Brazilian Journal of Analytical Chemistry
2023, Volume 10, Issue 40, pp 182-197
doi: 10.30744/brjac.2179-3425.TN-94-2022

BrJAC

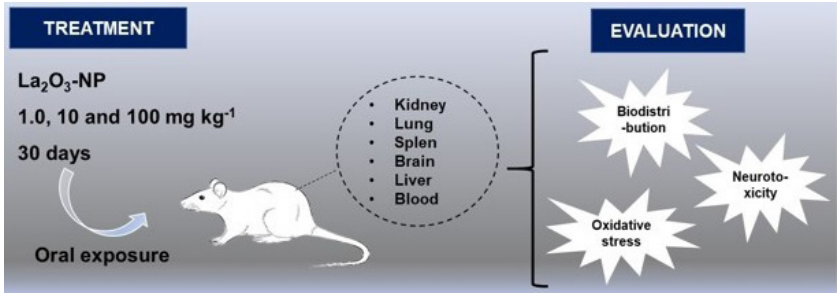
TECHNICAL NOTE

Lanthanum Oxide Nanoparticles Distribution in Wistar Rats after Oral Exposure and Respective Effects

Graciela M. Heidrich[1], Vinicius M. Neves[1], Naiara Stefanello[1], Vanessa V. Miron[1], Thauan F. Lopes[1], Sindy R. Krzyzaniak[1], Paola de A. Mello[1], Maria Rosa C. Schetinger[1], Dirce Pozebon[2], Valderi L. Dressler[1]

[1]*Universidade Federal de Santa Maria, Avenida Roraima, 1000, Campus de Camobi, Santa Maria, RS, 97105-900, Brazil*

[2]*Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves, 9500, Instituto de Química, 97501-970, Porto Alegre, RS, Brazil*



Adult Wistar rats were exposed to lanthanum oxide nanoparticles (La_2O_3-NPs). Animals were initially treated with single doses of La_2O_3-NPs suspensions at 5.0, 50, 300 and 2000 mg kg^{-1} per body weight (*bw*), which were orally administered. Behavior changes, symptoms of intoxication and mortality were not observed for individuals treated with the La_2O_3-NPs. However, the histological analysis of different organs of the treated rats revealed that 300 mg kg^{-1} and 2000 mg kg^{-1} *bw* La_2O_3-NPs caused hepatic lesions. Subsequently, 40 individuals were divided in four groups with 10 individuals in each group and daily treated with water only (control) and with 1.0, 10 and 100 mg kg^{-1} *bw* La_2O_3-NPs. After 30 days, it was observed that the La_2O_3-NPs did not affect the body weight and organs weight of the animals. The La_2O_3-NPs also did not change the levels of creatinine, urea, glutamyl transferase (γ-GT), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and thiobarbituric acid reactive substances (TBARS) in blood serum. Neurotoxicity, evaluated by the acetylcholinesterase (AChE) activity, was not observed as well. An increase of reactive oxygen species (ROS) was found in kidney of rats treated with 100 mg kg^{-1} *bw* La_2O_3-NPs. Conversely, protein oxidation decreased in the liver of those animals. The catalase (CAT) activity was not affected by La_2O_3-NPs and that of superoxide dismutase (SOD) was in the liver of animals treated with 10 mg kg^{-1} *bw* La_2O_3-NPs. Lanthanum was determined in organs and blood of the treated animals. The element was not detected in the blood but was in the organs, in higher concentration in liver, kidneys, and heart. Lanthanum present in the form of NPs or as free ion could not be detected. As such, it is worth investigating possible transformation of La_2O_3-NPs in the organism, their elimination routes, and effects of longer exposure times.

Cite: Heidrich, G. M.; Neves, V. M.; Stefanello, N.; Miron, V. V.; Lopes, T. F.; Krzyzaniak, S. R.; Mello, P. A.; Schetinger, M. R. C.; Pozebon, D.; Dressler, V. L. Lanthanum Oxide Nanoparticles Distribution in Wistar Rats after Oral Exposure and Respective Effects. *Braz. J. Anal. Chem.* 2023, *10* (40), pp 182-197. http://dx.doi.org/10.30744/brjac.2179-3425.TN-94-2022

Submitted 28 September 2022, Resubmitted 14 December 2022, Accepted 20 December 2022, Available online 03 February 2023.

Keywords: La_2O_3 nanoparticles, bioanalytic, Wistar rats, Lanthanum accumulation, toxicity, ICP-MS

INTRODUCTION

Nanoparticles (NPs) has been increasingly used with the advance of science and technology. However, due to their nanometric dimension, NPs may lead to different biological effects when compared with macromolecular, and monoelemental species.[1] Therefore, studies concerning NPs toxicity are important. It is worth citing the NPs toxicity can be influenced by the pH and ionic strength of the physiological medium.[2-4]

The NPs effects in living organisms involves interactions with cellular components, membrane, organelles, and macromolecules present in the cell. Different NPs, treatment doses and exposure periods can induce different responses.[5] Silver nanoparticles (Ag-NPs), one of the most used and studied NPs, with spherical form and size ranging from 30 to 90 nm, has shown cytotoxic effect against breast cancer cell lines.[6,7] In addition, 20 nm Ag-NPs administered orally to rats for 30 days had negatively affected their memory and cognitive coordination.[8] Rare earth elements (REEs) have been increasingly used whereas beneficial or harmful effects of them to animals and humans have been observed. However, most studies have basically dealt with lanthanum (La) and cerium (Ce) in ionic form.[9-11] Lanthanum oxide nanoparticles (La_2O_3-NPs) have been used in semiconductors, fuel cells, optic devices, magnetic data storage, ceramics, catalyst, automobiles, biosensors, water treatment and biomedicine. Magnetic properties of La_2O_3-NPs were evaluated for field-controlled markers for drug release and as a contrast agent in nuclear magnetic resonance imaging.[12] La_2O_3-NPs have also been applied as antimicrobial and antiviral agent.[13,14] Therefore, the exposure of living organisms to La_2O_3-NPs have increased with the increasing use of La_2O_3-NPs. In a study conducted by Dressler et al. to verify La_2O_3-NPs deposition in bone, La_2O_3-NPs were administered orally to Wistar rats, and it was demonstrated that the NPs were present only on the surface of the bone.[15]

Distribution of NPs in organism regions and cells (biodistribution) can be used to detect the NPs fate.[16] For example, NPs can act as carriers of drugs and target specific cells, improving medical diagnosis and treatment.[17] The NPs biodistribution can be influenced by the route of administration (intravenous, oral, pulmonary, and dermal), physical (mainly size and shape) and chemical (reactivity) properties, besides the physiological environment in contact with the NPs. The absorption and distribution of NPs are also affected by the NPs coating material and surface charge.[1] One of the main routes of exposure of humans to NPs is through the consumption of products containing NPs in their composition. When ingested, NPs can be absorbed through the gastrointestinal tract and, in contact with the bloodstream, they can interact with proteins and other biomolecules.[2] In cases where NPs have protein-like dimensions of less than 40 nm and appropriate surface composition they can form complexes, which may lead to changes in proteins structure and even alter their functions and enzymatic activities.[18] For example, the enzymes alanine aminotransferase (ALT), aspartate transaminase (AST) and gamma-glutamyl transferase (γ-GT) present in liver can be altered when this organ is injured.[18] Thus, alteration of these enzymes in blood provides information about liver function and identifies possible hepatic lesions due to absorption of NPs. Renal damage can be identified by creatinine and urea alteration in blood serum.[19] Antioxidants are produced by the organism's metabolism system to inhibit the damage caused by the action of free radicals or non-radical reactive species. The relationship among oxidants and antioxidants is equilibrated in normal metabolism but the equilibrium can be affected by the increase of external agents (oxidants) that cause oxidative stress.[20, 21]

Despite the wide application of La_2O_3-NPs, little is known about their effects on animals. Therefore, the effect and distribution of La_2O_3-NPs in Wistar rats were studied in the present work. To this end, AST, ALT and γ-GT activities, uric acid, creatinine, antioxidant enzymes (catalase (CAT) and superoxide dismutase (SOD)), oxidative stress (through thiobarbituric acid reactive substances (TBARS)) protein oxidation, reactive oxygen species (ROS) and neurotoxicity were evaluated. Absorption of La_2O_3-NPs was evaluated through La determination in kidney, spleen, brain, pancreas, lung, and blood.

