Toxicology Mechanisms and Methods

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/itxm20

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To cite this article: Mangaldeep Dey & Rakesh Kumar Singh (2022) Chronic oral exposure of aluminum chloride in rat modulates molecular and functional neurotoxic markers relevant to Alzheimer's disease, Toxicology Mechanisms and Methods, 32:8, 616-627, DOI: 10.1080/15376516.2022.2058898

To link to this article: https://doi.org/10.1080/15376516.2022.2058898

TOXICOLOGY MECHANISMS AND METHODS
2022, VOL. 32, NO. 8, 616–627
https://doi.org/10.1080/15376516.2022.2058898

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RESEARCH ARTICLE

Chronic oral exposure of aluminum chloride in rat modulates molecular and functional neurotoxic markers relevant to Alzheimer's disease

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ABSTRACT

Aluminum is an environmentally abundant potential neurotoxic agent that may result in oxidative damage to a range of cellular biomarkers. The potential sources of aluminum accumulation in the body include drinking water, food, medicines, vaccines, and aluminum cookware utensils, etc. The accumulation of aluminum in the brain is reported to be associated with cholinergic dysfunction, oxidative stress and neuronal damage, which may ultimately cause Alzheimer's disease. Since chronic exposure to aluminum leads to its accumulation in the brain, so this study was done by a long-term (24 weeks) low dose (20 mg/kg) oral exposure of aluminum chloride in rats. In this chronic model, we have evaluated the major hallmarks of Alzheimer's disease including amyloid-beta ($A\beta_{1-42}$) and phosphorylated-tau (p231-tau) protein in brain tissue. Furthermore, we evaluated the level of acetyl cholinesterase activity, inflammatory cytokines such as TNF-α, IL-6 and IL-1β, and oxidative stress biomarkers in the rat brain in this model. The neurobehavioral parameters were also assessed in animals by using spontaneous locomotor activity, passive avoidance, rotarod test and novel object recognition test to evaluate alteration in learning, memory and muscle co-ordination. We found that chronic oral exposure to aluminum chloride causes a significant increase in structural hallmarks such as $A\beta_{1-42}$ and p231-tau levels along with pro-inflammatory cytokines (TNF-α and IL-6), oxidative stress, and a decrease in antioxidant markers such as GSH and catalase in the brain tissue. These biomarkers significantly affected neurobehavioral parameters in animals. This study provides a mechanistic understanding of chronic aluminum-induced neuronal toxicity in the brain with relevance to Alzheimer's disease.

ARTICLE HISTORY
Received 17 October 2021
Revised 20 March 2022
Accepted 21 March 2022

KEYWORDS
Aluminum chloride; neuroinflammation; neurovegetative markers; Alzheimer's disease

1. Introduction

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative disorders, characterized by a progressive loss of neuronal and synaptic functions leading to impairment of memory and cognition. The aggregation of amyloid beta peptides (Aβ) in extracellular neuronal cells and the formation of intracellular neurofibrillary tangles are considered to be two classical hallmarks of AD (Armstrong 2013; Srivastava et al. 2021). In addition to these two classical hallmarks, chronic neuronal inflammation in the brain has emerged as a third important core pathological feature of AD including oxidative stress, neuroinflammation leading to amyloidogenesis (Cheignon et al. 2018). Despite extensive research on AD, there is still a gap in the mechanistic understanding of the disease pathophysiology.

Although the precise etiology of AD is not known, several genetic and environmental factors have been attributed as the causation of AD. Amongst environmental factors, particularly the accumulation of aluminum in the brain has been correlated with the increasing incidence of AD. Chronic exposure to aluminum is considered one of the important risk factors in the development of neurodegenerative diseases such as AD. Aluminum is the third most abundant element in the Earth's crust, but non-essential to human biological functions (Karbouj 2007). However, due to its ubiquitous nature, its exposure to humans is quite high mainly through cooking utensils, drinking water, contaminated air, food and food additives (Kumar and Gill 2014). It may also be due to excessive use of aluminum-containing compounds via dialysates, parenteral nutrition, and vaccines. This excessive aluminum exposure may lead to its accumulation in several tissues including the brain, bone, liver, kidney, skeletal systems, immune systems, reproductive systems (Exley 2013; Zhu et al. 2013; Cheraghi et al. 2017). Among these tissues, the brain is a potential target for aluminum accumulation and its resultant toxicity. Aluminum can easily enter the blood-brain barrier (BBB) through its high-affinity transferrin receptors present across BBB, and thereby gets its entry to enter and accumulate in the brain. Its accumulation takes place particularly in the hippocampus and frontal cortex regions of the brain, leading to neuronal degeneration as evidenced in AD (Crapper et al. 1973; Rizvi et al. 2016). Aluminum can enter the central nervous system following either its systemic administration or its chronic exposure through different routes leading to behavioral impairments and neurodegeneration (Shaw and Tomljenovic 2013). Many epidemiological studies have shown that increased aluminum levels in the brain lead to obvious neurotoxicity and can cause learning

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and memory dysfunction in humans and experimental animals. Several studies have also found that degenerating neurons in AD contain a higher level of aluminum in the brains (Crapper et al. 1973; Cheng et al. 2014; Rizvi et al. 2016). Thus, chronic aluminum accumulation in the brain is considered a contributing factor in the pathogenesis of neurodegenerative diseases (Exley 2013). However, the exact mechanism of aluminum-induced neuroinflammation and neurodegeneration in the brain during AD remains unclear. Based on the experimental evidences, it has been suggested that there is a relationship between high levels of aluminum in brain and increased risk of a number of neurodegenerative disorders (Berthon 1996; Becaria et al. 2002; Yokel 2002).

Hence, the objective of this study was to evaluate the mechanism of neurotoxicity and neurobehavioral effects caused by chronic exposure of aluminum chloride *in-vivo* at 20 mg/kg oral dose. The acute oral lethal dose (LD_{50}) of aluminum chloride has been found to be in the range of 3630 ± 400 mg/kg, as per World Health Organization (WHO) reports. Therefore, we selected $\sim 1/200^{th}$ dose of acute LD_{50} *i.e.* 20 mg/kg for chronic oral exposure for six months in this model. We have analyzed the effect of such chronic aluminum exposure on alteration of molecular and functional biomarkers, $A\beta_{1-42}$ and phosphorylated-tau (p231) protein level in rat whole brain. In addition, we have also investigated neurobehavioral studies relevant to AD pathogenesis *in-vivo*. Our study concluded that chronic oral exposure to aluminum chloride may cause an alteration of oxidative and pro-inflammatory biomarkers, leading to $A\beta_{1-42}$ aggregation, tau hyperphosphorylation and consequent neurobehavioral changes.

