Influence of Zinc on Calcium-Dependent Signal Transduction Pathways During Aluminium-Induced Neurodegeneration

Neha Singla · D. K. Dhawan

Abstract Metals perform important functions in the normal physiological system, and alterations in their levels may lead to a number of diseases. Aluminium (Al) has been implicated as a major risk factor, which is linked to several neurodegenerative diseases including Alzheimer's disease and Parkinson's disease. On the other hand, zinc (Zn) is considered as a neuromodulator and an essential dietary element that regulates a number of biological activities in our body. The aim of the present study was to investigate the effects of Zn supplementation, if any, in ameliorating the changes induced by Al on calcium signalling pathway. Male Sprague Dawley rats weighing 140–160 g were divided into four different groups viz.: normal control, aluminium treated (100 mg/kg b.wt./day via oral gavage), zinc treated (227 mg/l in drinking water) and combined aluminium and zinc treated. All the treatments were carried out for a total duration of 8 weeks. Al treatment decreased the Ca^{2+} ATPase activity whereas increased the levels of 3′, 5′-cyclic adenosine monophosphate, intracellular calcium and total calcium content in both the cerebrum and cerebellum, which, however, were modulated upon Zn supplementation. Al treatment exhibited a significant elevation in the protein expressions of phospholipase C, inositol triphosphate and protein kinase A but decreased the expression of protein kinase C, which, however, was reversed upon Zn co-treatment. Al treatment also revealed alterations in neurohistoarchitecture in the form of calcium deposits, which were improved upon zinc co-administration. The present study, therefore, suggests that zinc regulates the intracellular calcium signalling pathway during aluminium-induced neurodegeneration.

Keywords Aluminium · Zinc · Calcium · Neurodegeneration

Abbreviations
Al	Aluminium
Zn	Zinc
Ca	Calcium
PLC	Phospholipase C
IP3	Inositol triphosphate
PKA	Protein kinase A
PKC	Protein kinase C

N. Singla · D. K. Dhawan (✉)
Department of Biophysics, Panjab University, Sector-14, Chandigarh 160014, India
e-mail: dhawan@pu.ac.in

N. Singla
e-mail: nehasbph@gmail.com

Present Address:
N. Singla
Department of Biophysics, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Introduction

Alteration in metal homeostasis in the brain has been perceived as one of the key factors in the progression of neurodegenerative diseases. Various metals like aluminium, lead, iron, copper and mercury have been observed to be involved in the pathogenesis of neurological disorders [1, 2]. But the world over, among various central nervous system disorders, Alzheimer's has been adjudged as one of the most prevalent neurodegenerative diseases [3, 4]. Mounting evidences for the last few years have shown that Al is the most potential aetiological agent in causing Alzheimer's disease [5, 6]. Even several microscopic, spectroscopic as well as biochemical investigations have clearly elucidated the correlation between Al and Alzheimer's disease [7, 8]. Apparently, the exact mechanism through which Al manifests the progression of

disease and the molecular basis of its interaction is rather sparse and remains to be fully explored.

Human exposure to Al is mainly attributed to its wide availability in nature and through manmade sources [9–11]. Al has been reported to mediate its neurotoxicity either through its direct or indirect interaction with the neurons [12–14]. Studies have revealed that high accumulation of Al in the brain can cause several neuropathological alterations, impairment in various neurotransmitter systems and progressively leads to behaviour deficits [15–17]. Scientists have also proven that Al exposure can disturb the normal functioning of antioxidant defences system, apoptotic machinery as well as carbohydrate and lipid metabolism in the brain [18–21].

Recently researchers have found that Al can disrupt the calcium homeostasis in neurons by interacting with calcium binding sites [22, 23]. Further, studies have also indicated that disturbance in calcium concentration in the brain is associated with the development of distinct patterns of neurodegeneration as well as responsible for memory and learning deficits [15, 24, 25]. Although several theories have been put forward to unravel the mechanism behind aluminium and calcium induced neurodegeneration but the exact mechanism is not clear till date.

On the other hand, Zn is an essential micronutrient in the body and is the second important transition metal in the brain [26, 27]. It plays an important role in keeping the integrity of the blood–brain barrier and maintaining the composition of membrane lipids as well as proteins of neurons [28, 29]. Studies have also indicated its neuromodulatory role in various signal transduction processes and ion channels [30, 31]. Further, decrease in its levels is also responsible for various neurological deficits and diseased conditions [32, 33]. Evidences have also shown that low zinc status in the brain can also affect the uptake of calcium by neurons, which can progress into neurological disorders [34, 35]. Zn has also been proven as an efficient agent against various toxic conditions induced by various xenobiotics and metals in the brain [36–38]. Many in vivo *and* in vitro studies have also indicated that Zn possesses antioxidant as well as anti-apoptotic activity in the brain [39, 40]. The present study was aimed to evaluate the role of Zn, if any, during aluminium-altered calcium homeostasis and signal transduction pathways in the rat brain. Further, we are the first group which has shown an association between zinc, calcium and aluminium during neurodegeneration in experimental model.

Materials and Methods

Chemicals

Aluminium chloride and zinc sulphate were procured from Merck Ltd., whereas ^{45}Ca was procured from Board of Radiation Isotope Technology, Mumbai, (India). 3′, 5′-Cyclic adenosine monophosphate (cAMP) immunoassay kit and calpain activity assay kit were procured from Biovision (USA). Various antibodies were purchased from Merck (India), Genei (India) and Sigma (USA). All other chemicals were obtained from Sigma Chemicals Company (USA).

Experimental Design

Healthy male Sprague Dawley rats in the weight range of 140–160 g were obtained from the central animal house of Panjab University, Chandigarh, India. The animals were housed in polypropylene cages under a hygienic bed of husk in a well-ventilated animal room and were provided with standard animal feed obtained from Ashirwad Industries, Kharar, Punjab, India. The animals had a free access to diet and drinking water throughout the study. All the animals were kept at 25 °C under a standard regimen with a 12:12-h light–dark cycle (darkness 20:00–08:00 hours) and a relative humidity of 45 to 50 %. Before the beginning of various treatments, the rats were kept for acclimatization to experimental conditions for a period of 1 week. All the procedures were done in accordance with ethical guidelines for care and use of laboratory animals, which were approved by Institutional Animal Ethics Committee, Panjab University, Chandigarh, India.

To carry out the study, the animals of age 7–8 weeks were segregated into the following four groups and each group consisted of six animals: Animals of normal control group (untreated rats) were fed with normal diet and water ad libitum throughout the period of experimentation. Aluminium-treated animals were given aluminium chloride (AlCl$_3$ was dissolved in drinking water) orally at a dose level of 100 mg/kg body weight [41, 42]. Zinc-treated animals were supplemented with zinc in the form of ZnSO$_4$.7H$_2$O in drinking water, at a dose level of 227 mg/l [43, 44]. Combined Al- and Zn-treated animals were given a combined treatment of Al as well as zinc in a similar manner, as was given to aluminium- and zinc-alone-treated group animals, respectively. All the treatments continued for a period of 8 weeks.

At the end of the treatment schedule, the animals were sacrificed by decapitation after anaesthetization by diethyl ether. The brains were immediately removed, placed in ice-cold isotonic saline and dissected into cerebrum and cerebellum.

Preparation of Synaptosomes

Synaptosomes were obtained from the cerebrum and cerebellum regions of the brain by discontinuous sucrose gradient centrifugation method of Gray and Whittaker [45]. Briefly, homogenates of cerebrum and cerebellum 10 % (*w/v*) were prepared in 0.32 M sucrose in 20 mM Tris–HCl (pH 7.4) and were centrifuged at 1,000×*g* for 10 min. The supernatants

were further centrifuged at $12,500 \times g$ for 20 min to obtain crude mitochondrial pellets. The pellets were suspended in 0.32 M sucrose in Tris–HCl and layered over a gradient containing 0.8, 1.0 and 1.2 M sucrose. After centrifugation at $80,000 \times g$ for 2 h, the interfaces over 1.0 M sucrose containing synaptosomal fractions were removed. These were again pelleted by centrifugation at $80,000 \times g$ for 30 min and were re-suspended in physiological buffer, which constituted of synaptosomal preparation.

