

# Free radical scavenging in vitro and biological activity of diphenyl diselenide-loaded nanocapsules: DPDS-NCS antioxidant and toxicological effects

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**Abstract:** Selenium compounds, such as diphenyl diselenide (DPDS), have been shown to exhibit biological activity, including antioxidant effects. However, the use of DPDS in pharmacology is limited due to in vivo pro-oxidative effects. In addition, studies have shown that DPDS-loaded nanocapsules (DPDS-NCS) have greater bioavailability than free DPDS in mice. Accordingly, the aim of this study was to investigate the antioxidant properties of DPDS-NCS in vitro and biological activity in mice. Our in vitro results suggested that DPDS-NCS significantly reduced the production of reactive oxygen species and Fe(II)-induced lipid peroxidation (LPO) in brain. The administration of DPDS-NCS did not result in death or change the levels of endogenous reduced or oxidized glutathione after 72 hours of exposure. Moreover, ex vivo assays demonstrated that DPDS-NCS significantly decreased the LPO and reactive oxygen species levels in the brain. In addition, the highest dose of DPDS-NCS significantly reduced Fe(II)- and sodium nitroprusside-induced LPO in the brain and Fe(II)-induced LPO in the liver. Also,  $\delta$ -aminolevulinic acid dehydratase within the brain was inhibited only in the highest dose of DPDS-NCS. In conclusion, our data demonstrated that DPDS-NCS exhibited low toxicity in mice and have significant antioxidant characteristics, indicating that nanoencapsulation is a safer method of DPDS administration.

**Keywords:** diphenyl diselenide, DPDS-NCS, lipid peroxidation, antioxidant properties,  $\delta$ -ALA-D

## Introduction

Selenium (Se) is an essential trace element for mammals found within selenoproteins and has important health benefits.<sup>1,2</sup> However, several studies have reported that there is a narrow margin between the beneficial and the toxic effects of Se, stimulating the creation of new Se compounds, including diphenyl diselenide (DPDS) and ebselen.<sup>3,4</sup>

Accordingly, investigators have described that DPDS, a highly lipophilic organic selenium compound, has promising beneficial effects, which include neurological and hepatic protection against oxidative stress conditions.<sup>5,6</sup> In addition, most of the antioxidant properties of DPDS are associated with its ability to mimic the enzyme glutathione peroxidase.<sup>7</sup>

In contrast, studies have shown that DPDS has pro-oxidative effects in vivo, such as oxidation of sulfhydryl ( $-SH$ ) groups in proteins, reduction of glutathione levels (GSH), and inhibition of  $\delta$ -aminolevulinic acid dehydratase ( $\delta$ -ALA-D).<sup>8</sup> In addition, the lethal dose ( $LD_{50}$ ) for a single intraperitoneal (ip) administration of DPDS in mice is 210  $\mu\text{mol/kg}$ .<sup>9</sup>

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In this regard, nanotechnology is widely applied to produce significant advantages over prodrugs, including improving the bioavailability of lipophilic molecules and increasing the safe dose of toxic compounds.<sup>10,11</sup> In addition, nanoparticles help to enhance the stability of drugs and can efficiently control the drug release in a system, which can provide pharmacological efficacy by entrapping the drug molecules until they reach their target.<sup>12</sup>

Studies have shown that DPDS-loaded-nanocapsules (DPDS-NCS) have greater bioavailability than free DPDS in mice, which suggests an increase in drug absorption after nanoencapsulation.<sup>13</sup> However, DPDS-NCS antioxidant effects and the toxicity in mice remain unknown. Thus, the present study was designed to evaluate the antioxidant properties of DPDS-NCS against the generation of reactive oxygen or nitrogen species (ROS and RNS) *in vitro* and *ex vivo* and the biological effects following 72-hour exposure of DPDS-NCS exposure in mice.