2. Material and methods

2.1. Chemicals, reagents and kits

The general laboratory chemicals and reagents were procured from Sigma-Aldrich unless otherwise stated. Aluminum chloride ($AlCl_3$, catalogue no A0718), Acetylthiocholine iodide (ATCI, catalogue no. A5751), Glutathione (GSH, catalogue no. G6013)) 5, 5 o-Dithiobis (2-nitrobenzoic acid) (DTNB, catalogue no. D1830), Thiobarbituric acid (TBARS, catalogue no. T5500), 2, 7-dichlorofluorescin diacetate (DCFDA, catalogue no. 287810), modified Griess reagent (catalogue no. G4410) rat ELISA kits for proinflammatory markers TNF-α (catalogue no. RAB0480), IL-1β (catalogue no. RAB0278) and IL-6 (catalogue no. RAB0311) were procured from Sigma–Aldrich Chemicals Co., St. Louis (USA). The rat amyloid beta-peptide ($A\beta_{1-42}$) ELISA kit (catalogue no. MBS725269) and phosphorylated-tau 231 (pTau-231, catalogue no. MBS9346367) Elisa kits were purchased from MyBioSource.

2.2. Animals and study design

All *in-vivo* experiment protocols were approved by the Institutional Animal Ethics committee (IAEC) of the Institute. Male Wister rats (Rattus *norvegicus*), weighing about 80–100 g were used in this study. All the animals were kept in the institutional animal house facility under the automatically controlled condition of room temperature ($22 \pm 2\,^{\circ}C$), humidity ($55 \pm 5\%$) with of 12-hour light and 12-hour dark cycle. The animals were provided with standard commercial laboratory feed and water (aqua pure) *ad libitum*. All the animal studies were performed and executed according to the ethical committee guidelines.

2.3. Experimental design

The animals were acclimatized for a period of 1 week prior to the beginning of the experiment. All the animals were randomized, and ten animals were divided into two groups of five rats each and treated for twenty-four weeks as shown in Figure 1. The animals were provided chronic oral exposure (24 weeks duration) with a once-daily dose of 20 mg/kg aluminum chloride. This method was adopted to simulate a realistic situation of human exposure to aluminum salt through the oral route and evaluate the risk of aluminum-induced neuronal changes in the rat brain due to long-term continuous exposure.

Group I: Vehicle-treated control group
Group II: Aluminum chloride ($AlCl_3$, 20 mg/kg *per oral*)

After 24 weeks of exposure, the animals were sacrificed under anesthesia (urethane 1.5 gm/kg, intraperitoneal).

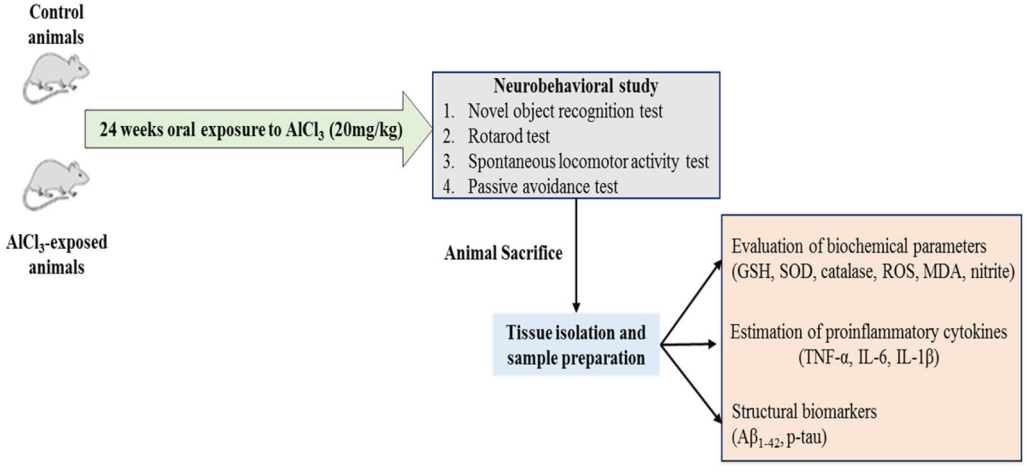

Figure 1. *In vivo* study design.

The brain tissue samples were removed and washed with chilled normal saline and stored at −80 °C for tissue-based biochemical estimations. The study plan is given in Figure 1.

3. Neurobehavioral studies in animals

3.1. Spontaneous locomotor activity

The spontaneous locomotor activity was determined by using the optovarimax apparatus. The open field activity of the rat was evaluated in a box having the dimension of 90 cm × 90 cm × 90 cm and it was subdivided into 19 equal squares by black lines. The rats were placed in the center of the open field during an experiment. The total distance traveled by the animal and its movement was recorded for 10 minutes duration (Masuo et al. 1997; Heredia-López et al. 2013).

3.2. Step-through passive shock avoidance task

The learning and memory of the animals were assessed using a step-through passive avoidance shuttle box, which is duly software operated (PACS-30) (Columbus Instruments, USA). In this equipment, there are two compartments, one compartment is illuminated, and the other one is dark consisting of a series of the metal grid, through which an electric shock may be provided to animals. These compartments are separated by a guillotine door. This experiment was performed for three successive days. On the first day, animals could explore for 30 seconds (s). After 30 s, the door was elevated, and animals could explore it freely. When the animal entered the dark compartment with all four paws, the guillotine door was closed, and the animals were returned to the home cage. On the second day, the animals were placed in the illuminated compartment and allowed to explore for 30 s. After 30 s, the door was elevated, so that the animals could explore freely. When the animal entered the dark compartment with all four paws, the guillotine doors were closed behind the animal and a foot shock (0.5 mA for 5 s) was given through the grid. After 10 s, the animal was returned to the home cage. On day 3, the short-term memory was assessed, the rats were placed in an illuminated chamber and 30 s later, the sliding door was raised and latency to step into the dark compartment was recorded. No shock was provided on the third day and the cutoff time was provided for 300 s (Elrod and Buccafusco 1988; Mohammadi et al. 2016).

3.3. Novel object recognition task

The novel object recognition test (NORT) evaluates the animal's ability to recognize a novel object in the environment. This test was performed in a top-opened square box (65 L × 65 B × 45 H cm). This procedure consists of three phases: habituation, familiarization, and test phase. In the habituation phase, each animal freely explored for 5 minutes in the open-field area in absence of any object. The animal was then removed from the open field and placed in its holding cage. During the familiarization phase, individual animals were placed in the open field containing two identical sample objects ($A + A$), for 10 minutes. During the test phase, an individual animal could explore for 10 minutes the open-field arena with two objects, one is identical to the sample and the other is novel ($A + B$). The time spent by the animal with the novel object and the familiar object was recorded manually (Leger et al. 2013; Lueptow 2017).

