Research Article

Aluminum Oxide Nanoparticles Induced Cognitive Deficits and Oxidative Stress in Frontal Cortex and Cerebellum of Rat -

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Submitted: 16 August 2017; Approved: 14 September 2017; Published: 15 September 2017


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ABSTRACT

Aluminum oxide nanoparticles (Al₂O₃-NPs) are widely used in industry. Nevertheless, the information about its toxicity on humans and environment is still deficient. The present study aimed to investigate the effect of four intravenous injections of Al₂O₃-NPs (20mg/kg body weight) on Wistar male rat brain. For this purpose we highlight behavioral consequences as well as oxidative response, Acetylcholinesterase (AChE) activity, Aluminum (Al) biodistribution, and histological changes in Frontal Cortex (FC) and Cerebellum (Cb). In anxiety related behaviors, Al₂O₃-NPs treated rats entered less frequently and spent more time in the plus maze’s enclosed arms than control rats. Al₂O₃-NPs exposure increased the Malondialdehyde (MDA) and thiol group levels in FC and decreased Catalase Activity (CAT) in this latter. Furthermore, Superoxide Dismutase (SOD) and AChE activities decreased both in FC and Cb. Glutathione Peroxidase Activity (GPx) decreased only in Cb. Sub-acute Al₂O₃-NPs exposure increased contents of Al and Magnesium (Mg) in FC and Cb. Nevertheless, calcium (Ca) contents decreased in the both same structures while iron (Fe) contents decreased only in FC compared to the control. The most pronounced histological changes were observed in FC tissue included astroglyosis, vascular congestion, neuronal degeneration, presence of edema and necrosis, degenerative neurofibrillary tangles and vacuolated cyttoplasm.

Considering the above results we suggest that accumulated Al in brain is the origin of histological changes, biochemical and mineral disturbances which lead to emotional disorders in Al₂O₃-NPs treated rats.

Keywords: Al₂O₃-NPs; Biodistribution; Rat; Brain; Emotional behavior; Oxidative response

INTRODUCTION

Nowadays pollution represents a serious problem which threatens our environment and has many several origins. Industrial revolution and excessive use of metals by men are classified as the main causes of pollution. Recently, a new technology field is fast growing: Nanotechnology. This technology uses Nanoparticles (NPs): a very tiny particles which diameter is < 100 nm [1,2]. This feature and the high catalytic properties make NPs widely used in several areas like medical applications, engineering, cosmetics... [3].

NPs are found everywhere in our environment, because they have high surface reactivity, they may have negative impacts on environmental and human health [2]. Their small sizes facilitate cellular uptake and transcytosis into the blood and lymph circulation [1]. When they can get into the bodies, they can be deposited in different organs such bone marrow, lymph nodes, spleen, and heart and can cause many diseases [4–6]. Some studies showed that NPs can also cross the blood brain barrier and reach the brain [3].

NPs was one of the most abundantly and commercially produced metal oxides NPs. It is used in various fields like domestic, consumer, medical product, industry of energetic systems [3,7] and military applications [8]. Aluminum Nanoparticles (Al-NPs) have been used as a fuel in propellants, procuring its high enthalpy of combustion and in pyrotechnic [8–11]. They were also utilized in electric components and batteries manufacture [12,13] and have been proposed as drug delivery system to increase solubility [10,13,14].

Previous studies have reported that Al-NPs can cause oxidative damages, cytotoxic and genotoxic effects, and inflammatory events [15–17]. Moreover, Al-NPs could cause neurotoxicity [18–20]. Furthermore, other studies have demonstrated that inhaled Al-NPs can get in respiratory tract and alveoli [21,22] and could be translocated via olfactory nerve and penetrate the brain. Also, Al-NPs could disrupt the Blood-Brain Barrier (BBB) [23].

