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COMPARATIVE STUDY OF BIOCONCENTRATION POTENTIAL AND BIOMARKERS IN *Biomphalaria glabrata* EXPOSED TO ZnO NANOPARTICLES AND BULK ZnO

Estudio comparativo del potencial de bioconcentración y de biomarcadores en *Biomphalaria glabrata* expuesta a nanopartículas de ZnO y su análogo no nano

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Key words: acetylcholinesterase, carboxylesterase, oxidative stress, nanotoxicology.

ABSTRACT

The uptake of nanoparticles (NPs) by aquatic invertebrates can lead to adverse health effects. The aims of the present study were: 1) to investigate the bioconcentration potential of ZnO NPs or its bulk analogous in *Biomphalaria glabrata* gastropods by measuring the soft tissue Zn content, and 2) to determine the responses elicited by the exposed snails through the analysis of recognized biomarkers of effect at the subcellular level. Snails exposed to 5 mg/L ZnO NPs or bulk ZnO for 48 h showed a significant higher soft tissue Zn content than control animals, with no significant differences between NPs- and bulk-treated snails. No significant differences were observed in the levels of lipid peroxidation and in the activities of acetylcholinesterase, carboxylesterase and catalase by 48 h-exposure to ZnO NPs or bulk ZnO with respect to control organisms. However, ZnO exposure induced a significant decrease in glutathione tissue content, without a nano-specific effect. The results as a whole encourage further studies to better understand the impact that engineered NPs may have on freshwater invertebrate species.

Palabras clave: acetilcolinesterasa, carboxilesterasa, estrés oxidativo, nanotoxicología.

RESUMEN

La incorporación de nanopartículas (NP) por parte de los invertebrados acuáticos puede tener efectos adversos para la salud de los mismos. Los objetivos del presente estudio fueron: 1) investigar el potencial de bioconcentración de NP de ZnO o de su análogo no nano en gasterópodos *Biomphalaria glabrata* midiendo el contenido de Zn en los tejidos blandos y 2) evaluar las respuestas generadas en los caracoles expuestos a través del análisis de reconocidos biomarcadores de efecto a nivel subcelular. Aunque los caracoles expuestos por 48 h a 5 mg/L de NP de ZnO o su análogo mostraron un contenido de Zn en los tejidos blandos significativamente más alto que los animales testigo, la acumulación de este metal fue similar entre los grupos tratados con ambas formas de ZnO. No se observaron diferencias significativas en los niveles de peroxidación lipídica y en las actividades de la acetilcolinesterasa, la carboxilesterasa y la catalasa luego de

48 h de exposición a NP de ZnO o su análogo con respecto a los organismos testigo. Sin embargo, la exposición a ZnO produjo una disminución significativa en el contenido de glutatión en los tejidos, sin un efecto nano específico. Los resultados en su conjunto fomentan el desarrollo de estudios con el fin de comprender mejor el impacto que las NP manufacturadas puedan tener en diversas especies de invertebrados de agua dulce.

INTRODUCTION

Nanoparticles (NPs) can exhibit different chemical reactivity, as well as optical, mechanical, electrical, and magnetic properties than the same compounds at the non-nano scale (Ju-Nam and Lead 2016). For this reason, they are particularly useful in diverse applications such as photocatalytic processes, sensors, environmental remediation and photodegradation of pollutants (Król et al. 2017, Khan et al. 2019). Within the vast world of NPs, zinc oxide NPs (ZnO NPs) have applications in the field of cosmetic products, especially in the formulation of sunscreens, as well as in the field of paints, due to their ability to block UVA and UVB rays, they are also used as catalysts, in the textile and food industry, and in the field of agriculture, to mention the most relevant (Espitia et al. 2012, Król et al. 2017, Yusefi-Tanha et al. 2020).

