Journal of Trace Elements in Medicine and Biology 53 (2019) 62–68

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

Zebrafish exposure to diphenyl diselenide-loaded polymeric nanocapsules caused no behavioral impairments and brain oxidative stress

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ARTICLE INFO

Keywords:
Organoselenium compounds
Nanoparticles
Nanotoxicology
Zebrafish
Novelty paradigm

ABSTRACT

Previous findings showed that the nanoencapsulation of diphenyl diselenide [$(PhSe)_2$], an organoselenium compound, provided superior biological effects and lower toxicological potential than its free form *in vitro*. However, few studies reported the behavioral and biochemical effects of this nanocapsules formulation *in vivo*. Zebrafish (*Danio rerio*) has emerged as a useful animal model to determine the pharmacological and toxicological effects of nanoparticles. Here, we evaluated the behavioral and brain oxidative effects after zebrafish exposure to $(PhSe)_2$-loaded nanocapsules. Formulations were prepared by interfacial deposition of preformed polymer method and later tested at concentrations ranging from 0.1 to 2.0 μM. Both locomotor and exploratory activities were assessed in the novel tank diving test. Moreover, brain oxidative status was determined by measuring thiobarbituric acid-reactive substance levels, glutathione peroxidase, glutathione redutase and glutathione S-transferase activities. $(PhSe)_2$-loaded nanocapsules showed no alteration on travelled distance, immobility, and erratic swimming, suggesting the absence of behavioral impairments. Interestingly, the higher concentration tested had anxiolytic-like effects, since animals spent more time in the top area and showed a decreased thigmotaxis behavior. Biochemical analysis demonstrated that the concentrations used in this study did not affect oxidative stress-related parameters in brain samples, reinforcing the low toxicological potential of the formulation. In conclusion, the exposure to $(PhSe)_2$-loaded nanocapsules caused no locomotor impairments as well as did not modify the oxidative status of zebrafish brain, indicating that this formulation is probably non-toxic and promising for future pharmacological studies.

1. Introduction

Selenium (Se) is an essential micronutrient for the human body and it is particularly important on the modulation of homeostatic oxidative status through endogenous antioxidant systems [1–3]. The Se biological importance motivated further studies concerning its potential application as a therapeutic tool. Diphenyl diselenide [$(PhSe)_2$] is an organoselenium molecule, that possess antioxidant, anti-inflammatory, antinociceptive, antitumor, neuro and hepatoprotective, antidepressant-like and anxiety-like actions [2,4]. Nevertheless, its high lipophilic features turns the preparation of aqueous parenteral solutions difficult, which reduces its complete dissolution in gastrointestinal tract and, in consequence, its oral bioavailability [5,6]. In addition, this molecule is known for its double character due to a contrasting behavior in biological systems, which depends on the conditions of exposure, among other factors (dose, administration route, animal species) [2,4,7], which negatively counterbalance its therapeutic application.

Over the last years, the nanotechnology field has been considered an important tool in the pharmaceutical research [8,9]. Undesired characteristics of active molecules such as high water insolubility and toxic effects can be masked and/or avoided by their incorporation into nanocarriers. Besides, the nanometric size promotes advantages such as drug targeting, controlled release, ease crossing of biological barriers and improved pharmacokinetic profile [9,10]. Furthermore, these systems can be administered via different routes (e.g., oral, intravenous, ocular, ophthalmic and cutaneous) [11]. Among the nanocarriers, polymeric nanocapsules have been studied for the encapsulation of lipophilic drugs due to their structural organization: a reservoir system

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https://doi.org/10.1016/j.jtemb.2019.02.005
Received 25 September 2018; Received in revised form 9 January 2019; Accepted 14 February 2019

wherein an oil core is surrounded by a polymeric wall and the drug can be adsorbed onto the polymeric surface and/or dissolved in the oil [10,12]. Of particular importance, the scientific literature shows studies regarding the $(PhSe)_2$ incorporation into nanocapsules [13,14]. Recently, in a previous study of our research group, $(PhSe)_2$-loaded nanocapsules were prepared using biocompatible materials such as medium chain triglycerides (MCT) and poly(ε-caprolactone) (PCL) and showed selective antimelanoma activity in a preliminary *in vitro* evaluation as well as no cytotoxic effects in human blood and keratinocytes cells [15].

