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Immunotoxicity of four nanoparticles to a marine bivalve species, *Tegillarca granosa*

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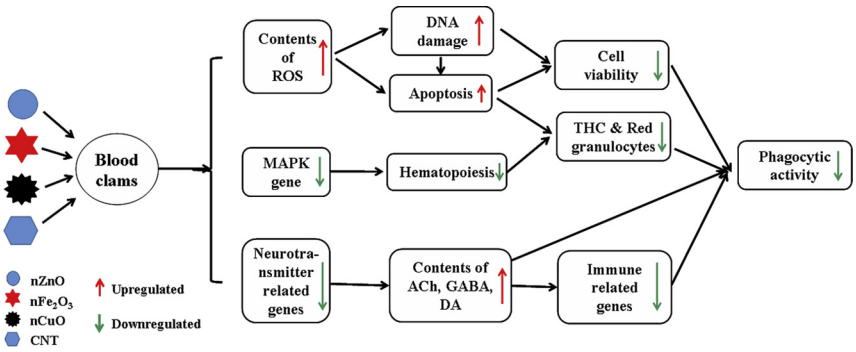
GRAPHICAL ABSTRACT



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ABSTRACT

The increasing application of nanomaterials drives the unintentional release of nanoparticles (NPs) into the ocean, which may pose a potential threat to marine organisms. It has been demonstrated that exposure to NPs could chanllenge the immune responses of marine species. However, the affecting mechanism behind remains poorly understood. In this study, the immunotoxic impacts and the mechanisms underpinning the effects of four major NPs, including nZnO, nFe_2O_3, nCuO, and carbon nanotube (MWCNT), were investigated in blood clam, *Tegillarca granosa*. The results showed that exposure to tested NPs resulted in reduced total counts, altered cell composition, and constrained phagocytic activities of haemocytes. The intracellular contents of reactive oxygen species (ROS) and the degree of DNA damage of haemocytes were significantly induced, whereas the haemocyte viability was suppressed. Furthermore, NP exposures led to significant increases in the *in vivo* contents of neurotransmitters. Down-regulations of the immune- and neurotransmitter-related genes were detected as well. Our data suggest that NP exposures hampered the immune responses of blood clams most likely through (1) inducing ROS, causing DNA damage, and reducing cell viability of haemocytes, (2) altering the *in vivo* contents of neurotransmitters, and (3) affecting the expression of immune- and neurotransmitter-related genes.

1. Introduction

Nanomaterials are materials with at least one dimension in the three-dimensional space at nanometer scale [1]. Due to intrinsic merits in material characteristics, engineered nanomaterials are currently widely used in various aspects of modern life [2]. For instance, zinc

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oxide nanoparticles (nZnO) are applied in the manufacturing of solar cells, sensors, semiconductors, paints, and cosmetics [3–5]. Iron oxide nanoparticles are gaining popularity in magnetic drug delivering, waste water treatment, and food/feed production [6–9]. Similarly, with excellent catalytic activity and thermal conductivity, copper oxide nanoparticles (nCuO) are used as catalysts and machine-parts [10,11]. In addition to metal oxide nanoparticles, carbon nanotubes (CNTs), one of the most important carbon-based NPs, are widely applied in industrial applications and drug delivery as well [12,13].

With the rapid boost of application and production of engineered nanomaterials [2,8,14], it has been predicted that increasing amount of nanoparticles (NPs) will be released into its ultimate sink, the ocean [1,15,16]. The concentrations of NPs are expected to reach as high as mg/L in the ocean, especially in the sediment [17,18], posing a potential threat to marine species [19]. In recent years, a series of adverse impacts of NPs, such as hindering fertilization [20–22], inhibiting larval development [23,24], arresting growth [25,26], and depressing immune responses [15,16] have been demonstrated in a variety of marine organisms [1]. For instance, exposure to 10 μg/L Ag-NP and nCuO for 15 days exerted significant DNA damage in mussel *Mytilus galloprovincialis* [27].

Since nanoparticles can be easily ingested by bivalve mollusks through the process of filter feeding [1,8], benthic sessile bivalves could be one of the most vulnerable species to NP contamination [22,28–30]. Essentially, arming a robust immune system is crucial for the survival of bivalve mollusks in the complicated marine environment [31,32]. However, in recent years, an increasing number of studies has demonstrated that exposure to NP pollution could hamper the immune responses of bivalve mollusks [14,15,28]. For example, it has been demonstrated that one day exposure of blue mussel *M. galloprovincialis* to different concentrations (0.05, 0.2, 1, 5 mg/L) of nano-carbon black (NCB), nano-fullerene (C60), nano-titanium dioxide ($nTiO_2$), and nano-silicon dioxide ($nSiO_2$) led to significantly depressed immune responses [15,28]. Similarly, obvious reductions in the total haemocytes count, phagocytic activity, and esterase and lysosomal contents upon 14-days exposure to $nTiO_2$ were observed in the thick shell mussel, *M. coruscus* [33]. In addition, it has been shown that 30-days of $nTiO_2$ exposure significantly constrained the total counts, phagocytic activity, red granulocytes ratio of the haemocytes, as well as the expressions of immune-related genes, in the blood clam *Tegillarca granosa* [16]. Though the immunotoxic effects of NPs have been increasingly revealed in bivalve mollusks, the underlying mechanism awaits further exploration.

Studies carried out in model species and/or cell lines have demonstrated that exposure to NPs could provoke *in vivo* oxidative stress, such as the induction of reactive oxygen species (ROS) [34,35]. Since the structural integrity and normal function of many biomolecules are sensitive to ROS [36,37], it is possible that NPs attack immunity of bivalve species by imposing oxidative stress. However this hypothesis deems further verification with more systematic experiment data.

More recently, it has been suggested that NPs could be neurotoxic to bivalve mollusks [39]. For example, both the *in vivo* contents of neurotransmitters and the activity of acetylcholinesterase (AChE) were found to be significantly altered by 96-h of exposure of blood clam, *T. granosa*, to $nTiO_2$ [39]. Since neurotransmitters, such as acetylcholine (ACh), γ-aminobutyric acid (GABA), and dopamine (DA), play crucial roles in modulating immune responses [40–42], the adverse impacts of NPs on bivalve species could be *via* neurotoxicity. However, with few reports on neurotoxicity of NPs, the claim/proposal of a general relationship between neurotoxicity and bivalve species and different types of NPs is still in its infancy and requires robust investigation. The blood clam, *T. granosa*, is a commercial marine bivalve species that is widely distributed along the coasts of Indo-Pacific [31,43]. As a typical benthic filter feeder inhabiting the intertidal coasts where pollutants, such as NPs, are often concentrated [32,44], the blood clam may expose to a high concentration of NP contaminations, with possible consequence unknown. Though evident immunotoxicity and neurotoxicity of $nTiO_2$ to blood clams has been reported [16,30,39], to the best of our knowledge, the toxic impacts of other NPs on *T. granosa* has yet to be described. Furthermore, the mechanism through which NPs manifest immunotoxicity is still poorly understood in marine invertebrates, such as the blood clam. Therefore, to gain a better understanding of the immunotoxic impacts and underpinning mechanism of NPs, the effects of 7-days exposure to four NPs, including nZnO, nFe_2O_3, nCuO, and MWCNT, on the immune responses, ROS contents, DNA damage, and cell viability of haemocytes in the blood clam. In addition, the *in vivo* contents of neurotransmitters and the expressions of immune- and neurotransmitter-related genes were analyzed after NP exposures.

Table 1
Exposure concentrations of NPs (mean ± SEM) in the seawater measured for each experimental group.

Nominal Concentrations of	0 (Control)	0.1 (mg/L)	1 (mg/L)	10 (mg/L)
nZnO	N / D	0.12 ± 0.01	1.04 ± 0.01	9.98 ± 0.01
nFe_2O_3	N / D	0.23 ± 0.03	1.04 ± 0.03	9.97 ± 0.01
nCuO	N / D	0.12 ± 0.01	1.01 ± 0.00	10.02 ± 0.00
MWCNT	N / D	0.14 ± 0.01	1.09 ± 0.01	10.07 ± 0.04

Table 2
Primer sequences and accession numbers for the internal reference and genes investigated.