^{45}Calcium Uptake in Synaptosomes

^{45}Ca uptake was studied in polarized (low K^+) and depolarized (high K^+) synaptosomes by the method of Edelfors and Ravn-Jonsen [46]. Addition of depolarized (high potassium) medium results in opening of voltage-operated Ca^{2+} channels, which facilitates the entry of ^{45}Ca into the synaptosomes. In this procedure, freshly prepared synaptosomal preparations (20 μl) were equilibrated at 25 °C for 10 min. Reaction was initiated by the addition of 100 μl of low K^+ medium, i.e. un-stimulated (136 mM NaCl, 4.9 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11 mM glucose and 20 mM Tris–HCl, pH 7.4) or high K^+ medium, i.e. stimulated (38 mM NaCl, 106 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11 mM glucose and 20 mM Tris–HCl, pH 7.4) to synaptosomes containing ^{45}Ca, respectively. The samples were incubated for 5 min and were stopped by the addition of ice-cold lanthanum medium and later filtered through presoaked millipore filters (0.45 μm pore size, Sartorius, USA) under vacuum. The filters were washed thrice by adding lanthanum medium and dried, and the radioactivity absorbed onto the filters was counted using the scintillation cocktail on a liquid scintillation counter. The scintillator (1,000 ml) used was comprised of 60 g naphthalene, 4 g PPO, 0.2 g POPOP, 20 ml ethylene glycol and 100 ml methanol, and the volume was made 1,000 ml with 1,4 dioxane [47]. The uptake at zero time represented the nonspecific binding of ^{45}Ca to the synaptosomes. The net potassium-induced ^{45}Ca uptake (ΔK) was obtained by subtracting the un-stimulated ^{45}Ca uptake from stimulated ^{45}Ca uptake.

Preparation of Synaptic Plasma Membrane

Synaptic plasma membranes were prepared from cerebrum and cerebellum of the rat brain for the assay of membrane-bound enzyme, $Ca^{2+}Mg^{2+}$ ATPase by using a discontinuous sucrose density gradient-ultracentrifugation method of Jones and Matus [48]. Briefly, cerebrum and cerebellum were homogenized separately in nine volumes of 10 % (w/v) sucrose. The homogenates were centrifuged at $800 \times g$ for 20 min, and the supernatants so obtained were centrifuged at $10,000 \times g$ for another 20 min. The pellets were then washed with 10 % (w/v) sucrose to yield the crude mitochondrial fractions. The pellets

were suspended in hypotonic buffer (5 mM Tris–HCl, pH 8.0) and were incubated at 0 °C for 30 min followed by homogenization. The lysates were made as 34 % (w/v) by the addition of appropriate amount of 48 % (w/v) sucrose solution. The upper phase of 28.5 % (w/v) sucrose was layered over the sample phase, and a small volume of 10 % (w/v) sucrose was overlaid onto this upper phase. The density gradients were centrifuged at $60,000 \times g$ for 110 min. The following fractions were recovered from each inter phase and the residual pellet. The upper white flocculent layer contained myelin, the middle gray band contained the synaptic plasma membrane and the brown pellet contained mitochondria.

Ca^{2+} ATPase

Ca^{2+} ATPase was assayed in synaptic plasma membranes according to the method of Desaiah et al. [49]. The $Ca^{2+}Mg^{2+}$ ATPase was assayed in a reaction mixture containing Tris–HCl buffer, $MgCl_2$, $CaCl_2$, sample and ATP. ATPase activity was determined in the presence of ethylene glycol tetraacetic acid (EGTA), and this was subtracted from the total $Ca^{2+}Mg^{2+}$ ATPase activity in order to obtain the net Ca^{2+} ATPase activity. The reaction mixture was incubated at 37 °C for 15 min, and the reaction was stopped by the addition of trichloroacetic acid (TCA). The contents were centrifuged at $3,000 \times g$ for 10 min, and Pi released was estimated in the supernatant by following the method of Fiske and Subbarow [50].

$Na^+ K^+$ ATPase

The enzyme activity was measured by using the method of Wallach and Kamat [51]. In this method, three test tubes were employed per sample. First tube was for test, second was for control and the third was for ouabain-insensitive enzymes. To the first test tube of each sample, homogenate was added which was followed by buffer and then substrate (ATP) and finally double distilled water was added. To the second tube of each sample, double distilled water was added instead of sample, but in addition, TCA was added. To the third tube, sample was mixed with buffer, ouabain, substrate (ATP) and followed by addition of double distilled water. Tubes were then incubated for 15 min at 37 °C on water bath. Later TCA solution was then added to stop the reaction. The contents were further centrifuged at $3,000 \times g$, and the inorganic phosphorous released was estimated by following the method of Fiske and Subbarow [50].

cAMP Levels

cAMP is an important "second messenger" and is a critical component of signal transduction pathways that are involved in many physiological processes. The levels of cAMP were

estimated by using cAMP direct immunoassay kit (Biovision, USA). The assay is based on the direct competitive immunoassay for sensitive and quantitative determination of cAMP levels. Protein G-coated plates anchors polyclonal antibody to the plate. cAMP–horseradish peroxidase (HRP) conjugate directly competes with cAMP from the sample to bind to the cAMP-specific antibody on the plate. After incubation and washing, the amount of cAMP–HRP bound to the plate was determined by reading HRP activity at O.D. 450 nm. The intensity of O.D. 450 nm was inversely proportional to the concentration of cAMP in the samples.

$$\text{Absorbance } (450 \text{ nm}) \; \alpha \; 1/cAMP$$

Calpain Assay

Calpain is a degrading enzyme whose activation depends upon the intracellular calcium levels. The calpain activity assay kit (Biovision, USA) specifically extracts cytosolic proteins without contaminations of cell membrane and lysosome proteases. Calpain enzyme was extracted by treating the brain samples with extraction buffer. Further, the extracted mixture was diluted with reaction buffer, and calpain substrate was added to the reaction mixture, which led to the breakdown of the substrate. The cleaved calpain substrate Ac-LLY-AFC (acetyl-Leu-Leu-Tyr-7-amino-4-trifluoromethylcoumarin) was determined fluorometrically. Ac-LLY-AFC emits blue light ($\lambda_{max} = 400$ nm) upon cleavage of the substrate by calpain; free AFC emits a yellow-green fluorescence ($\lambda_{max} = 505$ nm), which was quantified by using a fluorescence plate reader. Comparison of the fluorescence intensity from a treated sample with a normal control allows determination of the changes in calpain activity.

Intrasynaptosomal Free Ca^{2+} Levels

Intracellular free Ca^{2+} was determined in synaptosomes with the calcium sensitive indicator dye Fura-2/AM according to the method of Adamson et al. [52]. In this method, isolated synaptosomes (in physiological buffer) were loaded with 1 mM Fura-2/AM and were incubated at 37 °C for 1 h. The excess Fura-2/AM was removed by centrifugation. The fluorescence (F) at 340 to 380 nm excitation and 510 nm emission was measured, and [Ca^{2+}]$_i$ was calculated according to the formula:

$$\left[Ca^{2+} \right]_i \; = \; \left[(F - F_{min})/(F_{max} - F) \right] \; \times \; K_d$$

The K_d for Fura free acid is 225 nm. Maximal fluorescence (F_{max}) was measured after lysis of synaptosomes with SDS, and minimal fluorescence (F_{min}) was measured in the presence of 5 mM EGTA.

Calcium Estimation

Calcium levels were estimated in both the cerebrum and cerebellum by using atomic absorption spectrometer (Perkin Elmer) following the wet acid tissue digestion method of Zumkley et al. [53]. Briefly, the brain samples were mixed with perchloric acid/nitric acid (1:4 in volume) mixture, followed by digestion in sand bath until a white ash or residue was formed. The residue so obtained was dissolved in 10 mM nitric acid and was used for elemental analyses.

Western Transfer Analysis

Western blotting analysis was done according to the method of Towbin et al. [54]. The protein expressions of stress marker genes were observed in the post-mitochondrial fractions using anti-phospholipase C (PLC) (1:500) (Merck, Bangalore), anti-inositol triphosphate (IP3) receptor (1:500) (Genei, Bangalore), anti-protein kinase A (PKA) (1:500) (Merck, Bangalore) and anti-protein kinase C (PKC) (1:250) (Sigma, Bangalore). Densitometric analyses of bands were done by using Image J software (NIH).

Light Microscopic Examination

To carry out the histological studies, small sections of cerebrum (cerebral cortex) and cerebellum from each of the normal control and treated animals were taken. Immediately after sacrificing the animals, perfusion was done with 4 % paraformaldehyde, and the brains were subsequently fixed in 10 % formalin. After fixation, the tissues were processed carefully for embedding with paraffin wax (58–60 °C). Five- to seven-micrometre-thick paraffin sections were cut and were then subjected to alizarin red staining. Further, the stained slides were analysed under light microscope at ×10 and ×40 magnifications for histological examination.

Alizarin Red Staining

Alizarin Red S is an anthraquinone derivative used to identify calcium in tissue sections. In this staining, calcium forms an Alizarin Red S-calcium complex through a chelation process, and the end product formed is birefringent. In this staining procedure, the brain sections were de-paraffinized and brought to distilled water. Slides were stained with alizarin red solution (2 min) and were observed under microscopic (red-orange-stained calcium deposits). Excess of dye was removed by blotting, and slides were further dehydrated in acetone (five dips) followed by acetone-xylene (1:1) solution (five dips) and finally mounted with DPX.

