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Title: Calcium Supplementation Ameliorates Cerebellar Oxidative Stress in Lactational Aluminium-Induced Neurotoxicity in Rats

Running Title: Calcium and Aluminium Neurotoxicity

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Abstract

Introduction: The neurotoxic effects of aluminium exposure during the critical period of neurodevelopment has been well documented. This study investigated the putative protective effects of calcium supplementation on the cerebellum of juvenile Wistar rats following aluminium-induced neurotoxicity during lactation.

Methods: Four groups of juvenile rats were exposed via lactation to distilled water (control group), aluminium (40 mg/kg/d), calcium supplement (50 mg/kg/d), and a combination of both aluminium and calcium from postnatal day 4 to 28. The cerebella of the animals were excised to access the levels of antioxidant enzymes (SOD and GPx), lipid peroxidation (MDA), histomorphological alterations (H and E), Nissl profile (CFV) and glial activation (GFAP immunohistochemistry).

Results: Lactational aluminium significantly decreased the activities of superoxide dismutase and glutathione peroxidase while exacerbating lipid peroxidation and reactive astrocyte in cerebellar lysates. Lactational calcium supplementation normalized the activities of SOD and GPx, thereby preventing excessive lipid peroxidation and glial activation. Despite no apparent changes in the general histology of the cerebellum, aluminium induced chromatolytic changes in the Purkinje cell layer, which was counteracted by the antioxidant propensities of calcium supplementation.

Conclusion: These findings support that calcium supplementation significantly protected the cerebellum against Al-induced oxidative stress, chromatolysis, and neuroinflammation.

Keywords: Aluminium, Calcium, Cerebellum, Lactation, Oxidative stress, Glial activation
1. Introduction

Aluminium is the third most abundant element in Earth’s crust, the most abundant metallic element, and the most commonly used non-ferrous metal globally. It usually occurs as compounds, such as aluminium oxide, aluminium hydroxide, and potassium aluminium sulfate, some of which are present at varying extent in rocks, vegetation and animals (Gandara, 2013).

Products manufactured from aluminium and its alloys include consumer durables such as air conditioners, refrigerators, electrical conductors, building materials, cooking utensils and food packaging equipment (Gandara, 2013). In the health sector, aluminium is used in the production of vaccine adjuvant, antacids, antiperspirants and other skincare products. Aluminium salts have been used as coagulants in water treatment to reduce organic matter, colour, turbidity and microbial levels (Sperczyńska et al., 2016). Despite the numerous benefits of aluminium, it could also constitute a hazard to health. Exposure to toxic levels of Aluminium could occur through ingestion of contaminated food or water (Singh and Goel, 2015; Niu, 2018). Breastfeeding infants are exposed to aluminium through consumption of contaminated infant milk formula or breast milk of exposed mother (Niu, 2018). Aluminium crosses the blood-brain barrier using transferrin-mediated transport (Singh and Goel, 2015) and accumulates in various parts of the brain (Kumar and Gill, 2014). Exposure to aluminium has been associated with cognitive impairment and neurodegenerative disorders (Niu, 2018).

The cerebellum functions in the maintenance of motor coordination and balance, and also has a role in processing signals for perception, cognition, and emotion (Manto and Marmolino, 2009; Pandolfo and Manto, 2013; Reebet al., 2013; Lackey et al., 2018). Neurological dysfunctions arising from injury to the cerebellum are not uncommon. The cerebellum is especially susceptible to metabolic, medicinal and environmental insults, arising from exposure to substances such as alcohol, drugs, chemical agents and other toxins (Manto, 2012; Alekseeva et al, 2014).

Calcium is an important mineral required for structural development and physiologic functions of the body. It is not produced in the body but must be consumed in diet and absorbed through the gastrointestinal tract (Thomas and Weisman, 2006). During pregnancy and lactation, calcium intake supports fetal and neonatal growth and development (Thomas and Weisman, 2006). Despite the beneficial role of calcium in growth and development,
certain adverse effects, especially in the elderly have been reported, including increased risk of developing dementia and cerebrovascular disease (Kern et al., 2016).

This study investigated the effect of calcium supplementation on the cerebellum of juvenile rats that were exposed to aluminium chloride during the period of lactation.

2. Materials and Methods

2.1 Chemicals and reagents

Aluminium chloride was purchased from Integrated Sunaf (Nig.) Ilorin, Nigeria. Calcium mineral supplement was purchased from Feolu Pharmacy, Ilorin, Nigeria. Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx) assay kits as well as primary antibodies were purchased from Sigma-Aldrich Co., St Louis, MO, USA.

2.2 Experimental Animals

Adult female Wistar rats were procured from a breeder in Ogbomoso, Nigeria. The animals were acclimatized for seven days at the Animal House of the Faculty of Basic Medical Sciences, University of Ilorin. The animals were maintained in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

2.3 Animal mating

Vaginal smear test was carried out between 8:00 am, and 9:00 am daily prior to mating; this was done to know the phase of the oestrous cycle of female rats before introducing the male rats. Female rats in their pro-estrus phase were exposed to male rats between 4:00 pm and 8:00 am the next day. Mating was confirmed by the presence of spermatozoa in the vagina smear the following morning and the day was taken as day zero of pregnancy (Marcondes et al., 2002; Omotoso et al., 2018).

