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Original Research Article

Role of Aluminium in Alzheimer's disease: Ultrastructural Study in Rat Hippocampus.

Buddhadeb Ghosh *[1], Akhtaruzzaman [2], Shukchand Hansda [3], Suman Yadav [4], Ravi Kant Sharma [5].

*[1] Department of Anatomy, Apollo Institute of Medical Science & Research, Hyderabad, India. **ORCiD:** 0000-0001-8688-3817

[2] Department of Anatomy, Jalpaiguri Government Medical College & Hospital, West Bengal, India. **ORCiD:** 0000-0002-5843-3904

[3] Department of Anatomy, Institute of Post Graduate Medical Education & Research, Kolkata, West Bengal, India. **ORCiD:** 0000-0002-6819-9904

[4] Department of Anatomy, Dr Rajendra Prasad Government Medical college, Kangra, Himachal Pradesh, India. **ORCiD:** 0009-0004-7902-6381

[5] Department of Anatomy, Government Medical College, Amritsar, Punjab, India.

ABSTRACT

Background: Exposure to high levels of aluminium (Al) leads to neurotoxicity. Hippocampus is one of the preferred sites of aluminium accumulation. Nevertheless, the role of Al in Alzheimer's disease (AD) remains controversial and there is little proof directly interlinking Al to AD.

Aims: The present study was undertaken to find out the occurrence of AD pathogenesis in Hippocampus under moderate aluminium exposure in rats.

Materials and Methods: Adult rats were divided into control (C) and aluminium treated (E) groups having eight animal each. The rats in group E were exposed to aluminium 4.2 mg/kg body weight for three months with due approval from Institute Animal Ethics Committee. The hippocampus was processed for histopathological and electron microscopy observation.

Results: Moderate Al intake produces significant reduction in the count of Pyramidal cells in hippocampus identified by shrunken cells as well as pyknosis in cell bodies. The differences between the cell numbers in all groups were found to be statistically significant ($P < 0.05$). Cornu Ammonis (CA) exhibited significantly reduced nissl bodies with a marked reduction in neuronal cell loss. Neurofibrillary tangle and plaques were not seen in the given dose of Al exposure. Electron microscopy from experimental group showed that the majority of neurons were disintegrating, the nuclear membrane has ruptured, and nucleoli appeared significantly distorted. The chromatin condensed and the mitochondria had disintegrated. Many vacuoles and lipofuscin sediment in cytoplasm, as compared to the control group noted.

Conclusion: Present data demonstrated that moderate chronic aluminium exposure 4.2mg/kg body weight induced neurodegeneration in hippocampus but not significant for Alzheimer's disease pathogenesis.

KEYWORDS: Aluminium, Hippocampus, Alzheimer's Disease, Neurodegeneration, Histopathology, Electron microscopy.

Corresponding Author: Dr. Buddhadeb Ghosh, Department of Anatomy, Apollo Institute of Medical Science & Research, Hyderabad, India. Mob +918894114210.
E-Mail: dr.ghosh_b@apolloimsr.edu.in / dr.ghosh86@gmail.com

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INTRODUCTION

Brain is a susceptible organ for both aluminium (Al) toxicity and oxidative injury. Neurotoxicity caused by Al exposure is well evidenced

of which is oxidative injury [1,2]. In the 70s of and one of the suggested possible mechanisms last century, the medical fraternity considered the toxicity of, so called 'biologically inert' metal, Al. A large volume of research, since then, is conducted to get insight and fight against the Al toxicity. While the awareness about the toxic effects is bringing down the daily and avoidable uses of the Al in developed countries, being the cheap, Al wares are the commonly used cooking utensils and containers in India. Neurotoxic effects of Al are well established and specific effects on human central nervous system such as memory loss and impaired coordination are documented. Al is used widely in our day to day life. Al consumption is through food, beverages, drinking water and aluminium containing drugs [3]. The other sources of Al are food additives, containers, cookware, utensils and food wrappings [4]. Al is also present in tea leaves. The reported concentration of Al is 0.3 per cent Al in older leaves and about 0.01 per cent in younger ones. A very recent study showed that glue sniffing is an important problem among teenagers [5].