MATERIALS AND METHODS

Reagents and standards

Nanoparticles of La_2O_3 with a diameter of 15-30 nm, 99.9% purity and spherical morphology were purchased from Nanoamor (Houston, USA). These particles had been previously characterized, as reported by Nunes et al.[22] Nitric acid (65%, 1.4 kg L^{-1}, Sigma Aldrich, USA) was purified by sub-boiling distillation (in a duoPUR 2.01E system, Millestone, Sorisole, Italy) and then used in samples and solutions preparation. Water used throughout the work was distilled and purified (final resistivity of 18.2 MΩ cm) using a Milli-Q system (Millipore Corp., Bedford, USA). Other reagents and chemicals used were of analytical grade. Suspensions of La_2O_3-NPs were prepared in water and homogenized for 10 min in an ultrasound bath (Transsonic TI-H-5, Elma, Germany; 100 W, 25/45 kHz). Additional information about preparation of the La_2O_3-NPs suspensions can be found elsewhere.[22] Calibration solutions (ranging from 0.0 to 10.0 μg L^{-1} La) were prepared by dilution of a 10 mg L^{-1} multi-element solution (SCP33MS, SCP Science, Canada). The calibration solutions were prepared in 5% (v v^{-1}) nitric acid.

Animals

Sixty-day-old nulliparous and non-pregnant female Wistar rats, weighing 237-268 g each, were used in acute toxicity tests. Sixty-day-old male Wistar rats, weighing 251–322 g each, were used in subacute toxicity tests. The individuals were from the Central Animal House of the Federal University of Santa Maria (UFSM), Brazil. They were acclimated for seven days before starting the experiments. During the acclimation and experiments periods, the rats were housed in polycarbonate cages (five rats per cage) in a room with controlled temperature (23 °C ± 1) under 12 h light/dark cycle. Food and water were served *ad libitum*. The study was approved by the animal ethics committee of UFSM (protocol 4250170317).

Acute toxicity tests

The toxicity tests were carried out following the Guideline 425 from the Organization for Economic Co-operation and Development (OECD).[23] Doses of La_2O_3-NPs suspension were orally administered to the animals; the initial dose was 5.0 mg kg^{-1} *bw*. If the individual that had received this dose was still alive after 24 h, a second individual was treated with 50 mg kg^{-1} *bw* La_2O_3-NPs, followed by third and fourth individuals receiving 300 and 2000 mg kg^{-1} La_2O_3-NPs *bw*, respectively. One individual was treated with water only and used as control. The treated animals were observed for fourteen days, and symptoms associated with intoxication, like tremor, convulsion, diarrhoea and salivation were monitored. In the absence of mortality and intoxication signals, the animals were weighed, anesthetized with halothane (1-bromo-1-chloro-2,2,2-trifluoroethane) and euthanized. Then, their heart, spleen and liver were collected for histopathological analyses.

Subacute toxicity tests

In this case, 40 rats were divided in four groups with 10 individuals in each group, as follows: (*i*) control (individuals received water only), treated with (*ii*) 1.0, (*iii*) 10, and (*iv*) 100 mg kg^{-1} *bw* La_2O_3-NPs, respectively. The La_2O_3-NPs were administered orally (only with water in the case of the control animals), and each animal was daily treated at 5:00 pm. Following 30 days of treatment, the animals were weighed, anesthetized with halothane, and then euthanized. Their blood, brain, spleen, liver, lungs, heart, pancreas, and kidneys were removed and separated. The blood was collected through cardiac puncture and transferred to tubes containing ethylenediaminetetracetic acid (EDTA). The collected organs were separated, weighed, transferred to polypropylene tubes, which were maintained at -20 °C until analysis. The relative percentage of organ weight in relation to the body weight (*bw*) was calculated as Equation 1:

$$Relative\ organ\ weight\ (\%) = (organ\ weight\ \times 100)/b\,w \qquad \text{Equation 1}$$

Blood and organs analyses

The blood samples were centrifuged at 3500 *g* for 15 min, the precipitated discarded and the serum frozen at -20 °C for further analysis. AST, ALT and γ-GT activities were evaluated in duplicate using a semi-automatic analyzer (TP Analyzer Plus, Thermoplate, China) and commercial kits (Labtest Diagnostica, Brazil). Lipid peroxidation, ROS, protein oxidation, CAT and SOD activities were determined in liver and kidney tissues. For that purpose, separated parts of the organs were kept in 60 mmol L^{-1} Tris·HCl buffer (pH 7.4) in proportions of 1:10 (1 g of tissue in 10 mL of buffer), except for CAT and SOD. In this case, the proportion was 1:60 (1 g of tissue in 60 mL of buffer). Subsequently, the samples were homogenized and centrifuged at 2000 *g* for 10 min. Then, the supernatant was removed for analysis. Samples were always kept refrigerated during the analysis. The acetylcholinesterase (AChE) activity was measured in the cerebelum, estriatum, cerebral cortex and hippocampus. Absorbance was measured using an UV-1800 spectrometer (Shimadzu, Japan) or a SpectraMax i3 Molecular Device (LLC, USA).

Lipid peroxidation

The lipid peroxidation determination (with thiobarbituric acid – TBARS) in liver and kidney homogenates followed the method described by Olas et al.[24] In short, the sample placed in a 15 mL-tube was mixed with 15% (m/v) trichloroacetic acid in 0.25 mol L^{-1} HCl and 0.37% (m/v) thiobarbituric acid in 0.25 mol L^{-1} HCl. Subsequently, the tube containing the mixture was immersed in boiling water for 10 min. After cooling, the absorbance of the obtained solution was measured at 535 nm and the calculated results expressed as nmol malondialdehyde (MDA) mg^{-1} protein.

ROS determination

The ROS were determined by fluorimetry (QUIMIS Q798FIL, Brasil) according to Ali et al., whose method was adapted.[25] In this method, 2´,7´-dichlorofluorescein diacetate (DCFH-DA) is oxidized to fluorescent dichlorofluorescein (DCF) by intracellular ROS. The emission intensity was measured at 525 nm, after 30 min of adding the DCFH-DA. The calculated results were expressed as U DCF (unity of dichlorofluorescein) mg^{-1} protein.

Protein oxidation

The determination was based on the Levine method; the proteins oxidation occurs through reaction with 2,4-dinitrophenylhydrazine (DNPH) and the absorbance of the product is measured at 370 nm.[26] The results are expressed as nmol oxidized protein mg^{-1} protein.

Activities of CAT and SOD

The SOD and CAT activities in liver and kidney were measured according to methodologies recommended by Misra and Fridovich and Nelson and Kiesow, respectively.[27,28] The oxidation of epinephrine occurs at pH 10.5 to produce adrenochrome (of red color) whose absorbance is measured at 480 nm. The oxidation of epinephrine is inhibited by the addition of a sample containing SOD, decreasing the absorbance. The CAT activity was evaluated from the absorbance variation at 240 nm due to hydrogen peroxide decomposition in the sample at pH 8.5 and temperature of 37 °C, after 10 min. The results were expressed in μmol of SOD or CAT mg^{-1} protein.

Acetylcholinesterase activity

The acetylcholinesterase (AChE) activity in brain tissue was measured following an adapted method that had been developed by Ellman et al., as described by Rocha et al.[29,30] Thiocholine was mixed with 100 mmol L^{-1} potassium phosphate buffer (pH 7.5) and 1.0 mmol L^{-1} 5,5´-dithiobisnitrobenzoic acid (DTNB). The DTNB was reduced to thionitrobenzoic acid, producing a yellow-colored anion whose absorbance was measured at 412 nm. The calculated results were expressed as μmol AChE min^{-1} mg^{-1} protein.

Histological analysis

The spleen, heart, and liver of rats submitted to acute toxicity were removed and kept in 10% v v^{-1} buffered formalin. After dehydration, samples of these organs were embedded in paraffin and sectioned in 6 µm-thickness slices using a manual microtome. The slices were mounted on glass microscope slides, stained with hematoxylin-eosin and Masson/Goldner trichrome and visualised in a microscope (OLYMPUS CX21 microscope with an OLYMPUS camera).