3.4. Rotarod test

The Rotamax apparatus (Rotamex-5; Columbus Instruments) was used to assess motor co-ordination and motor performance in animals. The unit consists of a rotating rod and a power source for turning the rod and place below the rotating rod, where the rat can fall without injury. All the animals were trained on this apparatus before conducting the actual study for proper performance. The training continued for 3 consecutive days, under an accelerating protocol starting at 4 rotations per minute (rpm) and reaching 40 rpm in 5 minutes. On the final day of the study, the latency to fall was recorded automatically by photocells and total latencies on the rod were analyzed (Carter et al. 2001; Zhang et al. 2019b).

4. Measurement of biochemical parameters in brain tissue homogenate

4.1. Preparation of brain tissue homogenate and protein estimation

The rats were sacrificed under urethane (1.5 gm/kg, intraperitoneal) anesthesia. The whole brains were separated from the animals, and 10% w/v of brain tissue homogenate was prepared in ice-cold phosphate buffer pH 7.4. The brain tissue homogenates were centrifuged at 10 000 rpm at 4 °C for 10 minutes. The tissue supernatants were collected and used to carry out protein and biochemical estimation.

The supernatant of the brain tissue homogenate samples (5 µL) were incubated with solution D (2% sodium carbonate, 0.4% sodium hydroxide, 2% sodium tartrate and 1% copper sulphate) for 10 minutes at 37 °C. The resulting solution was treated with Folin Ciocalteu (FC) reagent in a 1:1 ratio for 30 minutes at 37 °C. The absorbance of the blue color developed was measured at 660 nm. The protein concentration in homogenate samples was extrapolated from the standard curve of known concentrations of bovine serum albumin (BSA), treated in the same manner.

4.2. Determination of reduced glutathione, superoxide dismutase and catalase levels

The level of reduced glutathione (GSH) was estimated according to the method of Rahman et al. with some modifications (Rahman et al. 2006). The tissue homogenate samples were mixed with an equal volume of 5% sulfosalicylic acid, vortexed and kept for 30 minutes in an ice bath. After centrifugation, the supernatant was collected. The GSH content in

samples was measured by using Ellman's reagent 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) solution at 412 nm. The results were extrapolated from the standard curve of absorbance of known concentrations of GSH and were expressed in μM GSH/mg protein.

The level of superoxide dismutase (SOD) activity was determined according to the method of Weydert et al. with some modifications (Weydert and Cullen 2010). The reaction mixture containing 1.2 ml of sodium pyrophosphate, 0.3 ml of PMS (phenazine methosulfate), 0.3 ml of NBT (nitroblue tetrazolium chloride), 0.2 ml of supernatant, 0.8 ml of distilled water and 0.2 ml of NADH (reduced nicotinamide adenine dinucleotide) was prepared. The control reaction mixture was prepared and contained 1.2 ml of sodium pyrophosphate, 0.3 ml of PMS, 0.3 ml of NBT, 1 ml of distilled water and 0.2 ml of NADH. Both mixtures were incubated at 37 °C for 90 s and then the reaction was stopped by adding 1 ml of acetic acid and the mixture was allowed to stand for 10 minutes. The absorbance was measured at 560 nm using a multimode plate Reader (SynergyH1M, Biotech).

The activity of tissue catalase was assayed by following the procedure of Sinha (1972). The reaction mixture containing 0.1 ml of tissue homogenate, 1 ml of phosphate buffer, 0.4 ml of distilled water and 0.2 ml of H_2O_2 was prepared. The control mixture containing 1 ml of phosphate buffer, 0.5 ml of distilled water and 0.2 ml of H_2O_2 was prepared. Both the mixtures were incubated at 37 °C for 15 minutes and then the reaction was stopped by the addition of 2 ml of acetic acid with dichromate (1:3 ratio of 5% potassium dichromate in distilled water and glacial acetic acid respectively). The mixture was then boiled for 15 minutes, cooled, and the absorbance of the color developed was recorded at 570 nm using a multimode plate Reader (SynergyH1M, Biotech).

4.3. Measurement of nitrite content and reactive oxygen species in tissue homogenates

The tissue nitrite content was determined according to the method described by Giustarini et al., with some modifications (Giustarini et al. 2008). Briefly, equal volumes of Griess reagent and supernatant were added into a 96-well plate and incubated for 10 minutes in dark conditions with shaking. The absorbance of the color developed was measured at 540 nm. The nitrite contents in brain tissue homogenates were calculated using a standard curve obtained from the absorbance of known concentrations of sodium nitrite treated under similar conditions. The results were expressed as μM/mg protein

The amount of ROS was measured by using 2, 7-dichlorofluorescein diacetate (DCFDA). This DCFDA gets converted into highly fluorescent DCF by cellular peroxides (including hydrogen peroxide). It was determined by following the protocol of Socci et al. (1999); Wu and Yotnda (2011). The reaction mixture included 10 μl tissue homogenate sample with 5 μl of 5 μM DCFDA and 985 μL phosphate buffer (pH 7.4). After 30 minutes of incubation, the fluorescence was measured at excitation and emission wavelength of 485 nm and 525 nm respectively using a multimode plate Reader (SynergyH1, Biotek Instruments). The results were expressed as fluorescence units per milligram of protein.

Malondialdehyde (MDA) is the end product of lipid peroxidation and it was measured in brain tissues by using the thiobarbituric acid reactive substance (TBARS) method (Ohkawa et al. 1979). The brain tissues were collected and rinsed with ice-cold PBS (pH 7.4). The tissues were minced and homogenized in phosphate buffer (pH 7.4) containing EDTA (1 mM). 100 μl of supernatant of brain tissue homogenates were incubated with SDS (sodium dodecyl sulfate) for 10 minutes followed by the addition of 20% acetic acid. The reaction mixture was further mixed with 0.8% (w/v) thiobarbituric acid for 1 h in a boiling water bath. The absorbance of the pink color developed was measured at 532 nm. MDA levels in tissue homogenates were calculated from the standard curve using the 1, 1, 3, 3-tetramethoxy propane (TMP) (97%). The results were expressed as μM MDA/mg protein.

4.4. Determination of $A\beta_{1-42}$, p-tau protein and acetylcholinesterase in brain tissue of animals

The levels of $A\beta_{1-42}$ and p-tau (P231) in rat brain homogenates were estimated by using an ELISA kit (MyBioSource San Diego USA). The brain homogenates were prepared in lysis buffer solution provided in the kit, and the assay was performed as per the instruction given in the kit product insert. Briefly, The standards and test samples were added to 96 well plates along with the sample diluent and conjugated protein and incubated for 90 minutes at 37 °C. After that, the plates were thoroughly washed with wash buffer, and substrates (mixture of substrate A and substrate B solution) were added to each well, followed by incubation at 37 °C for 30 minutes in dark. The enzyme-substrate reaction was terminated by the addition of a sulfuric acid solution and the intensity of color developed was measured spectrophotometrically at a wavelength of 450 nm. A standard curve was plotted relating the optical density (OD) of the color developed to the concentration of standards ($A\beta_{1-42}$ or p231-tau). The concentration of $A\beta_{1-42}$ and phosphorylated-tau in each sample was interpolated from this standard curve.

