. The induction of SOD activity revealed that fish might suffer from severe oxidative stress. This result is consistent with a previous study performed with fish exposed to carbamazepine[38]. However, inhibitory effects of SOD activity were observed at higher concentrations (≥80 mg/L), with a gradual decrease in inhibition as concentrations increased. Similarly, Zhang et al.[39] found that SOD activity decreased gradually as the concentration of 2,4-dichlorophenol increased. As NPs are able to generate ROS in liver, the change in SOD activity may indicate that oxidative stress and tissue damage were induced in the liver by NPs through superfluous ROS[40]. In addition, the decrease of SOD may be the result of adaptation or loss in compensatory mechanisms[41].

CAT is mainly located in the peroxisomes and, along with glutathione peroxidase, is responsible for the reduction of H_2O_2 produced from the metabolism of long chain fatty acids in peroxisomes[42]. CAT has one of the highest turnover rates of all enzymes: one molecule of CAT can convert millions of molecules of hydrogen peroxide to water and oxygen per second[43]. In the current study, liver CAT activity in fish was significantly inhibited by high concentrations of NPs. This reduction demonstrates that NPs induce peroxidative damage in the liver by altering the levels of CAT. Such disruption of anti-oxidant systems would enhance the generation of ROS and produce more serious oxidative damage to tissues[44].

IBR index was calculated by combining different biomarkers into a single value, which can be a useful basis for interpreting ecotoxicological surveys[45]. Given that the IBR is an indicator of environmental stress, nCuO was the most stressful chemical for fish, followed by nZnO and $nCeO_2$. The IBR value of nCuO/nZnO mixture was higher as compared with that of individual compounds, and a synergistic effect was observed. The ternary mixture exhibited an additive effect, while there seemed to be a decreasing, or antagonistic, effect for the binary mixtures containing $nCeO_2$. The present results demonstrate that the IBR index can be a useful tool for quantifying the integrated responses induced by NPs toward fish. This is consistent with the results of previous studies. Li et al.[46] calculated the IBR index using multiple biomarkers to discuss the overall stress of propiconazole on fish blood system and found that the IBR index was a simple tool for determining the general ''health status'' of organisms. Kim et al.[47] also found that the IBR index was useful for the quantitative assessment of the toxicological effects of perfluorooctanoic acid and perfluorooctane sulfonate in the common carp.

In conclusion, the current study investigated the biological effects of nCuO, nZnO, and $nCeO_2$ (single compounds and mixtures) on the brain AChE, gill Na^+/K^+-ATPase activity, and liver SOD and CAT activities in *Carassius auratus*. Obvious concentration-response relationships were observed in all cases. IBR analysis allowed a good discrimination between different exposures of metal oxide NPs. With regard to IBR variation, coexposure of nCuO and nZnO produced a synergistic effect, whereas there was an additive effect for the ternary mixture. The binary mixtures containing $nCeO_2$ exhibited less than additive, or antagonistic, effects. The present study indicates that IBR might be a useful tool for quantifying the integrated negative effects induced by coexisting chemicals toward fish.

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