Previously, Al toxicity is relatively well known and has been discussed in many publications. However, the toxicity of Al-NPs in the brain remains relatively unknown and is still an object of experimental work. The aim of the present study was to investigate the toxicity of Al₂O₃-NPs in Wistar male rat brain. For this purpose we highlight the behavioral consequences, the oxidative response, AChE activity, aluminum bio distribution, and histological changes following four intravenous injections of Al₂O₃-NPs (20mg/kg body weight).

MATERIAL AND METHODS

Animals

Twenty four Wistar male rats (174-210g) were acquired from the central animal (SIPHAT, Ben Arous, and Tunisia). Before starting the experiments, animals were left to acclimatize for at least, one week. They were kept in groups of 6 in Plexiglas cages (58 cm in length x 36 cm in width x 20 cm in height). The floor of the cage was covered with softwood bedding. The room was well-ventilated, the temperature was maintained at 22 ± 4°C, under a 12:12 light/dark cycle (light on at 07:00 AM and light off at 07:00 PM), with free access to food and water. The experimental protocols were approved by the Medical Ethical Committee for the Care and Use of Laboratory Animals of Pasteur Institute of Tunis (approval number: LNFP/Pro 152012).

Drugs

Commercially available Al₂O₃-NPs (Cat No. 544833, Sigma-Aldrich, MO, USA, TEM) were used in our study in the form of dry powder. The supplier’s product specifications are as follows: gamma phase alumina NPs, particle size < 50 nm (TEM), surface area 40 m²/g (BET).

Characterization of aluminum oxide nanoparticles

Transmission electron microscopic (TEM) analysis: The particle size and shape of aluminum oxide particles were determined by TEM [(TEM) Tecnai G2-200KV with microanalysis]. The sample preparation for TEM observation was as follow: the powder was firstly put in EtOH, and the ultrasonic dispersed particles were then deposited onto the lacey-carbon-coated copper grid.

Powdered X-ray diffraction analysis: The crystal structures of the NPs were characterized by powdered XRD (XRD; Bruker D8 Advance; 40 KV, 30 mA). D8 Advanced X-ray diffractometer, Burker, scan with 2.2 kW Cu anode radiation at wave length 1.54 A’ produced by a Ceramic X-ray tube. About 250 mg of was deposited on the sample holder for scanning in the range 10°–100°.

Treatment

In this study, the dose of Al₂O₃-NPs was chosen on the basis of our previous works (not published). Because NPs (like titanium dioxide nanoparticles) administered intraperitoneally could alter the neurobehavioral performance of adult Wistar [24] we hypothesized that intravenous injections of Al₂O₃-NPs. (20 mg/kg body weight)
could disturb emotional behavior of rats. We choose intravenous injection because this route of administration disseminates directly Al₂O₃-NPs into the blood. Al₂O₃-NPs were suspended in fresh sterilized physiological saline solution 9% sodium chloride at a concentration of 20mg/ml. This solution was sonicated using a sonicator (Sonics “vibra cell” model CV18) for 30 min before each injection to disperse the Al₂O₃-NPs in a solution was sonicated using a sonicator (Sonics “vibra cell” model CV18) for 30 min before each injection to disperse the Al₂O₃-NPs in a stable fashion [25]. To prevent -NPs agglomeration, the temperature of sonicator was kept below 30 °C. Animals were divided into two groups.

The control group (n=12): received a daily intravenous physiological saline solution injection (9% sodium chloride) during 4 consecutive days.

Al₂O₃-NPs group (n=12): received a daily intravenous Al₂O₃-NPs injection (20mg/kg body weight) during 4 consecutive days.

**Body weight**

Body weight was measured once a day during treatment days.

**Behavioral assessment**

Anxious related behavior was assessed using the plus maze test deviated from Pellow [26] 24 hours after the last injection. The experimental apparatus was shaped like a “plus” sign and consisted of a central platform 10 × 10 × 10 cm, two open arms 50 × 10 cm and two enclosed arms 50 × 10 × 50 cm (length, width, and height), opposite to each other. The maze was made of waterproof and odorless painted wood. The whole apparatus was elevated 60 cm above the floor. A test session starts by placing a rat in the center of the maze facing one of the open arms. The animal is considered inside one arm when the four paws are placed after the area marking line. The test lasted 5 minutes; after each trial the maze was cleaned with alcoholic solution (10% of volume).