Acetylcholinesterase (AChE) activity was determined in brain homogenate according to the method of Ellman et al. (1961). A 10% brain tissue homogenate (w/v) was prepared in phosphate buffer (pH 7.4). The brain homogenate samples were centrifuged at 10 000 rpm for 10 minutes at 4 °C and the supernatant was collected. 10 μL of the tissue homogenate samples were mixed with 190 μL of the mixture containing (10 mM DTNB, 75 mM ATCI and 50 mM phosphate buffer pH 7.4). The absorbance of the reaction mixture was measured at 412 nm on a kinetic loop using a multimode plate Reader (SynergyH1M, Biotek) for 20 minutes. The results were expressed as AChE enzyme activity in units/mg protein/minutes.

4.5. Evaluation of neuroinflammatory markers (IL-1β, IL-6, TNF-α) in brain homogenates

The level of neuroinflammatory markers in brain tissue samples was estimated by the respective ELISA kit

(Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instruction. The test samples and standards were added to the 96 well ELISA plates specified in the respective kits. The plates were incubated for 90 minutes at 37 °C. After incubation, the wells of the ELISA plates were washed thrice by using the wash buffer. This was followed by the addition of a biotinylated secondary antibody and incubation for 1 hour at 37 °C. After washing, the enzyme conjugate solution was added to each well and further incubated at 37 °C for 30 minutes. Finally, the ELISA plates were thoroughly washed with wash buffer thrice. Then the substrate reagents for color development by incubation at 37 °C for 30 minutes in dark. Further to this, a stop solution was added to each well to stop the reaction and the absorbance of the color intensity developed was measured at 450 nm.

5. Statistical analysis

The data analysis for this study was done using GraphPad Prism 8.0.2 (GraphPad Software, Inc., La Jolla, CA, USA) for the plotting of graphs and statistical evaluation. All the results were expressed as mean ± standard error of the mean (SEM) for all these experiments. The statistical analyses of the results were made using one-way analysis of variance followed by Dunnett's test for multiple comparisons and Student's t-test for single comparison. A p-value <0.05 was considered statistically significant.

6. Results

6.1. Effects of oral aluminum chloride exposure on various neurobehavioral tests

Spontaneous locomotor activity (SLA) is an important parameter that reflects the physical and mental status of experimental animals. It is also used to assess the neuronal function along with neurotoxic effects of a toxicant or new chemical entity. A decrease in locomotor activity may be due to toxicant-induced motor impairment or altered neuronal functions. Interestingly, the chronic oral exposure of aluminum chloride for 24 weeks significantly reduced ($p < 0.01$)) the distance traveled as compared to control animals (Figure 2(A)). Moreover, the locomotor activity of the rats can easily be depicted in the tracking graphs as aluminum chloride-exposed rats showed significantly less movement as compared to the control (Figure 2(B)). The results of the rotarod test revealed a noticeable impairment in muscle strength and muscle coordination. The outcomes of this test showed a significant decrease ($p < 0.001$) in the fall-off time of the aluminum-treated group as compared to control group rats (Figure 2(C)).

The novel object recognition test is used for the assessment of novelty-based memory or nonspatial memory; whereas the passive avoidance, a test is used to assess the memory performance. Interestingly, aluminum-intoxicated rats spend significantly ($p < 0.05$) less time exploring the new object than the familiar one as compared with control group animals (Figure 2(D)). This neurobehavioral effect is imparted particularly by memory alteration. Chronic aluminum exposure also significantly decreased ($p < 0.05$) the passive avoidance response in animals as compared to control animals (Figure 2(E)).

6.2. Effect of aluminum chloride exposure antioxidant enzyme levels

Existing literature indicates that oxidative stress and nitrosative stress is a major causative factors in aluminum-induced neurotoxicity. The two main consequences of oxidative stress are increased pro-oxidant and decreased antioxidants levels. We investigated the levels of three antioxidant markers in the brain including GSH, SOD and catalase (Figure 3(A–C)). There was a significant difference in brain GSH level was observed in this study. Exposures of aluminum, for 24 weeks resulted in a significant depletion in GSH level as compared to the control group. SOD is one of the important enzymes in maintaining the intrinsic antioxidant defense system. Chronic exposure to aluminum chloride marginally decreased the SOD level; however, it was not found to be significant. Catalase is one of the most crucial antioxidant enzymes which mitigates oxidative stress by destroying the cellular hydrogen peroxide to water. Chronic exposure to aluminum significantly decreased the catalase level in the brain compared to control rats.

6.3. Effect of aluminum chloride on oxidative stress biomarkers

Oxidative stress elevates intracellular levels of reactive oxygen species (ROS) that cause damage to lipids, proteins, and DNA. Aluminum accelerates oxidative stress biomarkers in the brain by producing nitrite, TBARS and ROS (Figure 3(D–F)). Although aluminum exposure marginally increased nitrite level in the brain but this was not significant (Figure 3(D)). Thiobarbituric acid reactive substance (TBARS) are widely used markers for lipid peroxidation. Reactive oxygen species causes lipid peroxidation. In our study, we found that TBARS level was significantly increased after chronic aluminum intoxication (Figure 3(E)). We found that long-term aluminum exposure causes a significant increase in brain ROS levels (Figure 3(F)).

6.4. Alteration in the levels of $A\beta_{1-42}$ and phosphorylated tau (p231) protein and acetylcholinesterase enzyme in rat brain tissue

Our further study explored the effect of chronic aluminum chloride (20 mg/kg) exposure on the level of key structural pathological markers, $A\beta_{1-42}$ and phosphorylated tau, aggregation in the brain using rat ELISA kits. $A\beta_{1-42}$ was found to be significantly increased in an aluminum group as compared to the control (Figure 4(A)). Similarly, the level of phosphorylated tau (p231-Tau) was found to be significantly higher in aluminum-treated groups (Figure 4(B)). Evaluation of these structural biomarkers of AD clearly demonstrates

