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IN VITRO EXPOSURE TO FULLERENE C_{60} INFLUENCES REDOX STATE AND LIPID PEROXIDATION IN BRAIN AND GILLS FROM *CYPRINUS CARPIO* (CYPRINIDAE)

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Abstract—Studies concerning the impact of nanomaterials, especially fullerene (C_{60}), in fresh water environments and their effects on the physiology of aquatic organisms are still scarce and conflicting. We aimed to assess in vitro effects of fullerene in brain and gill homogenates of carp *Cyprinus carpio*, evaluating redox parameters. A fullerene suspension was prepared by continued stirring under fluorescent light during two months. The suspension concentration was measured by total carbon content and ultraviolet–visible spectroscopy nephelometry. Characterization of C_{60} aggregates was performed with an enhanced dark-field microscopy system and transmission electronic microscopy. Organ homogenates were exposed during 1, 2, and 4 h under fluorescent light. Redox parameters evaluated were reduced glutathione and oxidized glutathione, cysteine and cystine, total antioxidant capacity; activity of the antioxidant enzymes glutathione S-transferase and glutathione reductase (GR), and lipid peroxidation (TBARS assay). Fullerene induced a significant increase ($p < 0.05$) in lipid peroxidation after 2 h in both organs and reduced GR activity after 1 h (gills) and 4 h (brain) and antioxidant capacity after 4 h (brain). Levels of oxidized glutathione increased in the brain at 1 h and decreased at 2 h as well. Given these results, it can be concluded that C_{60} can induce redox disruption via thiol/disulfide pathway, leading to oxidative damage (higher TBARS values) and loss of antioxidant competence. Environ. Toxicol. Chem. 2012;31:961–967. © 2012 SETAC

Keywords—Nanotoxicology Fullerene Glutathione Lipid peroxidation Redox state

INTRODUCTION

The production of nanomaterials is growing exponentially because of the enormous potential for applications in virtually all areas of industry, from goods and electronics to pharmaceutics, including personal care products, fuel cells, optical products, drug delivery devices, nonviral DNA vectors, and myriad other applications [1,2]. This massive production of engineered nanosubstances and nanoproducts has become a serious concern, because their potential toxicity is far from being clearly understood in terms of mechanisms and effects in biological systems [3,4].

In fact, the term *nanoparticles* includes substances with heterogeneous chemical structures, of which the only common characteristics are the size-specific properties that they acquire when at least one of their dimensions is on the nanoscale (less than 100 nm). These properties differ substantially from the bulk state of the same substance, and its effects depend on many parameters, such as size, surface area, shape, chemical composition, lattice structure, surface chemistry, surface charge, and aggregation state [5]. Generally, these materials are classified as carbon-derived materials (fullerenes and nanotubes) and inorganic substances such as metals, metal oxides, and quantum dots [6].

Among fullerenes, pristine C_{60} is the most common, possessing an aromatic cage-like structure of 60 carbons that allows it to be easily functionalized but also to react with biomolecules. Because of its high affinity for lipids (water solubility is less than 10^{-9} mg/L [7]) and its ability to cross the blood–brain barrier, fullerene C_{60} is considered a useful tool in biomedicine to carry and deliver drugs for treating many diseases, including cancer and neurodegenerative disorders [8]. In terms of its potential risk to the environment, the recent review of Kahru and Dubourguier [2] has analyzed fullerene toxicity data from several organisms and considered this nanomaterial as "very toxic," with LC50 and EC50 ranging from 0.1 to 1.0 mg/L.

In terms of generating oxidative stress, controversial evidence about fullerene toxicity can be found. Some studies have associated lipid peroxidation with C_{60} toxicity in human cell lines of fibroblasts, liver carcinoma, and astrocytes [9]. The classic study by Oberdörster et al. [10] reported increased lipid peroxides content for brain and the opposite results for gills of the fish *Micropterus salmoides*. In another study, in which gills and brain of goldfish were exposed to nC_{60}, a reduction in reduced glutathione (GSH; pro-oxidant condition) and a parallel decrease in content of lipid peroxides (antioxidant condition) were observed. Only in the liver of fish exposed to the highest fullerene concentration (1 mg/L) was a significant increment of lipid peroxidation registered [11]. These and other results from

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in vivo assays suggest that this kind of exposure is under the influence of several parameters, such as actual concentrations of the nanoparticle taken by animals or organs and physical and chemical modifications of particles resulting from the exposure medium (i.e., ionic strength). Because it is believed that toxicity of fullerenes is due to inducing oxidative stress, these are critical points when redox state markers are being analyzed.