The hippocampus is a region in which neurogenesis persist throughout life. In the hippocampus, neural progenitor cells (NPCs) located along the sub granular zone (SGZ) produce new granule neurons in the hippocampal dentate gyrus (DG), which play an important role in spatial learning and memory [6]. Chronic exposure to Al not only causes neurologic signs, which mimic progressive neurodegeneration, but also results in neurofilamentous changes in the cerebral cortex, hippocampus. Al or aluminium chloride (AlCl3) role in exacerbating neurodegenerative disorders like Alzheimer's disease (AD) and Parkinsonism dementia are under debate [7].

AlCl3 exposure leads to impairment in learning, memory and cognition function as observed both from clinical and from animal experiments [8]. Deposition of Amyloid beta (Aβ) is an early and critical event in the pathogenesis of AD first forming in temporal cortical regions including the hippocampus a region implicated in memory formation [9]. It was proposed that Aβ aggregates to form neurotoxic plaques, which lead to neurode generation accompanied by dementia. The present study was undertaken to find out the occurrence of histopathological changes in the adult rat hippocampus following moderate aluminium consumption.

MATERIALS AND METHODS

Sixteen wistar rats include equal number of male and female rats of an average weight of 200 gm and an average age of 120 days were used in this study. Animals were kept individually in plastic cages in noise free, air-conditioned animal house with temperature maintained at 75°F and on a light dark cycle of 12: 12 hours. Humidity was maintained with a minimum of 50%. Rats were fed on diet pellets, tap water ad libitum and treated with utmost humane care. The experimental study was carried out from 2015 to 2022 after due approval from the Institute Animal Ethics Committee, Registration no 668/02/C/CPCSEA and the procedures were performed according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, India). After one week of acclimatization, rats were randomly divided (with the help of Random Allocation Software Version 1.0, May 2004) into 2 groups namely-

Group C (Control group, 8 animals) received the normal saline water (Sodium chloride) for 3 months.

Group E (Experimental group, 8 animals) received aluminium chloride (4.2mg/kg body weight) dissolved in drinking water containing sodium chloride (0.9%) for 3 months.

The treatments were carried out through oral feeding gavage once daily. Their weights were recorded daily. After 3 months, the animals were anaesthetized with pentobarbitone (i.p) and an intracardiac perfusion of normal saline followed by 4% paraformaldehyde saline was performed. The brains of both groups of animals were dissected out and blotted. The brains of both groups of animals were extracted on an ice chilled tray and sliced. Right hippocampus was fixed in formalin for histopathological staining and left

hippocampus fixed in Karnovsky solution (4° celsius) for electron microscopy observation.

Histological study: Formalin fixed right brain tissue was carefully dissected to isolate the hippocampus. The tissue was processed for routine paraffin embedding and stained with haematoxylin eosin (H&E) staining and Cresyl Violet staining according to John D Bancroft Theory and Practice of Histological Techniques [10].

For haematoxylin eosin staining slide were deparaffinized through xylene (2-3 min) and absolute alcohols (1-2 min) then dipped in 95 % alcohol followed by 70% alcohol 50% alcohol 30% alcohol. Then washed thoroughly with distilled water and placed in haematoxylin for 3-5 min and then examined the section after rinse with distilled water under low magnification of microscope to confirmed its over stained. Differentiate in acidified alcohol confirming the desired destaining with microscope. After confirmation of nuclei, Blueing the section in running tap water. Then rinsed in distilled water and placed the slide in another jar of 30% alcohol for 3 min. Then placed in 50% alcohol and followed by 70% alcohol and 95% alcohol. Then the slide was counter stained in 0.5-1 % eosin in 90% alcohol for 30 second to 1 min until the cytoplasm take deep pink stain. Then dipped in 95% alcohol for few second and placed it to absolute alcohol for 3 min. To ensure full dehydration it was kept in next absolute alcohol for 3 min. Then the slide was transferred in xylene for 2 min and followed by next xylene for 2 min until the section appears absolutely clear or transparent. The slide was mounted in DPX (Dibutyl phthalate Polystyrene Xylene) after rinsing well in xylene.