Lanthanum determination

For La determination, liver, heart, kidney, pancreas, brain, spleen, and lung samples were decomposed in a microwave oven system (Speedwave 4, Berghof, Germany). The samples decomposition was carried out in trifluoromethyl-modified polytetrafluoroethylene (TFM-PTFE) flasks, where 4.0 mL of HNO_3 were added to 250 mg of sample and allowed to stand for 30 min at ambient temperature. Subsequently, the mixture was heated at 900 W for 40 min. After cooling, the digestate was transferred to a polypropylene flask (Sarstedt, Germany), where the sample solution volume was completed to 20 mL by adding water.

Lanthanum was determined by means of inductively coupled plasma-mass spectrometry (ICP-MS), being employed an Elan DRCII (PerkinElmer Sciex, Canada) instrument. This instrument was equipped with a concentric nebulizer (Meinhard Associates, USA), a baffled cyclonic spray chamber (from Glass Expansion) and a quartz torch fitted to a 2.0 mm internal diameter injector tube. Argon (99.996%, from Glass White Martins/Praxair, Brazil) was used as plasma, nebulizer, and auxiliary gas. The ICP-MS instrument was operated according to the manufacturer's recommendations. The conditions set are summarized in Table I.

Table I. Operating conditions set for lanthanum determination using ICP-MS

Parameter	Setting
Plasma power, W	1300
Plasma gas flow rate, L min^{-1}	15
Auxiliary gas flow rate, L min^{-1}	1.2
Nebulizer gas flow rate, L min^{-1}	1.16
Dwell time, ms	40
Readings	5
Readings per replicate	3
Replicates	3
m/z monitored	139

Accuracy, limits of detection and quantification

Accuracy was checked by analyzing the certified reference material (CRM) NIST 1566a (oyster tissue, from National Institute of Standards and Technology, USA). A *t*-test at 95% confidence level was applied to compare the La concentration found with that certified value. To check if the differences were significant in the other determinations, when appropriate, *t*-test or one-way ANOVA (analysis of variance) both at 95% confidence level were applied. The limits of detection (LOD) and quantification (LOQ) for La were calculated through $B + 3s$ (LOD) and $B + 10s$ (LOQ), respectively, where B is the blank concentration value and s is the standard deviation of 10 consecutive measurements of the blank. The LOD and LOQ were calculated following recommendations from the International Union of Pure and Applied Chemistry (IUPAC).

RESULTS AND DISCUSSION

Acute toxicity effects

Behavior alterations of the animals monitored for fourteen days after oral administration of 5, 50, 300, and 2000 mg kg^{-1} *bw* La_2O_3-NPs were not observed. They were weighed before and fourteen days after the La_2O_3-NPs administration and it was observed their weight had increased. Since the animals were in the growth phase, the weight increase was considered normal and not caused by the La_2O_3-NPs given to them. The ratio of the heart, spleen and liver weight to the body weight was not different for the different doses of La_2O_3-NPs – these organs and the animals were weighed before and 14 days after the La_2O_3-NPs administration. Figures 1, 2 and 3 show the histology of the liver, spleen and heart, respectively.

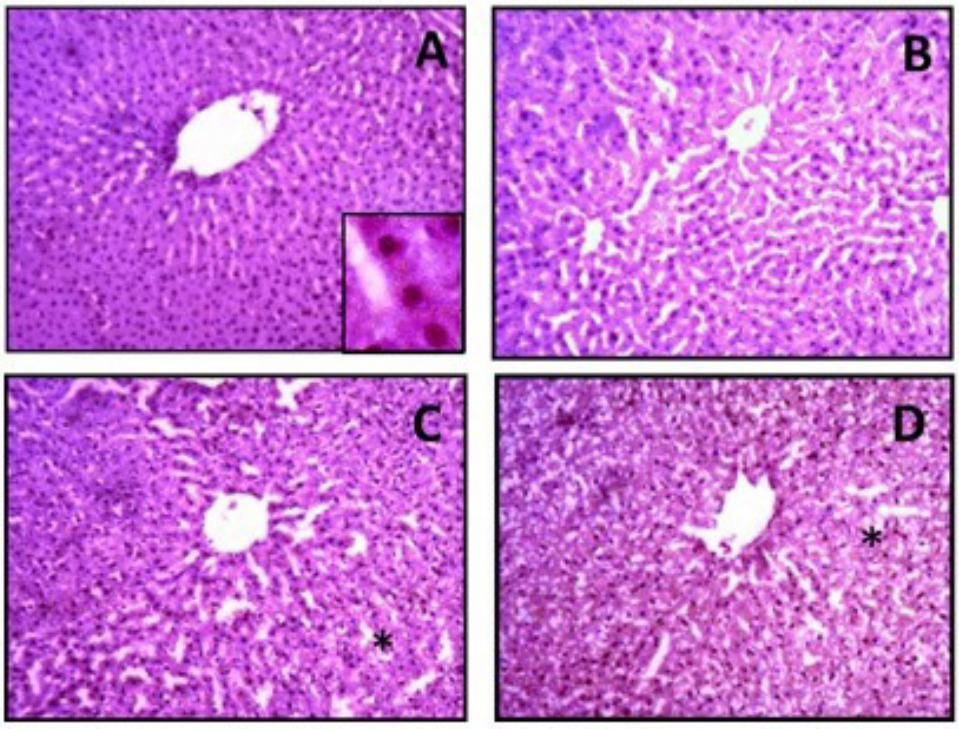


Figure 1. Histology of the rat's livers: control (A), treated with 5 (B), 300 (C), and 2000 (D) mg kg^{-1} *bw* La_2O_3-NPs. Magnification: 10x; 40x in the inset of Figure (A). * Denotes changes observed.

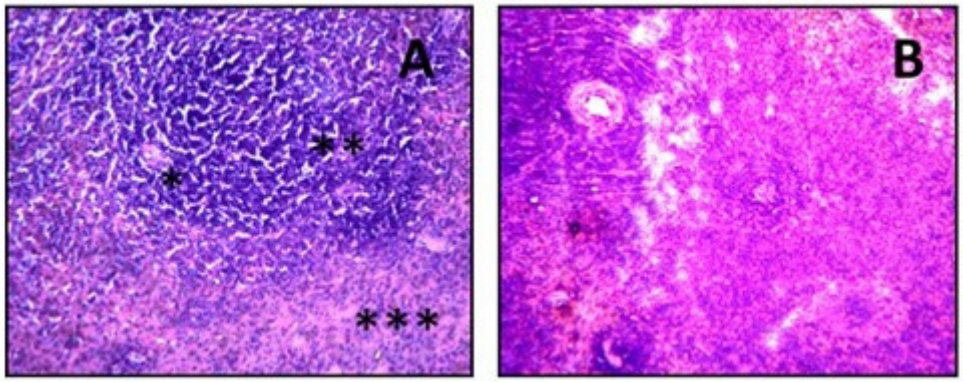


Figure 2. Spleen histology: control rat (A) and a rat treated with 2000 mg kg^{-1} *bw* La_2O_3-NPs (B). Magnification: 10x; * central arteriole; ** blank pulp; and *** red pulp.

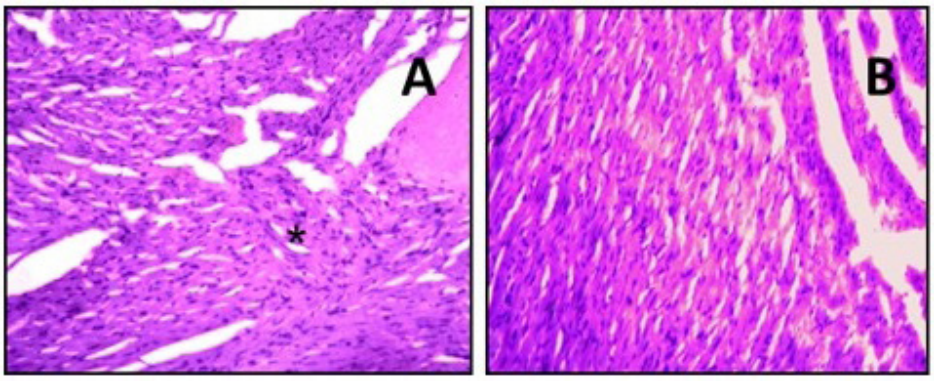


Figure 3. Heart histology: control rat (A) and a rat treated with 2000 mg kg^{-1} *bw* La_2O_3-NPs (B). Magnification: 10x (B); * muscle cells with central nuclei.