Moreover, there is enough evidence that reactive oxygen species (ROS) play a pivotal role in cellular redox signaling and control, possessing well-defined pathways or nodes that include the balance between redox couples such as GSH or oxidized glutathione (GSSG) and cysteine (Cys)/cystine (CySSyC) [12,13]. From this perspective, it is possible that nanoparticles can alter the redox state of cells, with the toxicity or benefits observed being a consequence, at least in part, of their interaction with redox nodes.

In light of this, we choose an in vitro approach based on the work of Shinohara et al. [14], using the same fish species (*Cyprinus carpio,* Cyprinidae) that these authors employed. The method takes advantage of the direct exposure of organ homogenates to C_{60} to diminish interfering variables, a condition that is interesting when biochemical markers of oxidative stress are being evaluated. The main hypotheses that govern the present study were associated with evaluating oxidative stress generation of fullerene suspensions in the gills and brain of carp, *C. carpio,* taking into account that a byproduct of glutathione metabolism, GSSG, has not previously been evaluated in assays conducted to analyze the pro-oxidant effects of fullerene. Thus, the main objective of present study was to analyze biochemical redox responses in two organs of the fish *C. carpio* after exposure to fullerene under fluorescence light, in order to promote photoexcitation of this nanomaterial, leading to a pro-oxidant condition [14].

MATERIALS AND METHODS

Preparation of fullerene solutions

Previous evidence has shown that preparing fullerene suspensions with organic solvents such as tetrahydrofuran enhances the toxicity [15]. We used a methodology employed by Lyon et al. [7] in which an aqueous fullerene C_{60} suspension (200 mg/L; SES Research; 99% purity, average C-C distance 1.44 Å, mean ball diameter of C_{60} 6.83 Å, mass density 1.72 g/cm^3) was stirred in Milli-Q water for two months under constant fluorescent light. After this, the suspension was centrifuged at 25,000 *g* for 1 h at 15°C and filtered sequentially through 0.45- and 0.20-μm filters. Fullerene concentration was estimated by measuring total carbon concentration with a TOC-V CPH (Shimadzu) total organic carbon analyzer and by ultraviolet–visible spectroscopy nephelometry. This last measurement was performed in a spectrofluorimeter (Shimadzu RF-5301PC) with an appropriately 1-cm-thick quartz cuvette [16]. After the concentration measurements, suspensions were concentrated through evaporation in a SPD SpeedVac® system centrifugal vacuum concentrator (Thermo Savant). Fullerene suspensions were analyzed by using a JEOL JSM 1200 EX II transmission electron microscope (TEM) operating at 100 kV. Samples of about 30 μl of C_{60} suspension were placed onto 300 mesh TEM grids (SPI) coated with Formvar. Analysis was performed after 24 h to allow sample evaporation [7]. As showed in *Results*, the two methodologies employed to measure fullerene concentration give results that are very different. We opted to express fullerene concentration measured through the determination of total carbon concentration.

Preparation of brain and gills extracts

Adult carp (*Cyprinus carpio*, Teleostei, Cyprinidae) with a mean weight of 27.9 ± 3.8 g were obtained from local suppliers. Once in the laboratory, fish were acclimated in a 300-L aerated freshwater (pH 7.0, 7.20 mg O_2/L, 20°C) tank equipped with a filtering system for at least two weeks prior to the experiments. Organisms were fed with food pellets at a rate of 1% of the body weight per day. For organ dissection, fish were cryoanesthetized and gills and brains extracted. Organs were homogenized (1:3 w/v) in KCl 1.15% for in vitro experiments to measured lipid peroxidation and redox state. For the measurement of parameters associated with antioxidant enzymes and total antioxidant capacity, organs were homogenized according to Gallagher et al. [17] and then exposed in vitro to fullerene. In this case, organs were homogenized (1:3) in Tris-HCl buffer (100 mM, pH 7.75) plus EDTA (2 mM) and Mg^{2+} (5 mM). Samples were centrifuged at 10,000 *g* for 20 min at 4°C, and the supernatant was employed for all measurements describe here. Previously, total protein content was determined through the Biuret method (550 nm), in triplicate, using a microplate reader (BioTek LX 800).