Genes	Sequence (5′ - 3′)	Accession no.
18S-F	CTTTCAAATGTCTGCCCTATCAACT	JN974506.1
18S-R	TCCCGTATTGTTATTTTTCGTCACT	
IKKα-F	ATATTGTGCTGGTGGAGATT	JZ898319
IKKα-R	GCTTCAGATCACGGTGTATA	
TAB2-F	CCACCAAGAATCCACCAT	JZ898321
TAB2-R	TCGCAGCATTCACACTTA	
MAPK-F	CACCAACAACTACCTATGAAG	MK463600
MAPK-R	TGAATAATGCCTGCTGAATG	
NFκβ1-F	AATCAAGCAGGTGTAGTAGAC	KY623561
NFκβ1-R	CAGACAGGACAGCCAGAT	
RAS-F	CGGACGCTATTATGCTAGT	MK450341
RAS-R	TGTGTTGCCTCTGTATCG	
GABAB-F	GGCTCTAAGTATCCATCAGTT	MF463599
GABAB-R	GACCGTCCAGGTTCTCTA	
Gi/o-F	AGAAGATCCTGCCTCCAA	MK450339
Gi/o-R	GGTCAATGCTCATAACATCA	
PI3K-F	ACTCGCTACCAGAGAATAAG	MK463601
PI3K-R	TTCCACTTCCTTCGTAACC	
Gq-F	GCACAGGTTACTCAGAAGA	MK450340
Gq-R	TCATAATCCACTTGGCGTAT	
PKA-F	AGAGACCTGAAGCCTGAA	MK463602
PKA-R	AAGTCCAAGTTCTGTCCTC	

2. Materials and methods

2.1. Collection and acclimation of animals

According to Shao [45], adult blood clams with a shell height of 23.82 ± 0.83 mm were collected in July 2018 *via* a dredge harvest method using a clam harrow from the intertidal zone of Yueqing Bay, Wenzhou, China (28°28′ N and 121°11′ E), a relative pollution free site where many potential pollutants (*i.e.* heavy metals, persistent organic pollutants, and nanoparticles) were found to be under the detection limit [46,47]. Prior to experiment, clams were acclimated for 14 days in a 1000-L plastic tank filled with 800 L sand filtered seawater (temperature at 26.37 ± 0.08, pH at 8.06 ± 0.01, and salinity at 21.90 ± 0.01‰). Following the methods described by Su [48] and Zhao [49], clams were fed with microalgae *Platymonas subcordiformis* at the satiation feed rate and seawater in the tank was replaced daily

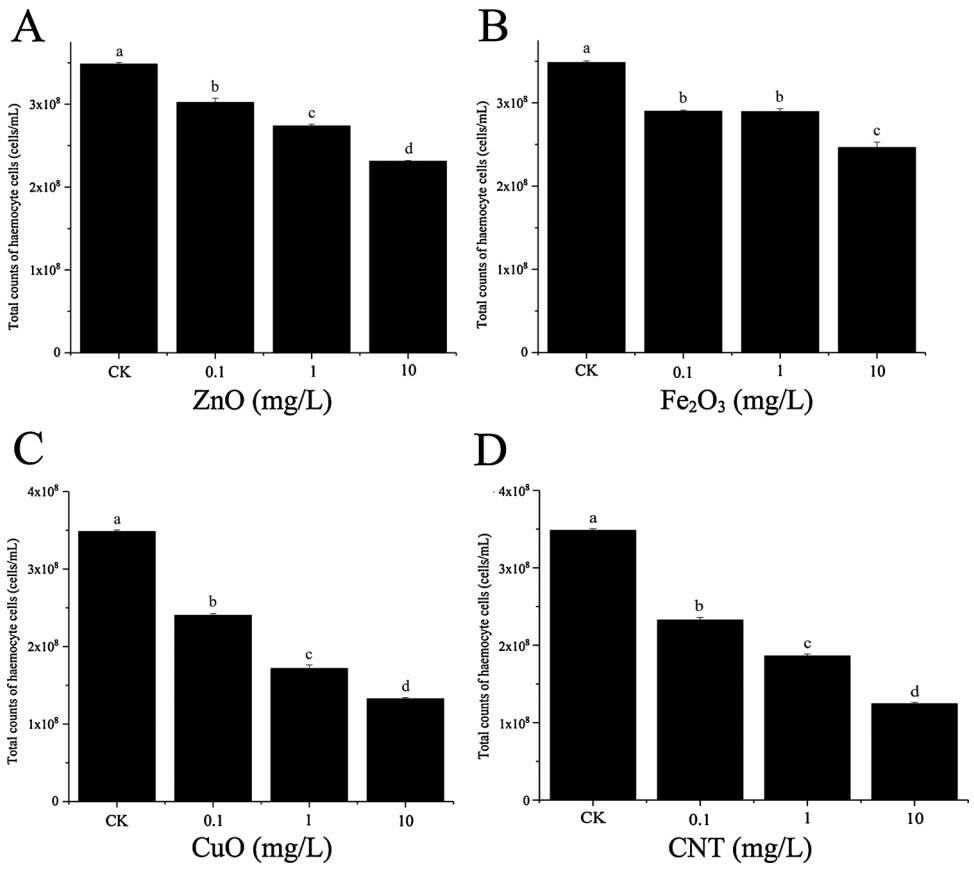

Fig. 1. The total counts of haemocytes cells for each treatment in relation to the exposed nanoparticles (A: nZnO, B: nFe_2O_3, C: nCuO, and D: MWCNT). All data are presented as the mean ± SEM, and the mean values that do not share the same superscript are significantly different at $p < 0.05$.

during the acclimation period.

2.2. Characterization of NPs and preparation of stock solutions

The nZnO and nFe_2O_3 with purity ≥ 99.5% were purchased from Klamar Reagent Co. Ltd. (Shanghai, China). The nCuO and MWCNT with purity ≥ 99.9% and ≥ 99.13%, respectively, were purchased from Ziqibio Co. Ltd. (Shanghai, China). According to the reported methods [14,15,30], the NPs tested in this study were characterized by a combination of analytical techniques. In brief, transmission electron microscopy (TEM, JEM - 1230, JEOL, Tokyo, Japan) was used to determine the particle morphology and size. The crystalline structure of the NP particles was identified by X-ray powder diffractometry (XRD, Rigaku D/MAX 2550/PC, Tokyo, Japan). The specific surface area in m^2/g (Brunauer-Emmett-Teller, BET) was measured with Micrometrics Analytical Services (Micromeritic TriStarII 3020, Micrometritics Instrument Corp, Norcross, GA). The parameters of the tested NPs were provided in the supplementary material (Figure S1; Table S1). Stock suspensions of NPs (1 g/L) were prepared daily by dispersing the NPs in distilled water followed by ultrasonication at 70% amplitude for 20 min.

2.3. Exposure experiment and sampling

To assess the impacts of NPs, clams (35 individuals per tank) were exposed to 30 L seawater containing nominally 0 (control), 0.1, 1, or 10 mg/L of the tested NPs according to the methods of Gottschalk [18] and Rocha [1]. The designed exposure concentrations were obtained by adding corresponding stock solutions into the 30 L experimental seawater. Following previous studies [31,50], an exposure duration of 7 days was adopted and three replicates were conducted for each treatment. During experiment, seawater was replaced daily with freshly prepared seawater containing the corresponding concentrations of NPs. Water samples (10 mL) were collected from each experimental trial before the assignment of clam individuals and the working concentrations of corresponding NP were determined with the water samples *via* inductively coupled plasma mass spectrometry (ICP-MS, PE NexION 300X, USA) following the methods described by Orians and Boyle [51], The measured concentration of NP in the seawater of each exposure group is presented in Table 1. No individual mortality was observed during the exposure.

2.4. Haemocyte count and cell composition analysis

After 7 days of corresponding treatment, haemocyte samples were collected for total count analysis according to the methods of Liu [30] and Shi [16]. Briefly, three clam individuals were randomly taken from each experimental treatment and rinsed with 0.1 M phosphate buffer saline (PBS, pH at 7.4) solution to remove impurities. Next, 100 μL haemolymph were extracted from the cavity of each individual using a 2-mL syringe and immediately transferred to a 1.5-mL centrifuge tube prefilled with 100 μL 2.5% glutaraldehyde. After adding 800 μL PBS, the total count of haemocytes was estimated with a Neubauer's haemocytometer (XB-K-25, Anxin Optical Instrument) under a Nikon eclipse E600 microscope at a magnification of 400 × .