The nanocarriers toxicology is still a challenge field for their pharmaceutical applications and represents an obstacle toward the clinical studies of nanoformulations. The main aspects regarding such condition are the possible unknown interaction with biological molecules due to their nanometric size scale, shape and surface charge [16,17]. There are several experimental approaches to estimate nanoparticles toxicity, including *in vitro* analyses as well as cellular assays, multi-cellular organisms and *in vivo* models employing different animal species, mainly rodents [16,17]. In this context, zebrafish (*Danio rerio*) has become an alternative animal model in the last years [18,19]. This species exhibits genetic and physiologic similarities with the human organism, which reinforces its application as an experimental tool. Importantly, protocols using zebrafish models are fast and low cost due to the fish small size, high reproducibility and quick development [18]. Interestingly, behavior evaluations are the most important parameters to identify toxic effects through modification of swimming and exploratory activity [19]. Moreover, zebrafish specie has been used as animal model to assay the safety, toxicity and/or biological effects of nanocarriers [20].

Previously, Ibrahim and co-workers used behavior parameters to evaluate the free $(PhSe)_2$ effects in zebrafish animal model employing the novel tank test. The free compound elicited anxiolytic-like effects at low concentration. However, beyond the pharmacological effects, the high concentrations tested caused adverse effects to the animals [21]. Thus, considering the advantages of this animal model to elucidate the toxicity and pharmacological properties of drugs/formulations, the present study was designed to evaluate whether the acute exposure of zebrafish to $(PhSe)_2$-loaded polymeric nanocapsules would cause locomotor and exploratory behaviors as well as would modify some oxidative stress-related parameters in brain samples.

2. Materials and methods

2.1. Materials

PCL (MW: 80 KDa), Span 80® (sorbitan monooleate) were acquired from Sigma Aldrich (Brazil). Tween 80® (polysorbate 80) and MCT (medium chain triglycerides) were furnished by Delaware (Brazil). All other were obtained from standard commercial suppliers. Other solvents and reagents were analytical grade and used as received.

2.2. Compound

$(PhSe)_2$ was obtained by the method described by Paulmier [22]. Chemical purity was determined by hydrogen and carbon nuclear magnetic resonance, as well as gas chromatography (99.9%).

2.3. Diphenyl diselenide-loaded polymeric nanocapsules

Nanocapsule suspensions containing $(PhSe)_2$ were prepared by interfacial deposition of preformed polymer method [23] and characterized in terms of mean diameter, polydispersity index, zeta potential, drug content and encapsulation efficiency according to Ferreira and coworkers [15]. Briefly, an organic phase composed by PCL (0.1 g), Span 80® (0.077 g), MCT (330 µL) and $(PhSe)_2$ (0.05 g) was prepared in acetone (27 mL), and magnetically stirred until solubilize the components. Then, it was injected into a Tween 80® (0.077 g) aqueous dispersion (53 mL) and magnetically stirred for 10 min longer. Next, the organic solvent and the water excess were evaporated under reduced pressure to 10 mL of final volume, which corresponds to a $(PhSe)_2$ concentration at 5.0 mg/mL (NC $(PhSe)_2$). For comparison purposes, formulations without $(PhSe)_2$ were also prepared (NC B).

2.4. Animals

Adult zebrafish (*Danio rerio*) of mixed genders (50:50 male:female ratio) of 4 to 6 months old from a heterogeneous wild-type stock, weighing 0.6 ± 0.1 g and measuring 3.0 ± 1.0 cm in length were obtained in a local commercial supplier (Hobby Aquários, RS, Brazil) and housed in 50-L aquariums (80–100 fish per aquarium) for at least 2 weeks prior to the experiments. All tanks were filled with non-chlorinated water previously treated with 132 µL/L AquaSafe (Tetra, VA, USA) and maintained under mechanical and chemical filtration at 26 ± 2 °C. Water temperature, pH, and conductivity were set at 26 ± 2 °C, 7.0–8.0, and 1500–1600 µS/cm, respectively. The room illumination was provided by ceiling-mounted fluorescent lamps on a 14/10 light/dark photoperiod (lights on at 7:00 a.m.) and animals were fed twice a day with a commercial flake fish food (Alcon BASIC, Alcon, Brazil), which is recommended by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animals were used in compliance with the Council for Control of Animal Experiments (CONCEA), in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the experiments were approved by the Ethics Committee for the Use of Animals-CEUA from the Federal University of Santa Maria (protocol number: 2301271116/2017). All efforts were made to minimize the number of animals used in the experiments and their stress.