Considering the rise of nanotechnology, the release of ZnO NPs into aquatic ecosystems, due to accidental and/or intentional causes, may occur (Bundschuh et al. 2018). Once they reach the aquatic systems, NPs can interact with other contaminants, with dissolved organic or particulate matter, and they can also be incorporated by biological species (Bundschuh et al. 2018). It has been estimated that water environmental concentrations are 100 µg/L (Chen et al. 2022). However, it should be considered that much higher concentrations could be generated in effluents of treatment plants (Gottschalk et al. 2013).

According to previous results, molluscs can incorporate Zn after acute exposure to ZnO NPs (Ali et al. 2012, Montes et al. 2012, Fahmy et al. 2014, Trevisan et al. 2014, Gagné et al. 2019). However, to our best knowledge, there are no reports on the freshwater snail *Biomphalaria glabrata* (Planorbidae, Mollusca). The incorporation of NPs by the organisms can lead to bioaccumulation process and can induce a series of biological responses that account for adverse health effects, including an imbalance in the cellular redox state and other effects that reflect a process of neurotoxicity (Ma and Lin 2013, Almeida et al. 2019, Canesi et al. 2019). Interestingly, it has been reported that in several aquatic organisms exposed to ZnO NPs or its non-nano analogous, toxicity and influx rate may differ (Heinlaan et al. 2008, Wong et al. 2010, Khan et al. 2013).

Oxidative stress manifests itself when the generation of reactive oxygen species (ROS) overcomes the antioxidant defenses of cells, triggering damage to membrane lipids (lipid peroxidation), damage to the genetic material (DNA/RNA), damage to proteins and/or enzyme inactivation (Juan et al. 2021). Organisms have various antioxidant defences, of enzymatic and non-enzymatic nature, such as catalase (CAT) and glutathione (GSH), and these are commonly used as oxidative stress biomarkers (Bhagat et al. 2016, Almeida et al. 2019). On the other hand, the measurements of thiobarbituric acid reactive substances (TBARS) and acetylcholinesterase (AChE) activity are frequently used as biomarkers of lipid peroxidation and neurotoxicity, respectively (Domingues et al. 2009, Nikinmaa 2014, Almeida et al. 2019). In addition, carboxylesterase (CES) activity has also been proposed as a useful biomarker of toxicity for several pollutants such as carbamates and organophosphates (Cacciatore et al. 2018), some pharmaceuticals and personal care products (Solé and Sanchez-Hernandez 2018) or polycyclic aromatic hydrocarbons (Mrdaković et al. 2023).

For several decades, various species of molluscs have been used as bioindicators in metal toxicity assessment tests (Elder and Collins 1991, Aisemberg et al. 2005, Ruiz et al. 2018). In particular, de Freitas Tallarico (2015) has proposed the use of the freshwater gastropods of the genus *Biomphalaria*, as bioindicator species of relevant importance in Latin American countries, where they are widely distributed. The gastropods *B. glabrata* belong to the Planorbidae Family, and are of medical importance because they can be intermediate hosts of *Schistosoma mansoni*, the parasite that causes schistosomiasis (Cavalcanti et al. 2012). These snails have a relatively short life cycle and are highly sensitive to toxic substances (de Freitas Tallarico et al. 2014, 2016). This species has already been widely used as an experimental model in classical toxicology tests, and more recently, it has also been selected for nanotoxicological studies (Verrengia Guerrero et

al. 2000, Aisemberg et al. 2005, Cochón et al. 2007, Oliveira-Filho et al. 2017, 2019, de Vasconcelos Lima et al. 2019, Caixeta et al. 2020, Garate et al. 2020, De-Carvalho et al. 2022).

The aims of the present study were: 1) to investigate the bioconcentration potential of ZnO NPs or its bulk analogous in *B. glabrata* by measuring the Zn content, and 2) to determine the responses elicited by the exposed snails through the analysis of recognized biomarkers of toxicity at the subcellular level.