Animal behavior in the plus maze device was recorded by a video camera placed above the apparatus and connected to a computer. Data were then analyzed by a video tracking software Any-maze (stooling diffusion*)

**Tissue preparation**

The day following behavioral testing, Rats were scarified by decapitation. Brains were carefully excised on an ice cooled glass plate, immediately rinsed with physiological solution and dried with filter paper. Different regions of rat’s brain FC and Cb were immediately isolated, weighed and set in liquid nitrogen then stored at −80°C until analysis. Each region was homogenized in PBS solution (phosphate buffered saline; pH 7.4). The homogenates were centrifuged at 13 000rpm for 20 minutes at 4°C. The supernatant was divided in aliquots and then used to determine protein concentration, MDA and thiol group levels, CAT, SOD, GPx, AChE activities and trace elements content.

**Antioxidant biomarkers and enzymes assays**

Total protein was determined according to the method of Hartree [27] with bovine serum albumin as standard. The lipoperoxidation was estimated spectrophotometrically at 532 nm by measuring brain MDA according to the method cited by [28], results were expressed as nmoles of MDA/mg of protein. CAT activity was assayed by ultraviolet spectrophotometry at 240 nm according to the method of Aebi [29], results were expressed as U/mg of protein. SOD activity was determined according to Misra and Fridovich [30] by spectrophotometry at 480 nm, results were expressed U/mg of protein. GPx activity was measured by the method of Flohe and Gunzler [31], GPx activity was expressed as U/mg of protein. Tissue thiol groups were determined spectrophotometrically at 412 nm according to Ellman [32], levels were expressed as Mm.

**Acetylcholinesterase activity**

AChE activity was assessed by the Ellman method [33]. The change in absorbance was measured for 15 min at 30 s intervals at 412 nm with microplate. Results were expressed as U AChE (μmoles of acetylthiocholine iodide hydrolyzed/min/mg of protein).

**Aluminum levels**

Al levels were determined in FC and Cb of control and treated animals as following: samples were incinerated at 550°C in oven muffle (STUART) for 48 h to obtain white residue. After being cooled to room temperature, each sample was recovered by 1.25 ml concentrated nitric acid and was brought to 12.5 ml with ultra-pure water. Al brain levels were measured by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES).

**Iron, Calcium and magnesium levels**

Free iron was determined in FC and Cb by the ferrozine method [34] using a commercially kit from Biomaghreb. At acidic pH 4.8 ion Fe²⁺ is released from transferrine, which is reduced to Fe⁺⁺ by ascorbic acid. Fe⁺⁺ reacts with ferrozine and gives a colorful complex measurable at 560 nm. Ionizable Ca and Mg were determined using commercially available kits from Biomaghreb (Tunisia).

**Histological study**

Immediately after decapitation, for each group brain samples were fixed in 10% formalin for 10 days. Fixed tissues were dehydrated in a graded series of ethanol and xylene solutions, and embedded in paraffin. Then they were processed for microtomy. Sections were cut of about 5 μm thick, then they were deparaffinized, rehydrated in a graded series of ethanol, and stained with Hematoxylin and Eosin (H&E).

The slides were then washed, dried and viewed under a light microscope and photomicrographs were taken.

**Statistical analysis**

Results were presented as mean ± SEM. Behavioral data were analyzed using one-way ANOVA with Al₂O₃-NPs treatment as principal factor. Oxidative stress data obtained from groups were analyzed using t-test. The level of significance was set at \( p \leq 0.05 \).