Cresyl violet staining slide were deparaffinized through xylene (2-3 min) and absolute alcohols (1-2 min) then dipped in 95 % alcohol followed by 70% alcohol 50% alcohol 30% alcohol. Then placed in with 0.5 % filtered Cresyl fast violet; stain for 10 minutes. And then rinsed with distilled water. Then the slide was differentiated in 0.25% acetic alcohol until most of the stain has been removed (4 - 8 seconds). The slide was pass through the absolute alcohol into xylene and it was checked microscopically for appropriate staining. The slide was mounted in DPX (Dibutyl phthalate Polystyrene Xylene) after rinsing well in xylene. The slides were then air dried and viewed under a light microscope and photomicrographs were taken at a total magnification of 40X, 100X and 400X.

Electron microscopy study: Left brain hippocampus samples of control and aluminium treated group were fixed in 2 % paraformaldehyde and 2.5 % glutaraldehyde (karnovsky solution) in 0.1 M PB, pH 7.3 for 4 hrs at 4°C. After wash, samples were post fixed in 1 % osmium tetroxide for 1 h, dehydrated in acetone, infiltrated and embedded in Araldite CY212. Thick sections were cut in 1 mm and stained in toluidine blue and examined under a light microscope. Thin sections (70–80 nm) were cut, and every tenth sections were mounted on copper grids. The grids were stained with uranyl acetate and lead citrate and viewed under a Morgagni 268D transmission electron microscope (FEI Company, Eindoven, The Netherlands). Digital images were acquired at magnification range 2500–10000 using iTEM software (Soft Imaging Systems, Munster, Germany).

Statistical Analysis: The treated group were compared to control group using T test. The collected data were expressed as the mean ± SD. Difference with values of $p < 0.05$ was considered statistically significant.

RESULTS

Representative photomicrographs of hippocampus from control groups in Figure.1 (A, B & C) and Figure.2 (A, B & C) showing cornu ammonis (CA) with its structure stratum oriens (SO), stratum pyramidalis (SP), stratum radiatum (SR) and stratum moleculare (SM) and dentate gyrus (DG) with its three layers molecular (M), granule (G) and plexiform (P). The pyramidal cell layer in hippocampus of Al exposed rats showing reduced number of neuronal cells with dead neurons that are identified by shrunken cells and as well as nonhomogeneous acidophilic stained cytoplasm in Figure.1 (D, E & F). Cytomegaly and enucleation also seen in the experimental groups. Cornu ammonis of Al exposed rats

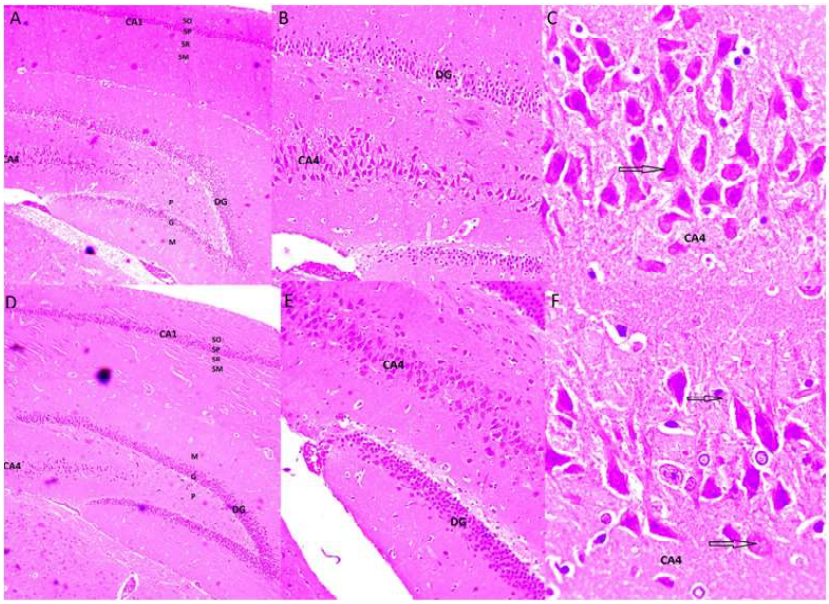

Fig. 1: Photomicrograph of the hippocampus from the control group in figure A (40X), figure B (100X) & figure C (400X) and aluminium treated group in figure D (40X) & figure E (100X) & figure F(400X). CA (Cornu ammonis), SO (statum oriens), SP (stratum pyramidalis), SR (stratum radiatum), SM (stratum molecular). DG (Dentate gyrus), P (plexiform cell layer) G (granular cell layer) M (molecular cell layer). Normal pyramidal neurons (arrow) in figure C and dead neurons (arrow) in figure F. Haematoxylin & Eosin staining.