MATERIALS AND METHODS

Materials

ZnO NPs of commercial origin (Sigma-Aldrich, USA; CAS No. 1314-13-2; lot number, MK-BT6312V), average size less than 35 nm, were used. Initially, a stock suspension was prepared in bi-distilled water, containing 700 mg NPs/L. To investigate the possible differential behavior of the nanoparticulate material with that of the same composition, but on a macro scale, ZnO (Mallinckrodt J.T. Baker, USA; CAS No. 1314-13-2) of analytical purity quality was used. Initially, a stock suspension was prepared in bi-distilled water, containing 200 mg non-nano ZnO/L. Both suspensions were prepared according to the NPs suspensions preparation guide (OECD 2012) in order to ensure homogeneity and minimize particle aggregation.

To perform the bioassays, and before being diluted in dechlorinated water, the stock suspensions of ZnO NPs and bulk ZnO were sonicated for 30 min at a frequency of 40 kHz and 160 W of ultrasonic power (ultrasonic bath; Testlab ®, Argentina) at room temperature so as to break up large aggregates.

Potable tap water was left to rest for at least 24 h to dechlorinate and then it was filtered through a carbon column to eliminate traces of dissolved organic matter and ensure the complete elimination of chlorine prior to use (USEPA 2002). The following physical and chemical parameters were recorded: total hardness = 48 ± 3 mg $CaCO_3$/L; alkalinity = 29 ± 2 mg $CaCO_3$/L; pH = 7.0 ± 0.2, conductivity = 250 ± 17 µS/cm and dissolved oxygen = 8.1 ± 0.4 mg O_2/L.

Reduced glutathione (GSH, CAS No. 70-18-8); glutathione reductase (CAS No. 9001-48-3), NADPH (CAS No. 2646-71-1) and 5,5´-dithio-bis (2-nitrobenzoic acid) (DTNB, CAS No. 69-78-3) were all of maximum purity (> 98%; Sigma-Aldrich, USA). All other chemicals and reagents used were of analytical grade.

Zinc oxide nanoparticles characterization

Particle size was determined by scanning electron microscopy (SEM), using a Carl Zeiss NTS SUPRA 40 operating at 5 kV. In order to perform stability studies, the stock suspension was characterized after being prepared (t = 0 day) and after 15 days. At each time, the suspension was sonicated at a frequency of 40 kHz and 160 W of ultrasonic power for 30 min, before characterization.

According to SEM analyses, a 20 µL drop was deposited on a copper grid with carbon film and dried next to an oven (T = 100 ± 3 ºC), before being analyzed.

Bioassays

Cultures of *B. glabrata* were maintained in our laboratory under standard conditions in 17-20 L tanks, at T = 22 ± 2 ºC, with a 14:10 h light/dark cycle and constant aeration. The aqueous medium consisted of dechlorinated water. The organisms were fed three times a week with lettuce leaves ad libitum.

Bioassays were performed acutely (48 h of exposure) under static conditions (without medium renewal) at 22 ± 2 ºC in plastic containers with a capacity of 500 mL containing 300 mL of solution and a photoperiod of 14:10 h light/darkness. An adult snail with an average body weight of approximately 0.111 ± 0.050 g was placed in each container without food or aeration. The exposure solutions were prepared by adding the corresponding aliquots of the stock suspensions of NPs or non-nano ZnO to dechlorinated water. Depending on the experiment, three to six replicates were made for each exposure group, and in all cases, controls with dechlorinated water were performed.

Zn bioconcentration

According to Fahmy et al. (2014), the LC_{10} and LC_{50} in *B. alexandrina*, a freshwater snail of the same genus of *B. glabrata*, were 7 and 145 mg ZnO NPs/L, respectively. Based on those results, a preliminary experiment was performed to set the exposure concentration, and so the gastropods were exposed to 5 and 7.5 mg ZnO/L, as NPs or non-nano ZnO, for 48 h. At 5 mg ZnO/L, no changes in snail appearance or behavior with respect to untreated snails could be observed. In contrast, visceral mass retraction was observed in snails exposed to 7.5 mg/L ZnO NPs for 48 h. Therefore, a concentration of 5 mg ZnO/L was established for further experiments.