Protein Estimation

Protein contents were estimated by using the method of Lowry et al. [55].

Statistical Analysis

The statistical significance of the data was determined by using one-way analysis of variance and a multiple post hoc test (Student–Newman–Keuls). The results were represented as mean±SD of six observations. Comparisons were made as follows:

*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ by Newman–Keuls test when values of treated groups are compared with normal control group
****$p \leq 0.05$, *****$p \leq 0.01$, ******$p \leq 0.001$ by Newman–Keuls test when values of Al+Zn group are compared with Al-treated group

Results

Synaptosomal K^+ Stimulated $^{45}Ca^{2+}$ Uptake

Effect of Al exposure on Ca^{2+} influx through voltage-operated calcium channels under stimulated (high K^+) and unstimulated (low K^+) conditions was analysed during our study. Two months of Al exposure to rats resulted in a significant increase ($p \leq 0.001$) in Ca^{2+} influx in comparison to untreated control rats, which was observed via voltage-operated calcium channels, after incubation with radiolabelled calcium in both polarizing and depolarizing media (Table 1). Zn supplementation to Al-treated rats significantly reduced the calcium influx when compared to Al-treated rats and hence afforded protection in maintaining the low intracellular calcium levels. However, Zn treatment alone to normal rats

did not reveal any statistically significant change in the calcium influx when compared to untreated control rats.

Ca^{2+} ATPase

Ca^{2+}ATPase, a CaM-regulated membrane bound enzyme, is responsible for maintaining intracellular calcium levels in neurons. A significant decrease in the Ca^{2+} ATPase activity was observed following Al treatment in comparison to untreated control rats (Table 2). Interestingly, Zn co-treatment to Al-exposed rats caused a significant increase in the Ca^{2+} ATPase activity in cerebrum ($p \leq 0.010$) and cerebellum ($p \leq 0.050$) when compared to Al-treated rats. Further, no significant change was observed in the Ca^{2+}ATPase activity when rats were treated with Zn in comparison to normal control rats.

Na^+/K^+ ATPase

Al treatment significantly inhibited Na^+/K^+ ATPase enzyme activity in both cerebrum and cerebellum ($p \leq 0.001$) in comparison to untreated control rats (Table 3). However, when Al-treated rats were supplemented with Zn, a significant increase in the Na^+/K^+ ATPase activity was observed in cerebrum ($p \leq 0.050$) and cerebellum ($p \leq 0.010$) as compared to Al-treated rats. Further, zinc alone treatment did not cause any significant change in the Na^+/K^+ ATPase activity in both regions of the brain when compared to untreated control rats.

cAMP Levels

The levels of cAMP were found to be elevated in synaptosomes of cerebrum ($p \leq 0.010$) and cerebellum ($p \leq 0.001$) of Al-exposed animals in comparison to untreated normal control rats (Table 4). On the contrary, Zn supplementation to Al-treated animals significantly declined the cAMP levels in synaptosomes of both the cerebrum ($p \leq 0.050$) and cerebellum ($p \leq 0.001$) when compared to Al-treated rats. However, no

Table 1 Effect of zinc on net ^{45}Ca uptake in synaptosomes isolated from cerebrum and cerebellum of rats subjected to aluminium treatment

Groups	Cerebrum	Cerebellum
Normal control	0.58±0.17	1.26±0.14
Aluminium	2.01±0.29***	3.11±0.99***
Zinc	0.95±0.64	1.11±0.56
Aluminium+zinc	1.36±0.42**˙****	1.66±0.45******

^{45}Ca uptake: micromoles of Ca per minute per milligram protein. All the values are expressed as means±SD; $n=6$ for each treatment group

*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ by Newman–Keuls test when the values are compared with normal control group; ****$p \leq 0.05$, *****$p \leq 0.01$, ******$p \leq 0.001$ by Newman–Keuls test when the values of Al+Zn-treated group are compared with Al-treated group

Table 2 Effect of zinc on Ca^{2+} ATPase activity in cerebrum and cerebellum of rats subjected to aluminium treatment

Groups	Cerebrum	Cerebellum
Normal control	74.13±3.67	55.29±2.54
Aluminium	20.68±1.44***	24.40±1.02**
Zinc	74.59±3.98	54.98±2.19
Aluminium+zinc	43.85±2.59**˙*****	39.82±2.85*˙****

Ca^{2+} ATPase activity: nanomoles Pi liberated per minute per milligram protein. All the values are expressed as means±SD; $n=6$ for each treatment group

*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ by Newman–Keuls test when the values are compared with normal control group; ****$p \leq 0.05$, *****$p \leq 0.01$, ******$p \leq 0.001$ by Newman–Keuls test when the values of Al+Zn-treated group are compared with Al-treated group

Table 3 Effect of zinc on Na$^+$/K$^+$ ATPase enzyme activity in cerebrum and cerebellum of rats subjected to aluminium treatment

Groups	Cerebrum	Cerebellum
Normal control	18.24±2.41	20.08±1.94
Aluminium	8.06±1.08***	7.69±0.96***
Zinc	17.44±2.24	19.65±1.86
Aluminium+zinc	12.36±1.96**·****	12.91±0.03**·*****

Activity: micromoles of Pi liberated per minute per gram tissue. All the values are expressed as means±SD; $n=6$ for each treatment group

*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ by Newman–Keuls test when the values are compared with normal control group; ****$p \leq 0.05$, *****$p \leq 0.01$, ******$p \leq 0.001$ by Newman–Keuls test when the values of Al+Zn-treated group are compared with Al-treated group

significant change in the levels of cAMP was observed in rats treated with zinc alone as compared to control rats.

Calpain Activity

Calpain, a degrading calcium-dependent enzyme, is a cysteine protease, and its activity is directly related to the intracellular calcium levels. After 2 months of Al exposure, a significant increase ($p \leq 0.001$) in calpain activity was observed in both the cerebrum and cerebellum when compared to normal control rats (Table 5), whereas simultaneous Zn treatment to Al-treated rats significantly decreased the calpain activity in both regions of the brain in comparison to Al-exposed rats.

Intrasynaptosomal Free Ca^{2+} Levels

Al exposure resulted in an increased ($p \leq 0.001$) intracellular free calcium levels in synaptosomes of cerebrum and cerebellum when compared to free intracellular calcium levels of untreated control rats (Table 6). However, zinc co-administration was able to reverse the trend in synaptosomes of both regions of the brain, and the levels were appreciably reduced in comparison to Al-treated rats. Further, zinc alone

Table 4 Effect of zinc on cAMP levels in synaptosomes isolated from cerebrum and cerebellum of rats subjected to aluminium treatment

Groups	Cerebrum	Cerebellum
Normal control	22.20±0.05	21.66±0.02
Aluminium	37.17±0.09**	66.84±0.12***
Zinc	21.10±0.05	23.20±0.03
Aluminium+zinc	27.20±0.07****	30.22±0.06**·******

cAMP levels: picomoles per milligram protein. All the values are expressed as means±SD; $n=6$ for each treatment group

*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ by Newman–Keuls test when the values are compared with normal control group; ****$p \leq 0.05$, *****$p \leq 0.01$, ******$p \leq 0.001$ by Newman–Keuls test when the values of Al+Zn-treated group are compared with Al-treated group

Table 5 Effect of zinc on calpain activity in cerebrum and cerebellum of rats subjected to aluminium treatment

Groups	Cerebrum	Cerebellum
Normal control	33.45±2.94	17.37±1.11
Aluminium	94.84±8.69***	77.75±2.68***
Zinc	32.77±2.50	18.49±4.19
Aluminium+zinc	49.18±4.62*****	54.51±5.83**·****

Calpain activity: relative fluorescent unit per milligram protein. All the values are expressed as means±SD; $n=6$ for each treatment group

*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ by Newman–Keuls test when the values are compared with normal control group; ****$p \leq 0.05$, *****$p \leq 0.01$, ******$p \leq 0.001$ by Newman–Keuls test when the values of Al+Zn-treated group are compared with Al-treated group

treatment did not cause any statistically significant change in intracellular free calcium levels in synaptosomes of cerebrum and cerebellum when compared with normal control rats.

Calcium Levels

A significant increase in the levels of Ca was observed in cerebrum ($p \leq 0.010$) and cerebellum ($p \leq 0.001$) after 2 months of Al exposure when compared to the untreated normal control rats (Table 7). Zinc supplementation to Al-treated rats significantly reduced the levels of Ca in cerebrum ($p \leq 0.050$) and cerebellum ($p \leq 0.010$) in comparison to rats treated with Al alone. However, no significant change in the Ca levels was observed in Zn-treated animals when compared to untreated control rats.