2.5. Experimental design

Animals were randomly assigned to different groups (n = 8 to 12/group) for behavioral experiments and biochemical assays. Fish were randomly handled, removed from their home tanks, and individually transferred to beakers filled with the nanocapsule suspensions. Based on the study of free $(PhSe)_2$ conducted by Ibrahim and co-workers [21], fish were individually exposed to different NC $(PhSe)_2$ concentrations (0.1, 0.25, 0.5, 1.0 and 2.0 µM) during 30 min. To discard some effect from the formulation constituents, other group was exposed to NC B at the same volume used to prepare the NC $(PhSe)_2$ at 2 µM. The NC formulations were further diluted in water to achieve the desired concentrations. In addition, a control group was concomitantly carried out (without any exposure). For each exposure, a single fish was placed in each solution, and the solution was not used in subsequent experiments. In order to maintain the same experimental conditions, all experiments were performed from 9:00 a.m. to 4:00 p.m.

2.6. Locomotor and exploratory evaluations by novel tank test

Thirty minutes after NCs exposure, animals were individually placed in a rectangular tank (23.9 cm along the bottom × 28.9 cm at the top, 15.1 cm in height) filled with home system water to perform the locomotor and exploratory evaluations [21,24,25]. A webcam (Microsoft LifeCam 1.1 with Auto-Focus) connect to a laptop was placed in front and on the top of the novel tank, to monitoring the location and swimming activity of zebrafish during a 360-seconds trial [26]. The apparatus was virtually divided in two horizontal sections (bottom and top) to assess the vertical exploration, number of entries and time spent in bottom or top area. Travelled distance was used to measure motor pattern. Anxiety- like behaviors were determined by measuring the number of freezing episodes and erratic swimming. Behavioral parameters were automatically measured at a rate of 30 frames/s using appropriate video-tracking software (ANY-maze, Stoelting CO, USA)

and analyzed by different observers. To minimize handling stress and external influences, animals were gently moved from home tanks to beakers (exposure procedure) or to the novel tank. All experimental procedures were performed on a stable surface with the environmental distractions kept to a minimum. Each experimental group comprised individuals from multiple batches, and the tank water was replaced with clean system water for each trial.

2.7. Biochemical assays

2.7.1. Tissue preparation

After behavioral experiments, zebrafish were anesthetized with 0.25 g/L tricaine [27] and euthanized by punching the spinal cord behind the opercula. Brain samples were dissected out in ice, transferred to microtubes, and stored at - 80 °C. To perform the biochemical analyses, samples were homogenized in 150 μL of Tris-HCl 50 mM pH 7.4 buffer and then centrifuged (3000 *g* for 10 min, - 4 °C) to obtain the supernatant (S1), which was used in the assays. All experiments were performed in duplicate.

2.7.2. Protein determination

Protein was determined in the S1 by the Coomassie blue method using bovine serum albumin as analytical standard. Absorbance of samples was measured at 595 nm [28].

2.7.3. Lipid peroxidation

Thiobarbituric acid-reactive substance (TBARS) levels was the protocol of choice to estimate the lipid peroxidation [29]. Briefly, an aliquot of S1 was added to NaOH 3 M and incubated at 60 °C for 30 min. After, phosphoric acid 6% and thiobarbituric acid (TBA) 0.8% were added to the system and the mixture heated at 90 °C for 2 h. A volume of 10% sodium dodecyl sulfate (SDS) and n-butanol was then added in order to extract the TBA-malondialdehyde (MDA) adduct, which was analyzed on Shimadzu® HPLC equipment. The analytical procedure was composed by a column Phenomenex® ODS-2 C_{18} reverse-phase (250 mm × 4.6 mm, 5 μm; 100 Å, Allcrom, BR) and a mobile phase constituted by ultrapure water and methanol (50:50; v/v). The HPLC analyses were performed under isocratic conditions at a 0.6 mL/min flow rate and UV detector set at 532 nm with a 20 μL sample volume injection [30]. Results were expressed as nmol MDA/mg protein.