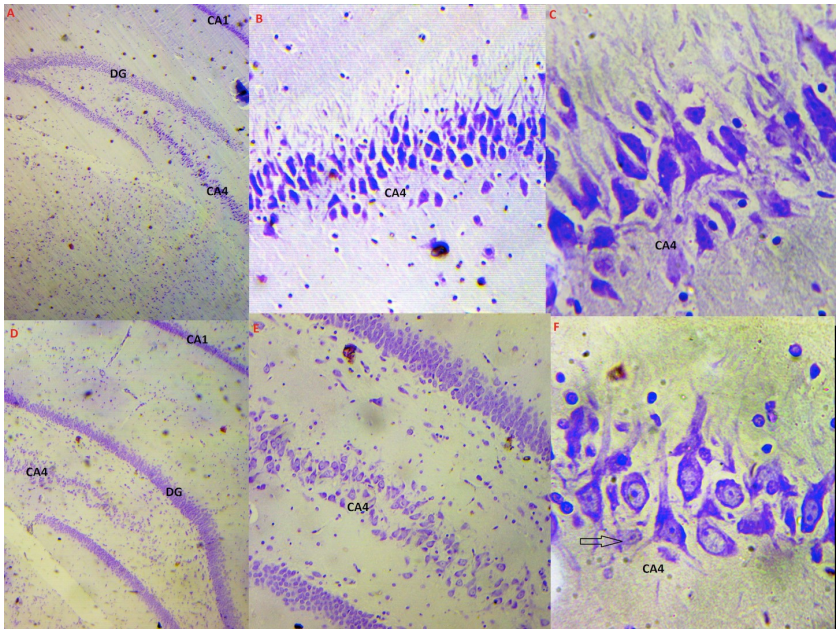

Fig. 2: Photomicrograph of the hippocampus from the control group in figure A (40X), figure B (100X) & figure C (400X) and aluminium treated group in figure D (40X) & figure E (100X) & figure F(400X). CA (Conu ammonis), DG (Dentate gyrus). Loss of Nissl substance (arrow) in the pyramidal cell noted in the treated group as compared to Control group. Cresyl Violet staining.

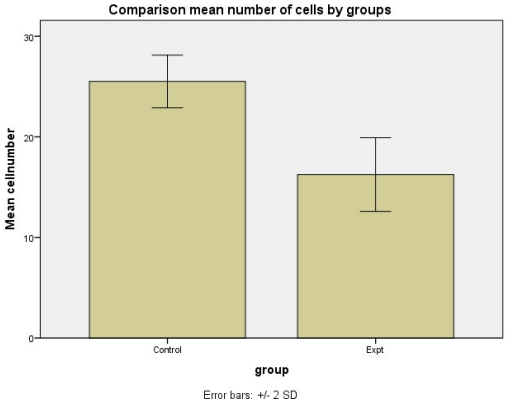

Fig. 3: Comparison of Pyramidal cell number per slice (under 40X magnification scale bar = 20 µm) between control and experimental group (Expt) of animals. The numbers of the cells (mean ± SD) decreased in the experimental groups. The result is significant at $p < 0.05$.