To study the comparative potential bioconcentration, gastropods were exposed to 5 mg ZnO/L, as NPs or non-nano ZnO, for 48 h. The incorporation of the

NPs and the non-nano equivalent was estimated by Zn analysis performed on the total soft tissue of each organism. To do this, snails were placed on a Petri dish in contact with ice until there was no response to external stimulus (approximately 5 min). After this time, and with the help of a slide and tweezers, the valve was discarded and the total soft tissue was removed. Then, the excess water was removed with filter paper and the mass (wet weight) was determined. The soft tissue was transferred to 25 mL borosilicate glass tubes and HNO_3 (c) was added to each tube in a 5:1 (volume:tissue) ratio. In order to verify the absence of contamination during the process, a digestion blank was prepared by placing HNO_3 (c) in an extra tube, without animals. The samples were then heated in a water bath at 100 ºC for 6-8 h until the complete destruction of the organic matter. The determination of Zn was carried out using an atomic absorption spectrophotometer (AAS) (Varian Assoc. Inc., USA; model AA-575) by direct aspiration of the samples in an air-acetylene flame according to the conditions recommended by the manufacturer. In all cases, a deuterium lamp was used for background noise correction. For Zn, a hollow cathode lamp was used, and the readings were made at one of the characteristic wavelengths of the element (λ = 213.8 nm). To determine the linearity range, standards were used (0.5 mg Zn/L - 2 mg Zn/L), prepared in HNO_3 (1% v/v) from a commercial standard solution of Zn containing 1000 mg/L (Titrisol ®; $ZnCl_2$ in 0.06% HCl; Sigma-Aldrich, USA; CAS No. 7646-85-7). The detection limit was equal to 0.1 mg Zn/L.

NPs and bulk ZnO exposure concentrations were checked by AAS. In all cases, they were within 95% of nominal concentrations.

Biomarker parameters

Gastropods were exposed to ZnO NPs or bulk ZnO for 48 h to evaluate AChE, CES, CAT, GSH, and TBARS. Then, the snails were placed on a Petri dish in contact with ice for 5 min, and the valves were removed. The head-foot region was separated from the other tissues (pulmonary region, digestive gland, and gonads), which were weighed and processed together. This criterion was adopted to avoid excessive variability in the results since the tissue that makes up the head-foot region is very dense, and its degree of homogenization is highly variable (Garate et al., 2020).

All the results were standardized by protein content measured according to the method of Lowry et al. (1951), using bovine serum albumin as a standard.

AChE and CES

The soft tissues were homogenized on ice in a 1:5 ratio (tissue:volume) in 20 mM Tris-HCl buffer at pH = 7.5 with 0.5 mM ethylenediaminetetraacetic acid (EDTA) and then centrifuged at 11 000 x g for 20 min at 4 ºC. AChE was measured following the protocol described in Garate et al. (2020), in which the activity is measured at 412 nm, in a 100 mM sodium phosphate buffer solution pH = 8.0 containing 0.2 mM of DTNB, as chromogenic agent and 0.75 mM of acetylthiocholine iodide as substrate. CES was measured following the protocol described in Cacciatore et al. (2018) in which the activity is measured at 400 nm using 1 mM p-nitrophenyl butyrate as substrate.

CAT

The methodology (with minor modifications) described by Cochón et al. (2007) was followed. The soft tissues were homogenized on ice in a 1:5 ratio (tissue:volume) with 20 mM Tris-HCl buffer at pH = 7.5 containing 0.5 mM EDTA. They were then centrifuged at 11 000 x g for 20 min at 4 ºC, and the supernatants were diluted (1:2) with 50 mM sodium phosphate buffer at pH = 7.0, containing 0.1% Triton ® X-100 (Sigma-Aldrich, USA; CAS No. 9036-19-5). Finally, they were sonicated for 5 min at a frequency of 40 kHz and a power of 160 W and used as enzyme source. To determine the activity of the enzyme, H_2O_2 was used as a substrate, recording the drop in absorbance at 240 nm due to its decomposition into H_2O and O_2 (Cochón et al. 2007).