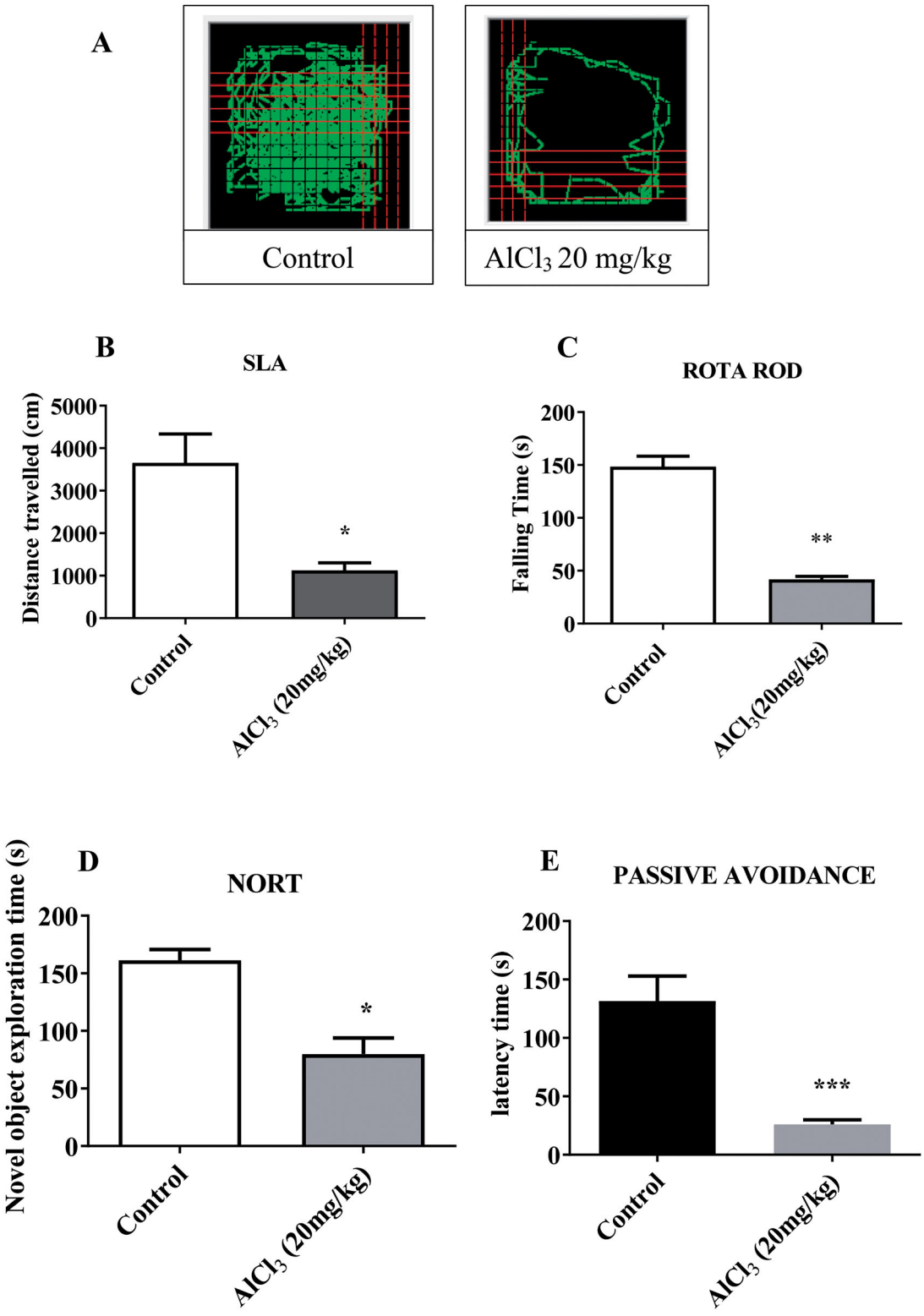

Figure 2. Effect of chronic $AlCl_3$ exposure on (A) locomotor path track of animals in animal activity meter (B) spontaneous locomotor activity, (C) neuromuscular coordination using Optovarimax apparatus in Rota rod test, (D) novel object exploration and (E) passive avoidance test. Values are presented in mean ± SEM ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs control.

that chronic aluminum intoxication has possible neurotoxic effects *in-vivo*.

We also observed that there was an increase in enzyme activity of acetylcholinesterase in brain tissue of animals exposed to aluminum as compared to the control group (Figure 4(C)). Brain cholinergic functions are primarily dependent on the neurotransmitter acetylcholine (ACh) which is one of the major cholinergic neurotransmitters hydrolyzed and inactivated by acetylcholinesterase (AChE). Increased AChE activity may lead to a decreased level of ACh