Similarly, haemolymph was collected individually from three clams from each treatment using a 2-mL syringe. After mixing 700 μL freshly extracted haemolymph with 300 μL 2.5% glutaraldehyde, the mixture was used to determine the cell type composition of haemocytes following the methods of Liu [30] and Zha [52]. The mixture was centrifuged at 4000 rpm for 4 min and a blood smear was subsequently made with 50 μL blood precipitates. Once air dried, the blood smears

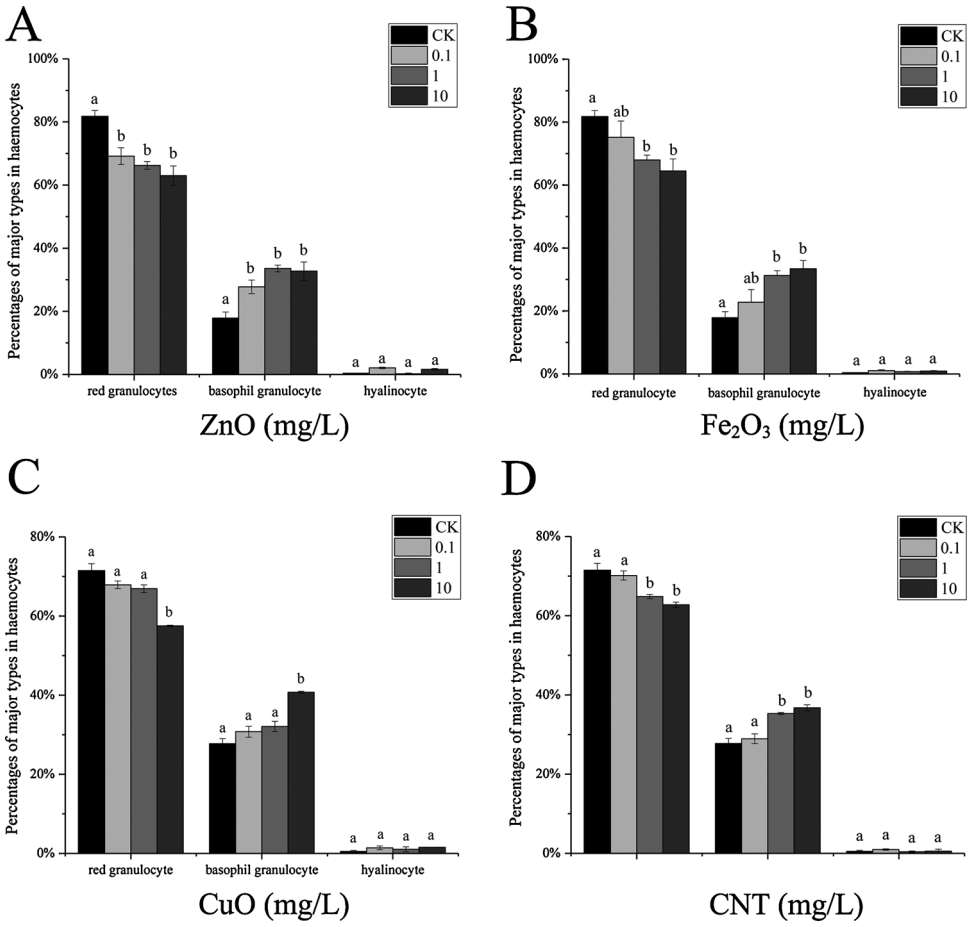

Fig. 2. Percentages of three major types of haemocytes for each treatment in relation to the exposed nanoparticles (A: nZnO, B: nFe_2O_3, C: nCuO, and D: MWCNT). All data are presented as the mean ± SEM, and the mean values that do not share the same superscript are significantly different at $p < 0.05$.

were stained with Wright-Giemsa stain (G1020, Solarbio). The counts for various cell types were determined at 400 × magnification under a Nikon eclipse E600 microscope. To ensure the accuracy of estimation, at least 100 haemocyte cells were scored for each sample.

2.5. Phagocytosis assays

Phagocytosis assays were performed as described by Shi and Su [16,48]. In brief, yeast suspension containing (2.87 ± 0.13) × 10^7 yeast cells were prepared by dissolving 7 mg yeast powder (Instant Dry Yeast, Angel Yeast) in 10 mL PBS followed by supersonic homogenization. A volume of 200 μL of haemolymph was first extracted from each individual. After determining the haemocyte concentration with 20 μL haemolymph using a Neubauer's haemocytometer, 100 μL haemolymph were mixed with 100 μL precooled Alsever's solution (Solarbio, China) in a 1.5-mL centrifuge tube. After spinning at 1000 rpm for 15 s, supernatant was removed and a yeast suspension was added at a yeast-haemocyte ratio of 10 : 1. The yeast-haemocyte mixture was incubated at room temperature (25 °C) for 30 min and then fixed with 100 μL 2.5% glutaraldehyde. Blood smears were subsequently prepared, stained with Wright-Giemsa stain, and used for the estimation of phagocytic rate with a Nikon eclipse E600 microscope at a magnification of 400 × . Three clams from each treatment and more than 200 haemocytes for each individual were scored.

2.6. Determination of intracellular ROS contents

Following the manufacturer's instructions, intracellular contents of ROS in haemocytes were measured with commercial fluorescence kits (Nanjing Jiancheng Bioengineering Institute, China). Briefly, after 7 days of the corresponding treatment, haemocyte samples were collected individually from 6 clams from each treatment. Haemocytes were then incubated with the fluorochrome (provided by the kit) in the dark at 37 °C for 30 min. Blood smears were prepared and images were captured with an Olympus BH-2 fluorescence microscope (Olympus, Melville, NY) at the excitation and emission wavelengths of 520 and 590 nm, respectively. Following the methods of Guo [53] and Su [48], intracellular contents of ROS were estimated from the measured fluorescence intensities with the software Image-Pro Plus 6.0 (Rockville, MD, USA) using the following equation: F = ID / Area, where F is the fluorescence of the ROS stained, ID (integrated density) represents total grayscale density value of the stained haemocyte area, and 'area' is stained area of the haemocytes.

2.7. Comet and cell viability assays

The OxiSelect™ Comet Assay Kit (Cell BioLabs, San Diego, CA, USA) was used to estimate the degree of DNA damage following the protocol provided. Similarly, haemocyte samples were collected individually from three clams from each treatment. After mixing 15 μL of the haemocyte sample with 75 μL 0.5% preheated low-melting-point agarose, the mixture was transferred into the well of CometSlide™. The solidified sample was then incubated with lysis buffer (provided) at 4 °C in the dark for 50 min. After another 30 min incubation with the alkaline buffer (provided), electrophoresis was conducted at 30 V for 15 min. The sample slide was then washed twice (5 min each) with distilled water followed by dehydration with 99% ethanol. Once air dried, the slides were stained with Vista Green DNA dye for 15 min. Fluorescence