## Materials and methods

### Materials

Thiobarbituric acid (TBA), malondialdehyde (MDA), *O*-phthalaldehyde (OPT), *N*-ethylmaleimide, Tris-HCl, trichloroacetic acid, and sodium dodecyl sulfate (SDS) were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Fe(II) sulfate, sodium nitroprusside (SNP), and acetic acid were obtained from Merck (Rio de Janeiro, RJ, Brazil). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

### Compound

DPDS was prepared following the method previously described by Paulmier, and the chemical purity of this compound (99.9%) was assessed by hydrogen and carbon nuclear magnetic resonance and gas chromatography.<sup>14</sup>

### Preparation of nanocapsules

Nanocapsule suspensions were prepared by the interfacial deposition of preformed polymer method as described by Fessi et al.<sup>15</sup> Briefly, an organic solution consisted of the oily phase – canola oil (1.551 g), a low HLB (hydrophilic–lipophilic balance) surfactant – Span 80® (0.383 g), polymer (polycaprolactone [PCL] with molecular weight 70,000–90,000 Da) (0.5 g), and acetone (133.5 mL) at 40°C was added under moderated magnetic stirring to an aqueous solution (266.5 mL of Milli-Q water) containing a high HLB surfactant – Tween 80® (0.383 g) at 35°C. DPDS-NCS were prepared by dissolving DPDS in the organic solution during the preparation of the nanocapsules, according to the method described by Giordani et al.<sup>13</sup>

## Nanocapsules characteristics

The particle size, polydispersity indices, and zeta potential were determined using Zetasizer Nano Series (Malvern Instruments, Malvern, UK). The pH values were determined using a Digimed DM-20 calibrated potentiometer (Digimed, São Paulo, SP, Brazil). The encapsulation efficiency was determined by high-performance liquid chromatography as described by Giordani et al.<sup>13</sup>

### Animals

Male adult swiss albino mice (25–30 g) were used. They were obtained from a local breeding colony. The animals were maintained on a 12-hour light and 12-hour dark cycle at a room temperature of 22°C±2°C, with free access to food and water. The animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Santa Maria, Brazil (permit number 024745/2009).

### In vitro experiments

#### Tissue preparation

The mice were sacrificed by cervical dislocation. The brains and livers were removed and immediately placed on ice. Both tissues were homogenized in 10 mM Tris-HCl and centrifuged for 10 minutes at 2,000 rpm. The supernatant fraction (S1) was collected immediately for the *in vitro* assays.

#### Lipid peroxidation assay

Lipid peroxidation (LPO) was determined by measuring TBA-reactive substances as previously described by Ohkawa et al.<sup>16</sup> Aliquots of brain and liver supernatants (100 µL of S1) were incubated for 60 minutes with freshly prepared Fe(II) (10 µM) in the absence or presence of unloaded nanocapsules (vehicle) and DPDS-NCS (10 µM, 20 µM, 40 µM, 80 µM) in a medium containing 10 mM Tris-HCl buffer at pH 7.4. The reaction was stopped by the addition of SDS (8.1%), and LPO products were measured by the addition of acetic acid/HCl buffer, pH 3.4 and 0.6% TBA, pH 6.0. The color reaction was developed by incubating tubes in boiling water for 60 minutes. TBA-reactive substance levels were measured by spectrophotometer at 532 nm using a standard curve of MDA.

#### ROS production

The substrate 2',7'-dichlorofluorescein diacetate (DCFH-DA) was utilized to measure the intracellular formation of ROS, according to Myhre et al.<sup>17</sup> The brain and liver supernatants (S1) were incubated with unloaded nanocapsules (vehicle) and DPDS-NCS (10 µM, 20 µM, 40 µM, 80 µM). DCFH-DA

(1 mM) was added to the medium, and the incubation was continued for 60 minutes in the dark. The fluorescence was measured using 488 nm for excitation and 525 nm for emission. ROS levels were expressed as nanomoles of DCF per milligram of protein.

## In vivo experiments

### Lethal dose–response

Male adult Swiss albino mice were treated with an ip injection of saline (control), unloaded nanocapsules (vehicle), or DPDS-NCS (10  $\mu\text{mol/kg}$ , 50  $\mu\text{mol/kg}$ , 100  $\mu\text{mol/kg}$ , 500  $\mu\text{mol/kg}$ , and 1,000  $\mu\text{mol/kg}$ ). According to Nogueira et al, the death was observed for up to 72 hours to determine the lethal properties of the DPDS-NCS.<sup>9</sup>

## Ex vivo experiments

### Animal treatment

Ex vivo experiments were performed in mice treated with an ip injection of saline (control), unloaded nanocapsules (vehicle), or DPDS-NCS (10  $\mu\text{mol/kg}$ , 50  $\mu\text{mol/kg}$ , 100  $\mu\text{mol/kg}$ , 500  $\mu\text{mol/kg}$ , and 1,000  $\mu\text{mol/kg}$ ) after 72 hours of exposure. All mice were sacrificed by cervical dislocation. The brains and livers were removed, weighed, dissected, and kept on ice until the time of assay. A portion of both tissues were homogenized in 10 mM Tris-HCl and centrifuged for 10 minutes at 2,000 rpm. The supernatant fraction (S2) was collected immediately for the ex vivo analysis. The remaining portions of brain and liver were used for the measurement of reduced (GSH) and oxidized (GSSG) glutathione levels.