**RESULTS**

**Al₂O₃-NPs characterization**

The TEM and X-ray diffraction analysis were done for the characterization of Al₂O₃-NPs. The TEM measurements (Figure 1) have shown very thin particles (nanopowder, < 50 nm). The XRD results showed five dominant peaks [36.53u, 37.72u, 39.46u, 47.80u and 67.01u] confirming the crystalline nature of aluminium oxide nanoparticles the same peaks were obtained by Pakrashi et al. [35].

**Body weight**

Al₂O₃-NPs treatment did not affect the body weight of the injected rats (Figure 3).
Elevated plus maze test

Our results (Table 1) indicated that both distance travelled and number of entries in closed arms decreased in Al2O3-NPs treated rats compared to control rats (p ≤ 0.01). Furthermore statistical analyses also revealed that Al2O3-NPs group spent more time in the closed arms (p ≤ 0.05).

In a corollary manner the time spent in the open arms as well as the number of entries in these latter decreased significantly (p ≤ 0.001) in Al2O3-NPs group compared to control group. In the same way the time spent in center area and the number of entries in this latter decreased significantly in Al2O3-NPs treated rats compared to control ones (p ≤ 0.01).

Nevertheless, the results indicated that Al2O3-NPs group spent less time than control rats in the open arms (p ≤ 0.001) and in the center, and avoided the more anxiogenic parts of the apparatus (distal parts of the open arms) (p ≤ 0.001) preferring the distal closed arm sectors (p ≤ 0.05). Al2O3-NPs administered rats stayed immobile in the closed arms (see Table 1), they prostrated in the secure areas.

The average of speed decreased during the test in Al2O3-NPs group compared to the control ones (p ≤ 0.01) (see Table 1).

Oxidative stress

Activities of oxidative enzymes are represented in Table 2. Sub-acute Al2O3-NPs treatment caused significant inhibition of SOD activity both in FC and Cb, while CAT activity decreased only in FC. GPx activity decreased only in Cb of Al2O3-NPs treated rats. Furthermore MDA (Figure 4) and thiol group levels (Table 2) were increased in FC of Al2O3-NPs treated rats compared to control ones.

Acetylcholinesterase activity

Our findings showed that Al2O3-NPs inhibit the activity of AChE in FC and Cb compared to control (Figure 5).

Al content and mineral homeostasis

Al levels in control and Al2O3-NPs treated groups are shown in Table 3. Sub-acute Al2O3-NPs treatment caused a significant Al increase in FC and Cb tissues which disturbed the mineral balance in the two studied structures. Our data shows an increase in Mg levels in FC and Cb of Al2O3-NPs treated rats. However Ca levels deceased in the same structures in treated group while Fe levels decreased only in FC of Al2O3-NPs group.

Histological study

The histology of FC and Cb were viewed (Figures 6 and 7 respectively). Histological FC examination showed the presence of astrogliosis phenomena, vascular congestion in treated group. Moreover, photomicrographics revealed Al2O3-NPs intoxicated neuronal cell with pyknotic nuclei and cell without nuclei, presence of red neurons having eosinophilic cytoplasm. Furthermore we note the presence of edema, necrosis, a degenerative neurofibrillary tangles...
and vacuolated neuron cytoplasm. Cb sections revealed the presence of edema and lymphocytic infiltration in Al₂O₃-NPs group.

**DISCUSSION**

The aim of the present study was to investigate the effects of Al₂O₃-NPs on the behavioral performances, the oxidative response and biodistribution of aluminum in rat brain. In our study Al₂O₃-NPs exposure did not alter the general health of the rats. Indeed, no mortality was observed and no obvious significant differences were found in the body weight of the treated group. The emotional reactivity of rats’ was evaluated using a multiparametric analytical characterization of anxious index with the plus-maze test. This model is used as a general tool in neurobiological anxiety research. Our results showed that intravenous injection of Al₂O₃-NPs (20mg/kg body weight) impaired the emotional response of rats. This was supported by the results of the plus-maze test, in which Al₂O₃-NPs decreased significantly