GSH

The soft tissues were homogenized on ice in a 1:5 ratio (tissue:volume) with a 125 mM sodium phosphate buffer, 6.3 mM EDTA, at pH = 7.5. Then, 100 µL of 30% (v/v) trichloroacetic acid were added to 900 µL of homogenate, incubated on ice for 15 min in the dark, and centrifuged at 11 000 x g for 5 min at room temperature. Total GSH levels (GSHt) were determined following the protocol of Tietze (1969) which is based on the sequential oxidation of GSH by DTNB and the reduction by NADPH in the presence of the enzyme GSH reductase. The formation of 2-nitro-5- thiobenzoic acid (TNB) was monitored spectrophotometrically at λ = 412 nm.

TBARS

Lipid peroxidation was estimated as malondialdehyde (MDA) equivalents, using the assay of TBARS (Iummato et al. 2018). The soft tissues were homogenized on ice in a 1:5 ratio (tissue:volume) in 20 mM Tris-HCl buffer at pH = 7.5 with 0.5 mM EDTA and

then centrifuged at 11 000 x g for 20 min at 4 ºC. The reaction mixture contained 175 µL of sample and 25 mM thiobarbituric acid, 250 mM HCl, 0.92 M trichloroacetic acid and 0.7 mM butylhydroxytoluene in a final volume of 1.175 mL. The mixture was boiled for 1 h, cooled in ice, centrifuged at 8000 x g for 10 min and the absorbance of supernatant was measured at 535 nm.

Statistical analysis

Results were expressed as mean ± S.D. Data were submitted to one-way ANOVA followed by Tukey post-test by using Origin (Pro) 9 (OriginLab©, MA, USA). The level of significance used was 0.05. Prior to ANOVA, data were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene's tests, respectively.

RESULTS

Zinc oxide NPs characterization

SEM images obtained of the freshly prepared NPs suspension showed free particles that had an average size of 30 ± 8 nm, while agglomerates/aggregates were within the size range 200 to 1600 nm (**Fig. 1a**). After 15 days of being prepared, the average particle size was similar to the size in the freshly prepared stock solution (34 ± 7 nm) (**Fig. 1b**).

The results confirmed that NPs average size in diluted dechlorinated water suspensions coincide with the size reported by the manufacturer, and that it was not modified after 15 days of being prepared.

Zn levels in *B. glabrata* exposed to ZnO NPs or bulk ZnO

We investigated the potential bioconcentration of ZnO in snails exposed to ZnO NPs or bulk ZnO for 48 h. Treated animals showed significant higher soft tissue Zn levels than control animals (**Fig. 2**). However, no significant differences ($p > 0.05$) were found between NPs-and bulk-treated snails.

Biomarker parameters

B-esterases

No significant differences were observed in AChE (**Fig. 3a**) or CES (**Fig. 3b**) activity by exposure to ZnO NPs or bulk ZnO with respect to control organisms ($p > 0.05$).

Oxidative stress parameters

Comparing with controls, a significant decrease in GSHt levels (**Fig. 4a**) could be observed by exposure