### Thiobarbituric acid-reactive substance levels

Aliquots (200  $\mu\text{L}$ ) of brain and liver supernatants (S2) were mixed with 500  $\mu\text{L}$  TBA (0.6%), 200  $\mu\text{L}$  SDS (8.1%), and 500  $\mu\text{L}$  acetic acid (pH 3.4). The color reaction was developed by incubating tubes in boiling water for 60 minutes.<sup>16</sup> TBA-reactive substance levels were measured at 532 nm using a standard curve of MDA, and the results were expressed as nanomoles of MDA per milligram of protein.

### Fe(II) or SNP-induced lipid peroxidation

The end products of LPO were determined in tissue samples as previously described by Ohkawa et al.<sup>16</sup> Aliquots of brain and liver supernatants (100  $\mu\text{L}$  of S2) were incubated for 60 minutes with freshly prepared Fe(II) (10  $\mu\text{M}$ ) or SNP (5  $\mu\text{M}$ ). The assays were carried out as described in section in vitro experiments, except that the compounds were not added to the reaction medium.

## Measurement of intracellular reactive oxygen species production

Aliquots (20  $\mu\text{L}$ ) of brain and liver supernatants (S2) were added to a medium containing Tris-HCl buffer (10 mM; pH 7.4) and DCFH-DA (1 mM). The assay was conducted as described earlier to the in vitro experiments, except that the compounds were not added to the reaction medium.<sup>17</sup>

## Measurement of reduced (GSH) and oxidized (GSSG) glutathione levels

For the measurement of GSH and GSSG levels, we used a method previously described by Hissin and Hilf.<sup>18</sup> Briefly, 250 mg of brain and liver were homogenized in 3.75 mL phosphate EDTA buffer (pH 8) plus 1 mL  $\text{H}_3\text{PO}_4$  (25%). Homogenates were centrifuged at 4°C at 130,000 $\times$  *g* for 30 minutes, and the supernatants (S3) were separated in two different aliquots of 500  $\mu\text{L}$  each for measurement of GSH and GSSG. For GSH determination, 100  $\mu\text{L}$  of the supernatant (S3) was diluted in 1.8 mL of phosphate buffer and 100  $\mu\text{L}$  of OPT (1  $\mu\text{g}/\mu\text{L}$ ). The mixtures were incubated at room temperature for 15 minutes, and their fluorescent signals were recorded in the RF-5301PC Shimadzu spectrofluorometer (Shimadzu Corporation, Kyoto, Japan) at 420 nm of emission and 350 nm of excitation wavelengths. For the measurement of GSSG levels, a 250  $\mu\text{L}$  of the supernatant (S3) was incubated at room temperature with 100  $\mu\text{L}$  of *N*-ethylmaleimide (0.04 M) for 30 minutes at room temperature, and after that 140  $\mu\text{L}$  of the mixture was added to 1,760 mL of NaOH (0.1 N) buffer, following the addition of 100  $\mu\text{L}$  OPT and incubated for 15 minutes, using the above-outlined procedure for GSH assay.

### $\delta$ -ALA-D activity

The enzymatic activity was assayed according to the method of Sassa by measuring the rate of porphobilinogen (PBG) formation.<sup>19</sup> The incubation was initiated by adding 200  $\mu\text{L}$  of brain and liver supernatants (S2). Brain and liver samples were incubated for 180 minutes and 30 minutes, respectively, at 39°C. The reaction was stopped by the addition of 10% trichloroacetic acid containing 0.05 mol/L  $\text{HgCl}_2$ , and the PBG was measured with Ehrlich's reagent, using the molar absorption coefficient of  $6.1 \times 10^4$  for Ehrlich-PBG salt. The results were expressed as nmol PBG/h/mg protein.