2.7.4. Glutathione peroxidase (GPx)

GPx activity was assessed by spectrophotometric analysis following the rate of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm by the coupled reaction with glutathione reductase [31]. The system consisted of potassium phosphate buffer (100 mM, pH 7.0), NaN_3 1 mM, reduced glutathione 1 mM, NADPH 0.15 mM and 20 μL of S1 (40–60 μg of protein). The reaction was started by addition of H_2O_2 0.4 mM (30 μL). The GPx activity was expressed as nmol/min/mg of protein.

2.7.5. Glutathione reductase (GR)

GR activity was based on the consumption of NADPH and it was spectrophotometrically measured at 340 nm. An amount of 120 μL of S1 (40–60 μg of protein) was added to 250 μL of a system composed by TFK 0.15 M and NADPH 0.15 mM. The reaction was started by the addition of 30 μL of GSSG (oxidized glutathione substrate). The GR activity was determined using the extinction coefficient of 6.22 mM/cm and expressed as nmol/min/mg protein [32].

2.7.6. Glutathione S-transferase (GST)

GST activity was carried out according to Habig and co-workers [33]. Samples were consisted of 1-chloro-2, 4-dinitrobenzene (CDNB) 1 mM in ethanol, glutathione reduced 10 mM, potassium phosphate buffer (20 mM; pH 6.5) and 20 μL of S1 (40–60 μg of protein). The enzyme activity was calculated by the absorbance changes at 340 nm using a molar extinction coefficient of 9.6 mM/cm. One unit GST activity was defined as the amount of enzyme required to catalyze the conjugate 1 mol CDNB with GSH/min at 25 °C. The activity was expressed as μmol GS-DNB/min/mg protein.

2.8. Statistical analysis

Data were expressed as average ± standard error of the mean (S.E.M.). The data normality was assessed by the D'Agostino and Pearson omnibus normality test and analyzed by one-way ANOVA, followed by the Student-Newman-Keuls' multiple comparison test whenever necessary. The significance level was set at $p \leq 0.05$. Since NC B results were compared with control data by *t*-test and no significant differences were revealed, data obtained with the NC B exposure were used to perform statistical evaluation with the different NC $(PhSe)_2$ concentrations.

3. Results and discussion

Over the last years, the zebrafish emerged as a versatile model in behavioral neuroscience and toxicology [18]. When compared to mammals, this species shares evolutionarily conserved brain structures, neurotransmitters, receptors and hormones, suggesting its utility in preclinical toxicological screenings [18]. Moreover, due to their intermediate position between *in vitro* cell-based tests and *in vivo* mammalian models, the use of zebrafish in basic research reduces the existing gap between invertebrates and mammalian model systems [20,34]. The novel tank test has been employed as an experimental protocol to determine possible alterations in the normal behavioral activity of zebrafish and estimate the pharmacological and/or toxicological effects of substances [18,24,25]. Some parameters, such as travelled distance, number of immobile episodes, and erratic swimming are considered indicative of locomotor activity, while thigmotaxis and vertical activity (bottom and top areas) are used as exploratory endpoints [19,24].

The effects of free $(PhSe)_2$ in zebrafish were investigated elsewhere [21]. Similarly, the novel tank test was also employed to evaluate spatiotemporal behavior, locomotor parameters, vertical exploration, and homebase formation. The $(PhSe)_2$ was used at a concentration ranging from 0.1 to 1.0 μM and anxiolytic-like effects were observed at 0.25 μM. However, beyond the pharmacological effects, the high concentrations tested (0.5 and 1.0 μM) caused severe side effects. Here, we evaluated for the first time the effects of nanoencapsulated $(PhSe)_2$ on zebrafish, using the behavioral assessment as a potential indicative of toxicological effects. After the exposure to NCs formulation, brain oxidative status was determined to further investigate the actions of NC $(PhSe)_2$ on oxidative stress-related biomarkers.

Fig. 1 depicts the results of immobile episodes numbers and erratic swimming after the NC exposure. These behaviors indicate high stress situations and/or anxiety during habituation to novelty [19]. There were few episodes of immobility and erratic swimming (Fig. 1A and B) and no statistical differences were revealed among groups. Importantly, fish exposed to free $(PhSe)_2$ at 1 μM increased immobility episodes [21], a similar side effect when compared to benzodiazepines, such as diazepam and chlordiazepoxide [35]. Conversely, the nanoencapsulation abolished this negative effect, reinforcing the advantages of molecules incorporation in this system [10].