Normal histology with hepatocytes arranged radially from the central vein towards the periphery of the liver lobes was observed for the control animal (Figure 1A). Remarkable changes were not observed for the liver of an animal treated with 5 mg kg^{-1} *bw* La_2O_3-NPs (Figure 1B). On the other hand, treatment with 300 and 2000 mg kg^{-1} *bw* La_2O_3-NPs resulted in hydropic degeneration of the liver (Figures 1C and 1D), characterised by the presence of larger hepatocytes with a clear cytoplasm, small pale vacuoles and a normal nucleus. Any change was not observed in the spleen (Figures 2A and 2B) and heart (Figures 3A and 3B), even for the animals that had been treated with 2000 mg kg^{-1} *bw* La_2O_3-NPs (Figures 2B and 3B).

Subacute toxicity effects

The animals were daily observed and weekly weighed; physical modifications like hair loss, mucosal secretion, and weight loss were not detected during the treatment period (30 days) and all individuals survived. Figure 4 shows that the animals body weight increased, and according to ANOVA, there was not significant difference among the groups. As previously mentioned, the animals were in the growth phase and the weight increase was considered normal.

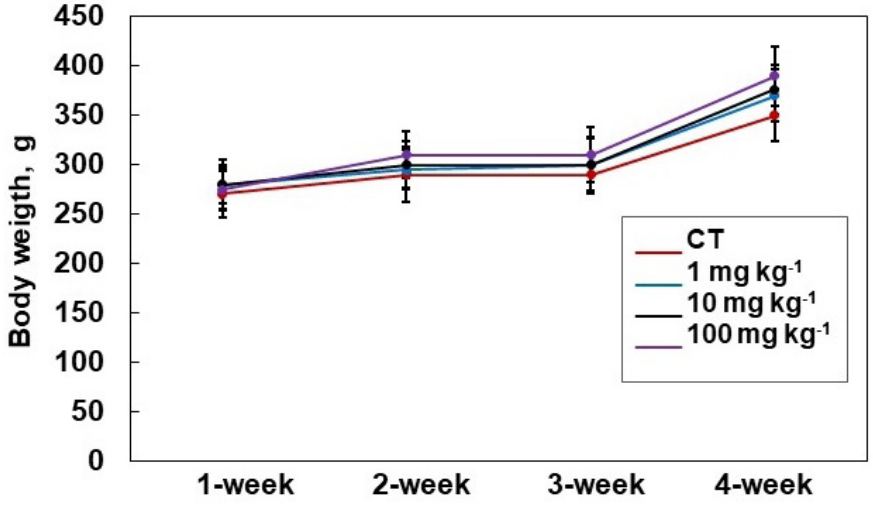


Figure 4. Body weight of the rats daily treated with La_2O_3-NPs for 30 days. The animals were weighed weekly. CT: control.

The animals were weighed and euthanized at the end of treatment period. Then, their organs were collected and weighed. The relative organ weigh (organ weight ×100)/*bw*) is given in Table II. According

to ANOVA, the relative organ weigh did not differ significantly among control rats and those treated with La_2O_3-NPs. However, a longer time exposure to La_2O_3-NPs would cause possible changes in the animals weight and must be studied em more details.

Table II. Relative organ weight for control rats (CT) and those treated with La_2O_3-NPs. Values are the mean ± standard deviation for 10 specimens or individuals.

	Relative organ weight, %			
Organ	**0.0 (CT)** (n = 10)	**1.0 mg kg^{-1} *bw*** (n = 10)	**10 mg kg^{-1} *bw*** (n = 10)	**100 mg kg^{-1} *bw*** (n = 10)
Brain	0.533 ± 0.038	0.539 ± 0.044	0.502 ± 0.182	0.536 ± 0.043
Liver	3.47 ± 0.11	3.66 ± 0.45	3.63 ± 1.29	3.80 ± 0.55
Left kidney	0.353 ± 0.150	0.357 ± 0.036	0.372 ± 0.036	0.368 ± 0.032
Right kidney	0.340 ± 0.144	0.351 ± 0.027	0.366 ± 0.038	0.362 ± 0.037
Spleen	0.177 ± 0.012	0.189 ± 0.016	0.182 ± 0.022	0.178 ± 0.015
Right lung	0.121 ± 0.018	0.124 ± 0.009	0.138 ± 0.024	0.135 ± 0.028
Heart	0.332 ± 0.031	0.340 ± 0.022	0.361 ± 0.054	0.337 ± 0.035
Pancreas	0.285 ± 0.027	0.245 ± 0.052	0.288 ± 0.031	0.255 ± 0.051
Body weight	359 ± 24	375 ± 19	386 ± 24	372 ± 18

Creatinine, urea, γ-GT, ALT and AST in blood serum

The creatinine, urea, γ-GT, ALT and AST levels found in blood serum of control rats and those treated with La_2O_3-NPs are given in Table III. The ANOVA revealed that levels were not statistically different.

Table III. Analysis of blood serum of control rats (CT) and those treated with La_2O_3-NPs. Values are the mean ± standard deviation for six individuals.

	Treated with La_2O_3-NPs, mg kg^{-1} *bw*			
Parameter	**0.0 (CT)** (n = 6)	**1.0** (n = 6)	**10** (n = 6)	**100** (n = 6)
Creatinine (mg dL^{-1})	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
Ureia (mg dL^{-1})	47 ± 7	52 ± 8	51 ± 8	48 ± 3
γ-GT (U L^{-1})	< 1	< 1	< 1	< 1
ALT (U L^{-1})	132 ± 35	157 ± 13	140 ± 41	161 ± 32
AST (U L^{-1})	48 ± 13	51 ± 6	49 ± 7	50 ± 8

Oxidative stress in kidney

Figure 5 illustrates the oxidative stress in kidney. According to Figure 5A, the ROS levels decreased in rats treated with 1.0 or 10 mg kg^{-1} *bw* La_2O_3-NPs and increased in those treated with 100 mg kg^{-1} *bw* La_2O_3-NPs. The ROS level for each group of animals treated with La_2O_3-NPs was significantly different when compared with the control group animals. Decreasing TBARS levels (Figure 5B) were observed for

all treatments but with significant differences for the groups of animals treated with 10 and 100 mg kg^{-1} *bw* La_2O_3-NPs when compared with the control group. On the other hand, significant difference was not observed in protein oxidation (Figure 5C), CAT (Figure 6A) and SOD (Figure 6B) levels.

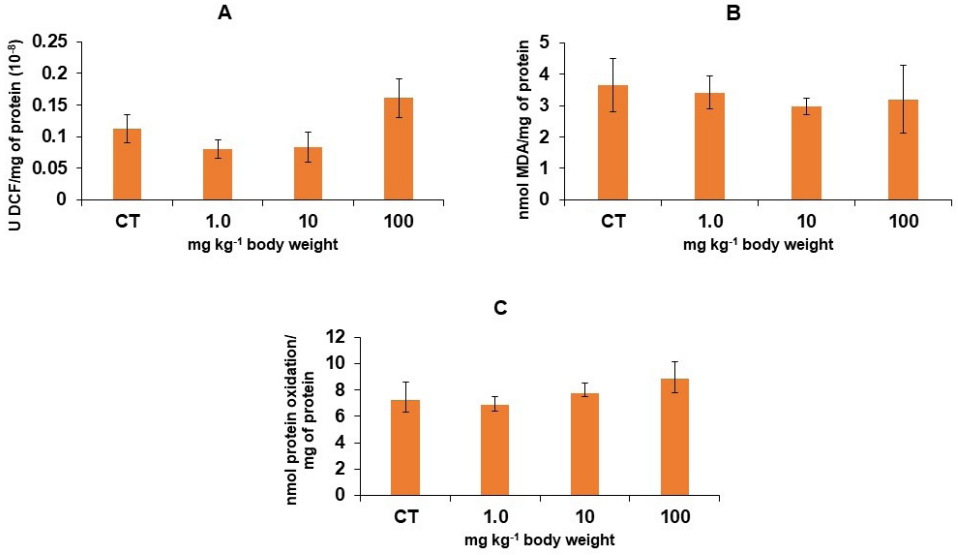


Figure 5. (A) Reactive oxygen species (ROS), (B) lipid peroxidation (as TBARS) and (C) protein oxidation levels in kidney of control rats and those treated with La_2O_3-NPs for 30 days. Each group comprised six individuals (*n* = 6).