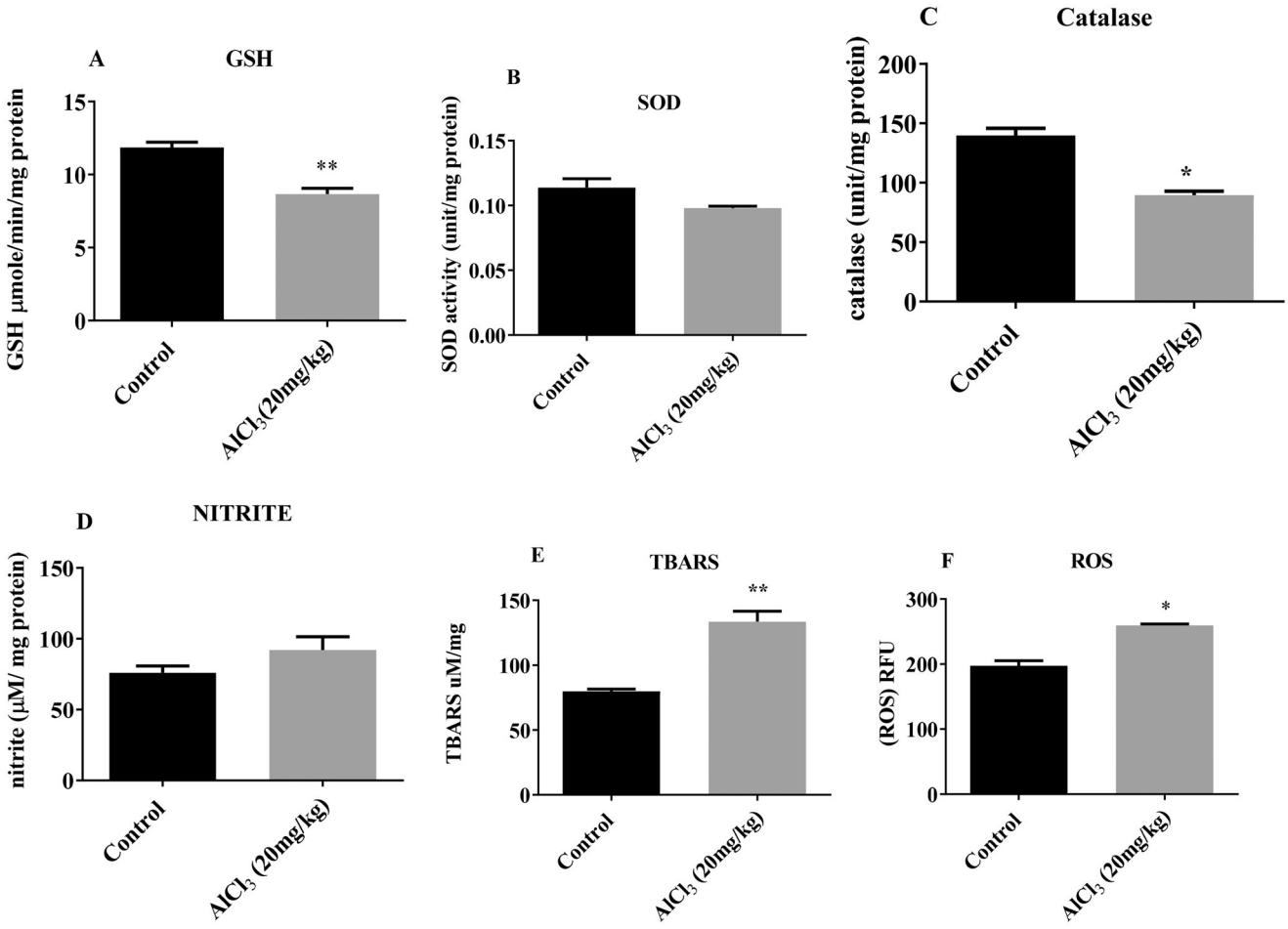

Figure 3. Effect of chronic exposures of aluminum chloride on (A) GSH, (B) SOD, (C) catalase, (D) nitrite (E) TBARS and (F) ROS in supernatants of brain homogenate. All the values are presented in mean ± SEM ($n = 5$). *$p < 0.05$, **$p < 0.01$ vs Control.

in the brain and is predominantly associated with cholinergic deficits in AD.

6.5. Levels of neuroinflammatory cytokines (TNF-α, IL-6 & IL-1β) in brain tissue of animals

We found that chronic aluminum chloride exposure in rats led to a significant increase in the levels of TNF-α, IL-6 & IL-β levels in the brain as compared to the control group (Figure 5(A–C) respectively). These findings are in concur with the evidence that aluminum exposure causes an increase in both oxidative stress and inflammatory events. Initially, Aβ aggregates and oxidative stress in the brain might be responsible for triggering the activation of inflammatory cytokines through brain macrophage and microglial cells. These cytokines may, in turn, be responsible for the exacerbation of AD pathology.

7. Discussions

Aluminum is a well-known neurotoxicant present in the environment and may contribute to cognitive dysfunctions and AD. The existing evidence indicates that there are three possible routes of aluminum entry into the brain from either systemic circulation or the site of absorption. Aluminum fluxes into the brain across the blood-brain barrier (BBB), the choroid plexuses and the nasal cavity (Yokel 2001; Wang 2018). However, the redistribution of aluminum out of the brain is slow, hence aluminum may get accumulated in the brain for a longer time (Wang 2018). Existing literature indicates that there is an increase in aluminum content in the aging brain tissue (Yokel 2001; Wang 2018). In past decades several efforts have been made to understand the mechanism of aluminum-induced neurological effects, but it is still not much clear. The neuronal effects due to long-term aluminum exposure are characterized by behavioral alterations and significantly increased oxidative stress. Herein, our main results showed that $AlCl_3$ exposure to animals led to a decrease in the recognition index and step-through latency in behavioral tests respectively. Also, it caused significant lipid peroxidation and diminished enzymatic and non-enzymatic antioxidant activities by decreasing levels of superoxide dismutase, catalase and GSH. Although greatly controversial issues about aluminum neurotoxicity mechanisms are existing, oxidative stress is considered as the potent reason for the occurrence of aluminum-induced neurotoxicity and the main pathological factor in the neurodegenerative mechanism of Alzheimer's disease. Aluminum ions have been shown to cause various adverse effects in the mammalian brain. Despite the single oxidation state of aluminum, Al^{3+}, has a strong affinity for negatively charged oxygen-donor ligands,

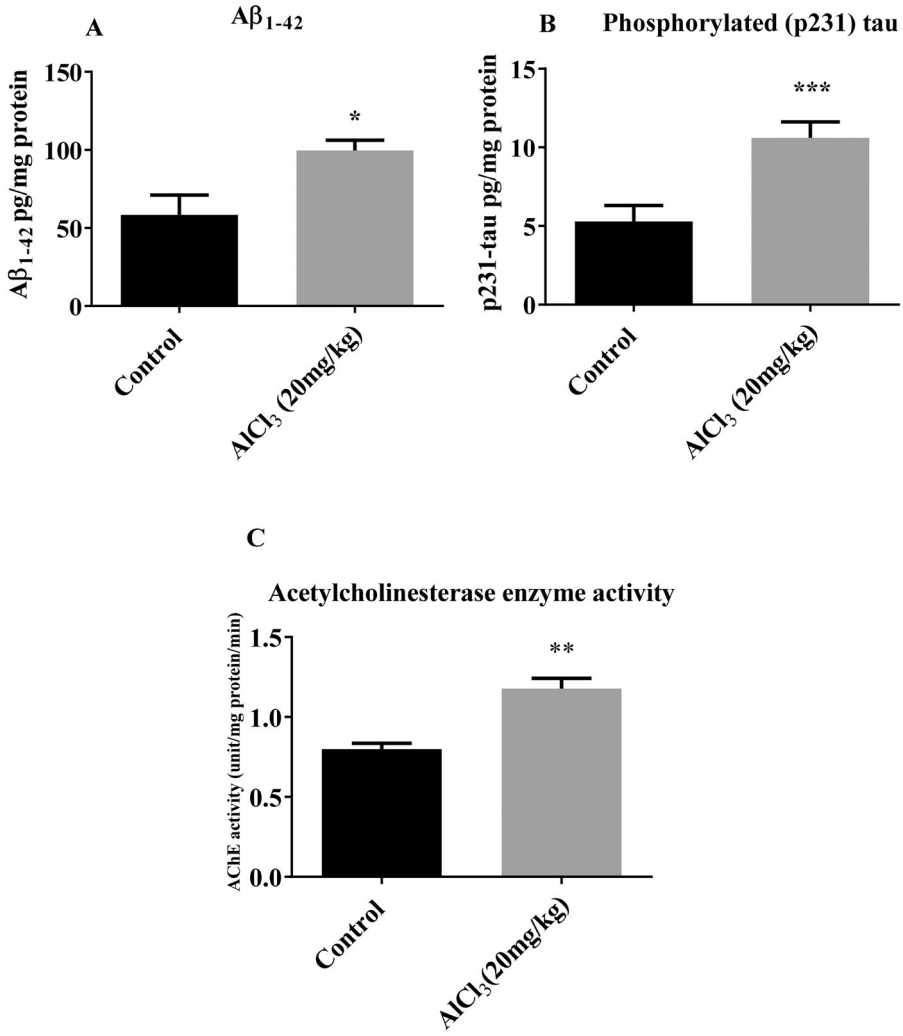

Figure 4. Effect of chronic exposures of aluminum chloride on levels of (A) $A\beta_{1-42}$, (B) p231-tau and (C) levels of AChE in brain tissue of animals. All the values are presented in mean ± SEM ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, vs control.