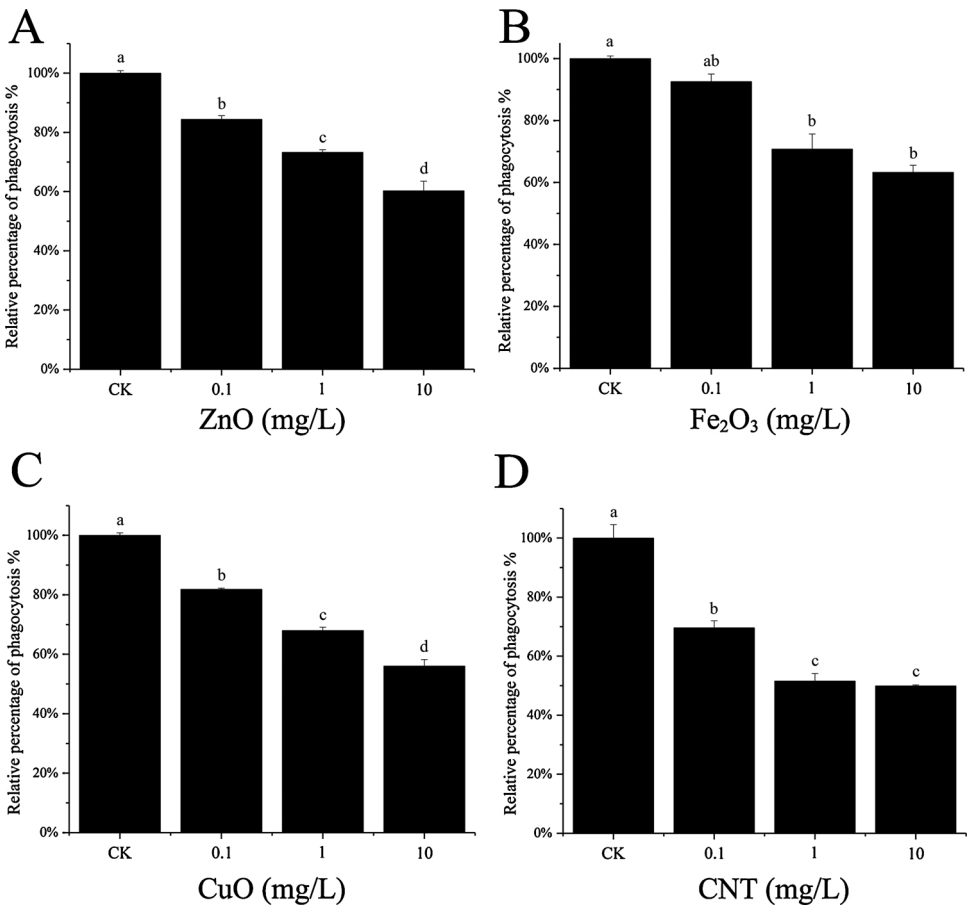

Fig. 3. Relative phagocytic rates of haemocytes for each treatment in relation to the exposed nanoparticles (A: nZnO, B: nFe_2O_3, C: nCuO, and D: MWCNT). All data are presented as the mean ± SEM, and the mean values that do not share the same superscript are significantly different at $p < 0.05$.

images were subsequently obtained with an Olympus BH-2 fluorescence microscope (Olympus, Melville, NY) at excitation and emission wavelengths of 520 and 590 nm, respectively. The degree of DNA damage was analyzed with Photoshop CS6 according to the methods of Gong [54]. For all the samples that were tested, at least 100 randomly selected cells were examined.

Following the methods by Parolini [55] and Zha [52], cell viability of haemocytes was measured with MTT cell proliferation and cytotoxicity assay kits (Beyotime Biotech, Hangzhou, China). Briefly, after extraction, 100 µL haemocytes were seeded into a 96-well plate and mixed with 10 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL). The mixture was incubated in the dark at 37 °C for 4 h and then mixed with 100 µL formazan solvent followed by another round of incubation in the dark at 37 °C until complete dissolution of the crystal of formazan dye. Absorbance of the sample was then determined at a wavelength of 570 nm using a microplate reader (Thermo Multiskan Go, USA). Relative percentages of cell viability were subsequently obtained according to the methods of Ong [56].

2.8. Determination of the in vivo contents of neurotransmitters

The concentrations of ACh, GABA, and DA were measured with commercial kits (MLBIO biotechnology Co. Ltd., Shanghai, China) following the manufacturer's instructions [39]. In brief, after dissection on ice, gill tissues where haemolymph is often concentrated were peeled off individually from six clams from each treatment group and immediately frozen in liquid nitrogen. Frozen tissues were homogenized in 0.1 mL precooled PBS (pH = 7.4) on ice with an electric homogenizer (ART, MICCRA D-1, Germany) and centrifuged at 2000 rpm for 20 min at 4 °C. Supernatants were collected for the measurement of neurotransmitters with a microplate reader (Thermo Multiskan Go, USA) at the absorption wavelength of 450 nm.

2.9. Gene expression analysis

After 7 days of exposure, three individuals were randomly picked out from each experimental treatment for gene expression analysis. Total RNA of each individual was extracted from the gill tissue with EASYspin Plus RNA exaction kit (Aidlab, China). The quality and concentrations of RNA were verified with gel electrophoresis and a NanoDrop 1000 UV/visible spectrophotometer (Thermo Scientific), respectively. Next, high-quality total RNA was reverse transcribed into first strand cDNA using a PrimeScript RT Reagent (TaKaRa, China). Real-time quantitative PCR (qRT-PCR) was performed with a Bio-Rad CFX96 Real Time System (BioRad Laboratories, USA) in triplicates. Denaturation at 95 °C for 5 min and 40 cycles of 98 °C for 10 s and 61 °C for 30 s were adopted in the amplification with a 10 µL qPCR system containing 0.5 µL of 10 mM forward and reverse primer, 5 µL AceQTM qPCR SYBR Green Master Mix (Vazyme, China), 3 µL double-distilled water, and 1 µL cDNA template. In total, the expressions levels of ten immune- or neurotransmitter-related candidate genes encoding the inhibitor of nuclear factor kappa-B kinase subunit alpha (IKKα), TAK1-binding protein 2 (TAB2), mitogen-activated protein kinase (MAPK), nuclear factor NF-kappa-B p105 subunit (NFκB1), ras-related protein R-Ras2 (RAS), gamma-aminobutyric acid type B receptor subunit 1 (GABAB), guanine nucleotide-binding protein subunit beta-5 (Gi/o), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), guanine nucleotide-binding protein (Gq), and protein kinase A (PKA) were investigated in this study. The gene 18S rRNA was uesd as an internal

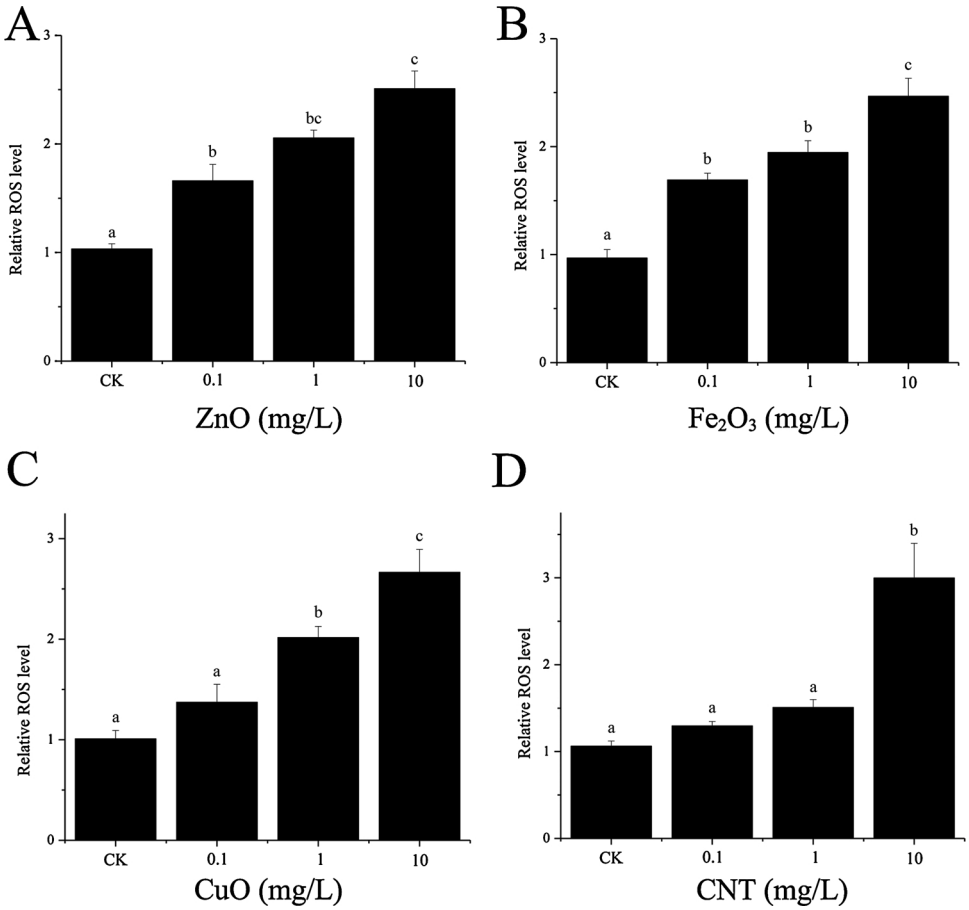

Fig. 4. Intracellular ROS contents for each treatment in relation to the exposed nanoparticles (A: nZnO, B: nFe_2O_3, C: nCuO, and D: MWCNT). All data are presented as the mean ± SEM, and the mean values that do not share the same superscript are significantly different at $p < 0.05$.

reference. All primers (Table 2) used were synthesized by Sangon Biotech (Shanghai, China). The melting curve analysis (MCA) was used to verify the specificity and reliability of qPCR products and the $2^{-\Delta\Delta}$CT method was applied to analyze the relative expression levels of genes [39,57].