## Protein quantification

The protein concentration was estimated by the Bradford method using bovine serum albumin as the standard.<sup>20</sup>

## Statistical analysis

All data are expressed as means  $\pm$  standard error of the mean for each experimental group. Determination of statistical significance was performed by an one-way analysis of variance, followed by Newman–Keuls multiple range test when appropriate. Differences between groups were considered to be significant when  $P < 0.05$ .

## Results

### In vitro results

#### Effect of DPDS-NCS on LPO induced by Fe(II) in the brains and livers of mice

LPO in mice brain and liver homogenates was induced with Fe(II) (10  $\mu$ M) (Figure 1), and the antioxidant effect of DPDS-NCS on these homogenates was investigated. The DPDS-NCS decreased LPO induced by Fe(II) at the highest concentration tested (80  $\mu$ M) in the brain sample (Figure 1A); however, no antioxidant effects were observed in the liver sample (Figure 1B).

#### Scavenging of normal ROS production

Brains and liver samples were incubated with different concentrations of DPDS-NCS to test the scavenging of ROS (Figure 2). All concentrations of DPDS-NCS tested significantly decreased normal ROS production in the brain (Figure 2A); however, no effects on the concentration of ROS were observed in the liver sample (Figure 2B).

### In vivo results

The administration of DPDS-NCS at any of the concentration tested (10  $\mu$ mol/kg, 50  $\mu$ mol/kg, 100  $\mu$ mol/kg, 500  $\mu$ mol/kg, and 1,000  $\mu$ mol/kg) was not lethal after 72 hours of exposure (data not shown).

## Ex vivo results

### Thiobarbituric acid-reactive substance levels

A significant reduction in brain MDA levels was observed with the administration of 1,000  $\mu$ mol/kg DPDS-NCS as compared to the control group (Figure 3A); however, significant decrease in the formation of MDA was not observed in the liver (Figure 3B). In addition, when LPO was induced by Fe(II) (10  $\mu$ M), the highest dose of DPDS-NCS led to significant antioxidant activity in both the brain and liver (Figure 4A and B). When LPO was induced with SNP (5  $\mu$ M), the DPDS-NCS (1,000  $\mu$ mol/kg) significantly reduced MDA formation in the brain (Figure 4C). The DPDS-NCS did not result in significant MDA reduction at any dose tested when LPO was induced with SNP in the liver (Figure 4D).

### Quantification ROS production

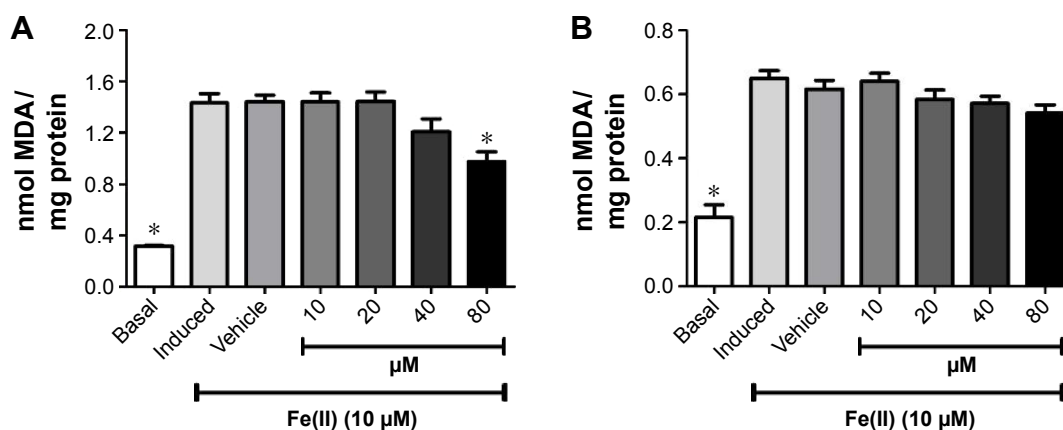
Figure 5A shows that significantly reduced ROS production in the brain by DPDS-NCS (1,000  $\mu$ mol/kg); however, exposure to DPDS-NCS did not change normal ROS production in the liver (Figure 5B).

### GSH and GSSG levels

All groups presented similar GSH and GSSG levels in the brain and liver, indicating that the DPDS-NCS did not interfere with these parameters after 72 hours of exposure (data not shown).