Fig. 2A shows the results of travelled distance in the novel tank test. Alterations on travelled distance may reflect hyper/hypoactivity and/or loss of motor patterns [19]. Here, none of the NC $(PhSe)_2$ concentrations tested showed modification on travelled distance when compared to NC B ($p > 0.05$). In contrast to the study conducted by Ibrahin and co-workers, zebrafish exposure to free compound caused a significant reduction in travelled distance at 0.5 and 1.0 μM, which represent an undesired side effect of lethargy [21]. These results indicate that nanoencapsulation reduces adverse effects on locomotion.

The habituation and the gradual exploration of novel environments

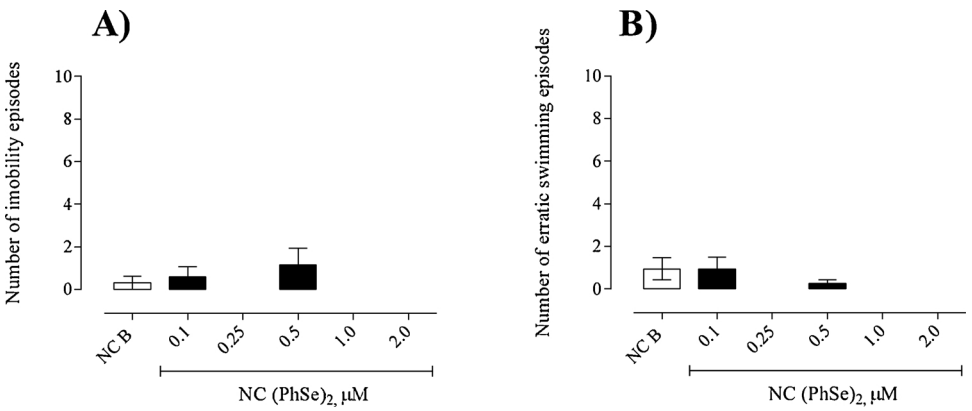

Fig. 1. Immobility (A) and erratic swimming (B) of zebrafish after exposure to the NC B and NC $(PhSe)_2$. Each column represents the mean ± S.E.M. Data were analyzed by one-way ANOVA and no significant differences were detected. For notes, NC $(PhSe)_2$ means nanocapsules containing $(PhSe)_2$; NC B means nanocapsules without $(PhSe)_2$.

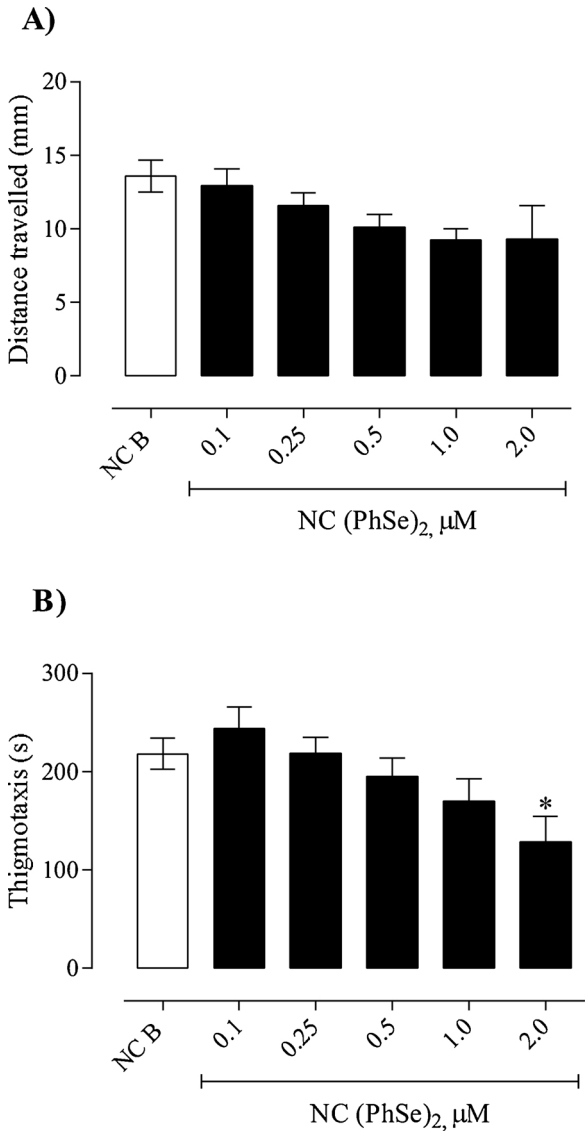

Fig. 2. Effects of NC $(PhSe)_2$ on distance travelled (A) and tigmotaxis (B). Each column represents the mean ± S.E.M. Asterisks denote significant differences (* $p < 0.05$) in comparison to the NC B group by one-way ANOVA, followed by Newman-Keuls' post hoc test. For notes, NC $(PhSe)_2$ means nanocapsules containing $(PhSe)_2$; NC B means nanocapsules without $(PhSe)_2$.