Western Transfer Analysis

Two months of Al treatment resulted in a significant increase ($p \leq 0.001$) in the protein expressions of PLC, IP3 and PKA whereas decreased expression of PKC in both the cerebrum and cerebellum when compared with untreated control rats (Fig. 1). On the contrary, Zn co-administration to Al-treated

Table 6 Effect of zinc on intrasynaptosomal free [Ca^{2+}]$_i$ in cerebrum and cerebellum of rats subjected to aluminium treatment

Groups	Cerebrum	Cerebellum
Normal control	137.37±17.25	141.00±26.88
Aluminium	625.24±87.93***	543.52±99.73***
Zinc	168.97±50.05	134.82±22.69
Aluminium+zinc	220.44±32.92**·******	278.32±37.93**·******

Intracellular free calcium level: nanomolar free [Ca^{2+}]$_i$. All the values are expressed as means±SD; $n=6$ for each treatment group

*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ by Newman–Keuls test when the values are compared with normal control group; ****$p \leq 0.05$, *****$p \leq 0.01$, ******$p \leq 0.001$ by Newman–Keuls test when the values of Al+Zn-treated group are compared with Al-treated group

Table 7 Effect of zinc treatment on the levels of calcium in cerebrum and cerebellum of rats subjected to aluminium treatment

Groups	Cerebrum	Cerebellum
Normal control	0.22±0.05	0.16±0.02
Aluminium	0.38±0.05**	0.45±0.09***
Zinc	0.23±0.04	0.17±0.05
Aluminium+zinc	0.28±0.01****	0.30±0.04**·*****

Total calcium level: micrograms per gram tissue. All the values are expressed as means±SD; $n=6$ for each treatment group

*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ by Newman–Keuls test when the values are compared with normal control group; ****$p \leq 0.05$, *****$p \leq 0.01$, ******$p \leq 0.001$ by Newman–Keuls test when the values of Al+ Zn-treated group are compared with Al-treated group

rats resulted in a significant decrease in the protein expressions of PLC, IP3 and PKA and increased expression of PKC in comparison to Al-alone-treated rats. Further, Zn alone treatment caused no significant change in the protein expressions of PLC, IP3, PKA and PKC when compared to untreated normal control rats.

Light Microscopic Observations

Figures 2a and 3a show normal control sections of cerebrum and cerebellum. Calcium staining with alizarin red S in Al-treated brain sections showed increased red-orange-coloured deposits of calcium in both regions of the brain (Table 8). Elevated alizarin red-positive staining revealed the high content of calcium in the brain tissue of Al-treated animals, which suggests the neurodegenerative conditions in the brain due to calcium dyshomeostasis (Fig. 2b and 3b). Zinc-treated brain sections of cerebrum and cerebellum had similar morphology, as that for normal control sections of the brain (Fig. 2c and 3c). Further, combined Al and Zn rat brain sections showed lesser deposits of calcium in comparison to Al-treated brain sections (Fig. 2d and 3d).

Discussion

Accumulated evidences have shown that altered calcium ion concentration in the brain can affect the functioning of numerous metabolic processes of central nervous system [25, 56,
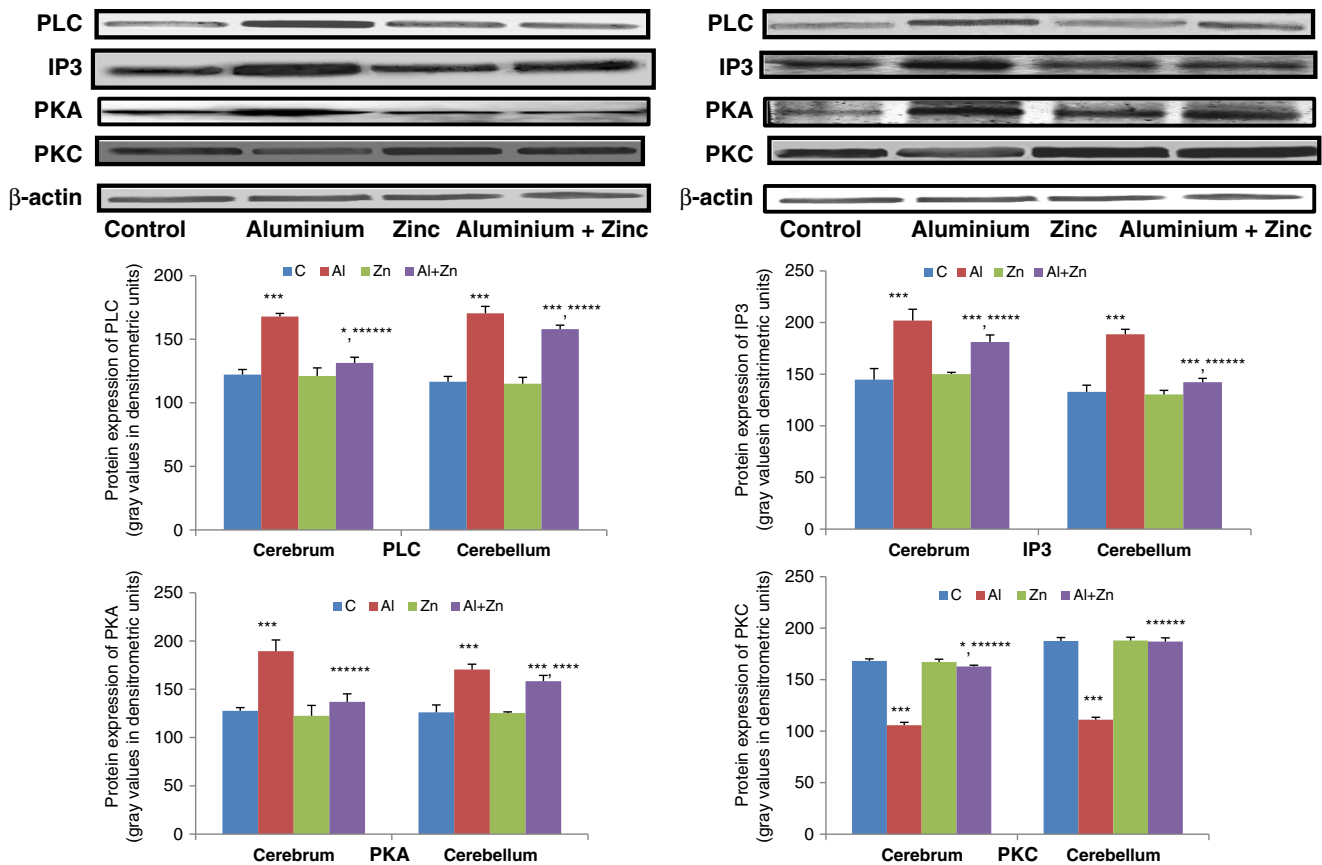

Fig. 1 Western blot and densitometric analyses of phospholipase C (*PLC*), inositol triphosphate (*IP3*), protein kinase A (*PKA*), protein kinase C (*PKC*) and β-actin in the cytosol isolated from cerebrum and cerebellum of normal control, aluminium-, zinc- and aluminium+zinc-treated animals