Exposure to C_{60}

Brain and gills extracts were exposed in vitro for 1, 2, and 4 h to 1 mg C_{60}/L. The concentration of 1 mg C_{60}/L was selected based on previous in vivo experiments performed with the fish *Micropterus salmoides* [10]. Control groups were also run in parallel, without adding fullerene suspension. After each exposure time, aliquots were stored, and then lipid peroxidation was measured (see *Measurement of lipid peroxidation*). At the three different exposure times, extract aliquots were stored to measure redox parameters (GSH/GSSG and Cys/CySSyC), total antioxidant capacity, and activities of glutathione S-transferase (GST) and glutathione reductase (GR). In all cases, fullerene exposure was performed under fluorescent light (556.3 ± 77.4 lux), taking into account that fullerene induction of lipid peroxidation is enhanced under this condition [14]. A previous study also showed fullerene's ability to induce lipid peroxidation under UV or tungsten lamp irradiation [18].

Measurement of lipid peroxidation

Lipid peroxides content was estimated by measuring thiobarbituric acid-reactive substances (TBARS) [19]. Brain and gills extracts (10 μl) were added to a reaction mixture made with 150 μl 20% acetic acid, 150 μl thiobarbituric acid (0.8%), 50 μl Milli-Q water, and 20 μl sodium dodecyl sulfate (SDS; 8.1%). Samples were heated at 95°C for 30 min and were then cooled for 10 min. Then, 100 μl Milli-Q water and 500 μl *n*-buthanol were added. After centrifugation (3,000 *g* for 10 min at 15°C), the organic phase (150 μl) was placed in a microplate reader and the fluorescence registered after excitation at 520 nm and emission of 580 nm. The concentration of TBARS (nmols/g wet tissue) was calculated by employing a standard curve of tetramethoxypropane (TMP; Acros Organics).

Measurement of reduced and oxidized glutathione and cysteine

The key redox state parameters considered were GSH, GSSG, Cys, and CySSyC concentrations. Levels of each were simultaneously measured through a high-performance liquid chromatography system [20] consisting of one ESA584 pump connected to a quaternary solvent-delivery system (ESA 582LPG) and a Shimadzu Prominence DGU-20A5 degasser system. This system was coupled to an ESA Coulochem III

electrochemical detector (EC) to detect the compounds with a guard cell (M5020) set at +100 mV and a double analytical cell (M5011) maintained at +650 mV (first cell, for GSH and Cys) or +850 mV (second cell, for GSSG and CySSyC). Twenty microliters of homogenates prepared in KCl 1.15% as previously described was injected into the high-performance liquid chromatography system to analyze the compounds. The thiolic compounds were separated through an ACE5-C18 column (25 cm × 4.6 mm, 5 μm) before detection on the EC detector. The mobile phase consisted of sodium phosphate 50 mM, pH 2.7, and 50 μM octanesulfonic acid with 2% acetonitrile, and was pumped isocratically at 1 ml/L. The signal of the peaks was recorded and monitored with EZChrom Elite software. Quantification of the compounds in samples was performed based on standard calibration curves previously constructed for each compound.

Measurement of total antioxidant capacity

Total antioxidant competence against peroxyl radicals was analyzed through ROS determination in organ samples treated or not with a peroxyl radical generator [21]. Peroxyl radicals were produced by thermal (35°C) decomposition of 2,2′-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Sigma-Aldrich) [22]. For ROS determination, we employed the fluorogenic compound 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA; Invitrogen) at a final concentration of 40 μM, according to the methodology described by Amado et al. [21]. Readings were carried in a fluorescence microplate reader (Victor 2; PerkinElmer) in a medium containing 30 mM HEPES (pH 7.2), 200 mM KCl, 1 mM $MgCl_2$, and 40 μM DCF-DA. Total fluorescence generation was calculated by integrating the fluorescence units (FU) along the time of the measurement, after adjusting FU data to a second-order polynomial function. The results were expressed as area difference of FU × minutes in the same sample with and without ABAP addition and standardized to the ROS area without ABAP (background area). The relative difference between ROS area with and without ABAP was considered a measure of antioxidant competence, with high area difference meaning low antioxidant capacity, because high fluorescence levels were obtained after adding ABAP, meaning low competence to neutralize peroxyl radicals [21].