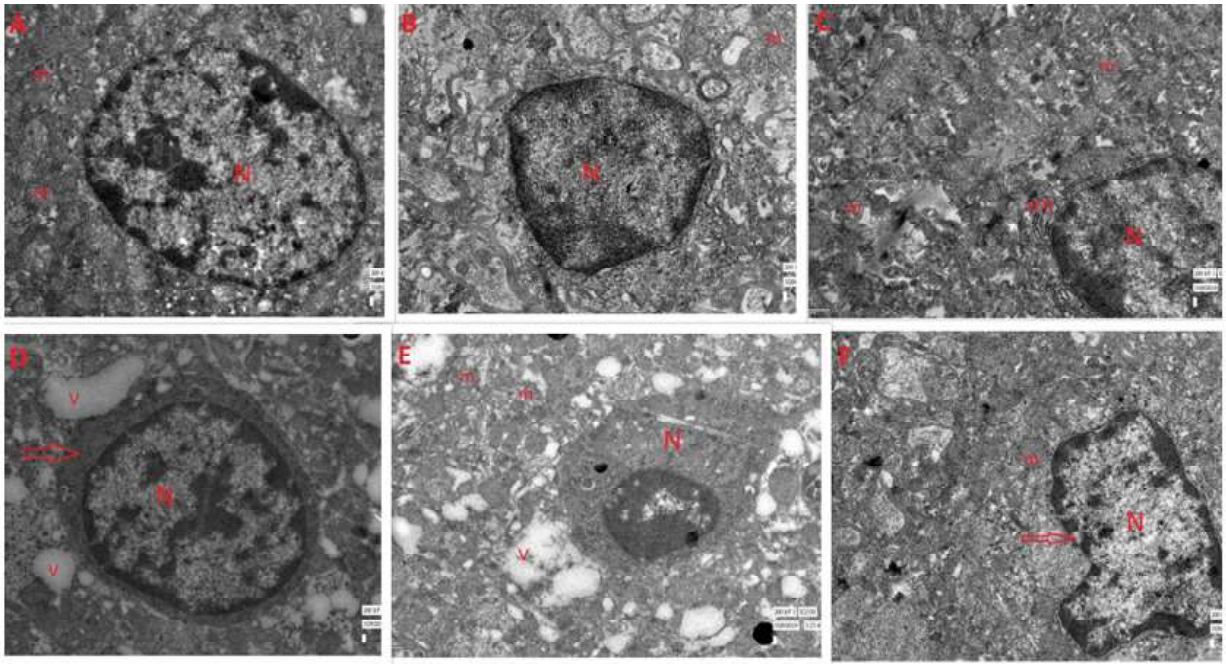

Fig. 4: Electron microscopy photograph of the hippocampus from the CA region control group in figure (A, B & C) and aluminium treated group in figure (D, E & F). Nucleus (N), rough endoplasmic reticulum (rER), mitochondria (m), Intracytoplasmic granulovacuolar degeneration (arrow) in figure D, Ruptured nuclear membrane (arrow) in figure F. vacuoles (v). Scale bar = 1µm.

exhibited significantly reduced nissl bodies with a marked reduction in neuronal cell loss as well as pyknosed cell bodies in Figure.2 (D, E & F). The numbers of the cells (mean ± SD) decreased in the experimental groups found in graph Figure.3 and to be statistically significant ($P < 0.05$).

Electron microscopy of CA pyramidal neurons obtained from control group in Figure.4 (A, B & C) and aluminium (Al) treated in Figure.4 (D, E & F). The control group showing a large round nucleus with the homogeneous karyoplasms and chromatin was uniformly distributed and contains well developed rough endoplasmic reticulum and several dense bodies and mitochondria. In the Al treated group, the majority of neurons were disintegrating, the nuclear membrane has ruptured, and nucleoli appeared significantly distorted (Figure.4F). The chromatin condensed or dissolved, and the mitochondria had disintegrated and nucleoli appeared significantly distorted and the cells represent advanced states of apoptosis (Figure.4E). Many vacuoles and lipofuscin sediment in cytoplasm seen, as compared to the control group. Perinuclear accumulation of swollen mitochondria and intracytoplasmic granulovacuolar degeneration seen (Figure. 4D).

DISCUSSION

Brain is particularly vulnerable to Al and is the primary target organ of Al toxicity. The accumulation of Al in brain might result from the increased permeability of the blood brain barrier [11,12]. Hippocampus is one of the preferred sites of Al accumulation. Following chronic Al exposure, significant Al accumulation in hippocampus had been reported in hippocampus. In susceptible subjects, through promotion of oxidative stress, Al can produce hippocampal lesions characterized by dysfunctional microtubule. In addition, damaged neuritis, loss of synapse along with depletion of pyramidal cells were observed in the hippocampus of Al exposed animals. Shrunken cytoplasm with deeply stained eccentric nucleus along with degenerating neurons were also reported in the hippocampus of Al intoxicated rats. Apart from neurofibrillary degeneration and elevated amyloid precursor protein accumulation granulovacuolar degeneration had been documented in hippocampus upon exposure to Al. Typical neuropathological changes were also observed in the hippocampus of AD model rats with oral exposure to Al and suggested to be associated with higher Al accumulation. In this study, we demonstrated morphological changes of