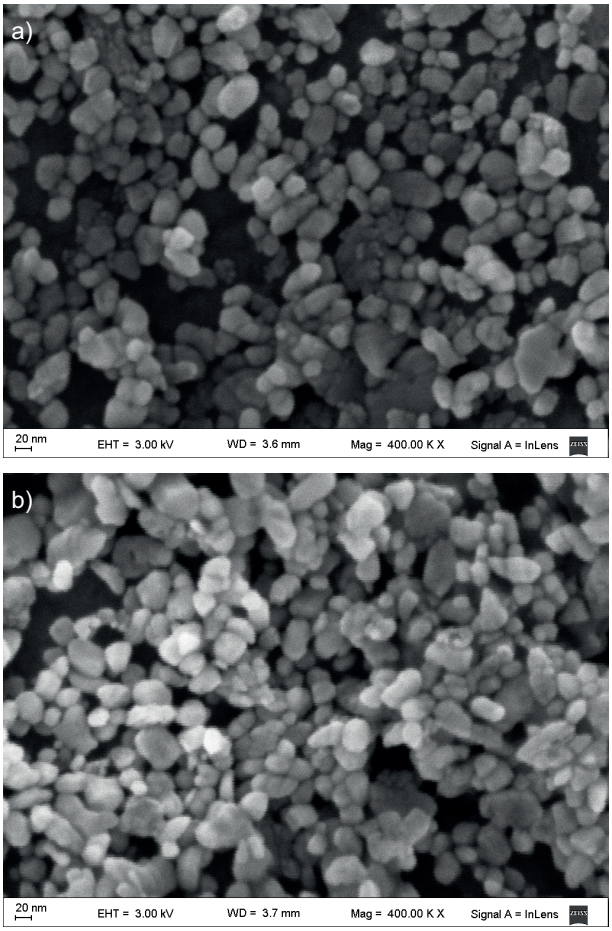

Fig. 1. Scanning electron microscopy images of a) a freshly prepared (magnification: 400000X) and b) 15 days aged (magnification: 400000X) ZnO nanoparticles suspension.

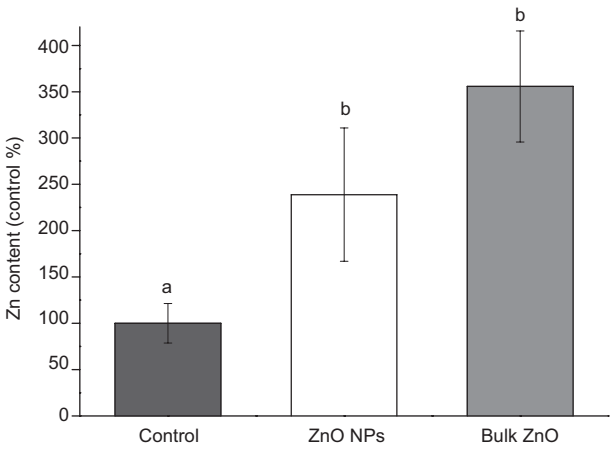

Fig. 2. Zn levels (expressed as control%) in *B. glabrata* exposed to ZnO nanoparticles (NPs) or bulk ZnO for 48 h. Each data represents the mean value ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$). Control levels: ZnO nanoparticles (39 ± 8) µg Zn/g (wet weight); bulk ZnO (19 ± 3) µg Zn/g (wet weight).

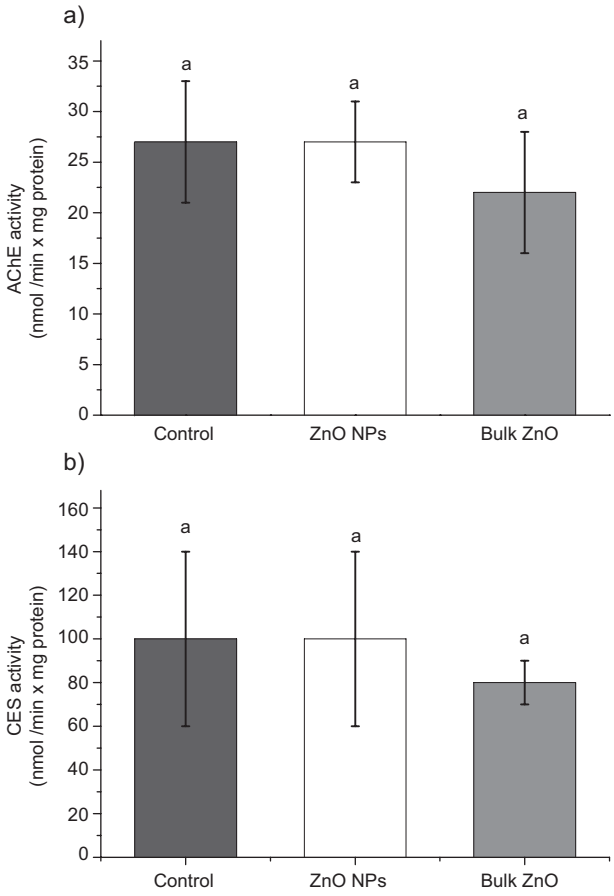

Fig. 3. (a) Acetylcholinesterase (AChE) and (b) carboxylesterase (CES) activity in *B. glabrata* exposed to ZnO nanoparticles (NPs) or bulk ZnO for 48 h. Data are expressed as the mean value ± SD (n = 6 for AChE and n = 4 for CES). Different letters indicate significant differences ($p < 0.05$).