Measurement of glutathione S-transferase and glutathione reductase activity

Glutathione S-transferase (GST) activity was determined after conjugation of 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene at 340 nm [23]. Glutathione reductase activity was measured by using sodium phosphate buffer (200 mM, pH 7.4), GSSG (1 mM), and NADPH (0.1 mM) and then by monitoring the reduction in NADPH absorbance at 340 nm [24].

Statistical analysis

All variables were analyzed by means of ANOVA [25]. Previously, normality and variance homogeneity were verified, and mathematical transformation was applied if at least one assumption was violated. Means comparisons were performed with the Newmann–Keuls method and in some cases with orthogonal contrasts. In all cases, type I error probability was fixed at 0.05 ($\alpha = 0.05$).

RESULTS

Concentration and characterization of fullerene particles

Figure 1a shows the size of fullerene particles obtained from TEM, showing that the size of particle aggregates are on the order of 200 nm. A conspicuous difference was registered for determining the fullerene concentration measured by total carbon concentration (5.01 ± 0.88 mg/L) and by ultraviolet–visible spectroscopy nephelometry (0.094 ± 0.023 mg/L), as shown in Figure 1b.

Effects of fullerene on lipid peroxidation and redox parameters

A significant increase ($p < 0.05$) in lipid peroxidation (LPO) content in both brain (Fig. 2a) and gills (Fig. 2b) was observed after 2 h of exposure to fullerene. This effect appears be attenuated in the fourth hour of exposure and was similar on both organs. Reduced glutathione concentrations were not affected ($p > 0.05$) by fullerene in either organ (data not shown). Concentration of GSSG was augmented ($p < 0.05$) in brain tissue after 1 h, followed by a decrease at 2 h (Fig. 3a). No differences in GSSG content ($p > 0.05$) were registered for gills (Fig. 3b).

Neither Cys nor CySSyC was affected by fullerene in either brain or gills ($p > 0.05$; data not shown). Total antioxidant

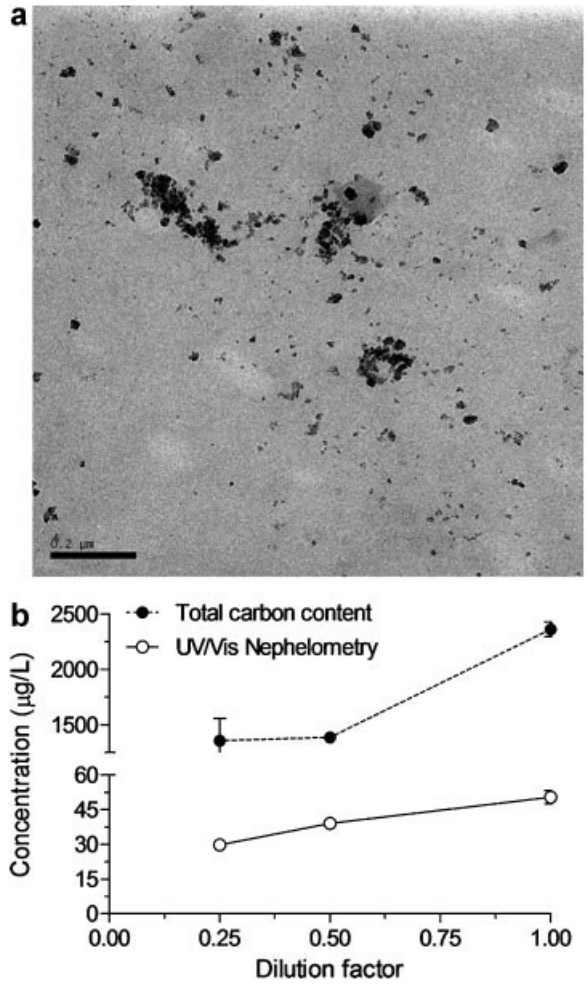

Fig. 1. **(a)** Transmission electronic microscopic image of the aqueous fullerene (C_{60}) suspensions employed in the bioassays. **(b)** Concentration of the C_{60} suspensions used in the assays. A dilution curve shows values obtained through total organic carbon content and ultraviolet–visible (UV/VIS) spectroscopy nephelometry. Data are expressed as mean and one standard error. Some error bars in the figure are hidden by the symbol.