depend on anxiety-like behaviors, which can be measured by the time spent in each horizontal sections and vertical areas of tank [19]. Moreover, thigmotaxis refers to a behavioral preference by tank edge/sides, where animals avoid the central areas [19]. Fig. 2B shows that animals exposed to NC $(PhSe)_2$ at concentrations 0.1, 0.25, 0.5 and 1.0 µM had a similar thigmotaxis when compared to NC B ($p > 0.05$), suggesting that no behavioral impairments concerning the aquarium spatial preference were observed. Nevertheless, 2.0 µM NC $(PhSe)_2$ decreased the time in the peripheral area ($p < 0.05$), suggesting the absence of toxic effects and increased exploratory activity, which indicates anxiolytic-like behavior. Reinforcing such hypothesis, this behavioral modification was already reported in zebrafish exposed to escitalopram, a well-recognized anxiolytic drug [36].

Usually, non-treated fish have a tendency to homebase formation, establishing a safe place in a specific tank area, where animals often return after exploring other areas [19,21,37]. In this sense, the spatiotemporal behavior of zebrafish exposed to a range of NC $(PhSe)_2$ concentrations were also determined. Fig. 3A shows that the highest concentration tested (2.0 µM) reduced time spent in the bottom area ($p < 0.05$). Furthermore, zebrafish exposed to 2.0 µM NC $(PhSe)_2$ showed a significant increase in time spent in the top area, as demonstrated in Fig. 3B ($p < 0.05$). The other NC $(PhSe)_2$ concentrations showed no change in the respective parameters ($p > 0.05$). Fig. 4C and D presents the transition numbers to the top area and the latency to enter the top, respectively. No statistical differences were found among the groups ($p > 0.05$). In the study of Ibrahim and co-workers, the animal group exposed to 1.0 µM $(PhSe)_2$ spent more time in the aquarium top area, suggesting anxiolytic-like effects. However, the concentration that promoted this potential pharmacological action also triggered undesirable effects [21].

Our data suggest that differently than observed to free $(PhSe)_2$ [21], NC $(PhSe)_2$ showed no potential toxicological effects in the evaluated parameters. The respective study did not test $(PhSe)_2$ concentrations upper to 1 µM probably due to the compound low water solubility in higher concentrations. On the other hand, we showed the behavioral and biochemical effects of 2 µM, that were possible due to the aqueous character of NC suspension. It is important to highlight that both $(PhSe)_2$ forms (free and nanoencapsulated) elicited anxiolytic-like effects in zebrafish. However, while the free compound showed such effect at 0.25 µM, the NC $(PhSe)_2$ triggered anxiolytic-like effects only at 2.0 µM. This difference could be attributed to the $(PhSe)_2$ controlled release, conferred by nanocapsules. Such characteristic is an advantageous property that prevents the abrupt organism exposure of to the selenium compound, reducing its toxicity. Moreover, such nanocapsules property is well demonstrated by scientific reports, especially in formulations prepared with the components used here [10,38,39].

Biochemical markers of oxidative status were assessed in brain samples of the animals after zebrafish exposure to NC $(PhSe)_2$. Fig. 4 depicts the MDA levels (A) and the activity of GPx (B), GR (C) and GST (D). One-way ANOVA analysis revealed that regardless the NC $(PhSe)_2$ concentration tested, the parameters remained unchanged. The oxidative stress is an important mechanism associated with the toxicity