### $\delta$ -ALA-D activity

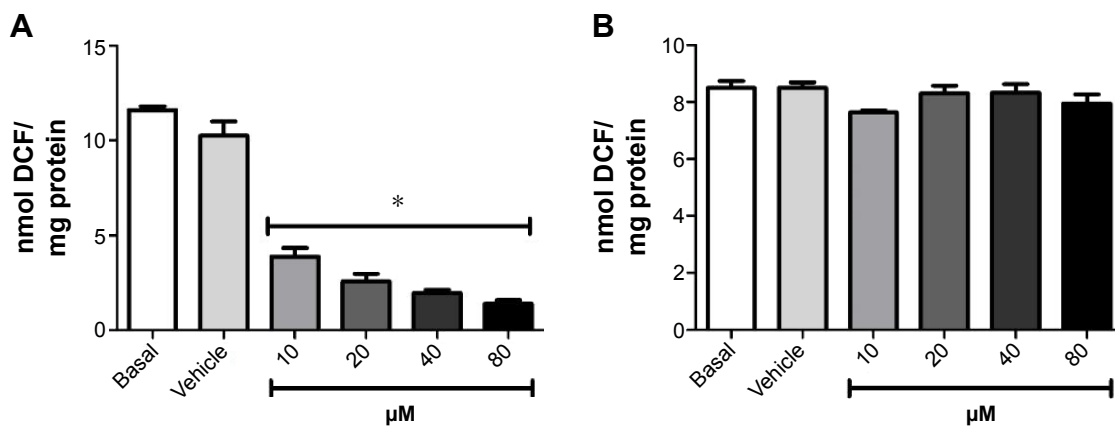
The highest dose of the DPDS-NCS (1,000  $\mu$ mol/kg) led to a significant decrease in brain  $\delta$ -ALA-D activity (Figure 6A); however, none of the doses of DPDS-NCS tested changed the activity of  $\delta$ -ALA-D in the liver (Figure 6B).



**Figure 1** Effect of DPDS-NCS in the TBARS induced by Fe(II) in vitro in the brain (A) and in the liver (B).

**Notes:** Data are shown as mean  $\pm$  SEM,  $n=4$ . \*Represent a significant difference if compared with the induced group by Newman–Keuls test,  $P < 0.05$ .

**Abbreviations:** DPDS-NCS, diphenyl diselenide-loaded nanocapsules; TBARS, thiobarbituric acid-reactive substances; SEM, standard error of the mean; MDA, malondialdehyde.



**Figure 2** Effect of DPDS-NCS in the ROS production in vitro in the brain (A) and in the liver (B).

**Notes:** Data are shown as means  $\pm$  SEM, n=4. \*Represent a significant difference if compared with the basal group by Newman-Keuls test,  $P < 0.05$ .

**Abbreviations:** DPDS-NCS, diphenyl diselenide-loaded nanocapsules; ROS, reactive oxygen species; SEM, standard error of the mean; DCF, dichloro-dihydro-fluorescein.

## Discussion

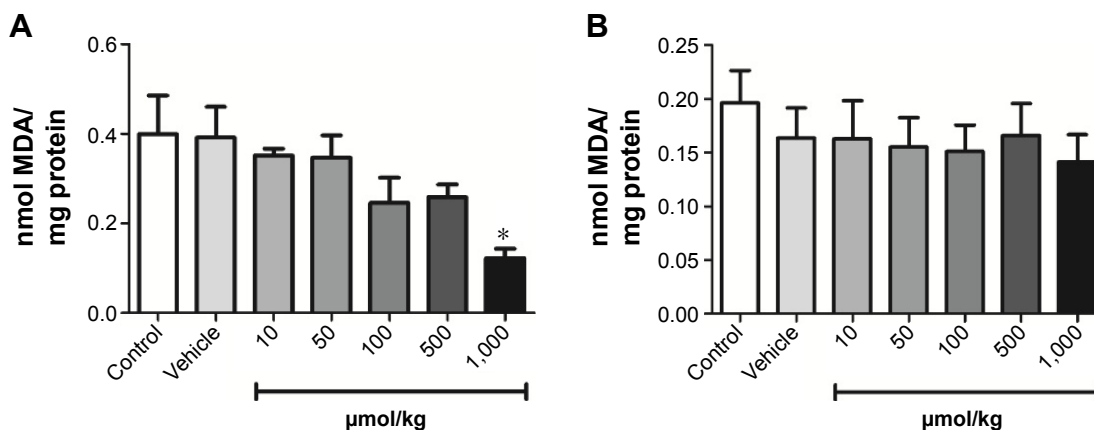
In this study, we performed some antioxidant and toxicological assays to characterize DPDS-NCS in vitro, ex vivo, and in vivo properties. To the best of our knowledge, this is the first study that evaluated DPDS-NCS in the mentioned assays. In addition, DPDS was considering as a promising therapeutic compound of oxidative stress-induced tissue damage.<sup>21–23</sup> However, DPDS has a narrow barrier between beneficial and the advent of toxicological effect, which is a limiting factor in their current usage.<sup>9,24</sup>