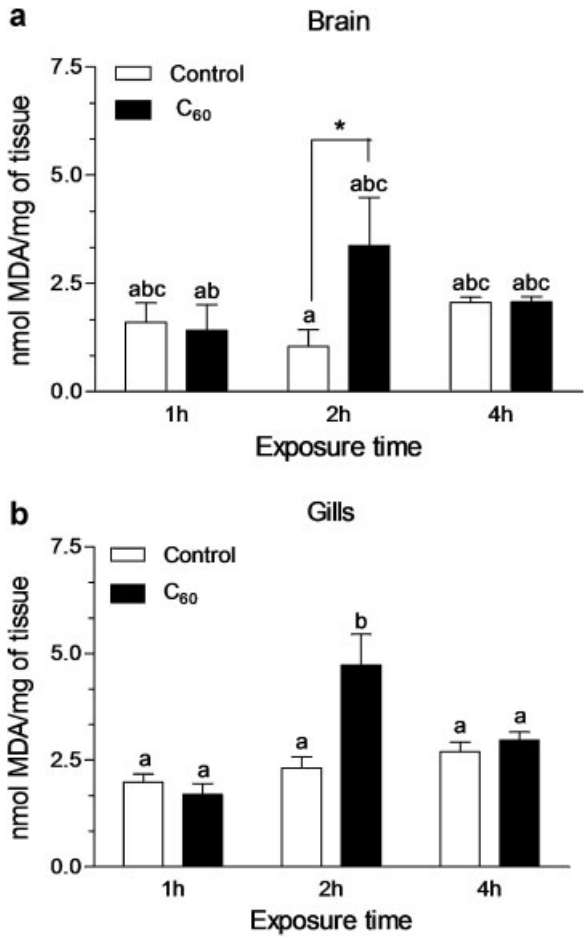

Fig. 2. Concentration of thiobarbituric acid reactive substances (TBARS) in **(a)** brain and **(b)** gill carp extracts after 1, 2, or 4 h exposure to 1 mg/L fullerene (C_{60}). Data are expressed as mean and one standard error. Numbers of analyzed samples were four for each treatment. Identical letters mean absence of statistical differences ($p > 0.05$). $^{*}p < 0.05$, employing orthogonal contrast. TBARS data are expressed in nmoles tetramethoxypropane/mg tissue.

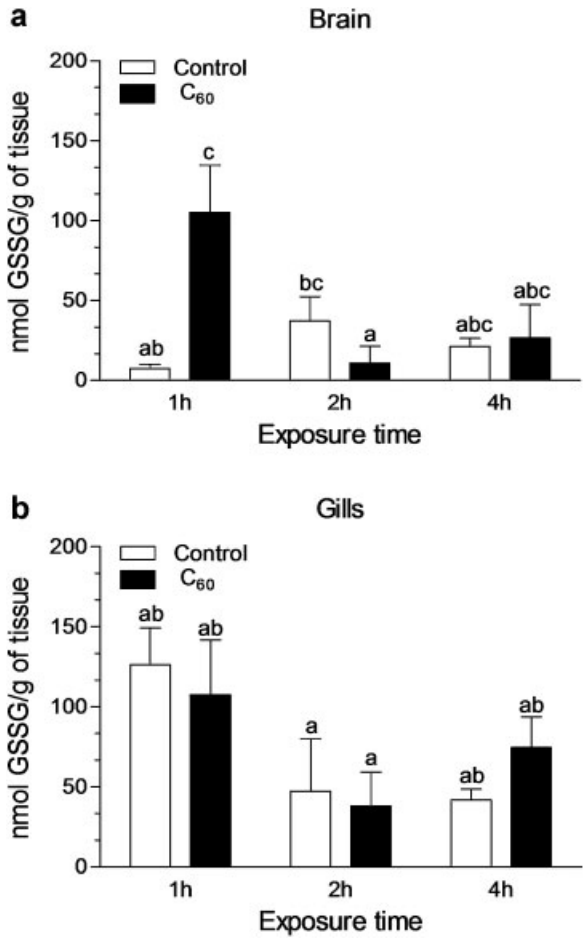

Fig. 3. Concentration of oxidized glutathione (GSSG) in **(a)** brain and **(b)** gill carp extracts after 1, 2, or 4 h exposure to 1 mg/L fullerene (C_{60}). Data are expressed as mean and one standard error. Number of analyzed samples varied from three to five for each treatment. Identical letters mean absence of statistical differences ($p > 0.05$).

capacity of the brain diminished ($p < 0.05$) only in the fourth hour of exposure (Fig. 4a). In gills (Fig. 4b), fullerene was not able to induce differences at any of the exposure times ($p > 0.05$).