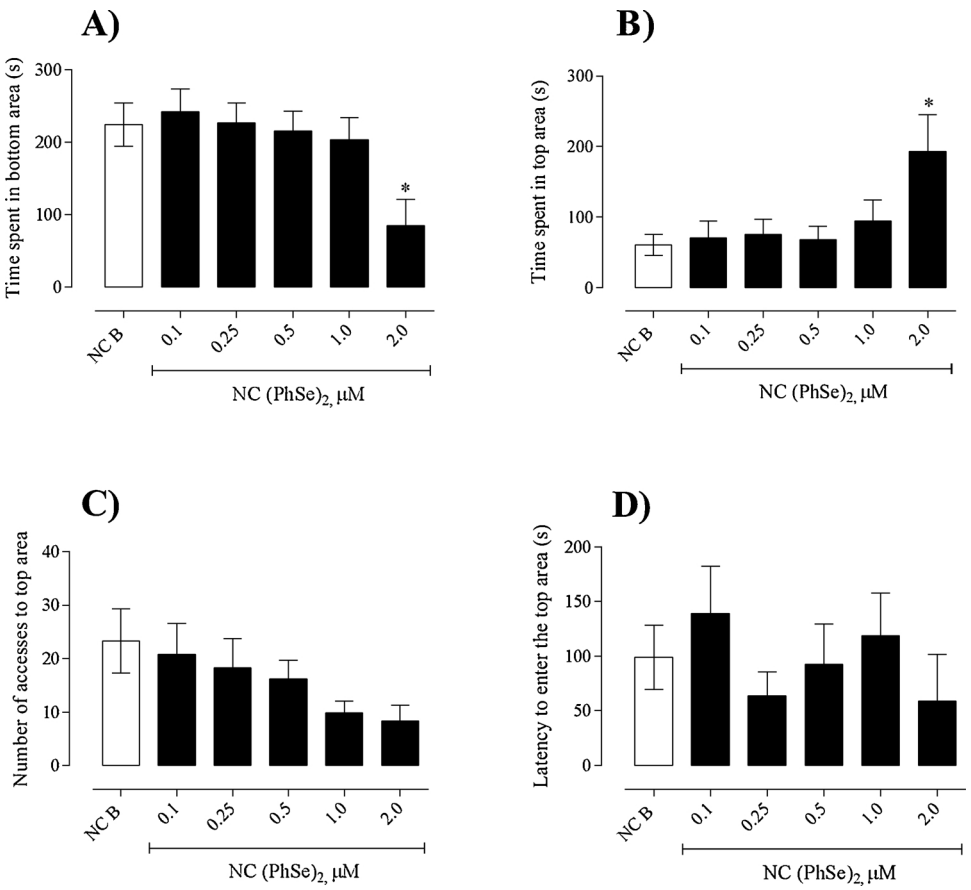

Fig. 3. Effects of NC $(PhSe)_2$ on vertical exploratory parameters: Time spent in the bottom area (A), time spent in the top area (B), entrance numbers on the top area (C) and latency to enter to the top area (D). Each column represents the mean ± S.E.M. Asterisks denote significant differences (* $p < 0.05$) in comparison to the NC B group by one-way ANOVA, followed by Newman-Keuls' post hoc test. For notes, NC $(PhSe)_2$ means nanocapsules containing $(PhSe)_2$; NC B means nanocapsules without $(PhSe)_2$.