including inorganic and organic phosphates, carboxylate, hydroxyl groups and amino acid side chains which facilitates its binding to many cellular molecules, e.g. DNA, RNA, proteins, ATP, amino acids (Mujika et al. 2014). As a result, exposure to aluminum may lead to altered gene expression, which ultimately elevates apoptotic events and neuronal death and in the brain. AD is a multifactorial disease caused by cholinergic system disruption, oxidative stress, inflammation, mitochondrial dysfunction, and neuronal apoptosis (Bamberger and Landreth 2002; Selkoe 2002; Ferreiro et al. 2012). Therefore, the present study was aimed to investigate the mechanism of *in-vivo* neurotoxic effects of aluminum and its role in the development of AD.

Our results demonstrate the effect of 24 weeks of chronic oral administration of aluminum on neurobehavioral, neuroinflammatory and neurodegenerative changes in an animal model. Such chronic exposures caused significant activation of structural biomarkers and neuroinflammatory alterations in the rat brain along with neurobehavioral changes. It was observed that exposure to aluminum chloride (20 mg/kg) alters the locomotor activity of animals, falling time in Rotarod and retention time in passive avoidance was decreased. Novel object exploration time was also reduced in the aluminum exposed group as compared to the control group. All these results support the past findings, long-term exposures of aluminum impairs memory and locomotor dysfunction (Kaizer et al. 2008; Erazi et al. 2011; Farhat et al. 2017).

Oxidative damage is not only an important pathogenic factor in AD but also a common inducement of neuronal death. Several studies have demonstrated that oxidative/nitrosative stress plays a pivotal role in the pathogenesis of neurodegenerative disorders. Oxidative stress is induced by excessive production of free radicals and reactive oxygen species (ROS) and imbalance in the antioxidant system. It has been reported that cumulative oxidative stress alters mitochondrial dysfunction, causes oxidation of protein, lipids and DNA, which in turn cause cellular damage and death (Hoogendam et al. 2017). To study the toxic manifestations of aluminum, oxidative stress variables were studied. Our result demonstrates that there was dyshomeostasis of the antioxidant and pro-oxidant levels in brain tissue in the form of decreased GSH, SOD and catalase levels with a simultaneous increase in the levels of ROS, nitrite and MDA in an aluminum-treated group. So, our study demonstrates chronic exposure to aluminum causes oxidative stress and

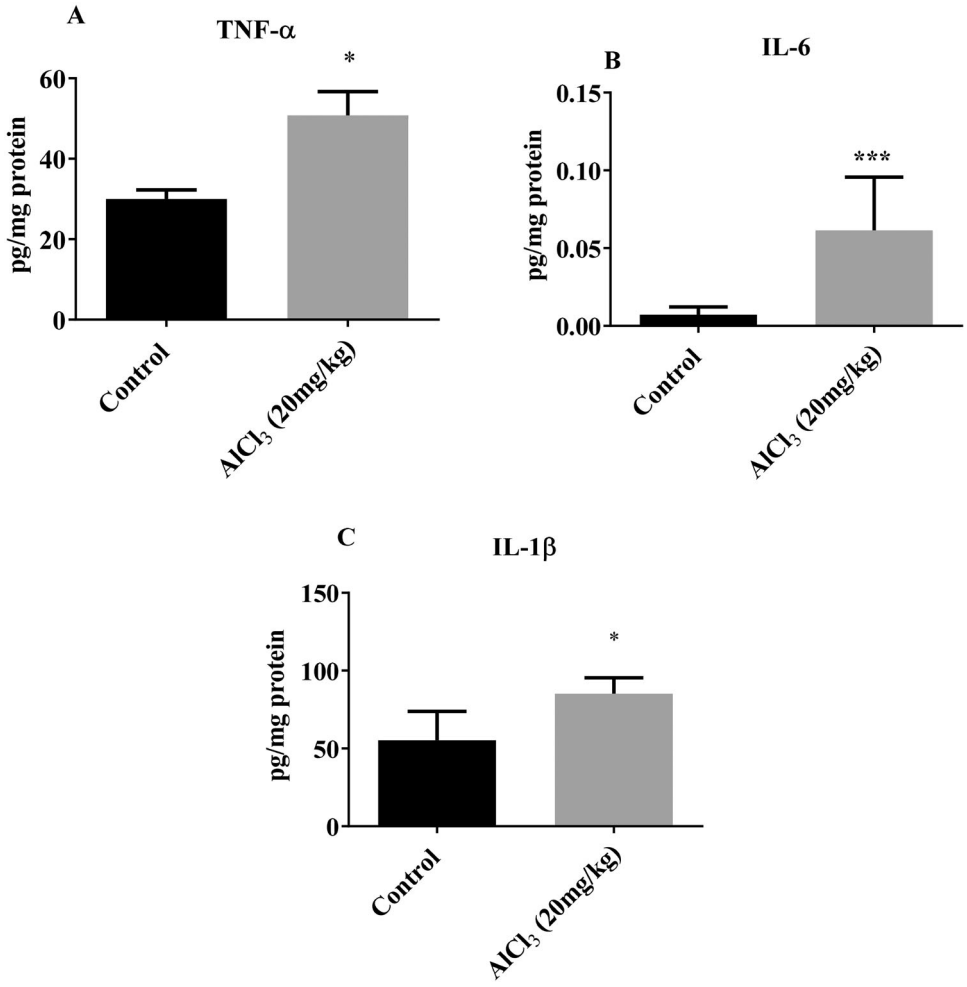

Figure 5. Effect of chronic exposures of aluminum chloride on proinflammatory cytokines including (A) TNF-α, (B) IL-6, and (C) IL-1β in supernatants of brain tissue homogenate. All the values are presented in mean ± SEM ($n = 5$), $^{*}p < 0.05$, $^{***}p < 0.001$ vs Control.

exacerbates neuronal structural and inflammatory biomarkers in the brain tissue of animals.