Glutathione reductase activity data are shown in Figure 5. The activity was significantly reduced ($p < 0.05$) in brain after 4 h of exposure to fullerene (Fig. 5a) and in gills exposed after 1 h ($p < 0.05$; Fig. 5b). Glutathione S-transferase activity showed no significant differences ($p > 0.05$) between treatments for either organ (data not shown).

DISCUSSION

Particle characterization by TEM confirmed that the suspensions employed in the assays were in fact nanoaggregates. However, a huge discrepancy in values was seen between the two methodologies used to measure fullerene concentration (total organic carbon content and ultraviolet-visible spectrometry nephelometry), as shown in Figure 1b. This result points to the need to address not only preparing and characterizing fullerene suspensions but also the methodology employed to assess concentration. With regard to the latter, many researchers have warned about the implications of comparisons among studies with the same type of nanoparticle that might not have been properly characterized and yet proceeded under the same conditions [26,27]. However, with respect to the different techniques of concentration measurements, no studies to our knowledge have pointed out this problem. Our first attempt to measure fullerene concentration following a previously described methodology [10] failed in several trials. We suspect this was the case because of the very low C_{60} concentration obtained through the toluene-water extraction process, which the espectrophotometric technique was not able to detect. We subsequently adopted total organic carbon (TOC) content measurements to obtain information about the fullerene concentration in the working suspension. It is important to mention that if ultraviolet-visible spectrometry nephelometry values are considered, C_{60} toxicity is enhanced once TOC values were 53.3-fold higher. If true, this means that the results presented here were obtained with concentrations very much lower than those used in most previous fullerene nanotoxicological studies, a situation that urgently needs confirmation.

The generation of oxidative stress by fullerene is due, at least in part, to photoactivation (ultraviolet and visible range), as shown by Kamat et al. [18]. These authors have inferred that photoactivated fullerene induces the generation of several ROS, including singlet oxygen and hydroxyl radicals, among others. Differences in lipid peroxidation in carp brain with and without fluorescent light incidence have also been reported [14]. In this

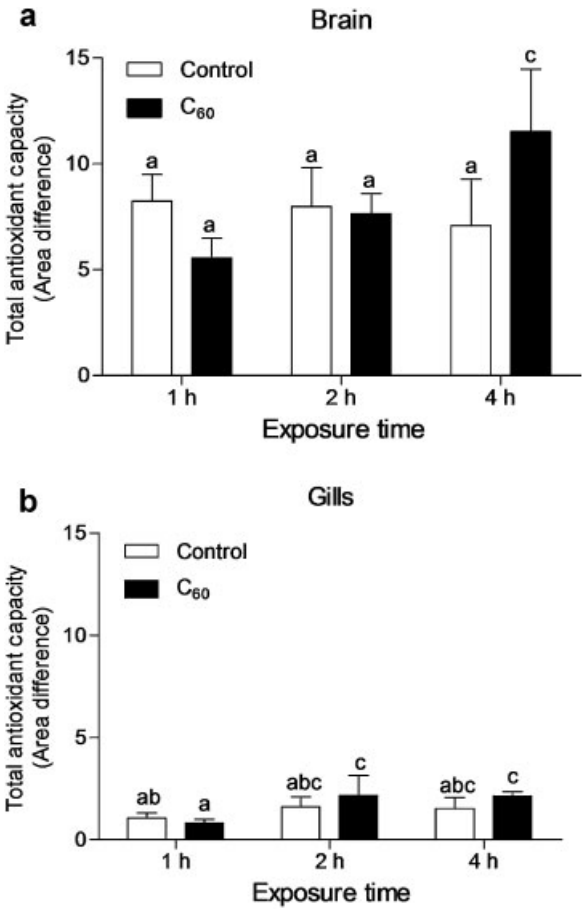

Fig. 4. Total antioxidant capacity against peroxyl radicals in **(a)** brain and **(b)** gill carp extracts after 1, 2, or 4 h exposure to 1 mg/L fullerene (C_{60}). Data are expressed as mean and one standard error. Numbers of analyzed samples were five for each treatment. Identical letters mean absence of statistical differences ($p > 0.05$).