Fig. 2 The calcium localisation in cerebrum section of rat from different treatment groups. **a** Normal histoarchitecture of cerebrum with no calcium deposits in normal control group. **b** The section represents the deposition of calcium (marked as *black arrows*) after aluminium treatment whereas **c** a cerebrum section from zinc-treated group with no calcium burden. Cerebrum section from combined treatment group **d** shows improvement with reduced calcium deposits
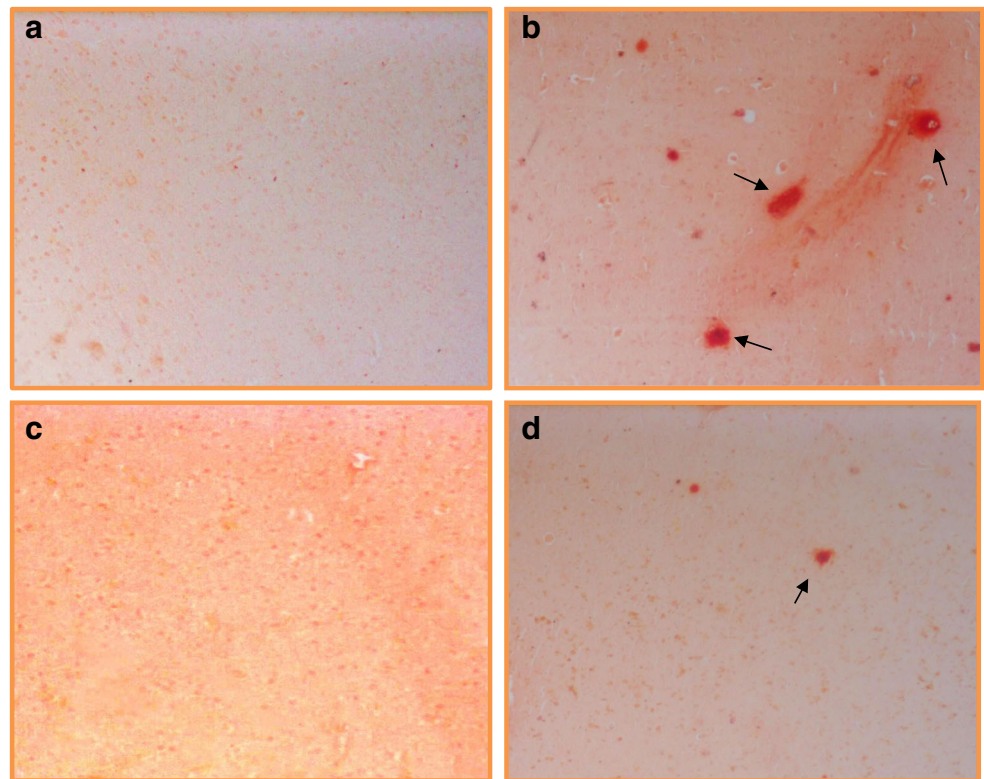
Fig. 3 **a** Section from normal control rat represents normal morphology of cerebellum with no calcium burden; **b** cerebellum from aluminium-intoxicated rat with red-coloured alizarin-positive deposits of calcium (*black arrows*); **c** section of cerebellum from zinc-treated group with normal histoarchitecture of the brain similar to that of control **d** rat cerebellum section from Al+Zn-treated group with lesser deposits of calcium in comparison to Al-alone-treated brain section
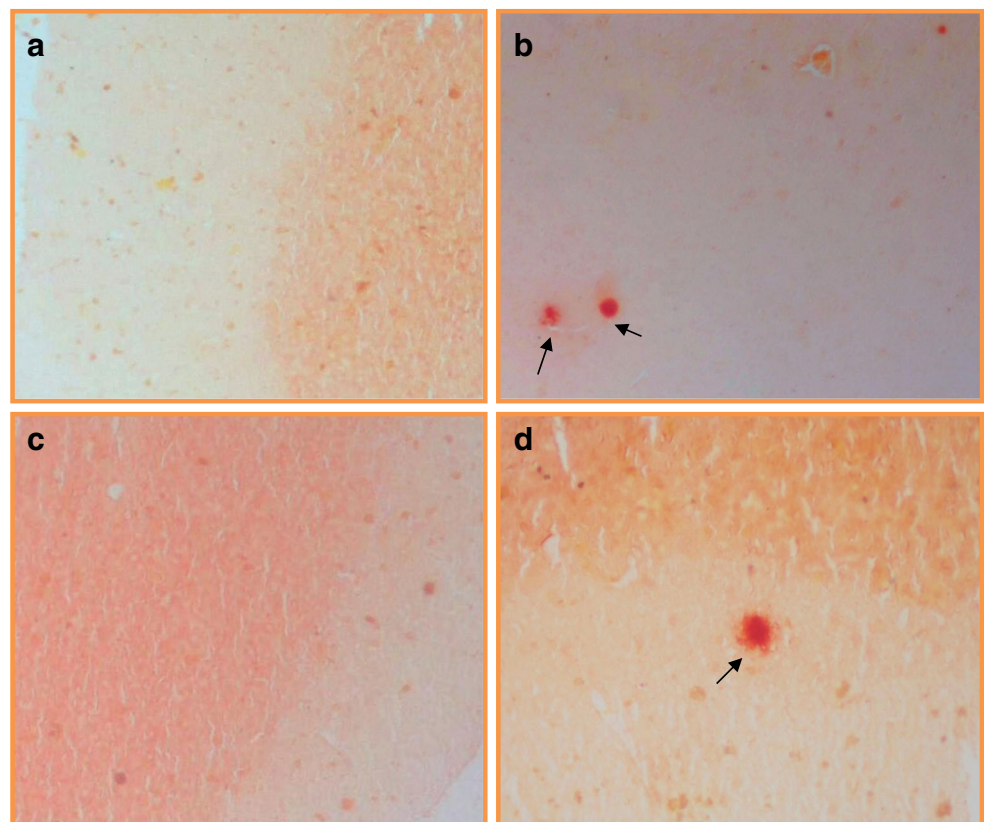
Table 8 Number of calcium deposits in the brain of rats subjected to different treatments

Groups	Cerebrum	Cerebellum
Normal control	Nil	Nil
Aluminium	27	14
Zinc	Nil	Nil
Aluminium+zinc	13	9

Number of alizarin red-stained calcium deposits (for quantification, five different slides in each group were observed at 40-fold magnification using bright field microscopy)

*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ by Newman–Keuls test when the values are compared with normal control group; ****$p \leq 0.05$, *****$p \leq 0.01$, ******$p \leq 0.001$ by Newman–Keuls test when the values of Al+ Zn-treated group are compared with Al-treated group

57]. Calcium plays an important role in regulating various neuronal processes, including excitability, neurotransmitter release, gene transcription, cell proliferation and synaptic plasticity [58, 59]. Further, maintaining normal calcium content in a cell either through influx or efflux mechanism has been considered as a vital phenomenon for normal neuronal activity. On the other hand, aluminium after crossing the BBB is known to antagonistically replace various essential elements such as calcium, magnesium, zinc and phosphorus from various enzymes and proteins that are present in the brain [1, 9, 60]. This impairment in elemental status induced by Al has been reported to cause adverse effect in the brain's neurophysiology by altering a series of biochemical cascades [15, 16, 21]. The present study has been designed to assess the effect of Al on calcium signalling pathway and also to evaluate the possible protection, if any, provided by zinc in such conditions.

Ca^{2+} ATPase is a membrane-bound transport protein that helps in regulating intracellular calcium levels by removing excess of Ca^{2+} from neuronal cells. In the present study, the activity of Ca^{2+} ATPase was found to be significantly decreased after Al exposure that reflects the impairment in Ca^{2+} extruding capacity of the cell and is understandably due to direct interaction of Al on the active site of Ca^{2+} ATPase [61]. It has been reported that Ca^{2+} ATPase requires $Mg^{2+}ATP$ complex as a cofactor to attain its normal activity [62]. Al due to its high affinity for Mg^{2+} (possess same physiochemical characteristics) would have replaced it from Mg^{2+} ATP complex and formed Al-ATP complex, which is further a potent inhibitor of Ca^{2+} ATPase [63]. Studies have also suggested that Al can decrease the activity of calcium ATPase by altering the conformation/activity of calcium binding protein calmodulin [22, 64, 65]. However, Zn treatment was significantly able to improve the activity of calcium ATPase in both regions of the brain (Table 2). This is because Zn might have prevented the attachment of Al to the active binding site of Ca^{2+} ATPase/Mg^{2+}ATP or its supplementation

has regulated the disturbed activity of calmodulin. Law et al. [66] have also shown a significant role of zinc in regulating calmodulin activity during zinc-deficient conditions.

Further, our results showed a significant increase in intrasynaptosomal free calcium level in cerebrum and cerebellum after aluminium treatment. This substantial increase is apparently due to decreased Ca^{2+} exuding ability as a result of decreased calcium ATPase activity. The increase in calcium levels may also be due to release of calcium from intracellular stores, a process reported to be triggered in the presence of metals [67]. Further, increase in total calcium levels has also been observed in our study after Al treatment, which suggests the overall disturbance in calcium content of neurons (Table 7). Further, increased calcium ions stress in neurons can also trigger the process of lipid peroxidation, which also justifies our findings as increased LPO levels have also been seen in our study after Al exposure (data not shown). However, Zn treatment to Al-exposed animals was able to decrease the elevated calcium levels in the brain, which is due to scavenging activity of Zn. Hence, Zn by quenching the Al-induced free radicals as well as free calcium ions from neurons has resulted in the reduction of cellular stress and accelerated a number of calcium- as well as non-calcium-dependent cellular mechanisms [29, 39, 68]. Further, its role in maintaining metal ionic concentration has also been studied in various neurotoxic conditions, which supports our findings [69, 70].

Reports have also suggested that raised intracellular calcium concentration in the brain can also be due to default in influx mechanism [57, 59]. So, we have also studied the effect of Al treatment on $^{45}Ca^{2+}$ influx through voltage-operated calcium channels under stimulated (high K^+) and unstimulated (low K^+) conditions. Aluminium exposure for 2 months resulted in a significant increase in ^{45}Ca influx in both the cerebrum and cerebellum. These findings suggest the harmful effect of Al^{3+} on voltage-operated calcium channels, which plays a crucial role in coupling electrical activity during the process of neurotransmission and open transiently in response to membrane depolarization. This enhancement could further lead to the raised levels of intracellular calcium, thus causing calcium stress in neurons and thereby leading to altered calcium homeostasis [61]. Another possible reason for this stress is due to Al^{3+}-induced perturbations in the structure of the channel molecule or in a phospholipid domain, which has potential to alter the calcium transport capacity and causing oxidative injury in neurons [71]. Recently, Walton [72] proposed that Al by inhibiting the activity of Ser/Thr phosphatase impedes Ca^{2+} influx mechanism in NMDA receptors. Further, Zn co-treatment with Al proved beneficial as it was able to reduce the calcium influx in both regions of the brain. It seems reasonable that Zn understandably has protected protein thiol groups of calcium channel from getting oxidized as increased calcium ion concentration in neurons might have caused oxidation of calcium channels [73].