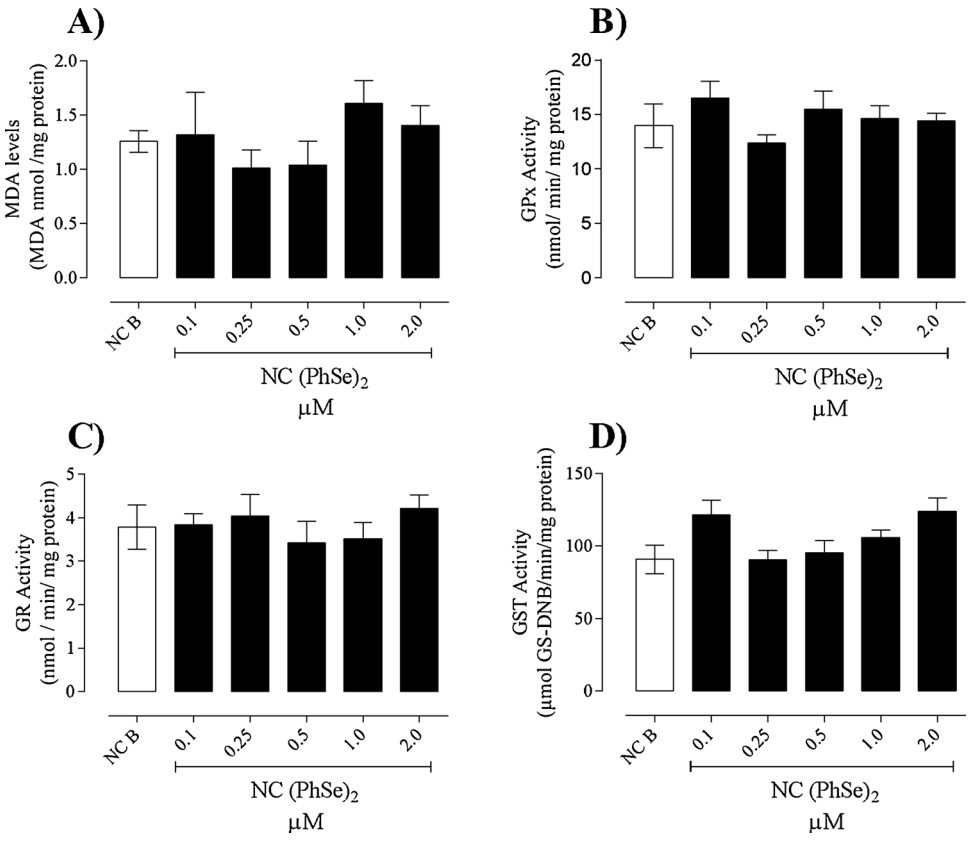

Fig. 4. Oxidative status of zebrafish brain following NC $(PhSe)_2$ exposure. TBARS levels (A), glutathione peroxidase (B), glutathione reductase (C) and glutathione S-transferase (D) activities. Each column represents the mean ± S.E.M. One-way ANOVA did not reveal significant differences among groups. For notes, NC $(PhSe)_2$ means nanocapsules containing $(PhSe)_2$; NC B means nanocapsules without $(PhSe)_2$.

triggered by nanostructures [40]. The small size could facilitate nanoparticles uptake by cells, promoting superior interaction between the formulation and cellular components, which may cause oxidative injury by overproduction of reactive species [40,41]. In addition, the brain is the most susceptible organ when it comes to oxidative stress, mainly because of its high oxygen consumption [42]. Based on the current results, it is possible to suggest that the exposure of zebrafish to NC $(PhSe)_2$ did not trigger neither behavioral impairments nor brain oxidative imbalance.

Currently, the $(PhSe)_2$ bioavailability in mice and rats was already demonstrated, but no reports in this regard were found for zebrafish. Interestingly, a recent study showed that zebrafish have several similarities with superior mammalians (rodents and humans) concerning the pharmacokinetic profile of drugs [43]. In this study the authors highlighted that zebrafish species has a similar profile of absorption, distribution and metabolization processes to human and rats [43]. Furthermore, significant amount of the drug was also determined in brain. Regarding the free $(PhSe)_2$ distribution, Prigol et al. showed that after a single intragastric administration the compound has a large distribution among the organs, mainly to kidneys, liver and fat as well as brain of mice and rats [6]. Later, Giordani et al. showed that the $(PhSe)_2$ encapsulation into nanocapsules provided a modification in the biodistribution profile, expanding the compound amount in the organs, including the brain [13].

Several reports demonstrated that the administration of $(PhSe)_2$ elicited an anxiolytic-like effect in different murine animal models [44–46] and in zebrafish model [21], suggesting that such action could be mediated by central mechanisms. In this sense, to the best of our knowledge, the current study is the first in reporting the evaluation of *in vivo* $(PhSe)_2$-loaded polymeric nanocapsules toxicological potential as well as to show its pharmacological action in an animal model. Importantly, the exposure of zebrafish to the formulation caused an anxiolytic-like effect without triggering any signal of toxicity, differently from the free compound, which provided toxic effects to the animals. Since zebrafish physiology is very similar to mammalians as rodents, the free $(PhSe)_2$ anxiolytic effect could be possibly attributed to the cerebral distribution, mainly due to its high lipophilic character.

4. Conclusion

In summary, the nanocapsules formed by PCL and MCT reduced the $(PhSe)_2$ apparent toxicity in zebrafish, broadening the perspective of its therapeutic application. In this sense, the nanocapsules suspension containing $(PhSe)_2$ may be considered a promising formulation for future studies in pharmacological action.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

Acknowledgements

L. M. Ferreira acknowledges the grant from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (grant number: 1530525).

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