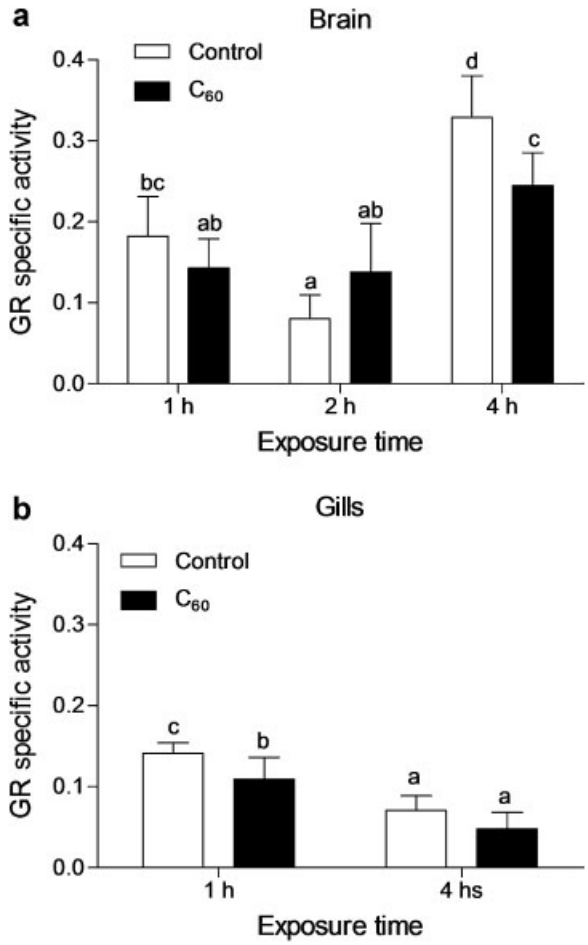

Fig. 5. Glutathione reductase (GR) activity (in nmoles NADPH/min/mg proteins) in **(a)** brain and **(b)** gill carp extracts after 1, 2, or 4 h exposure to 1 mg/L fullerene (C_{60}). Data are expressed as mean and one standard error. Numbers of analyzed samples were five for each treatment. Identical letters mean absence of statistical differences ($p > 0.05$).

way, the in vitro assay conditions of the present study should contribute to oxidative stress generation by fullerene.

It is interesting to note that fullerene provoked no effect on GSH content or on GST activity in either tissue, indicating that C_{60} detoxification, if it occurs, probably does not happen via phase II transferases such as the GST family. Furthermore, this means that exposure of homogenates to the nanoparticle did not induce any other reaction that could deplete GSH stocks, in contrast to an in vivo study with goldfish [11]. In spite of this, we also found augmented GSSG levels in brain after 1 h and decreased levels after 2 h (Fig. 4a). Furthermore, total antioxidant capacity was diminished in the brain after 4 h (Fig. 5a), indicating that some other component of the antioxidant defense system (but not GSH) was affected by fullerene.

With the biphasic response in brain GSSG content, the high values observed after 1 h of exposure can be ascribed to oxidative stress generation by fullerene. The lowering of GSSG after 2 h of exposure can be considered a result of its reaction with sulfhydryl groups of thiol-containing amino acid residues in proteins. This reaction can lead to protein glutathionylation [28], eventually leading to damaging effects such as enzyme inactivation, susceptibility to proteolytic degradation, and cross-linkage of protein surface thiols [29]. If some of these glutathionylated proteins are taking part in the antioxidant system or thiols redox state, this should decrease total antioxidant capacity, as observed after 4 h of exposure in brain.

Another biphasic response was registered for TBARS content, which rose at 2 h, decaying to basal levels at 4 h of exposure both in brain and in gills (Fig. 3). It is known that malondialdehyde can also induce protein carbonylation in acidic pH [30], so it is possible that polypeptides and proteins acted as scavengers, decreasing TBARS values. In addition, low levels of TBARS at 4 h could be maintained by the spent antioxidants, also explaining the lowering of total antioxidant capacity observed in carp brain after 4 h.