2.10. Statistical analysis

One-way ANOVAs followed by Tukey's post hoc tests were conducted to compare ROS contents, cell viabilities, and neurotransmitters concentrations. For these analyses, the assumptions of normality and homogeneity were assessed using Levene's and Shapiro-Wilk's tests, respectively. Nonparametric Kruskal-Walis ANOVAs followed by pairwise Mann-Whitney tests were performed to compare THCs, percentages of different haemocytes, phagocytic activities, and degrees of DNA damage [58]. The expression levels of each gene under query were compared with the control by a *t*-test. All the statistical analyses were performed with OriginPro 2017 and a *p* value less than 0.05 was accepted as statistically significance.

3. Results

3.1. Impacts of NP exposures on total count (THC), cell composition, and the phagocytic activity of haemocytes

Exposure of blood clams to the tested NPs resulted in a significant reduction in THC (Fig. 1, $p < 0.05$). The levels of THCs dropped to approximately 86.71%, 78.59%, and 66.35% compared to the control for clams exposed to 0.1, 1, and 10 mg/L nZnO, respectively (Fig. 1A, $\chi^2 = 10.46$, df = 3, $p = 0.02$). Similarly, after 7 days of exposure to nFe_2O_3, the THCs decreased to approximately 83.17%, 83.08%, and 70.75% of the control for 0.1, 1 and 10 mg/L nFe_2O_3 treatment groups, respectively (Fig. 1B, $\chi^2 = 9.43$, df = 3, $p = 0.02$). In addition, the THCs were only approximately 68.93%, 49.33% and 38.05% of the control for clams exposed to 0.1, 1 and 10 mg/L nCuO, respectively (Fig. 1C, $\chi^2 = 10.38$, df = 3, $p = 0.02$). Treatment with 0.1, 1 and 10 mg/L MWCNT revealed a significant THCs reduction to approximately 66.73%, 53.44% and 35.76% of the control, respectively (Fig. 1D, $\chi^2 = 10.42$, df = 3, $p = 0.02$).

While the percentages of red granulocytes and basophil granulocytes were unaffected by lower doses of nZnO, nFe_2O_3, nCuO, and MWCNT, exposure of clams to higher concentrations of these NPs led to a significant decrease in the percentage of red granulocytes (Fig. 2, $\chi^2 = 9.67$, df = 3, $p = 0.02$ for nZnO; $\chi^2 = 9.36$, df = 3, $p = 0.02$ for nFe_2O_3; $\chi^2 = 9.15$, df = 3, $p = 0.02$ for nCuO; and $\chi^2 = 9.36$, df = 3, $p = 0.02$ for MWCNT, respectively) whereas an increase in that of basophils granulocyte (Fig. 2, $\chi^2 = 9.46$, df = 3, $p = 0.02$ for nZnO; $\chi^2 = 8.77$, df = 3, $p = 0.03$ for nFe_2O_3; $\chi^2 = 8.23$, df = 3, $p = 0.04$ for nCuO; and $\chi^2 = 8.44$, df = 3, $p = 0.04$ for MWCNT, respectively). According to the Kruskal-Walis ANOVA analysis, the percentage of hyalinocyte was not significantly affected by NP exposure.

Nanoparticle exposures exerted a significant dose-dependent arrest impact on the phagocytosis of haemocytes (Fig. 3, $p < 0.05$). The phagocytic rates were approximately 84.39%, 74.25%, and 60.28% of the control for clams exposed to 0.1, 1 and 10 mg/L nZnO (Fig. 3A, $\chi^2 = 10.38$, df = 3, $p = 0.02$), respectively. Though the phagocytic rate of haemocytes was not affected by nFe_2O_3 exposure at a concentration of 0.1 mg/L, it was reduced to approximately 70.78% and 63.31% of the control for clams exposed to 1 and 10 mg/L nFe_2O_3 (Fig. 3B, $\chi^2 = 9.67$, df = 3, $p = 0.02$), respectively. Similarly,

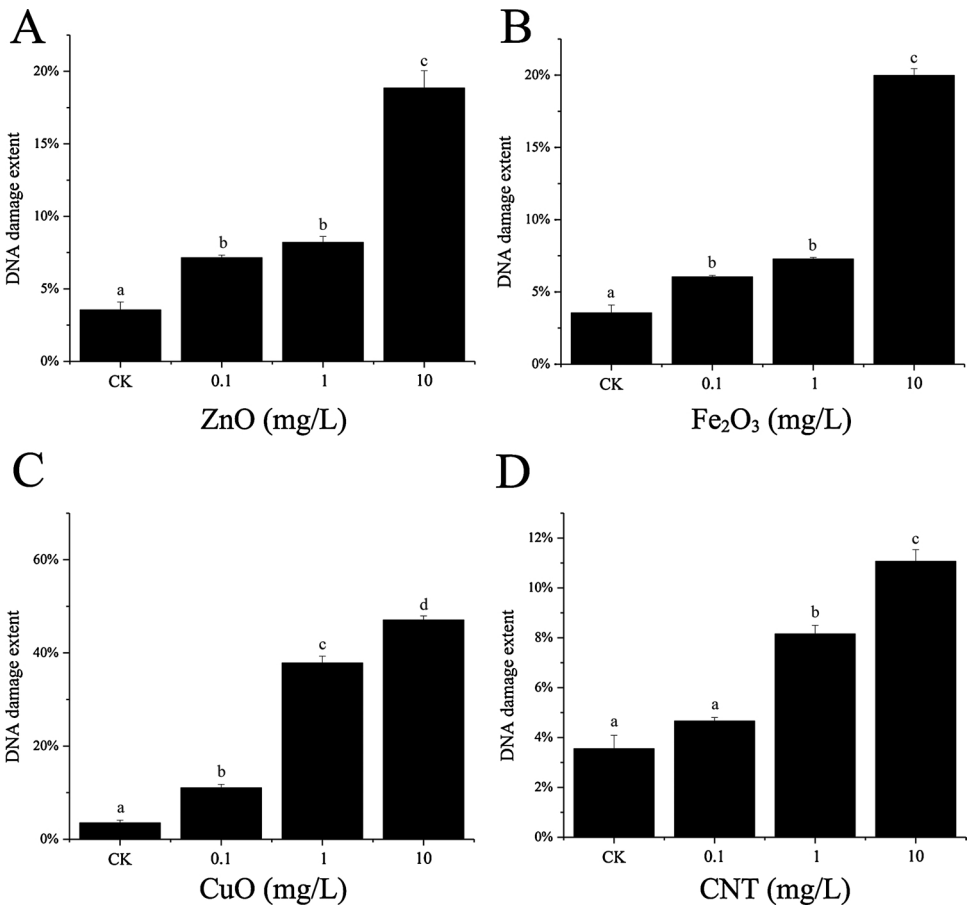

Fig. 5. Degrees of DNA damage for each treatment in relation to the exposed nanoparticles (A: nZnO, B: nFe_2O_3, C: nCuO, and D: MWCNT). All data are presented as the means ± SEM, and the mean values that do not share the same superscript are significantly different at $p < 0.05$.

compared to the controls, 18.15%, 32.01%, 43.97%, and 30.39%, 48.47%, 50.08% reductions in phagocytic rates were detected after 7 days exposure of clams to 0.1, 1 and 10 mg/L of nCuO and MWCNT (Fig. 3C, D, $\chi^2 = 10.38$, df = 3, $p = 0.02$ for nCuO and $\chi^2 = 9.36$, df = 3, $p = 0.02$ for MWCNT), respectively.