2.4 Animal Grouping and Drug Administration

Immediately after delivery, the pups were randomly divided into four groups of six rats each (alongside their mothers for breastfeeding to continue). The mother rats were treated in order
to deliver the administered substances into the pups through breast milk. Group A (Control) was fed on normal chow and water; Group B received aluminium chloride (40 mg/kg/b.wt./d) (Olajide et al., 2017b) via oral gavage; Group C received calcium supplement (50 mg/kg/b.wt./d) (Weingarten et al., 2008) via oral gavage; while Group D was co-administered with aluminium chloride and calcium at doses stated above. The pups were allowed to remain with their mothers for breastfeeding up to postnatal day 28, while treatment commenced from postnatal day (PD) 4 to 28 (that is a total of 25 consecutive days).

2.5 Preparation of Brain samples
At the end of the experiment, the young rats were sacrificed in two categories from each group. Rats in the first category were anaesthetized with ketamine and perfused transcardially first with a flush of phosphate buffer saline (PBS, pH 7.0) and followed by 4% paraformaldehyde (PFA). The cerebellum was excised from the brain and subsequently fixed in 4% PFA. The cerebellar tissues were then processed and routinely stained with haematoxylin and eosin (H&E) and using Cresyl fast violet (CFV) staining techniques. PFA-fixed tissues were also processed to immunohistochemically characterize reactive astrocytes using anti-glial fibrillary acidic protein (GFAP) antibody. The second category of animals in each group was sacrificed by cervical dislocation to prevent ketamine from meddling with biochemical redox. The rats were then decapitated and dissected quickly to obtain the cerebellum. Cerebellar tissues were homogenized in 0.25 M sucrose with an automated homogenizer at 4°C to obtain the cerebellar lysates for biochemical studies. The homogenate was centrifuged (12,000 g, 10 min) and the supernatant was separated for estimation of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzyme levels using enzyme-linked immunosorbent assay (ELISA) technique as stipulated by the manufacturer’s guide.

2.5 Photomicrography and Statistical Analysis
Photomicrographs of the cerebellum were obtained using an Amscope microscope camera attached to a light microscope. The data obtained from the neurobehavioral and enzymatic assay were subjected to statistical analysis using Graphpad Prism (version 6). The values were plotted in ANOVA with Tukey’s multiple comparison tests. Data obtained were presented as mean ± standard error of mean with the level of significance set at a p-value less than 0.05, 0.01 and 0.005. The results obtained were represented in bar charts with error bars to show the mean and standard error of mean respectively.
3. Results

3.1 Calcium supplementation normalizes Al-induced oxidative stress

In the present study, we assayed for the levels of cerebellar antioxidant enzymes, SOD and GPx. The level of SOD enzyme reduced significantly in the cerebellum of aluminium-induced rats when compared to the control (p<0.01) and other treated groups (p<0.05) (Fig. 1). The rats that received only calcium supplement (group C) had an increase in SOD level compared with the aluminium-induced rats (p<0.05); however, SOD level in calcium-treated rats was still lower than that seen in the Control and the rats co-treated with aluminium and calcium, though not statistically significant. Although the tissue level of SOD appeared higher in animals co-treated with aluminium and calcium compared to those that received only calcium, the difference was not statistically significant (p>0.05). Aluminium toxicity caused a significant reduction in the cerebellar levels of GPx when compared to the control (p<0.05) and other treated groups (p<0.05) (Fig. 2). On the other hand, rats that received only calcium supplementation had the highest level of cerebellar GPx, though a significant difference only existed between the former and the aluminium-induced rats (p<0.05), and not with the control or rats co-treated with calcium and aluminium. Administration of calcium during aluminium-induced neurotoxicity increased the level of GPx significantly compared to rats exposed to only aluminium (p<0.05); although the level was higher than the Control too, this was not statistically significant (Fig. 2).

3.2 Calcium supplementation counterbalances Al-induced lipid peroxidation

The cerebellar level of malondialdehyde (a marker of lipid peroxidation) was assayed for in the present study. The cerebellar level of MDA was elevated significantly in animals administered with aluminium (p<0.05) compared with the control and calcium supplementation groups (Fig. 3). Calcium supplementation significantly reduced MDA level compared with the Control (p<0.05), aluminium-administered rats (p<0.01) and those rats that received both calcium and aluminium (p<0.01). Rats that were treated with both calcium and aluminium had a markedly raised MDA level, though not as high as that seen in rats that received only aluminium.