Further, Zn has been considered as a potent antioxidant during various adverse physiological conditions and therefore could have afforded protection against calcium-induced neuronal injury by regulating the normal functioning of calcium channels [28, 36, 39, 43]. Moreover, our previous findings have also shown that Zn is a potential agent for alleviating Al-induced oxidative DNA injury [41]. Other probable alternative is that Zn by competing with calcium for its binding site within the channel or close to its vicinity has inhibited the calcium influx and accelerated the process of synaptic transmission, which is also observed in our study (data not shown). Further, Kitamura et al. [74] and Franco-Vidal et al. [75] have also shown that Zn helps in reducing calcium influx mechanism during ischemic neuronal injury and pneumolysin-induced toxicity in rats.

Further, to assess the effect of Al on intracellular signal transduction pathways, cAMP levels were also determined in the present study. cAMP, a second messenger, plays an important role in many biological processes such as regulating the functions of ion channels. In the present study, after 2 months of Al exposure, a significant increase in the levels of cAMP was found in both regions of the brain which is apparently due to indirect effect of raised intracellular Ca levels (Tables 4 and 6). The plausible explanation for this is that increased intracellular calcium levels in neurons after Al treatment could have activated Ca/calmodulin-dependent adenyl cyclase activity, which further triggered the cAMP [76]. Few researchers have also observed disturbed cAMP activity after aluminium exposure in rats and in other in vivo studies [65, 76]. However, Zn co-treatment was able to decrease the raised calcium levels in both regions of the brain. The decrease in calcium levels due to zinc supplementation might have led to deactivation of calcium/calmodulin dependent adenyl cyclase, thereby reducing the levels of cAMP. Similar inhibitory effect of Zn on cAMP levels has also been reported by Klein et al. [77].

Calpain is a calcium-dependent degradative enzyme, which belongs to cysteine protease family and gets activated during adverse conditions such as mitochondrial dysfunctioning and disruption in calcium homeostasis. Our data indicate that after 2 months of aluminium exposure, there was a significant increase in the enzyme activity in both the cerebrum and cerebellum, which is attributed to increased level of calcium in neurons (Table 5). It may be argued that enhanced activity of this degradative enzyme leads to neuronal degeneration as a consequence of Al exposure. Similar effect on calpain activity has also been observed by Kaur and Gill [61] in different regions of the brain after intragastrical administration of Al in rats. However, Zn supplementation proved to be effective as it was able to reduce the calpain activity, thereby indicating maintenance of calcium homeostasis by zinc. Similar inhibitory activity of Zn on calpain activity has been reported by Whipple and Koohmaraie [78]

during in vitro studies. In addition, increased calpain activity in the present study is an indicator of Al-induced neuronal apoptosis, which, however, was contained appreciably by Zn (data not shown). Zn also exhibits its anti-apoptotic property and thus could have afforded protection by acting at calpain, thereby stabilising the mitochondrial integrity as well as the resistance of neurons towards free radical-induced apoptosis [27, 40].

Phospholipase C pathway is one of the signalling pathways, which can cause increase in calcium content in the cells. PLC is a class of enzymes that cleaves phospholipids. It hydrolyses the membrane phospholipid PIP2 and cleaves into two second messengers via inositol 1,4,5-trisphosphate and diacylglycerol (DAG). IP3 is soluble in nature due to which it can diffuse into the cytosol and binds to its receptor where it stimulates the release of calcium ions. On the other hand, DAG remains in the plasma membrane and is a physiological activator of PKC. During our study, Al treatment increased the activity of PLC, IP3 and PKA whereas it down-regulated the activity of PKC in both regions of the brain (Fig. 1). The probable mechanism behind this is that Al induced membrane depolarization that might have activated the G-protein cascade and led to activation of catalytic subunit of PLC (PLCγ1), which could have resulted in the breakdown of PIP2. Further, G-proteins are involved in the regulation of ion channels, metabolism and cytoskeletal structures that were also found to be altered in the present study. Increased activity of IP3 is further responsible for release of calcium into the cell from intracellular stores (ER and mitochondria), which ultimately causes increased calcium concentration in the cell. Further, IP_3 can also activate Ca^{2+} channels on the cell membrane indirectly by increasing the intracellular Ca^{2+} concentration as observed in our study following Al exposure [79]. Several studies have also shown the adverse effects of Al on G-protein signalling pathways that further caused calcium dyshomeostasis [72, 80]. Further, increased activity of protein kinase A has also been observed in the present study after Al treatment. PKA belongs to a family of enzymes whose activity depends on cellular levels of cAMP. Furthermore, increased levels of cAMP have also been noticed in our study, which clearly indicates the reason for raised activity of PKA [81]. Zundorf and Reiser [56] have also suggested similar findings during various neurodegenerative conditions. During our study, Al treatment caused a decline in PKC activity, which is a consequence of direct inhibitory action of Al on active sites of PKC. Al apparently has attacked on any of the three binding sites (Mg^{2+}-ATP, DAG and calcium) of PKC and has led to a decrease in the PKC activity by successfully competing with Mg^{2+} for ATP and also by blocking Ca^{2+} binding to the carboxyl group on PKC [82]. Another possible reason for decreased activity is due to reduced activity of DAG, which further has caused declined activity of PKC. Further, similar effect of Al exposure on PKC activity has been shown by

Cochran et al. [83] and Katsuyama et al. [84]. Therefore, from these findings, it can be inferred that Al treatment creates an imbalance in the calcium ion homeostasis, by affecting the voltage-activated calcium release, Ca^{2+}ATPase activity and PLC activation, thus disrupts the second messenger pathways.

Zinc supplementation, however, regulated the disturbed calcium homeostasis and G signalling cascade in the neurons. Decreased PLC and IP3 activity after Zn treatment shows its regulatory effect on G protein signalling, which is reasonably due to reduction of Al-induced calcium load and cellular stress that is very apparent from our previous findings. Similarly, Jansen et al. [85] have also shown the effectiveness of Zn in regulating PLC activity in C6 glioma cells, whereas declined PKA activity is directly correlated to the decreased cAMP levels after Zn treatment. The possible reason for improvement in activity of PKC is due to displacement of Al from active binding sites of PKC by zinc. Moreover, protein kinase C is a Zn metalloenzyme, and its supplementation might have also up-regulated its declined activity.

Further, decrease in Na^+/K^+-ATPase activity has also been observed in both the cerebrum and cerebellum after Al treatment, which reflects an imbalance in ion homeostasis of neurons. The decline in this transmembrane protein activity is possibly due to direct attachment of Al with the subunits of Na^+/K^+ ATPase, or Al-induced free radicals have caused energy-depleted conditions in a cell [20, 23]. Failure of the Na^+/K^+ ATPase has been implicated in the pathophysiology of neurodegenerative diseases. Various studies have also described the critical role of Na^+/K^+ ATPase in signal transduction and cell death pathways (apoptosis, necrosis and hybrid cell death) [86, 87]. However, Zn treatment to Al-treated rats significantly increased the Na^+/K^+ ATPase enzyme activity (Table 3). This might be the result of displacement of Al by zinc from the active binding site of enzyme, or its strong antioxidant behaviour has regulated ionic metabolism in neurons. Also, Lovell et al. [88] have shown the protective effect of Zn against Abeta neurotoxicity by increasing the activity of Na^+/K^+ ATPase that helps in maintaining the calcium homeostasis and prevents cell death.

Histopathological studies have also revealed the increased content of calcium in the brain after Al treatment for 2 months (Fig. 2, Fig. 3 and Table 8). A number of alizarin-stained red-coloured calcium deposits were seen after Al treatment, which complements the observed increase in intracellular calcium content, activated proteases and lipases in the present study. On the other hand, stained sections from Zn co-treated group have shown reduced number of calcium deposits, thereby suggesting the regulatory role of zinc on calcium content. Further, improvement in calcium levels and activities of various calcium regulating proteins also suggests the positive influence of Zn on calcium homeostasis.

In conclusion, the findings of the current study suggest that zinc is a potential neuromodulatory agent and effectively maintains calcium homeostasis during Al-induced neurodegeneration.

Conflict of Interests The authors declare that there are no conflicts of interest.