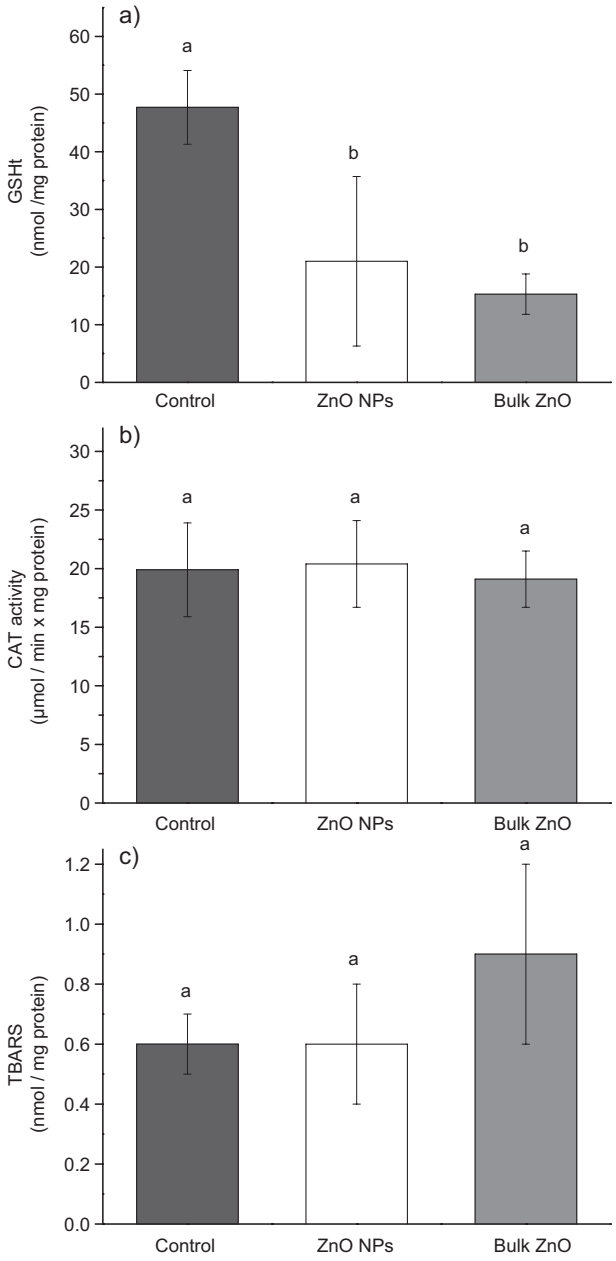

Fig. 4. (a) Total glutathione content (GSHt, n = 3), (b) catalase (CAT, n = 6) activity and (c) lipid peroxidation (TBARS, n = 3) in *B. glabrata* exposed to ZnO nanoparticles (NPs) or bulk ZnO for 48 h. Data are expressed as the mean value ± SD. Different letters indicate significant differences ($p < 0.05$).

to ZnO NPs and bulk ZnO ($p < 0.05$). However, no significant differences were found between nano and non-nano ZnO. This decrease in GSH levels was neither reflected by an alteration of CAT activity (**Fig. 4b**) nor in the increase of lipid peroxidation levels (**Fig. 4c**).