hippocampus nerve cells in Al exposed groups. We also showed the reduced nissl bodies with a marked reduction in neuronal cell loss in cresyl violet staining. The layer of pyramidal cells in hippocampus of Al exposed rats became thinner with dead neurons that are identified by shrunken cells and nucleus as well as pyknosed cell bodies. Neurofibrillary tangle and plaques were not seen in the given dose of Al exposure.

Blood Al levels were positively related with hippocampal Al levels, which has been clearly confirmed. The blood Al contents in AlCl3 treated rats gradually increased with the increasing AlCl3 dose while the hippocampus coefficient decreased accordingly [13]. High dose Al exposure to rats have a high success rate, mimic AD, and a cost-effective animal model, with memory impairment, neuron loss, neurogliocyte proliferation, and senile plaque (SP) and neurofibrillary tangle (NFT) aggregation in the brain. However, the high dose of Al may cause rat liver injuries and anorexia, accompanied with decreased weight [14].

Previous research showed that AD brains contain increased levels of Al. Atom absorption spectrography revealed that Al content is (3.6±2.9) mg/g dry weight in AD brain, higher than the content (1.8±0.8) mg/g in normal human brain. Brain cell degeneration can be produced when Al level is above 4 mg/g [15]. Free Al may enter the brain via olfactory nerves and by crossing the blood-brain barrier. Al can replace calcium and magnesium, binding to glutamic acid and arginine in the amino acid chain, which yields to the formation of stable compound of Al glutamate or Al arginine that precipitates in the cerebral cortex, hippocampus and amygdala where glutamatergic neurons are abundant. Al binds transferrin in the plasma. Abundant transferrin receptors exist in cerebral cortex, hippocampus, septal nuclei and amygdala, where Al is prone to form deposits [16].

AD is a neurodegenerative disease with cognitive and memory dysfunctions. The main pathological features of AD are the formations of SPs and NFT, as well as loss of cholinergic neurons in the basal forebrain [17]. The studies on a variety of Al salts such as Al lactate, AlCl3, Aluminium fluoride and aluminium silicate on aged rabbits, showed that neurofibrillary aggregates (NFAs) are most striking in the nucleus motoris medialis and substantia grisea intermedia: the large neurons of the nucleus of the motoris lateralis are minimally involved. These results indicate that Al inorganic complexes do not mimic AD neuropathology in its distribution of pathology.

However, Al-organic and Al-inorganic complexes administered to different animal groups like cats, ferrets and dogs also did not mimic the AD neuropathology. But, Al maltolate treated aged rabbits displayed NFTs in the axons imaged in hippocampal neurons, which follows the distribution of these lesions in AD [18]. Garruto et al studied hippocampus in guamaniam patients using a method of computer controlled electron beam X-ray microanalysis and wavelength dispersive spectrometry. The elemental images showed that Al deposits occur within the same NFT bearing hippocampal neuron, suggesting this element involvement in NFT formation [19]. The interesting work carried out by Savory and his coworkers on the quantitation of Al in the brain and spinal cord and its effects on neurofilament protein expression and phosphorylation provided new evidence for the involvement of Al in AD [7]. It has been noted that intraventricular administration of Al maltolate resulted neurofibrillary degeneration in brain stem and spinal cord [20].