Additionally, DPDS-NCS was previously characterized as macroscopic homogeneous aspect, as well as submicronic sizes, low polydispersity indices, negative zeta potentials, and slightly acid or neutral pH values.<sup>13</sup> Giordani et al also demonstrated that nanoencapsulation improves the DPDS-NCS pharmacokinetics when compared to the free DPDS.

In this regard, we evaluated the toxicity in mice, following exposure to DPDS-NCS, when administered intraperitoneally in mice. Accordingly, we showed that the administration of DPDS-NCS in doses fivefold higher than the LD<sub>50</sub> of free DPDS were not toxic and did not cause seizures in mice, indicating that nanoencapsulation reduces the toxicity of DPDS.

Another important property of DPDS and its analogs is their antioxidant activity in vitro and ex vivo.<sup>21,25</sup> In the most frequently cited mechanisms of organic selenium antioxidant activity, the selenium compound acts as substrate of the enzyme thioredoxin reductase or mimics glutathione peroxidase.<sup>5,26</sup>

Similarly, DPDS protected some tissues against the prooxidative effects of ROS and LPO.<sup>25,27,28</sup> In addition, LPO and ROS were widely associated with several oxidative diseases

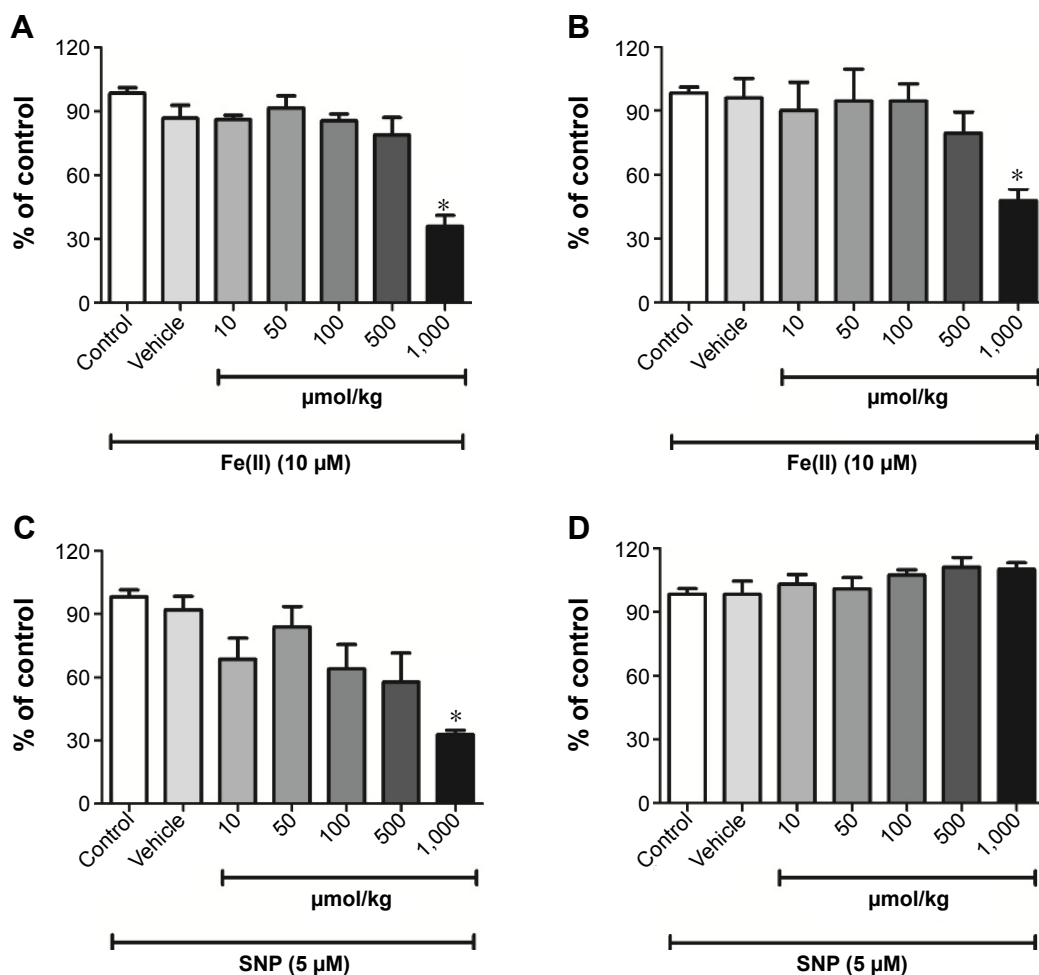


**Figure 3** Effect of DPDS-NCS in the TBARS ex vivo in the brain (A) and in the liver (B).

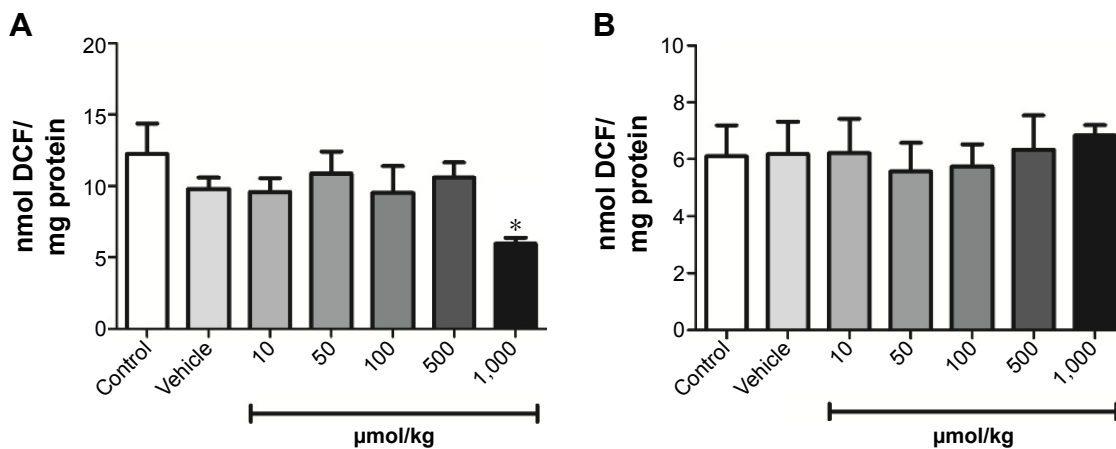
**Notes:** Data are shown as means  $\pm$  SEM, n=4. \*Represent a significant difference if compared with the control group by Newman-Keuls test,  $P < 0.05$ .

**Abbreviations:** DPDS-NCS, diphenyl diselenide-loaded nanocapsules; TBARS, thiobarbituric acid-reactive substances; SEM, standard error of the mean; MDA, malondialdehyde.

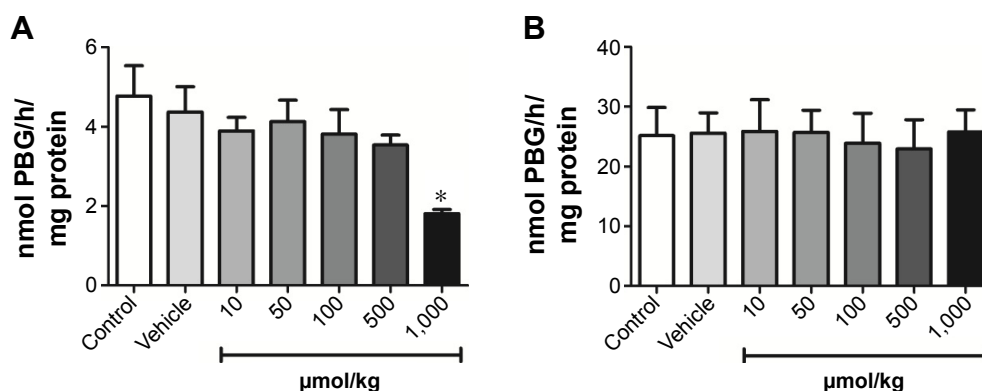




**Figure 4** Effect of DPDS-NCS administration in the lipid peroxidation production induced in the brain by Fe(II) (A) and SNP (C) and in the liver by Fe(II) (B) and SNP (D). **Notes:** Data are shown as means  $\pm$  SEM, n=4. \*Represent a significant difference if compared with the control group by Newman-Keuls test,  $P < 0.05$ . **Abbreviations:** DPDS-NCS, diphenyl diselenide-loaded nanocapsules; SNP, sodium nitroprusside; SEM, standard error of the mean.