References

1. Campbell A (2006) The role of aluminum and copper on neuroinflammation and Alzheimer's disease. J Alzheimers Dis 10:165–172
2. Weiss B (2010) Lead, manganese and methylmercury as risk factors for neurobehavioral impairment in advanced age. Int J Alzheimers Dis. doi:10.4061/2011/607543
3. Selkoe DJ, Lansbury PJ Jr. (1999) Alzheimer's disease is the most common neurodegenerative disorder. In: Siegel GJ, Agranoff BW, Albers RW et al (eds) Basic neurochemistry: molecular, cellular and medical aspects, 6th edn. Lippincott-Raven, Philadelphia. Available via: http://www.ncbi.nlm.nih.gov/books/NBK27944/
4. Brookmeyer R, Johnson E, Ziegler-Graham K, Arrighi HM (2007) Forecasting the global burden of Alzheimer's disease. Alzheimers Dement 3:186–191
5. Miu AC, Benga O (2006) Aluminum and Alzheimer's disease: a new look. J Alzheimers Dis 10:179–201
6. Walton JR (2010) Evidence for participation of aluminum in neurofibrillary tangle formation and growth in Alzheimer's disease. J Alzheimers Dis 22:65–72
7. Moore PB, Day JP, Taylor GA, Ferrier IN, Fifield LK, Edwardson JA (2000) Absorption of aluminium-26 in Alzheimer's disease, measured using accelerator mass spectrometry. Dement Geriatr Cogn Disord 11:66–69
8. Yumoto S, Kakimi S, Ohsaki A, Ishikawa A (2009) Demonstration of aluminum in amyloid fibers in the cores of senile plaques in the brains of patients with Alzheimer's disease. J Inorg Biochem 103:1579–1584
9. Cooke K, Gould MH (1991) The health effects of aluminium—a review. J R Soc Health 111:163–168
10. Cao H, Qiao L, Zhang H, Chen J (2010) Exposure and risk assessment for aluminium and heavy metals in Puerh tea. Sci Total Environ 408:2777–2784
11. Yokel RA, Hicks CL, Florence RL (2008) Aluminium bioavailability from basic sodium aluminium phosphate, an approved food additive emulsifying agent, incorporated in cheese. Food Chem Toxicol 46:2261–2266
12. Kaizer RR, Corrêa MC, Spanevello RM, Morsch VM, Mazzanti CM, Gonçalves JF, Schetinger MR (2005) Acetylcholinesterase activation and enhanced lipid peroxidation after long-term exposure to low levels of aluminum on different mouse brain regions. J Inorg Biochem 99:1865–1870
13. Lukiw WJ (2001) Aluminum and gene transcription in the mammalian central nervous system-implications for Alzheimer's Disease. In:

Exley C (ed) Aluminium and Alzheimer's disease: the science that describes the link. Elsevier Science, Amsterdam, pp 147–169

14. Sumathi T, Shobana C, Kumari BR, Nandhini DN (2011) Protective role of *Cynodon dactylon* in ameliorating the aluminium-induced neurotoxicity in rat brain regions. Biol Trace Elem Res 144:843–1853

15. Li XP, Yang YJ, Hu H, Wang QN (2006) Effect of aluminum trichloride on dissociated Ca^{2+} in hippocampus neuron cell as well as learning and memory. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi 24:161–163

16. Walton JR (2007) An aluminum-based rat model for Alzheimer's disease exhibits oxidative damage, inhibition of PP2A activity, hyperphosphorylated tau, and granulovacuolar degeneration. J Inorg Biochem 101:1275–1284

17. Abu-Taweel GM, Ajarem JS, Ahmad M (2012) Neurobehavioral toxic effects of perinatal oral exposure to aluminum on the developmental motor reflexes, learning, memory and brain neurotransmitters of mice offspring. Pharmacol Biochem Behav 101:49–56

18. Moumen R, Ait-Oukhatar N, Bureau F, Fleury C, Bouglé D, Arhan P, Neuville D, Viader F (2001) Aluminium increases xanthine oxidase activity and disturbs antioxidant status in the rat. J Trace Elem Med Biol 15:89–93

19. Ghribi O, Herman MM, Spaulding NK, Savory J (2002) Lithium inhibits aluminum-induced apoptosis in rabbit hippocampus, by preventing cytochrome c translocation, Bcl-2 decrease, Bax elevation and caspase-3 activation. J Neurochem 82:137–145

20. Singla N, Dhawan DK (2012) Regulatory role of zinc during aluminium-induced altered carbohydrate metabolism in rat brain. J Neurosci Res 90:698–705

21. Zatta P, Lain E, Cagnolini C (2000) Effects of aluminum on activity of krebs cycle enzymes and glutamate dehydrogenase in rat brain homogenate. Eur J Biochem 267:3049–3055

22. Kurita H, Nakatomi A, Shimahara H, Yazawa M, Ohki SY (2005) Al(3+) interaction sites of calmodulin and the Al(3+) effect on target binding of calmodulin. Biochem Biophys Res Commun 333:1060–1065

23. Silva VS, Nunes MA, Cordeiro JM, Calejo AI, Santos S, Neves P, Sykes A, Morgado F, Dunant Y, Gonçalves PP (2007) Comparative effects of aluminum and ouabain on synaptosomal choline uptake, acetylcholine release and (Na+/K+) ATPase. Toxicology 236:158–177

24. Bojarski L, Herms J, Kuznicki J (2008) Calcium dysregulation in Alzheimer's disease. Neurochem Int 52:621–633

25. Berridge MJ (2013) Dysregulation of neural calcium signaling in Alzheimer disease, bipolar disorder and schizophrenia. Prion 7:2–13

26. Popescu BF, Robinson CA, Rajput A, Rajput AH, Harder SL, Nichol H (2009) Iron, copper, and zinc distribution of the cerebellum. Cerebellum 8:74–79

27. Adamo AM, Zago MP, Mackenzie GG, Aimo L, Keen CL, Keenan A, Oteiza PI (2010) The role of zinc in the modulation of neuronal proliferation and apoptosis. Neurotox Res 17:1–14

28. O Dell BL (2000) Role of zinc in plasma membrane function. J Nutr 130:1432S–1436S

29. Song Y, Xue Y, Liu X, Wang P, Liu L (2008) Effects of acute exposure to aluminum on blood–brain barrier and the protection of zinc. Neurosci Lett 445:42–46

30. Trombley PQ, Blakemore LJ, Hill BJ (2011) Zinc modulation of glycine receptors. Neuroscience 186:32–38

31. Amico-Ruvio SA, Murthy SE, Smith TP, Popescu GK (2011) Zinc effects on NMDA receptor gating kinetics. Biophys J 100:1910–1918

32. Takeda A (2001) Zinc homeostasis and functions of zinc in the brain. Biometals 14:343–351

33. Bitanihirwe BK, Cunningham MG (2009) Zinc: the brain's dark horse. Synapse 63:1029–1049

34. Browning JD, O'Dell BL (1994) Low zinc status in guinea pigs impairs calcium uptake by brain synaptosomes. J Nutr 124:436–443

35. Kiedrowski L (2012) Cytosolic acidification and intracellular zinc release in hippocampal neurons. J Neurochem 121:438–450

36. Rudolf E, Cervinka M (2006) The role of intracellular zinc in chromium (VI)-induced oxidative stress, DNA damage and apoptosis. Chem Biol Interact 162:212–227

37. Bhalla P, Chadha VD, Dhawan DK (2007) Effectiveness of zinc in modulating lithium induced biochemical and behavioral changes in rat brain. Cell Mol Neurobiol 27:595–607

38. Joshi D, Mittal DK, Shukla S, Srivastav AK (2012) Therapeutic potential of *N* acetylcysteine with antioxidants (Zn and Se) supplementation against dimethylmercury toxicity in male albino rats. Exp Toxicol Pathol 64:103–108

39. Franco JL, Posser T, Mattos JJ, Trevisan R, Brocardo PS, Rodrigues AL, Leal RB, Farina M, Marques MR, Bainy AC, Dafre AL (2009) Zinc reverses malathion-induced impairment in antioxidant defenses. Toxicol Lett 187:137–143

40. An WL, Pei JJ, Nishimura T, Winblad B, Cowburn RF (2005) Zinc-induced anti-apoptotic effects in SH-SY5Y neuroblastoma cells via the extracellular signal-regulated kinase 1/2. Brain Res Mol Brain Res 135:40–47

41. Singla N, Dhawan DK (2013) Zinc, a neuroprotective agent against aluminum-induced oxidative DNA injury. Mol Neurobiol 48:1–12

42. Kakkar V, Kaur IP (2011) Evaluating potential of curcumin loaded solid lipid nanoparticles in aluminium induced behavioural, biochemical and histopathological alterations in mice brain. Food Chem Toxicol 49:2906–2913

43. Sidhu P, Garg ML, Dhawan DK (2004) Protective role of zinc in nickel induced hepatotoxicity in rats. Chem Biol Interact 150:199–209

44. Goel A, Dani V, Dhawan DK (2007) Zinc mediates normalization of hepatic drug metabolizing enzymes in chlorpyrifos-induced toxicity. Toxicol Lett 169:26–33