3.2. Impacts of NP exposures on the intracellular contents of ROS of haemocytes

Except for clams exposed to lower doses of nCuO (0.1 mg/L) and MWCNT (0.1 mg/L), the intracellular contents of ROS were significantly induced upon NP exposures (Fig. 4, $F_{3,24} = 28.51$, $p =$ 2.01E-7 for nZnO; $F_{3,24} = 32.28$, $p =$ 7.34E-8 for nFe_2O_3; $F_{3,24} = 21.05$, $p =$ 2.12E-6 for nCuO; and $F_{3,24} = 17.76$, $p =$ 7.36E-6 for MWCNT, respectively). When exposed to 1 mg/L of the tested NPs, the intracellular levels of ROS were approximately 1.99, 2.01, 2.00, and 1.42 times higher than the controls for nZnO, nFe_2O_3, nCuO, and MWCNT exposure groups, respectively, and the levels reached as high as 2.43 (nZnO), 2.54 (nFe_2O_3), 2.64 (nCuO), and 2.82 (MWCNT) times of the control for exposure groups at a concentration of 10 mg/L.

3.3. Impacts of NP exposures on the DNA damage and cell viability of haemocytes

Except individuals exposed to 0.1 mg/L MWCNT, exposure of clams to 7 days of nZnO, nFe_2O_3, nCuO, and MWCNT at all of the other concentrations tested led to significantly aggravated DNA damage (Fig. 5, $\chi^2 = 10.20$, df = 3, $p = 0.02$ for nZnO; $\chi^2 = 10.38$, df = 3, $p = 0.02$ for nFe_2O_3; $\chi^2 = 10.38$, df = 3, $p = 0.02$ for nCuO; and $\chi^2 = 10.38$, df = 3, $p = 0.02$ for MWCNT, respectively). The degrees of DNA damage increased to approximately 2.01, 2.31, and 5.30 times of the control after 7 days of exposing clams to 0.1, 1, and 10 mg/L nZnO, respectively. Similarly, significantly greater degrees of DNA damage ranged from 1.70 to 5.61 and from 3.11 to 13.23 times the control were detected upon nFe_2O_3 and nCuO exposure, respectively. In addition, compared to the control, the degree of DNA damage was significantly aggravated to approximately 1.31 and 3.11 times after 7 days of exposing clams to 1 and 10 mg/L MWCNT, respectively.

Data obtained from MTT assays showed that 7 days of exposing blood clams to nZnO, nFe_2O_3, nCuO, and MWCNT at all of the concentrations tested resulted in significant reductions in cell viability of haemocytes in a dose-dependent manner (Fig. 6, $F_{3,36} = 117.35$, $p =$ 0.00 for nZnO; $F_{3,36} = 48.49$, $p =$ 5.24E-12 for nFe_2O_3; $F_{3,36} = 58.72$, $p =$ 4.15E-13 for nCuO; and $F_{3,36} = 10.78$, $p =$ 4.73E-5 for MWCNT, respectively). Compared to control, the cell viability of haemocytes declined to approximately 17.00–36.00%, 11.67–21.89%, 19.78–36.67%, and 17.17–31.01% for clams exposed to the tested doses (0.1, 1, and 10 mg/L) of nZnO, nFe_2O_3, nCuO, and MWCNT, respectively.

3.4. Impact of NP exposures on the in vivo concentrations of neurotransmitters

The *in vivo* concentrations of the three investigated neurotransmitters (ACh, GABA, and DA) were significantly induced by exposure of clams to nZnO, nFe_2O_3, nCuO, and MWCNT (Fig. 7, $p < 0.01$). Compared to the control, approximately 1.47–1.85, 1.36–1.70, 1.21–1.58, and 1.59–1.99 times larger amounts of *in vivo* contents of ACh were detected for clams exposed to the tested doses of nZnO ($F_{3,24} = 109.48$, $p =$ 1.40E-12), nFe_2O_3 ($F_{3,24} = 84.01$, $p =$

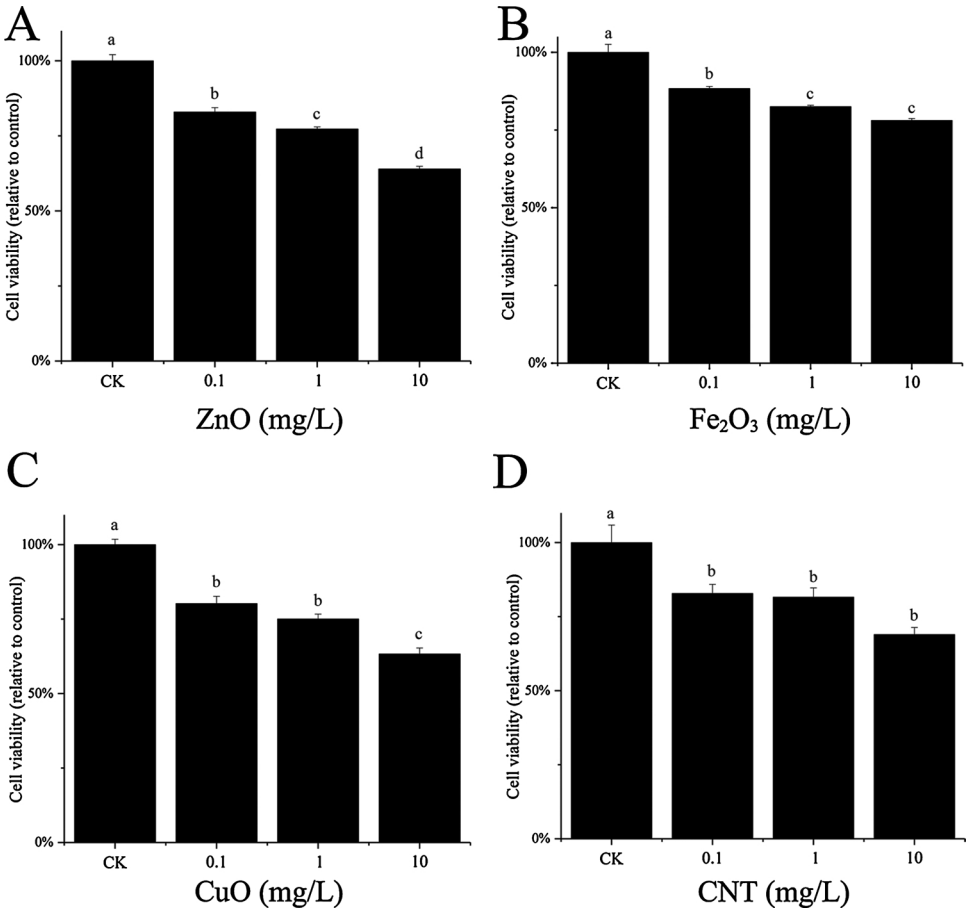

Fig. 6. Relative cell viability for each treatment in relation to the exposed nanoparticles (A: nZnO, B: nFe_2O_3, C: nCuO, and D: MWCNT). All data are presented as the means ± SEM, and the mean values that do not share the same superscript are significantly different at $p < 0.05$.

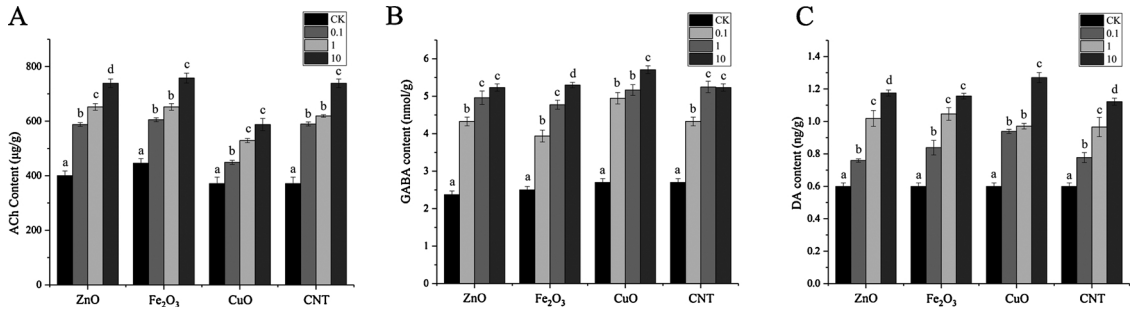

Fig. 7. *In vivo* concentrations of ACh (A), GABA (B), and DA (C) for each treatment in relation to the exposed nanoparticles (nZnO, nFe_2O_3, nCuO, and MWCNT). Data are presented as the means ± SEM and the mean values that do not share the same superscript are significantly different at $p < 0.05$.