To find out the influence of oxidative stress and neuroinflammation in amyloid and tau pathology we determined the level of amyloid-beta and tau in the rat brain. Several past findings have demonstrated that aluminum influences Aβ aggregation *in-vitro* and *in-vivo* (Clauberg and Joshi 1993; Exley et al. 1993; Zhang et al. 2019a). Tau proteins are microtubule-associated proteins, which stabilize the neuronal cytoskeleton. Aluminum significantly promotes tau phosphorylation and aggregation in the cortex and hippocampus, this may lead to memory and learning deficits (Justin Thenmozhi et al. 2016; Prema et al. 2016). In the present study, we found that aluminum exposure promotes Aβ and p231-Tau aggregation significantly. These findings emphasized the mechanistic role of aluminum as a relevant contributory factor (Figure 6) in the progression of AD pathology.

Acetylcholine is involved in the cholinergic transmission in the brain and acts as a neuromodulator in the brain (Farhat et al. 2017). Impairment in the cholinergic system manifests dementia in patients with AD (Haam and Yakel 2017). Currently, acetylcholinesterase enzyme (AChE) inhibition is targeted as a treatment strategy for slowing down the progression of AD by inhibiting its degradation at the synapse, hence enhancing the cholinergic transmission and delay in the progression of the disease (Ravi et al. 2018). We noted an increase in the acetylcholinesterase activity in the brain in aluminum-treated group compared to the control group. In recent findings increase in AChE is reported in mouse brains after long-term exposures to aluminum (Ravi et al. 2020). This suggests a notable cholinergic deficit function after chronic aluminum exposure due to degradation of Ach by AChE.

Neuroinflammation may trigger the secretion of a range of different pro-inflammatory cytokines which further aggravate the process of neurodegeneration during AD and other neurodegenerative diseases (Lu et al. 2009; Leng and Edison 2021). Recent findings demonstrated that there is an elevation in TNF-α in cerebrospinal fluid (CSF) and serum during the progression of disease in AD patients (Chang et al. 2017). The proinflammatory cytokines such as IL-1β, IL-6 induce oxidative stress, resulting in neuronal dysfunction and neurodegenerative disorders (Wang et al. 2015). In recent studies it has been shown that orally administered aluminum chloride increased the mRNA levels of TNF-α, IL-6, IL-1β in the hippocampal region of the rat brain (Cao et al. 2016). Aluminum has a role in the induction of neuroinflammation during the pathogenesis of Alzheimer's disease, and it has been shown

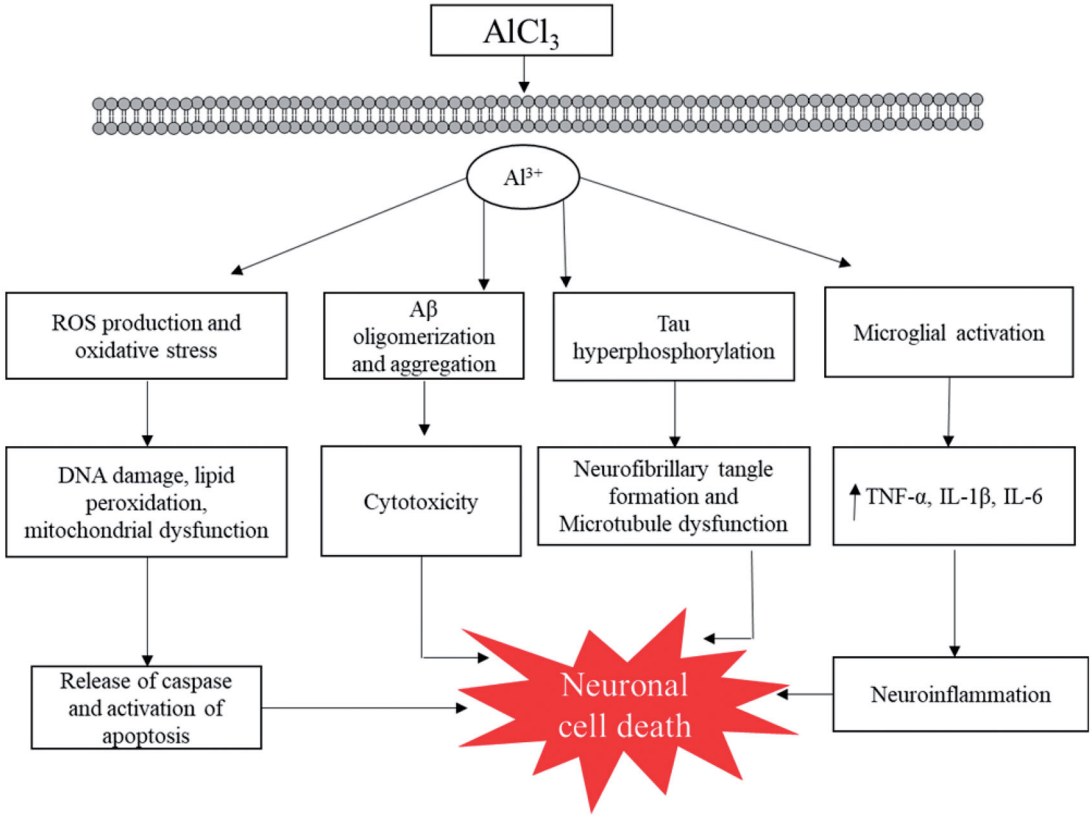

Figure 6. Mechanistic pathway of aluminum-induced neurotoxicity.

to dose-dependently increase TNF-α and IL-1α in mouse brain. In our study, we found a significant elevation of these pro-inflammatory markers (TNF-α, IL-6 and IL-1β) in the aluminum exposed group as compared to the control group.

Taken together, our study provides a basic understanding of aluminum-induced toxicity in the brain on chronic exposure through the oral route. This model also provides a close representation of a realistic situation of low dose, chronic exposure of aluminum in animal models. Further studies may be planned in this model to evaluate the alleviating effects of probable phytochemical or synthetic pharmaceutical therapeutic agents of aluminum-induced neurotoxicity in the brain.

8. Conclusions

The present study confirmed that chronic aluminum exposure may lead to induction of oxidative stress, and increased levels of structural and functional hallmarks (AChE, $A\beta_{1-42}$, phosphorylated-tau) and neuroinflammatory cytokines in rodent brain tissue. This is largely attributed to the neurodegenerative and neuroinflammatory effects of aluminum chloride and may be responsible for neurobehavioral and biochemical alterations *in-vivo*. Further molecular mechanistic studies may be performed to find a suitable target of intervention in aluminum-induced neurotoxicity.

Acknowledgement

The authors acknowledge the Department of Pharmaceuticals, Ministry of Chemicals and Fertilizers, Government of India, for the financial support and providing necessary infrastructures and facilities.

Disclosure statement

The authors declare no conflict of interest.

Funding

The author(s) reported there is no funding associated with the work featured in this article.

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