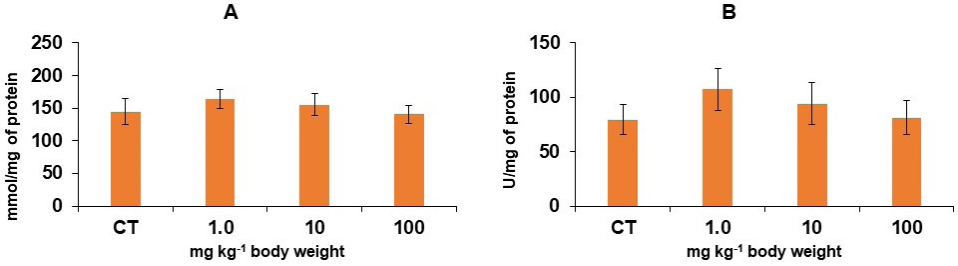


Figure 6. (A) Catalase and (B) superoxide dismutase activities in kidney of control rats and those treated with La_2O_3-NPs for 30 days. Each group comprised six individuals (*n* = 6).

Oxidative stress in liver

The oxidative stress in liver due to La_2O_3-NPs administration is depicted in Figure 7. The ROS levels in the liver (Figure 7A) of the control group animals and in those of the group treated with 10 mg kg^{-1} *bw* La_2O_3-NPs were significantly different. The difference was also significant for protein oxidation (Figure 7C) when the control group was compared with the group of rats treated with 100 mg kg^{-1} *bw* La_2O_3-NPs. However, for all groups the lipid peroxidation levels (Figure 7B) was not significantly different.

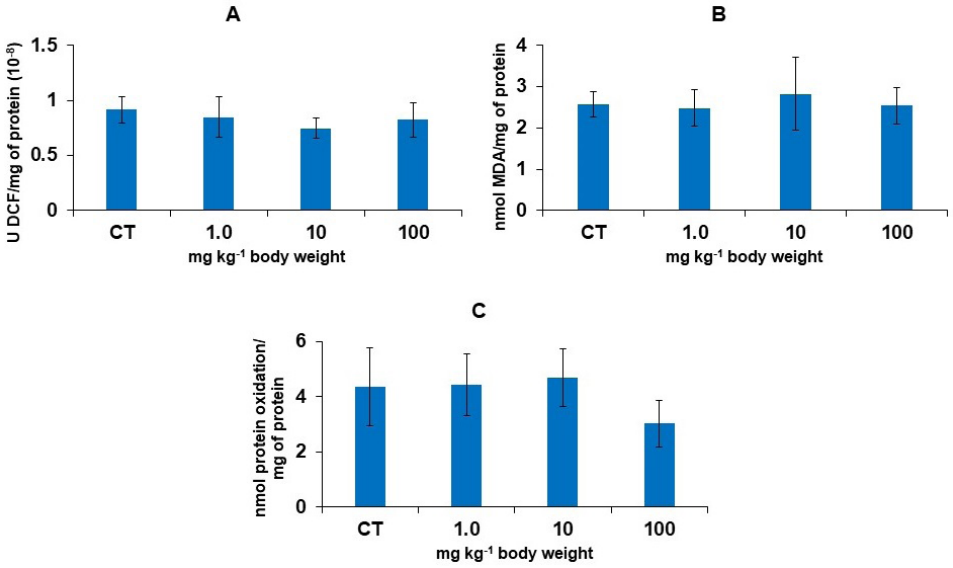


Figure 7. (A) reactive oxygen species, (B) lipid peroxidation, and (C) protein oxidation levels in liver homogenates of control rats and those treated with La_2O_3-NPs for 30 days. Each group comprised six individuals (n = 6).

The CAT and SOD activities were also not significantly different among the control and treated animal groups (Figure 8), excepting SOD for the group of animals treated with 10 mg kg^{-1} *bw* La_2O_3-NPs (Figure 8B).

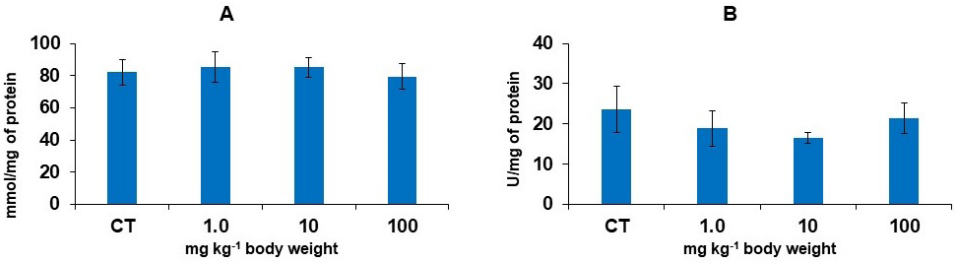


Figure 8. (A) catalase and (B) superoxide dismutase activities in liver homogenates of control rats and those treated with La_2O_3-NPs for 30 days. Each group comprised 6 individuals (n = 6) for CAT and 5 individuals for SOD (n = 5).

Acetylcholinesterase activity

Variation of the AChE activity was observed (Figure 9), but it was not significantly different for control and treated animals.

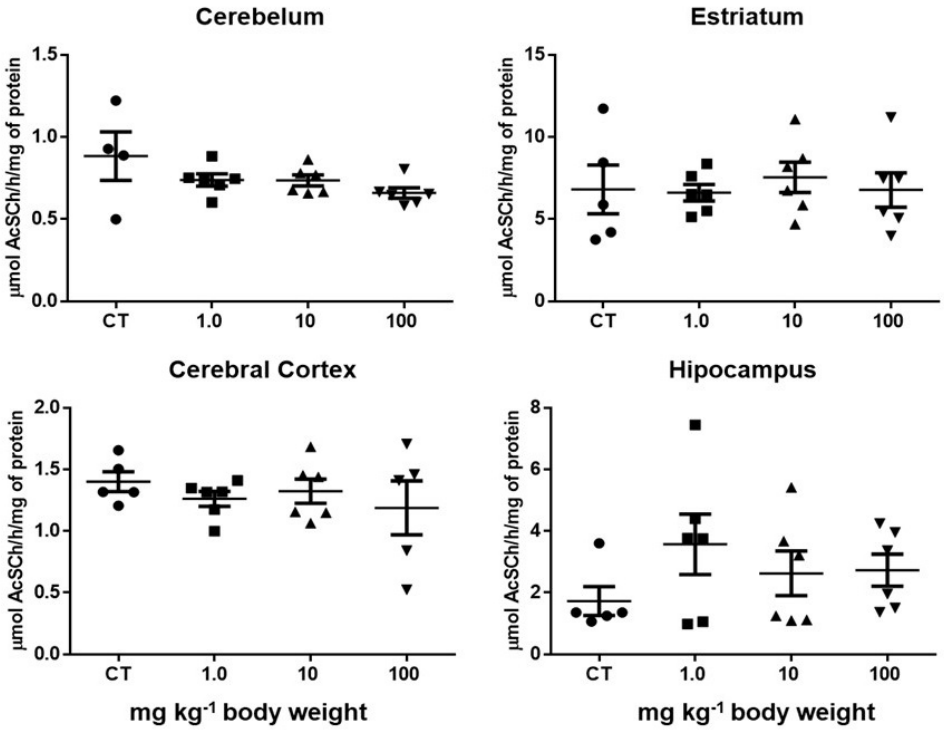


Figure 9. Acetylcholinesterase activity in brain of control rats and those treated with La_2O_3-NPs for 30 days. Each group comprised six individuals (n = 6).