<table>
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<tr>
<th>Table 2: Effect of Al₂O₃-NPs injection on CAT, SOD, GPx activities and Thiol groups levels in cortex and cerebellum.</th>
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<tr>
<td><strong>Frontal cortex</strong></td>
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<tr>
<td>Control</td>
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<td>SOD (U/mg protein)</td>
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<td>CAT (U/mg protein)</td>
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<td>GPx (U/mg protein)</td>
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<td>Thiol groups (Mm)</td>
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Values are given as mean ± SEM; *p ≤ 0.05

| Table 3: Al content and mineral distribution (μg/g fresh weight) in frontal cortex and cerebellum after four Al₂O₃-NPs injection. |
|-------------------|-----------------|-----------------|-----------------|
|                    | **Frontal cortex** | **Cerebellum** |
| Al                 | 0.55 ± 0.20     | 1.42 ± 0.11*   | 1.24 ± 0.25     | 2.02 ± 0.11*   |
| Fe                 | 6.18 ± 1.10⁻³   | 0.65 ± 8.10⁻⁵  | 1.89 ± 1.10⁻⁴  | 1.97 ± 1.10⁻⁴  |
| Ca                 | 36.64 ± 2.10⁻³  | 22.49 ± 2.10⁻⁵ | 34.23 ± 3.10⁻³ | 20.65 ± 2.10⁻⁵ |
| Mg                 | 75.08 ± 5.10⁻³  | 115.19 ± 0.01* | 68.86 ± 7.10⁻³ | 93.84 ± 7.10⁻³ |

Values are given as mean ± SEM. *p ≤ 0.05; **p ≤ 0.01

Figure 3: Body weight changes for rats treated with Al₂O₃-NPs. Values are given as mean ± SEM of 12 animals per group.

Figure 4: Effect of Al₂O₃-NPs injections on MDA levels in FC and Cb. Values are given as mean ± SEM. (*) significant mean Al₂O₃-NPs treatment effect (p ≤ 0.05).

Figure 5: Effect of Al₂O₃-NPs injections on ACHE activity in FC and Cb. Values are given as mean ± SEM. (*) significant mean Al₂O₃-NPs treatment effect (p ≤ 0.05).

Figure 6: Photo micrographics of FC (H&E stain). Control group (A- B), no histological change was observed. Histological cuts of Al₂O₃-NPs treated group (C-H) (B, C, D, E, F X40, G and H X100). A: Astroglyosis, VC: Vascular Congestion, PN: Pyknotic Nuclei, *: Cells without nuclei, E: Edema, N: Necrosis, D: Degenerative neurofibrillary tangles, V: Vacuolated cytoplasm.
the distance travelled and the number of entries in open arms. Furthermore, statistical analyses also revealed that Al2O3-NPs treated group spent more time in the closed arms. Moreover, complementary analyses of results indicate that treated animals avoided the more anxiogenic parts of the apparatus (distal parts of the open arms) preferring the distal closed arm sectors. Our data are consistent with the finding reported by Abdel-Aal et al. [36] which showed that Al impaired spontaneous locomotive capacity and exploratory behavior when animals were evaluated in the open field test. Moreover, the emotional reactivity analyses by Sethi et al. [37] indicate an elevated anxiety in the form of high ambulation and increased defection index in Al-intoxicated young and old rats. Currently, there is insufficient evidence to confirm the idea that Al2O3-NPs can cross the BBB. In our opinion, Al2O3-NPs injected in the systemic pathways appear to be absorbed in the brain in an ionic form. Our findings indicated that Al2O3-NPs enhance the bioaccumulation of Al in the FC and the Cb. In a recent study performed by Vilella et al. [38] the author found that NPs were widely distributed across the regions of the brain after the intraperitoneal injection on mice. This finding was further confirmed by our previous study in which Zn concentrations in rat brains increased significantly after an intravenous injection of ZnO-NPs suspension for 14 consecutive days [25]. The dissolved Al could partially contribute to the toxicity of Al2O3-NPs in biological system. This was confirmed, in the present study, by the positive relationships between the bioaccumulation of Al in brain structures (FC and Cb) and the disruption of the emotional behavior of Al2O3-NPs treated rats. Additionally, our results have also demonstrated a significant inhibition of AChE activity in FC and Cb of treated rats. Kumar et al. [39] reported that increased Al concentration could affect the neurons, leading to the depletion of AChE. Al or Al2O3-NPs could alter ion homeostasis including Ca2+ homeostasis, decreasing release of acetylcholine and consequential decrease in levels of AChE. This was confirmed in our results by the significant decrease of Ca2+ levels in FC and Cb of Al2O3-NPs-treated rats.