Al toxicity is more readily induced in adult and aged than in juvenile animals, and susceptibility to the behavioral toxicity of Al increases steadily with age [21]. Following Al lactate or tartrate subcutaneous injection for 30 days in rabbits, formations of intraneuronal NFTs are detected in brain stem, cerebral cortex and hippocampus. Administration of aluminium trichloride in rats for 3 months induces â-amyloid protein like

immune reactivity (APLI) neurons in all sectors of the dorsal hippocampal formation without significant plaques [22]. This set of data strongly indicates that Al plays a significant role in AD. In the present study we didn't find any NFT and plaques in the hippocampus. Al

has a specific toxic potential for cytoskeletal structures of brain cells. The neuronal specific markers microtubule associated protein type 2 (MAP2) and neurofilament light subunit (NF68KD) are inhibited at lower Al concentrations (IC50 180–630 mmol/L) than GFAP (IC50 700–1 000 mmol/L), demonstrating that neurons have a particularly high sensitivity to Al in comparison to astrocytes [23]. Chronic exposure to Al reduces the basal activity of guanylate cyclase and impairs the glutamate-nitric oxide-cyclic guanosine monophosphate (cGMP) pathway in vivo and in vitro. Al reduces the cerebellar content of calmodulin and nitric oxide synthase by 34% and 15%, respectively. In Al treated rats, the basal activity of soluble guanylate cyclase decreases by 66%, whereas the basal cGMP in cerebellar extracellular space decreases by 50% [24].

Following Al lactate or tartrate subcutaneous injection for 30 d in rabbits, formations of intraneuronal NFTs are detected in brain stem, cerebral cortex and hippocampus. In Alzheimer's disease (AD), the content of Al in brain is considerably high, and Al accumulates in its characteristic lesions including senile plaques and neurofibrillary tangles [25].

Hippocampus possesses the structural and functional plasticity at the synaptic level [26]. Our observation showed that Al induced morphological abnormalities of hippocampal cells in rats. As the AlCl3 dose increased, the impairment worsened gradually and led to decreased cell number, disordered cell arrangement and reduced dendrites. Hippocampus morphology alterations at tissue level might be due to the accumulation of Al in this brain region, which was consistent with the reduced hippocampus coefficient in AlCl3-treated rats.

Moreover, structure integrity is important in neuronal metabolism, and the structure damage is linked to some neurological disorders. Abnormal neurons have an adverse influence on spatial navigation in rats [27]. According to World Health Organization (WHO) provisional tolerable weekly intake is 2 mg aluminum/kg body Weight. Biological tolerance value at the workplace derived on the basis of neurotoxicity as the critical endpoint 50 ìg/g creatinine (BAT value); not classified into a carcinogenicity category according to German Research Foundation. The tolerable weekly intake set by the European Food Safety Authority (EFSA) is 1 mg aluminium/kg body weight can be reached through dietary exposure alone [28]. In this study, we observed that Al at 4.2 mg/kg body weight damaged neuronal ultrastructure in hippocampus, including shrunk nucleus areas, condensed nuclear density, increased heterochromatin and expanded cytoplasmic vacuolar areas in the hippocampus. These neuronal ultrastructure and morphology changes in hippocampus both indicated neuronal karyopyknotic and degeneration and could be the physiological basis of Al impairing long term memory. Researchers have confirmed that AlCl3 (40 mg/kg/day for 6 months) had obvious adverse effects on learning and memory capacity of rats [29].

Previous studies also demonstrated that Al exposure for 3 months could damage the behavioural performance of rats in water maze test [26]. In agreement with previous studies, our results in this study also showed that sub chronic Al exposure could similarly impact on neuronal cell damage. Moreover, the present data explain that Al exposure with moderate dose damage the neuronal cells in the hippocampus.

CONCLUSION

The results discussed here have broad implications for the role played by aluminium in neurodegenerative diseases, and suggest that long-term exposure of these metals should be avoided. Present data demonstrated that moderate chronic aluminium exposure 4.2mg/kg body weight induced neurode generation in hippocampus but not significant for Alzheimer's disease pathogenesis.

Author Contributions

Dr. Buddhadeb Ghosh: Conceptualization, Data acquisition, Data analysis or interpretation, Drafting of the manuscript, Approval of the final version of the manuscript.

Dr. Akhtaruzzaman: Data analysis or interpretation, Approval of the final version of the manuscript.

Dr. Shukchand Hansda: Data analysis or interpretation, Approval of the final version of the manuscript.

Dr. Suman Yadav: Critical revision of the manuscript, Approval of the final version of the manuscript.

Dr. Ravi Kant Sharma: Critical revision of the manuscript, Approval of the final version of the manuscript.

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Conflicts of Interests: None

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