1.65E-11), nCuO ($F_{3,24} = 30.44$, $p =$ 1.19E-7), and MWCNT ($F_{3,24} = 125.79$, $p =$ 3.77E-13), respectively. Similarly, the *in vivo* concentrations of GABA increased to approximately 1.82–2.21, 1.58–2.12, 1.83–2.11, and 1.60–1.94 times the concentrations of the control after nZnO ($F_{3,24} = 102.96$, $p =$ 2.49E-12), nFe_2O_3 ($F_{3,24} = 113.69$, $p =$ 9.80E-13), nCuO ($F_{3,24} = 107.05$, $p =$ 1.73E-12), and MWCNT ($F_{3,24} = 166.54$, $p =$ 2.60E-14) exposure, respectively. After exposed to nZnO, nFe_2O_3, nCuO, or MWCNT contaminated seawater for 7 days, the *in vivo* contents of DA were 1.27–1.96, 1.40–1.93, 1.57–2.12, and 1.30–1.87 times greater than the control, respectively ($F_{3,24} = 80.15$, $p =$ 2.54E-11 for nZnO; $F_{3,24} = 55.96$, $p =$ 6.57E-10 for nFe_2O_3; $F_{3,24} = 155.72$, $p =$ 4.94E-14 for nCuO; and $F_{3,24} = 44.33$, $p =$ 5.06E-9 for MWCNT, respectively).

3.5. Effects of NP exposures on the expressions of immune- and neurotransmitter-related genes

The expression levels of the test immune-related genes were generally suppressed by 7 days of exposing of blood clams to nZnO, nFe_2O_3, nCuO, and MWCNT (Fig. 8). When treated with nZnO for 7 days at the tested concentrations, the expressions of IKKα, TAB2, NFκB1, MAPK and Ras were all significantly downregulated. While the effect is negligible at the lowest dose (0.1 mg/L), exposing clams to higher concentrations (1 and 10 mg/L) of nFe_2O_3 significantly suppressed the expressions of all the immune-related genes tested. Similarly, except for IKKα and Ras, of the clams that were exposed to 0.1 mg/L nCuO, the expression levels of the immune-related genes investigated were significantly downregulated upon nCuO exposure. The expression of IKKα was not affected by exposing the clams to MWCNT. In addition, the

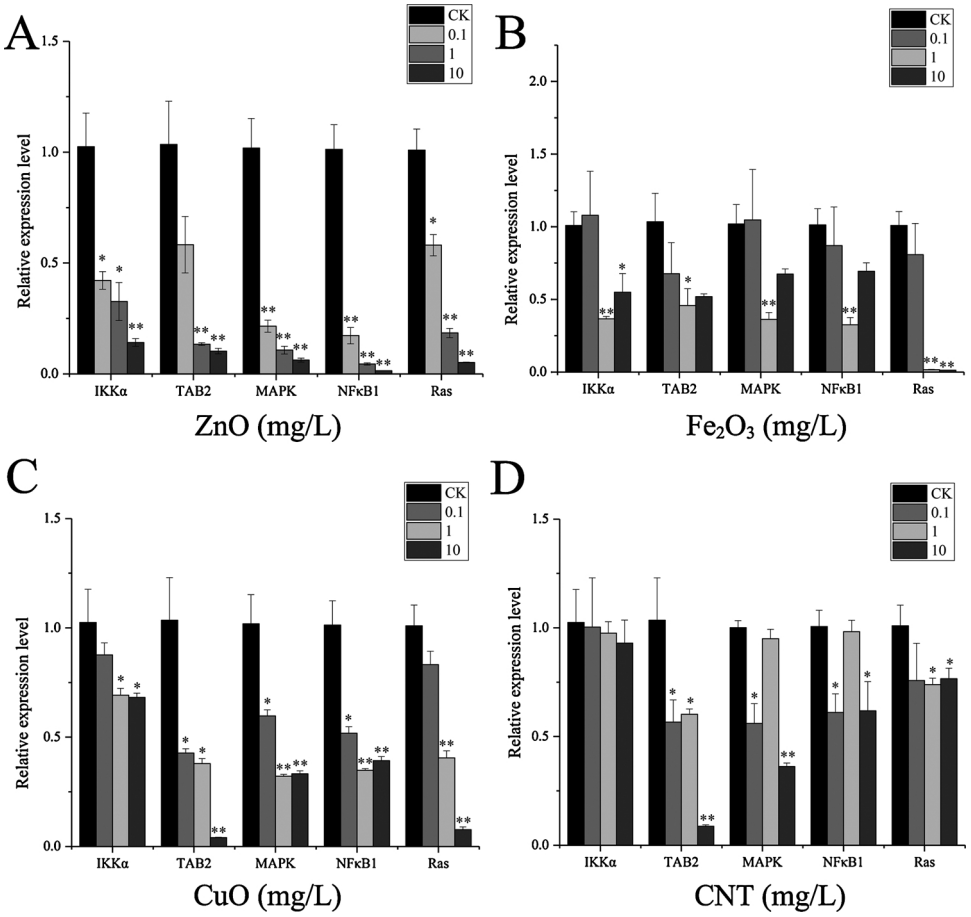

Fig. 8. Expression levels of immune-related genes for each treatment in relation to the exposed nanoparticles (A: nZnO, B: nFe_2O_3, C: nCuO, and D: MWCNT). Data are presented as the means ± SEM, and the mean values that do not share the same superscript are significantly different at $p < 0.05$.

expression levels of MAPK and NFκB1 as well as Ras were not affected by exposure of clams to 0.1 mg/L and 1 mg/L MWCNT, respectively. The expression levels of immune-related genes were significantly downregulated by MWCNT exposure at all of the other tested.

Similarly, the expression levels of neurotransmitter-related genes were generally suppressed by NP exposures (Fig. 9). Treatment with nZnO at all of the tested exposure concentrations significantly constrained the expressions of GABA, Gi/o, PI3K, Gq, and PKA. While no observable effect was seen at the lowest dose (0.1 mg/L), exposing the clams to higher concentrations of nFe_2O_3 significantly suppressed the expression levels of all the neurotransmitter-related genes tested. In clams exposed to 10 mg/L nCuO, the expression levels of neurotransmitter-related genes were significantly downregulated, except for PI3K. While the expression levels of GABA, Gi/o, and PI3K were unaffected by lower doses of MWCNT (0.1 mg/L for GABA, 0.1 and 1 mg/L for Gi/o, and 0.1 mg/L for PI3K), the expression levels of these neurotransmitter-related genes were significantly suppressed by MWCNT exposure at all of the other tested.

4. Discussion

Due to the lack of antigen-antibody mediated immune responses, marine bivalve mollusks, similar to many other invertebrates, mainly rely on haemocytes to fight against pathogen invasion [30,59,60]. In the present study, evident immunotoxicity indicated by reduced THC, altered cell type composition, and suppressed phagocytic activity of haemocytes, was detected for nZnO, nFe_2O_3, nCuO, and MWCNT. These findings are comparable to the reported for other NPs on other bivalve species [1,4,28], suggesting that immunotoxic effects could be universal phenomenon across different types of NPs in bivalve mollusks, rendering individuals higher susceptibility to pathogen challenges and therefore posing a potential threat to bivalve species. Furthermore, the data obtained in this study indicate that NPs may exert immunotoxicity through complicated cellular, biochemical, and molecular processes.