**Figure 5** Effect of DPDS-NCS administration in the ROS production in the brain (A) and in the liver (B). **Notes:** Data are shown as means  $\pm$  SEM, n=4. \*Represent a significant difference if compared with the control group by Newman-Keuls test,  $P < 0.05$ . **Abbreviations:** DPDS-NCS, diphenyl diselenide-loaded nanocapsules; ROS, reactive oxygen species; SEM, standard error of the mean; DCF, dichloro-dihydrofluorescein.



**Figure 6** Effect of DPDS-NCS administration in the brain (A) and in the liver (B)  $\delta$ -ALA-D.

**Notes:** Data are shown as means  $\pm$  SEM,  $n=4$ . \*Represent a significant difference if compared with the control group by Newman-Keuls test,  $P<0.05$ .

**Abbreviations:** DPDS-NCS, diphenyl diselenide-loaded nanocapsules;  $\delta$ -ALA-D,  $\delta$ -aminolevulinic acid dehydratase; SEM, standard error of the mean; PBG, porphobilinogen.

and many types of antioxidants play an important protection role through the redox mechanism.<sup>29,30</sup>

In this regard, we observed a significant *in vitro* antioxidant activity in the brains of mice, including a reduction of MDA levels (Figure 1) and ROS production (Figure 2). Huang and Cols have shown significant *in vitro* free radical scavenger activity of nano red elemental selenium, despite its small particle size.<sup>31</sup> Our findings corroborate with the findings of that study; although DPDS-NCS exhibit a small particle size, some DPDS *in vitro* antioxidant activity was maintained.

Also, the exposure of mice to DPDS-NCS reduced MDA levels (Figure 3) and ROS production (Figure 5) in the brain, demonstrating significant antioxidant activity following 72 hours of administration. In this way, we induced *ex vivo* LPO by Fe(II) to produce ROS and by SNP to generate RNS. We found that the highest dose of DPDS-NCS led to a significant reduction in the oxidant levels in the brain when induced by both pro-oxidants (Figure 4A and C). In addition, the highest DPDS-NCS dose also significantly decreased liver LPO when induced by Fe(II) (Figure 4B).

Another important DPDS characteristic is its ability to interact with and oxidize the sulfhydryl groups of proteins within biological systems. This is the most likely mechanism leading to the inhibition of  $\delta$ -ALA-D.<sup>6</sup> Several studies have shown that exposure to DPDS can induce toxicity by oxidizing cysteinyl residues for important enzymes.<sup>6,32</sup> Thus, our findings demonstrate that the DPDS-NCS at the highest dose tested were able to inhibit  $\delta$ -ALA-D in the brain, indicating that this dose maintained the enzyme inhibition 72 hours after administration of the DPDS-NCS (Figure 6A). However, we also verified that exposure to the DPDS-NCS did not inhibit the liver  $\delta$ -ALA-D (Figure 6B).

Moreover, DPDS-NCS was absorbed faster than the free compound, which demonstrated that the metabolism of DPDS-NCS in the liver can occur earlier than 72 hours of exposure.<sup>13</sup>

In addition, our results established that DPDS-NCS in all tested doses did not present any significant effect on the GSH and GSSG levels in the brain and liver, indicating that even if the thiol groups of the compound are oxidized, normal levels can reestablished.

## Conclusion

We developed *in vitro*, *in vivo*, and *ex vivo* assays to evaluate the antioxidant and the biological effects of DPDS-NCS in mice. The results of this study have demonstrated that antioxidant activity of DPDS-NCS can significantly influence the physico-chemical characteristics of the nanomaterial. Consequently, DPDS-NCS presented greater *in vitro* and *ex vivo* antioxidant effects in the brain than in the liver. Furthermore, the DPDS-NCS were not lethal to mice following 72 hours of exposure. Thus, DPDS exposure is safer after nanoencapsulation. In conclusion, further studies can be beneficial in evaluating the efficacy of DPDS-NCS as therapeutic option for the treatment against oxidative stress-associated diseases.

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## Disclosure

The authors report no conflicts of interest in this work.

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