Glutathione reductase activity was affected by fullerene in the brain at 4 h and in gills at 1 h (Fig. 5). Indeed, inhibition of this enzyme has been reported in many studies employing different toxicants and animal models, including freshwater fish [31–33]. Although its activity does not control GSH stocks directly because of the relative low concentration of GSSG compared with GSH, many toxicants can affect GR activity in fish, showing that its disturbance is indicative of oxidative stress [31]. In the present study, inhibition of GR in brain after 4 h was matched with a decrease in antioxidant competence in the same period. The expected increase in GSSG concentration at 4 h, however, was not observed (Fig. 5). This suggests that the excess of oxidized glutathione could be reacting with some substrate (e.g., protein sulfhydryl groups), possibly leading to glutathionylation, as stated above. This hypothesis is being investigated.

Table 1. Minimum fullerene (C_{60}) concentrations that induce significant effects on several organisms

Species	Observed effect	Fullerene concentration	Experimental conditions	Reference
Fish (*Carasius auratus*)	Lowering of GSH in brain, liver, and gills	1 mg/L (ASS)	In vivo, 32 d of exposure	[11]
Crustacean (*Daphnia pulex)*	Lowering of GST activity	0.5mg/L (ASS)	In vivo, 24 h exposure	[36]
Crustacean (*Daphnia magna*)	No offspring production (fifth brood)	2.5 mg/L (ASS)	In vivo, 21 days of exposure	[37]
Crustacean (*Daphnia magna*)	Augmented heart rate	0.26 mg/L (THF)	In vivo, 1 h exposure	[38]
Crustacean (*Daphnia magna*)	Mortality and fullerene accumulation	0.2 and 0.4 mg/L (THF)	In vivo, 48 h exposure	[39]
Bacteria (*Bacilus subtilis)*	Growth impairment	0.5 mg/L (ASS)	In vivo, exposure time not specified	[7]
Fish (*Cyprinus carpio*)	Augmented GSSG in brain	1 mg/L (ASS)	In vitro, 1 h exposure	Present study
Fish (*Cyprinus carpio*)	Augmented TBARS in brain and gills	1 mg/L (ASS)	In vitro, 2 h exposure	Present study
Fish (*Cyprinus carpio*)	Lowering of GR activity in gills	1 mg/L (ASS)	In vitro, 1 h exposure	Present study

GSH = reduced glutathione; GSSG = oxidized glutathione; GST = glutathione S-transferase; TBARS = thiobarbituric acid reactive substances; ASS = aqueous stirred suspension; THF = fullerene suspension prepared with the organic solvent tetrahydrofuran.

An important concern about the degree of toxicity of fullerene is its interaction with ions and proteins present in the medium (e.g., aquatic environments or physiological medium). Whereas ion salts increase particle size, causing agglomeration, it has been reported that nanoparticles can adsorb onto proteins, avoiding this effect [4,34]. These bindings can shift zeta potential, conferring stability on nano-sized particles. Once the particles are more stable, the effects may be enhanced, which has relevant ecotoxicological and physiological consequences. Some studies have reported that human and bovine serum albumin stabilized the particle size of fullerene by forming a protective layer, preventing coagulation in the presence of phosphate-buffered saline [35]. Thus, from the present work, it can be inferred that the in vitro exposure of brain and gill protein extracts should favor the maintenance of fullerene as nanoaggregates, instead of the time-dependent increase in agglomeration provoked by the ionic strength of sample homogenates.

As shown in Table 1, a comparison of the responses elicited by fullerene in several organisms clearly indicates that in vitro exposure (1–2 h) shows biochemical responses at fullerene concentrations very similar to those of used in in vivo experiments, in which longer exposure times (24 h or even 21–32 d) are required. The fact that the preparation of aqueous fullerene suspensions is now accepted as the better option for analyzing toxic responses, rather than using organic solvents such as tetrahydrofuran that augment fullerene toxicity [15], imposes a restriction in terms of time (at least four weeks of continuous stirring) and the low concentration of the suspensions obtained. These limitations can be attenuated, in part, by using an in vitro test in which low volumes of fullerene suspensions are required.

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