The THC of haemocytes was significantly reduced after exposure of blood clams to nZnO, nFe_2O_3, nCuO, and MWCNT, which may be accounted for the followings. First, similar to those reported for other NPs in other invertebrate species [15,28,61], the *in vivo* contents of ROS were significantly induced by exposure to NPs in this study. It is generally accepted that overproduction of ROS will upregulate Bcl-2-associated X (BAX) while downregulate apoptosis regulator Bcl-2 (BCL2) and therefore triggering apoptosis *via* activating the caspase pathway [62,63]. For instance, reports from *in vitro* assays suggest that carbon black NPs induce apoptosis process *via* activating caspase-3, caspase-7, and caspase-8 [62]. Therefore, haemocytes apoptosis could be aggravated by the overproduction of ROS upon NP exposure and therefore result in a decrease in the total number of haemocytes. Second, it has been suggested that NP contamination may cause damage in tissues, such as the gills and digestive glands of bivalve mollusks, through inducing ROS production [64]. Under these circumstances, a proportion of haemocytes may participate in the restoration of injured tissues leading to a reduction in the total number of haemocytes circulating in the haemolymph [40]. Third, except for modulating immune responses through the downstream NFκB signaling pathway, MAPK, being one of the key members of MAPK signaling pathway, also plays crucial roles in hematopoiesis regulation [61]. Since the expression of gene encoding MAPK was found to be significantly suppressed by NP exposure in this

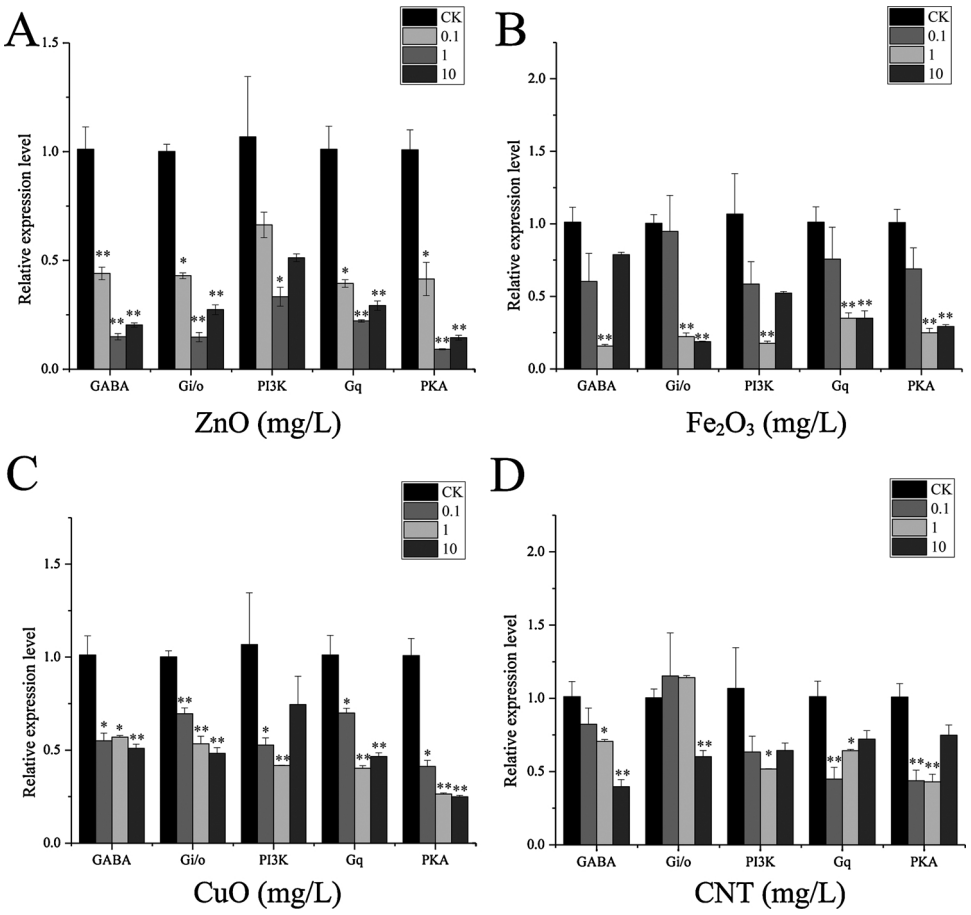

Fig. 9. Expression levels of neurotransmitter-related genes for each treatment in relation to the exposed nanoparticles (A: nZnO, B: nFe_2O_3, C: nCuO, and D: MWCNT). Data are presented as the means ± SEM, and the mean values that do not share the same superscript are significantly different at $p < 0.05$.

study (Fig. 8), it is postulated that hematopoiesis of the clams was also disturbed by NP exposure.

In addition to reductions in THC, the phagocytic activities of haemocytes of blood clams were found to be significantly constrained by NP exposures, which may be due to reduced proportion of red granulocytes, disturbed cellular function, and disrupted immune-related molecular pathways. According to previous studies, red granulocytes exhibit the highest phagocytic activity among the different types of haemocytes found in blood clam [30,31]. Therefore, the reduction in the proportion of detected red granulocytes could offer one explanation for phagocytosis suppression after NP exposures. In addition, it has been suggested that biomolecules, including DNA, membrane, and cytoskeleton were sensitive to oxidative stresses, such as ROS [48]. Therefore, the overproduction of *in vivo* ROS after NP exposure could result in damages to membrane and cytoskeleton as well as aggravated DNA damage. While an increased degree of DNA damage would impose on the viability of haemocytes and therefore result in suppressed phagocytic activity. On the other hand, it is known that membrane and cytoskeleton of haemocytes play crucial roles during the process of phagocytosis, such as mediating the recognition and engulfment of non-self particles [38,48], We reason that a decrease in phagocytic activity could be due to affected cellular alterations in membrane and cytoskeleton. Furthermore, the immune activity of haemocytes, including phagocytosis, was modulated by molecular pathways, such as the NFκB and Toll-like receptor signaling pathways [30–32]. Our data showed that the expression levels of IKKα, TAB2, NFκB1, MAPK and Ras from the immune-related pathways were markedly altered upon NP exposures, which may constrain phagocytosis and lead to reduced phagocytic activity.

Evident neurotoxicity of nZnO, nFe_2O_3, nCuO, and MWCNT to blood clam, indicated by the altered neurotransmitter contents and gene expressions, were detected in this study. While requiring more empirical data, the current results suggest that neurotoxicity could be a universal characteristic for different types of NPs in bivalve species. Since the expression levels of GABA, Gi/o, PI3K, Gq, and PKA were found to be significantly suppressed by NP exposure, the increase in the *in vivo* contents of the three neurotransmitters tested may be due to the downregulation of their corresponding modulatory enzymes, hindering hydrolysis of the corresponding neurotransmitters. For instance, it has been suggested that the increase in the intracellular contents of ACh, GABA, and DA in blood clam upon TiO_2 NP exposure could be caused by the downregulation of corresponding modulatory enzymes, the cacetylcholinesterase (AChE), gamma-aminobutyric acid transaminase (GABAT), and monoamine oxidase (MAO), respectively [39].

Recently, increasing evidence has suggested that neurotransmitters, including the ACh, GABA, and DA investigated in this study, are important molecules regulating the immune responses of invertebrate species, including bivalve mollusks [41,65–67]. For example, it has been shown that ACh can bind to corresponding receptors on the surfaces of immune cells *in vivo* and subsequently inhibit the NFκB signaling pathway, which results in unfavorable immune responses [41,42,68]. Therefore, the disrupted neuroendocrine-immune (NEI) regulation, as indicated by the significant alterations in the *in vivo* contents of neurotransmitters, could be one of the reasons underlying immune suppression after NP exposure. In addition, it has been demonstrated that neurotransmitters also play important roles in regulating the intracellular content of ROS [42,69]. For example, the expression levels of antioxidant enzymes, such as superoxide dismutase

and catalase in the haemocytes of scallop *Chlamys farreri* were significantly downregulated by ACh [42]. Since antioxidant enzymes are crucial for the removal of ROS, alterations in neurotransmitters may also hamper immune responses indirectly through disrupting intracellular regulation of ROS.

Given the current understanding of NP pollution is generally obtained *via* model prediction and laboratory experiments, it remains questionable whether the negative impacts of NPs, such as the immunotoxicity, is occurring in the present natural oceanic environment. However, with the rapid and constant increase in the production and application of engineered nanomaterials, NPs pollution, with their negative impacts, is becoming an environmental challenge to be tackled in the near future.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.05.071.

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