Lanthanum determination

The La concentration in blood of control and treated animals were lower than LOQ, which was 10 ng g^{-1}. The LOD and LOQ for La in the organs were 1.0 ng g^{-1} and 3.0 ng g^{-1}, respectively. Lanthanum was detected in all analyzed organs, where higher concentration was found in liver, kidney, and heart (Figure 10). Accumulation of La in pancreas, brain, spleen, and lung in control animals and those treated with La_2O_3-NPs was not significantly different. Results for the more concentrated and diluted samples solutions were similar, which indicated there was not matrix interference. A CRM (NIST 1566a, Oyster Tissue) was analyzed in parallel. The La concentration found (0.28 ± 0.02 µg g^{-1}) agreed with that informed on the CRM certificate (0.3 µg g^{-1}), considering a t-test at 95% confidence level. In addition, possible matrix effect was checked by diluting the sample solution.

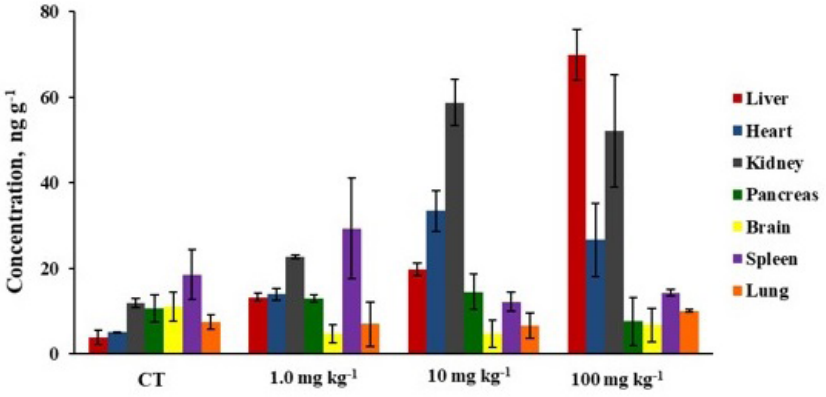


Figure 10. Lanthanum concentration found in organs of control (CT) rats and those treated with La_2O_3-NPs. Each bar represents the mean and standard deviation for three organs.

Discussion

Conditions as close as possible to those in real exposure should be considered in evaluating effects and toxicity of NPs, following *in vitro* and *in vivo* assay models. In the *in vitro* assay, cells are exposed to NPs of interest and parameters like inhibition of cell growth and respiration, free radicals production, lesions in DNA and other biochemical and cellular manifestations. *In vivo* assays use animal models, where growth, mortality, organs injury, enzymes activity, oxidation of proteins, production of ROS and other parameters are monitored.[31] Such parameters may indicate changes in normal body functioning due to the presence of NPs. Therefore, studies about the NPs toxicity are mandatory for a safer use of them. Possible effects of La_2O_3-NPs were investigated in the present study, considering physical and biochemical parameters and La bioaccumulation in different organs. Toxicity signals, body weight and ratio organ weight/body weight alterations were not induced by 5.0, 50, 300, and 2000 mg kg^{-1} *bw* La_2O_3-NPs. Histological analysis revealed that the spleen and heart of rats treated with La_2O_3-NPs at these concentrations were not affected. However, 300 and 2000 mg kg^{-1} *bw* La_2O_3-NPs injured the liver. As such, the investigation was continued, and the rats were treated with La_2O_3-NPs at concentration lower than 300 mg kg^{-1} *bw*; the welfare and safety of the animals were deemed in this case.

Although the body weight of the animals increased (Figure 3) during the treatment period (30 days), this was expected because they were 60-120 days old and still in the growth phase. The La concentration found in the analyzed rat organs (Figure 4) revealed the La_2O_3-NPs had accumulated in them. For each group, the La accumulation degree in the liver, kidney and heart ranged from 0.0002% to 0.005% of the administered La_2O_3-NPs amount in the period of treatment. These results are in accordance with the distribution degree of poorly soluble NPs reported by Geiser and Kreyling.[32] Similar distribution degree of administered nickel oxide NPs has been reported, with maximum accumulation of Ni in liver and kidney of rats.[33] Nevertheless, the NPs absorption is generally unfavorable. Agglomeration of the NPs may also difficult their absorption. The absorption of orally administered NPs can occur by specialized intestinal cells and accumulation in tissue might occur through the lymphatic system and blood circulation, as discussed by Silva et al. for titanium oxide NPs in humans.[34]

Lanthanum was not detected in blood probably because it was collected 24 h or more time after the last administration of La_2O_3-NPs. The accumulation of La in the liver can be explained by its cellular composition: it contains Kupffer cells, which are responsible for the removal of any “foreign” substance from blood in contact with the organ.[25] Perhaps this explains why greater accumulation of La occurred in the liver, and the La concentration in this organ increased with the La_2O_3-NPs concentration increase (see Figure 11). In previous studies, it has been demonstrated that La_2O_3-NPs can be incorporated in plants and bone.[22,15] Thus, it is possible that La_2O_3-NPs have been transported to the liver through the bloodstream and kept in the form of La_2O_3-NPs in the liver. Accumulation of La in the kidney may be related with the elimination of NPs through the urine, deeming that the renal system acts as a filter of impurities in the organism.[3] Accumulation of NPs in the heart remains unexplained. Park et al. observed accumulation of vanadium oxide-NPs in mice organs as follows: heart > liver > kidney > spleen.[35] These authors stated that additional studies were necessary to assess realistic causes and effects.

Creatinine, urea, γ-GT, ALT and AST increase may indicate toxicity effects of La_2O_3-NPs; creatinine and urea may indicate renal failure while γ-GT, ALT and AST indicate hepatic lesions. The creatinine, urea, γ-GT, ALT and AST levels (see Table III) in the blood of animals treated or not with La_2O_3-NPs were not different. Therefore, although La has accumulated in these organs, it can be affirmed that the La_2O_3-NPs administered did not damage the liver and kidneys during the treatment period (30 days). These findings corroborate those reported for organs of rats treated with copper oxide-NPs.[36] Exposure to metal oxide-NPs can induce excessive ROS generation, affects antioxidant enzymes and normal function of cell membrane due to imbalanced oxidant/antioxidant equilibrium.[37-39] An increase of ROS was found in kidney (Figure 5A) of rats treated with 100 mg kg^{-1} *bw* La_2O_3-NPs. Conversely, protein oxidation decreased in the liver of those animals (Figure 7C). Meanwhile, any significant effect of NPs on lipid peroxidation was not observed (Figure 7B).

The levels of ROS in the organism are controlled by protection mechanisms through the production of antioxidant enzymes like SOD and CAT for instance; SOD catalyzes reaction reduction of superoxide radicals to produce oxygen and hydrogen peroxide, while CAT catalyzes the hydrogen peroxide reduction to produce water and oxygen.[40,41] The CAT activity (Figure 8A) was not affected by La_2O_3-NPs and that of SOD (Figure 8B) was only in the liver of animals treated with 10 mg kg^{-1} *bw* La_2O_3-NPs.

Due to the small size of NPs they can cross the blood-brain barrier, interact with the central nervous system and induce neurotoxicity. The NPs can affect the AChE activity, the enzyme responsible for catabolizing acetylcholine, which is released into cholinergic synapses in the brain.[42] Inhibition of AChE induces acetylcholine accumulation and results in cholinergic toxicity and, therefore, the AChE activity is appropriate for evaluating the neurotoxicity of NPs.[43] For example, Canli et al. administered aluminum oxide-NPs (40 nm particles size) orally to rats (0, 0.5, 5 and 50 mg kg^{-1} *bw* NPs).[44] Significant decrease of the AChE activity in the brain of the animals was observed for all doses of NPs administered. Noor et al. observed reduction of AChE activity in the cortical and hippocampal brain regions in Wistar rats 24 h after receiving injection of gold-NPs (20 nm particles size).[45] However, in the present work the La_2O_3-NPs did not affect the AChE activity (Figure 9). The size of the administered La_2O_3-NPs was about 15-30 nm but these NPs can agglomerate, which would hinder their passage across the blood-brain barrier.[21] This is in accordance with the very low La concentration found the in brain (Figure 10), indicating that the NPs did not go into the brain easily.