To better understand the possible interaction of NPs with central nervous system, the current study analyzed the influence of Al2O3-NPs on brain oxidative response. Oxidative stress is considered as the most common mechanism of toxicity related to NPs exposure [40]. Our results showed a significant decrease in SOD and CAT activities and thiol group in FC. Moreover, Al2O3-NPs decreased the SOD and GPx activities in the Cb. Al2O3-NPs are involved in the production of ROS there by leading to a reduction in the enzyme activities, and state a condition of oxidative stress. In addition, our results showed that Al2O3-NPs could disrupt mineral elements metabolism in rat brain, which are necessary for antioxidant synthesis. Flora et al. [41] reported that ROS production will be related to the depletion of some essential elements such as iron Fe, Zn, Cu and Mn that function as cofactors to the antioxidant enzymes. Thus, in the present work, the most pronounced oxidative damage was observed in the FC tissue resulting in excessive MDA level in this structure. Al2O3-NPs might induce free radical generation that further initiated the process of lipid peroxidation and damaging cellular components. Accordingly, previous studies showed that Al could induce LPO in the brain leading to neurodegeneration [39,42], as confirmed by the increased levels of MDA and inhibition of the SOD, CAT and GPx activities in the brain [43]. The FC is the brain region that is most sensitive to the detrimental effects of stress exposure. Even quite mild acute uncontrollable stress can cause a rapid and dramatic loss of prefrontal cognitive abilities, and more prolonged stress exposure causes architectural changes in prefrontal dendrites [44]. Our data revealed that Al2O3-NPs intoxicated neuronal cell in the FC with pyknotic nuclei and cell without nuclei, presence of red neurons having eosinophilic cytoplasm. Moreover, we noted the presence of edema and necrosis, also a degenerative neurofibrillary tangles and vacuolated neuron cytoplasm. The nanosized Al is highly able to oxidize the neural membrane that lost its lipoprotein integrity [45] and partially cause damage to the BBB [46] and in turn facilitates the accumulation of Al to the brain tissues. The accumulated Al attach to the mitochondria and/or the nuclei causing the damage of cell and degradation of neurons [19]. Another study reporting the toxic effects of Al on mice brain, confirmed a damage in the hippocampus and cortex, including neurofibrillary degeneration, due to the accumulation of Al in these regions [47]. Researchers have conducted many in vivo and in vitro studies to explore the interactions between the nanomaterials and biological macromolecules, cells, organs, and tissues, and the majority of these studies have found that the effects of the biological toxicities of the nanomaterials may be induced by the mechanisms of oxidative stress and inflammatory reactions [18,48,49].

In this in vivo study we suggest that physiological stress response induced by Al2O3-NPs could coordinate changes in metabolic activity and behavior of animals. Short-term systemic exposures to Al2O3-NPs induced oxidative stress that could trigger cell apoptosis and architectural damage in the FC, this toxicity may result from the accumulation of aluminum in brain structures. Furthermore, this toxic effect may have a physiological impact on animal behavior, which was demonstrated in rats by the impairment of emotional reactivity.

REFERENCES