DISCUSSION

The chemical form of substances contributes to the interaction with biological molecules. Subcellular, cellular, or environmental factors can lead to a chemical element being reactive, having positive or adverse results in Zn homeostasis (Griscom et al. 2004). This comparative study shows that the freshwater gastropod *B. glabrata* can incorporate Zn from acute exposure to both NPs and bulk ZnO in a similar degree. Incorporation of Zn from acute exposure to ZnO NPs has also been reported in marine molluscs such as the oyster *Crassostrea*

gigas (Ostreidae, Mollusca) and the mussel *Mytilus galloprovincialis* (Mytilidae, Mollusca), which can incorporate the NPs through the gills and the digestive gland (Montes et al. 2012, Trevisan et al. 2014). Since *B. glabrata* are lung-bearing organisms, the possible routes of incorporation are limited to surface adsorption and ingestion (Kuehr et al. 2021).

Previous studies have suggested that under in vitro conditions, Zn can act as an inhibitor of AChE enzyme activity (Frasco et al. 2005). In addition, other metal oxide NPs (SiO_2 or Al_2O_3) have a low capacity to inhibit the activity of this enzyme (Wang et al. 2009). However, through in vivo studies with ZnO NPs, no significant modifications of AChE activity were reported in the aquatic polychaete *Hediste diversicolor* (Nereididae, Annelida), neither in the clam *Scrobicularia plana* (Semelidae, Mollusca) due to exposure to sediments contaminated with 3 g NPs/kg for 16 days (Buffet et al. 2012) nor in the clam *Ruditapes philippinarum* (Veneridae, Mollusca) exposed to 1 or 10 µg/L for three or seven days (Marisa et al. 2016). Similarly, no modifications in AChE activity, which could trigger a process of neurotoxicity, were found in ZnO (NPs or bulk) exposed *B. glabrata*. CES were proposed as toxicity markers in aquatic animals for several xenobiotics (Cacciatore et al. 2018, Solé and Sanchez-Hernandez 2018, Mrdaković et al. 2023). Nevertheless, no changes in CES activity were observed in *B. glabrata* under present assay conditions.

Regarding the oxidative stress biomarker parameters studied, no changes in CAT activity or in the levels of lipid peroxidation were observed, revealing a lack of damage to macromolecules due to acute exposition to the nano and non-nano forms of ZnO. However, it has to be considered that biomarker parameters, especially those related to oxidative stress processes, may undergo temporary changes. Therefore, it is important to consider the time in which they are determined (Ali 2015, Rocco et al. 2022). According to the literature, in the case of ZnO NPs, dissimilar results have been found, since CAT activity may or may not undergo changes, depending on the exposure concentration, time and the selected species (Buffet et al. 2012, Marisa et al. 2016, Fahmy and Sayed 2017). Noteworthy, total GSH levels decreased in exposed snails when compared to controls, without a nano-specific effect. Reduction of GSH levels after exposure to ZnO NPs have already been reported in other mollusc species (Ali et al. 2012, Fahmy et al. 2014, Fahmy and Sayed 2017). Taking into account that: a) In natural habits, NPs coexist with a wide variety of both natural and anthropogenic compounds, which may also alter the redox cellular status and b) GSH is implicated in the cellular antioxidant defense system, the maintenance of the intracellular redox environment, cellular signalling, and regulation of transcription factors (Circu and Aw 2012), the decrease in GSH levels observed in *B. glabrata* suggests that exposure to ZnO compounds could generate a higher susceptibility to co-exposure to prooxidants xenobiotics.

Our results, using a relevant mollusc from an ecological point of view due to its role in freshwater ecosystems, contribute to deepen the knowledge of the still incipient topic of the effects of NPs in invertebrates. Knowledge of the possible toxicity of different NPs allows the development of predictive models to determine which ones may pose a greater probability of risk and those that are expected to have little impact on human health and the environment.

CONCLUSIONS

The results show that *B. glabrata* gastropods could incorporate ZnO after exposure to a sublethal concentration for 48 h. Interestingly, Zn levels augmented in both ZnO NPs and bulk ZnO exposed *B. glabrata* snails similarly. After acute exposure, NPs and bulk ZnO did not induce neurotoxicity or macromolecular damage. However, they significantly decreased GSH tissue content without a nano-specific effect. The results encourage further studies to better understand engineered NPs' impact on freshwater invertebrate species.

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