The La concentration in blood of control and treated animals were lower than the LOQ. However, the La concentration was higher than the LOQ in the analyzed organs, at higher concentration in liver, kidney, and heart; the La concentration in liver, kidney and heart of animals treated with the NPs was higher than that in the organs of control animals. The accuracy was demonstrated through CRM analysis, revealing that interference were not insignificant since accurate results were obtained for La. Studies about La_2O_3-NPs transformation in the treated animals were not conducted. Nevertheless, in a previous study, NPs have been found on bones surface in rats treated with La_2O_3-NPs.[15] In this way, it can be inferred that La could also be present in the form of NPs in the analyzed organs.

CONCLUSIONS

Administration of La_2O_3-NPs at concentrations of 1.0, 10 and 100 mg kg^{-1} *bw* did not cause significant changes in biochemical parameters, body weight and organ weight of Wistar rats. Although the La concentration increased in liver, kidney and heart, oxidative stress and neurotoxicity were in general not induced by La_2O_3-NPs when administered at concentration lower than 100 mg kg^{-1} *bw* for 30 days.

Additional studies are necessary to demonstrate whether the La_2O_3-NPs are accumulated in the organs for longer period and if the toxicity will increase for longer time of exposure of the animals. It is also worth investigating possible agglomeration, solubilization or transformation of La_2O_3-NPs to free La (ionic form) during their metabolism in the organism.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgments

This work was supported by “Conselho Nacional de Desenvolvimento Científico e Tecnológico” (CNPq Proc. Nr. 480929/2011-4 and 306052/2017-2).

REFERENCES

(1) de Matteis, V. Exposure to inorganic nanoparticles: Routes of entry, immune response, biodistribution and in vitro/in vivo toxicity evaluation. *Toxics* **2017**, *5*, 29. https://doi.org/10.3390/toxics5040029

(2) Kermanizadeh, A.; Balharry, D.; Wallin, H.; Loft, S.; Møller, P. Nanomaterial translocation-the biokinetics, tissue accumulation, toxicity and fate of materials in secondary organs - a review. *Crit. Rev. Toxicol*. **2015**, *45*, 837-872. https://doi.org/10.3109/10408444.2015.1058747

(3) Iavicoli, I.; Fontana, L.; Nordberg, G. The effects of nanoparticles on the renal system. *Crit. Rev. Toxicol*. **2016**, *46*, 490-560. https://doi.org/10.1080/10408444.2016.1181047
(4) Geppert, M.; Schwarz, A.; Stangassinger, L.M.; Wenger, S.; Wienerroither, L.M.; Ess, S.; Duschl, A.; Himly, M. Interactions of TiO_2 nanoparticles with ingredients from modern lifestyle products and their effects on human skin cells. *Crit. Rev. Toxicol*. **2020**, *33*, 1215-1225. https://doi.org/10.1021/acs.chemrestox.9b00428
(5) Panariti, A.; Miserocchi, G.; Rivolta, I. The effect of nanoparticle uptake on cellular behavior: disrupting or enabling functions? *Nanotechnol. Sci. Appl*. **2012**, *5*, 87-100. https://doi.org/10.2147/NSA.S25515
(6) Durán, N.; Rolim, W. R.; Durán, M.; Fávaro, W. J.; Seabra, A. B. Nanotoxicology of silver nanoparticles: toxicity in animals and humans. *Química Nova* **2019**, *42*, 206-213. https://doi.org/10.21577/0100-4042.20170318
(7) Sadat, N. A.; Salehzadeh, A. Green biosynthesise of silver nanoparticles using gracilaria gracilis extract and its effect on breast cancer line. *The Breast* **2019**, *48*, S72. https://doi.org/10.1016/S0960-9776(19)30745-3
(8) Węsierska, M.; Dziendzikowsk, K.; Gromadzka-Ostrowsk, J.; Dudek, J.; Polkowska-Motrenko, H.; Audinot, J. N.; Gutlebe, A. C.; Lankoff, A.; Kruszewski, M. Silver ions are responsible for memory impairment induced by oral administration of silver nanoparticles. *Toxicol. Lett.* **2018**, *290*, 133–144. https://doi.org/10.1016/j.toxlet.2018.03.019
(9) Abdelnour, S. A.; El-Hack M. E. A.; Khafaga, A. F.; Noreldin, A. E.; Arif, M.; Chaudhry, M. T.; Losaccog, C.; Abdeenh, A.; Abdel-Daim, M. M. Impacts of rare earth elements on animal health and production: Highlights of cerium and lanthanum. *Sci. Total Environ*. **2019**, *672*, 1021-1032. https://doi.org/10.1016/j.scitotenv.2019.02.270
(10) Pagano, G.; Aliberti, F.; Guida, M.; Oral, R.; Siciliano, A.; Trifuoggi, M.; Tommasi, F. Rare earth elements in human and animal health: State of art and research priorities. *Environ. Res*. **2015a**, *142*, 215-22. https://doi.org/10.1016/j.envres.2015.06.039
(11) Pagano, G.; Guida, M.; Tommasi, F.; Oral, R. Health effects and toxicity mechanisms of rare earth elements – Knowledge gaps and research prospects. *Ecotoxicol. Environ. Saf*. **2015b**, *115*, 40-48. https://doi.org/10.1016/j.ecoenv.2015.01.030
(12) Brabu, B. S.; Haribabu, M.; Revathy, S.; Anitha, M.; Thangapandiyan, K. R.; Navaneethakrishnan, C.; Gopalakrishnan, S. S.; Murugan, T. S. K. Biocompatibility studies on lanthanum oxide nanoparticles. *Toxicol. Res.* **2015**, *4* (4), 1037-1044. https://doi.org/10.1039/C4TX00198B
(13) Balusamy, B.; Kandhasamy, Y. G.; Senthamizhan, A.; Chandrasekaran, G.; Subramanian, M. S.; Kumarave, T. S. Characterization and bacterial toxicity of lanthanum oxide bulk and nanoparticles. *J. Rare Earths* **2012**, *30*, 1298-1302. https://doi.org/10.1016/S1002-0721(12)60224-5
(14) Liu, J.; Mei, W.; Li, Y.; Wang, E.; Ji, L.; Tao, P. Antiviral activity of mixed-valence rare earth borotungstate heteropoly blues against influeza virus in mice. *Antivir. Chem. Chemother.* **2000**, *11*, 367-372. https://doi.org/10.1177/095632020001100603
(15) Dressler, V. L.; Ogunmodede, O. T.; Heidrich, G. M.; Neves, V. M.; Schetinger, M. R. C.; Morsch, V. M. Investigative analysis of lanthanum oxide nanoparticles on elements in bone of wistar rats after 30 days of repeated oral administration. *Biol. Trace Elem. Res*. **2019**, *196*, 153-167. https://doi.org/10.1007/s12011-019-01907-z
(16) Sa, L. T. M.; Albernaz, M. S.; Patricio, B. F. C.; Falcão Jr., M. V.; Coelho, B. F.; Bordim, A.; Almeida, J. C.; Santos-Oliveira, R. Biodistribution of nanoparticles: initial considerations. *J. Pharm. Biomed. Anal.* **2012**, *70*, 602-604. https://doi.org/10.1016/j.jpba.2012.06.008
(17) Ocsoy, I.; Tasdemir, D.; Mazicioglu, S.; Celik, C.; Katı, A.; Ulgen, F. Biomolecules incorporated metallic nanoparticles synthesis and their biomedical applications. *Mater. Lett*. **2018**, *212*, 45-50. https://doi.org/10.1016/j.matlet.2017.10.068
(18) Ozer, J.; Ratner, M.; Shaw, M.; Bailey, W.; Schomaker, S. The current state of serum biomarkers of hepatotoxicity. *Toxicol*. **2008**, *245*, 194-205. https://doi.org/10.1016/j.tox.2007.11.021