45. Gray EG, Whittaker VP (1962) The isolation of nerve endings from brain, an electron-microscopic study of cell fragments derived by homogenization and centrifugation. J Anat 96:79–88

46. Edelfors S, Ravn-Jonsen A (1991) Effects of simultaneous ethanol and toluene exposure on nerve cells measured by changes in synaptosomal calcium uptake and (Ca^{2+}/Mg^{2+})-ATPase activity. Pharmacol Toxicol 69:90–95

47. Butler FE (1961) Determination of tritium in water and urine. Liquid scintillation counting and rate drift determination. Anal Chem 33:409–414

48. Jones DH, Matus AI (1974) Isolation of synaptic plasma membrane from brain by combined flotation–sedimentation density gradient centrifugation. Biochim Biophys Acta 356:276–287

49. Desaiah D, Chetty CS, Rao KS (1985) Chlordecone inhibition of calmodulin activated calcium ATPase in rat brain synaptosomes. J Toxicol Environ Health 16:189–195

50. Fiske SH, Subbarow Y (1925) The colorimetric determination of phosphorous. J Biol Chem 66:375–400

51. Wallach DFH, Kamat VB (1966) Assay for membrane Na+–K+ ATPase. In: Colowick SP, Kaplan O (eds) Methods in enzymology. Academic, New York, pp 164–165

52. Adamson P, Hajimahammadreza I, Brammer MJ, Campbel IC (1989) Intrasynaptosomal free calcium concentration is increased by phorbol esters via a 1,4 dihydropyridine (L-type) Ca2+ channel. Eur J Pharmacol 162:59–66

53. Zumkley H, Bertram HP, Lison A, Knoll O, Losse H (1979) Aluminum, zinc and copper concentrations in plasma in chronic renal insufficiency. Clin Nephrol 12:18–21

54. Towbin H, Staehelin T, Gordon J (1992) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Biotechnology 24:145–149

55. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Follin-phenol reagent. J Biol Chem 193:265–275

56. Zundorf G, Reiser G (2011) Calcium dysregulation and homeostasis of neural calcium in the molecular mechanisms of neurodegenerative

diseases provide multiple targets for neuroprotection. Antioxid Redox Signal 14:1275–1288

57. Gleichmann M, Mattson MP (2011) Neuronal calcium homeostasis and dysregulation. Antioxid Redox Signal 14:1261–1273

58. Wang H, Zhang M (2012) The role of Ca^{2+}-stimulated adenylyl cyclases in bidirectional synaptic plasticity and brain function. Rev Neurosci 23:67–78

59. Thanawala MS, Regehr WG (2013) Presynaptic calcium influx controls neurotransmitter release in part by regulating the effective size of the readily releasable pool. J Neurosci 33:4625–4633

60. Lubkowska A, Chlubek D, Machoy-Mokrzyńska A, Noceń I, Zyluk B, Nowacki P (2004) Concentrations of fluorine, aluminum and magnesium in some structures of the central nervous system of rats exposed to aluminum and fluorine in drinking water. Ann Acad Med Stetin 50:73–76

61. Kaur A, Gill KD (2005) Disruption of neuronal calcium homeostasis after chronic aluminium toxicity in rats. Basic Clin Pharmacol Toxicol 96:118–122

62. Raftos JE, Lew VL (1995) Effect of intracellular magnesium on calcium extrusion by the plasma membrane calcium pump of intact human red cells. J Physiol 489:63–72

63. Sarin S, Julka D, Gill KD (1997) Regional alterations in calcium homeostasis in the primate brain following chronic aluminium exposure. Mol Cell Biochem 168:95–100

64. Siegel N, Haug AR (1983) Aluminum interaction with calmodulin: evidence for altered structure and function from optical and enzymatic studies. Biochim Biophys Acta 744:36–45

65. Julka D, Gill KD (1996) Altered calcium homeostasis: a possible mechanisms of aluminium-induced neurotoxicity. Biochim Biophys Acta 1315:47–54

66. Law JS, McBride SA, Graham S, Nelson NR, Slotnick BM, Henkin RI (1988) Zinc deficiency decreases the activity of calmodulin regulated cyclic nucleotide phosphodiesterases in vivo in selected rat tissues. Biol Trace Elem Res 16:221–226

67. Prabhu SD, Salama G (1990) The heavy metal ions Ag^+ and Hg^{2+} trigger calcium release from cardiac sarcoplasmic reticulum. Arch Biochem Biophys 277:47–55

68. Aimo L, Cherr GN, Oteiza PI (2010) Low extracellular zinc increases neuronal oxidant production through NADPH oxidase and nitric oxide synthase activation. Free Radic Biol Med 48:1577–1587

69. Gaetke LM, Chow CK (2003) Copper toxicity, oxidative stress, and antioxidant nutrients. Toxicology 189:147–163

70. Jaya Prasanthi RP, Hariprasad RG, Bhuvaneswari DC, Rajarami Reddy G (2005) Zinc and calcium reduce lead induced perturbations in the aminergic system of developing brain. Biometals 18:615–626

71. Koenig ML, Jope RS (1987) Aluminium inhibits fast phase of voltage-dependent calcium influx into synaptosomes. J Neurochem 49:316–320

72. Walton JR (2012) Aluminum disruption of calcium homeostasis and signal transduction resembles change that occurs in aging and Alzheimer's disease. J Alzheimers Dis 29:255–273

73. O Dell BL, Browning JD (2011) Zinc deprivation impairs growth factor-stimulated calcium influx into murine 3T3 cells associated with decreased cell proliferation. J Nutr 141:1036–1040

74. Kitamura Y, Iida Y, Abe J, Ueda M, Mifune M, Kasuya F, Ohta M, Igarashi K, Saito Y, Saji H (2006) Protective effect of zinc against ischemic neuronal injury in a middle cerebral artery occlusion model. J Pharmacol Sci 100:142–148

75. Franco-Vidal V, Beurg M, Darrouzet V, Bébéar JP, Skinner LJ, Dulon D (2008) Zinc protection against pneumolysin toxicity on rat cochlear hair cells. Audiol Neurootol 13:65–70

76. Johnson GV, Jope RS (1987) Aluminum alters cyclic AMP and cyclic GMP levels but not presynaptic cholinergic markers in rat brain in vivo. Brain Res 403:1–6

77. Klein C, Sunahara RK, Hudson TY, Heyduk T, Howlett AC (2002) Zinc inhibition of cAMP signaling. J Biol Chem 277:11859–11865

78. Whipple G, Koohmaraie M (1991) Degradation of myofibrillar proteins by extractable lysosomal enzymes and m-calpain, and the effects of zinc chloride. J Anim Sci 69:4449–4460

79. Pentyala S, Ruggeri J, Veerraju A, Yu Z, Bhatia A, Desaiah D, Vig P (2010) Microsomal Ca^{2+} flux modulation as an indicator of heavy metal toxicity. Indian J Exp Biol 48:737–743

80. Chen Y, Penington NJ (2000) Competition between internal AlF(4)(−) and receptor-mediated stimulation of dorsal raphe neuron G-proteins coupled to calcium current inhibition. J Neurophysiol 83:1273–1282

81. Chen L, Liu CJ, Tang M, Li A, Hu XW, Du YM, Shen JJ, Lu YL, Heschler J (2005) Action of aluminum on high voltage-dependent calcium current and its modulation by ginkgolide B. Acta Pharmacol Sin 26:539–545

82. Shafer TJ, Nostrandt AC, Tilson HA, Mundy WR (1994) Mechanisms underlying AlCl3 inhibition of agonist-stimulated inositol phosphate accumulation. Role of calcium, G-proteins, phospholipase C and protein kinase C. Biochem Pharmacol 47:1417–1425

83. Cochran M, Elliott DC, Brennan P, Chawtur V (1990) Inhibition of protein kinase C activation by low concentrations of aluminium. Clin Chim Acta 194:167–172

84. Katsuyama H, Saijoh K, Inoue Y, Sumino K (1989) The interaction of aluminium with soluble protein kinase C from mouse brain. Arch Toxicol 63:474–478

85. Jansen S, Arning J, Beyersmann D (2005) Zinc homeostasis in C6 glioma cells: phospholipase C activity regulates cellular zinc export. Biol Trace Elem Res 108:87–104

86. Yu SP (2003) Na(+), K(+) -ATPase: the new face of an old player in pathogenesis and apoptotic/hybrid cell death. Biochem Pharmacol 66:1601–1609

87. Xie Z, Cai T (2003) Na^+ K^+ ATPase mediated signal transduction: from protein interaction to cellular function. Mol Interv 3:157–168

88. Lovell MA, Xie C, Markesbery WR (1999) Protection against amyloid beta peptide toxicity by zinc. Brain Res 823:88–95