(19) Hauck, T. S.; Anderson, R. E.; Fischer, H. C.; Newbigging, S.; Chan, W. C. In vivo quantum-dot toxicity assessment. *Small* **2010**, *6*, 138-144. https://doi.org/10.1002/smll.200900626
(20) Canli, E. G.; Ila, H. B.; Canli, M. Response of the antioxidant enzymes of rats following oral administration of metal-oxide nanoparticles (Al_2O_3, CuO, TiO_2). *Environ. Sci. Pollut. Res*. **2019a**, *26*, 938-945. https://doi.org/10.1007/s11356-018-3592-8
(21) Hauser-Davis, R. A.; Silva, J. A. N.; Rocha, R. C. C.; Saint'Pierre, T.; Ziolli, R. L., Arruda, M. A. Z. Acute selenium selenite exposure effects on oxidative stress biomarkers and essential metals and trace-elements in the model organism zebrafish (Danio rerio). *J. Trace Elem. Med. Biol*. **2016**, *33*, 68-72. https://doi.org/10.1016/j.jtemb.2015.09.001
(22) Neves, V. M.; Heidrich, G. M.; Rodrigues, E. S.; Enders, M. S. P.; Muller, E. I.; Nicoloso, F. T.; de Carvalho, H. W. P.; Dressler, V. L. La_2O_3 Nanoparticles: Study of Uptake and Distribution in *Pfaffia glomerata* (Spreng.) Pedersen by LA-ICP-MS and μ-XRF. *Environ. Sci. Technol.* **2019**, *53*, 10827-10834. https://doi.org/10.1021/acs.est.9b02868
(23) OECD, Acute oral toxicity: Up and down procedure, Guideline for Testing of Chemicals 425 OECD, **2008**, pp. 1-2. Available at: https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecdtg425.pdf [Accessed in Dec. 2022].
(24) Olas, B.; Saluk-Juszczak, J.; Wachowicz, B. d-glucaro 1,4-lactone and resveratrol as antioxidants in blood platelets. *Cell Biol. Toxicol*. **2008**, *24*, 189–199. https://doi.org/10.1007/s10565-007-9028-8
(25) Ali, S. F.; LeBel, C. P.; Bondy, S. C. Reactive oxygen species formation as a biomarker of methylmercury and trimethyltin neurotoxicity. *Neurotoxicology* **1992**, *13*, 637–648. PMID: 1475065.
(26) Levine, R. L.; Garland, D.; Oliver, C. N.; Amici, A.; Climent, I.; Lenz, A. G.; Ahn, B. W.; Shaltiel, S.; Stadtman, E. R. Determination of carbonyl content in oxidatively modified proteins. *Meth. Enzymol*. **1990**, *186*, 464–478. https://doi.org/10.1016/0076-6879(90)86141-h
(27) Misra, H. P.; Fridovich, I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem*. **1972**, *247*, 3170-3175. PMID: 4623845.
(28) Nelson, D. P.; Kiesow, L. A. Enthalpy of decomposition of hydrogen peroxide by catalase at 25 degrees C (with molar extinction coefficients of H_2O_2 solutions in the UV). *Anal. Biochem*. **1972**, *49*, 474-478. https://doi.org/10.1016/0003-2697(72)90451-4
(29) Ellman, G. L.; Courtney, K. D.; Andres Jr., V.; Feather-Stone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol*. **1961**, *7*, 88-95. https://doi.org/10.1016/0006-2952(61)90145-9
(30) Rocha, J. B.; Emanuelli, T.; Pereira, M. E. Effects of early undernutrition on kinetic parameters of brain acetylcholinesterase from adult rats. *Acta Neurobiol. Exp.* **1993**, *53*, 431-437. PMID: 8249659.
(31) Hall, J. B.; Dobrovolskaia, M. A.; Patri, A. K.; McNeil, S. E. Characterization of nanoparticles for therapeutics. *Nanomedicine* **2007**, *2*, 789-803. https://doi.org/10.2217/17435889.2.6.789
(32) Geiser, M.; Kreyling, W. G. Deposition and biokinetics of inhaled nanoparticles. *Part. Fibre Toxicol*. **2010**, *7*, 1-17. https://doi.org/10.1186/1743-8977-7-2
(33) Dumala, N.; Mangalampalli, B.; Kamal, S. S. K.; Grover, P. Repeated oral dose toxicity study of nickel oxide nanoparticles in Wistar rats: a histological and biochemical perspective. *J. Appl. Toxicol.* **2019**, *39*, 1012–1029. https://doi.org/10.1002/jat.3790
(34) Silva, A. B.; Miniter, M.; Thom, W.; Hewitt, R. E.; Wills, J.; Jugdaohsingh, R.; Powell, J. J. Gastrointestinal absorption and toxicity of nanoparticles and microparticles: Myth, reality and pitfalls explored through titanium dioxide. *Curr. Opin. Toxicol.* **2020**, *19*, 112–120. https://doi.org/10.1016/j.cotox.2020.02.007
(35) Park, E. J.; Lee, G. H.; Yoon, C.; Kim, D. W. Comparison of distribution and toxicity following repeated oral dosing of different vanadium oxide nanoparticles in mice. *Environ. Res.* **2016**, *150*, 154-165. https://doi.org/10.1016/j.envres.2016.05.036
(36) Bugata, L. S. P.; Venkata, P. P.; Gundu, A. R.; Fazlur, R. M.; Reddy, U. A.; Kumar, J. M.; Mekala, V. R.; Bojja, S.; Mahboob, M. Acute and subacute oral toxicity of copper oxide nanoparticles in female albino Wistar rats. *J. Appl. Toxicol*. **2018**, *39*, 702-716. https://doi.org/10.1002/jat.3760

(37) Zhang, H.; Ji, Z.; Xia, T.; Meng, H.; Low-Kam, C.; Liu, R.; Pokhrel, S.; Lin, S.; Wang, X.; Liao, Y. P.; Wang, M.; Li, L.; Rallo, R.; Damoiseaux, R.; Telesca, D.; Mädler, L.; Cohen, Y.; Zink, J. I.; Nel, A. E. Use of metal oxide nanoparticle band gap to develop a predictive paradigm for oxidative stress and acute pulmonary inflammation. *ACS Nano.* **2012**, *6*, 4349-68. https://doi.org/10.1021/nn3010087

(38) Sarkar, A.; Ghosh, M.; Sil, P. C. Nanotoxicity: Oxidative Stress Mediated Toxicity of Metal and Metal Oxide Nanoparticles. *J. Nanosci. Nanotechnol.* **2014**, *14*, 730-43. https://doi.org/10.1166/jnn.2014.8752

(39) Lopaczyski, W.; Zeisel, S. H. Antioxidants, programmed cell death and cancer. *Nutr. J*. **2001**, *21*, 295–307. https://doi.org/10.1016/S0271-5317(00)00288-8

(40) Nita, M.; Grzybowski, A. The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults. *Oxid. Med. Cell. Longevity* **2016**. https://doi.org/10.1155/2016/3164734

(41) Powers, S. K.; Jackson, M. J. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiol. Rev.* **2008**, *88*, 1243-1276. https://doi.org/10.1152/physrev.00031.2007

(42) Sinis, S. I.; Gourgoulianis, K. I.; Hatzoglou, C.; Zarogiannis, S. G. Mechanisms of engineered nanoparticle induced neurotoxicity in Caenorhabditis elegans. *Environ. Toxicol. Pharmacol*. **2019**, *67*, 29-34. https://doi.org/10.1016/j.etap.2019.01.010

(43) Sandahl, J. F.; Jenkins, J. J. Pacific steelhead (*Oncorhynchus mykiss*) exposed to chlorpyrifos: benchmark concentration estimates for acetylcholinesterase inhibition. *Environ. Toxicol. Chem.* **2002**, *21*, 2452-2458. https://doi.org/10.1002/etc.5620211126

(44) Canli, E. G.; Ila, H. B.; Canli, M. Responses of biomarkers belonging to different metabolic systems of rats following oral administration of aluminium nanoparticle. *Environ. Toxicol. Pharmacol.* **2019b,** *69*, 72-79. https://doi.org/10.1016/j.etap.2019.04.002

(45) Noor, N. A.; Fahmy, H. M.; Mourad, I. M. Evaluation of the potential neurotoxicity of gold nanoparticles in the different rat brain regions. *Int. J. Sci.: Basic Appl. Res*. **2016**, *30*, 114-129. ISSN 2307-4531.