3.3 Histomorphology and Nissl profile of cerebellar cortex

The histoarchitectural manifestation of the cerebellar cortex showed a little variation in cellular morphological disposition and histoarchitecture across the groups. The control and the calcium supplementation group (Groups A and C respectively) (Fig. 4A and C) presented
with a typical cerebellar cortical array of cells characterised by a proper delineation of the molecular layer from the granular layer as separated by the Purkinje cell layer. The granular layer is made up of dense clusters of tiny granule cell soma while the molecular layer was composed of fewer cells. The adjoining single cell layer was made up of Purkinje cells characterized by large soma whose axonal projection ran into the molecular layer while the dendritic projections ran into the granular layer. The nuclei of cells in the aforementioned layers were typically stained, suggesting intactness and no apparent histopathological alterations. Furthermore, the Nissl profile of these cells in Groups A (Fig. 4 E, I) and C (Fig. 4 G, K) showed proper characterization of Nissl substance with no chromatolytic changes. However, the histological manifestation of the cerebellar cortex of animals postnatally exposed to aluminium (Group B: Fig 4B) showed perturbed histoarchitectural delineation, which was marked by poorly characterized Purkinje cells. Correspondingly, the Nissl substance of the Purkinje cells presented with peripheral chromatolysis as indicated by the poorly stained Purkinje cells (Group B: Fig 4F, J). Animals co-treated with calcium and aluminium chloride showed fine arrays of cells across the cerebellar cortex similar to those of the control groups (Group D: Fig 4H, L). However, the Nissl profile of the Purkinje cells showed mild chromatolytic changes. These findings suggest that calcium supplementation plays a neuroprotective role in maintaining the structural integrity of the cerebellum against Al-induced neurotoxicity.

3.4 Effects of lactation aluminium and calcium exposure on astrocyte activation.

To check the roles aluminium and calcium supplementation on glial activation, reactive astrocytes were immunohistochemically labelled using GFAP antibody. Glial expression in the control group appears characteristically normal. Increased GFAP immunopositive cells were observed in aluminium-treated groups, with the presence of increased reactive astroglia within the granule cell layer (Fig. 5). In rats co-treated with aluminium and calcium, expression of astrocytes appeared similar to those of the Control and Calcium groups. Cell count of the glial density showed a significant increase in glial expression in the Al-treated group relative to the control (p<0.05). Calcium supplementation in group D prevented excessive glial activation.
4. Discussion

Despite the enormous benefits of aluminium, its exposure to the body could affect brain morphology, biochemistry, and behaviour (Kumar and Gill, 2014). Oxidative stress stands out as one of the mechanisms of neurotoxicity associated with aluminium exposure (Andrade et al., 2017). Utilising this pathway especially, the current study explored the role of calcium supplementation in protecting the cerebellum against aluminium-induced oxidative stress. The present study exposed the inability of the antioxidant capacity of the cell to cope with the free radicals generated as a result of aluminium toxicity, using SOD and GPx as oxidative markers and MDA as a marker for lipid peroxidation.

Oxidative stress occurs whenever there is a breakdown of the dynamic equilibrium that exists between the production of free radicals and the antioxidant capacity of the cell, resulting in damage to nucleic acids, biomembrane lipids, proteins and other macromolecules (McCord, 2000; El-Bahr, 2013). Although normal metabolic processes generate non-pathogenic free radicals, these are usually transported or neutralized by the electron transport chains. This ability is compromised in diseased states, leading to the release of endogenous reactive oxygen species (ROS) by the mitochondria which compound oxidative stress and promote cell death. Oxidative stress arising from generated free radicals has been linked to the development of some diseases in the body, including neurodegenerative disorders (Phaniendra et al., 2015).

The antioxidant system of the body, which comprises the enzymatic and the non-enzymatic, is responsible for the removal of ROS from the body. The primary enzymes involved, the antioxidant enzymes, include superoxide dismutase, glutathione peroxidase, and catalase, while the non-enzymatic components include glutathione, selenium, vitamin C and E (El-Bahr, 2013).

Activities of antioxidant enzymes such as SOD and GPx have previously been reported to be reduced following aluminium-induced cytotoxicity (Ghorbel et al., 2016). SOD is a metal enzyme found in prokaryotic and eukaryotic cells and is the first line of defence against ROS (El-Bahr, 2013). Hence, to evaluate the molecular mechanisms underlying cerebellar damage in aluminium neurotoxicity and calcium protective mechanisms, activities of SOD were assayed in the cerebellum. This was significantly reduced in the cerebellum of juvenile rats that were exposed to aluminium during lactation, signifying the occurrence of oxidative
stress, which was significantly corrected by lactational calcium supplementation. Aluminium has the capability to cross the blood-brain barrier to reach the central nervous system (CNS) to effect its specific action (Yokel, 2012). Calcium has an important role to play during brain development, and its usefulness in mitigating aluminium toxicity could improve brain development in exposed subjects (Yarlagadda, et al., 2007). Malondialdehyde is a biomarker commonly used for the measurement of oxidative stress, being a naturally occurring product of lipid peroxidation (Marnett, 1999). The severity of lipid peroxidation is directly proportional to an increase in MDA level, and this was the case with aluminium neurotoxicity. Lipid peroxidation further impairs the equilibrium that exists between ROS generation and the antioxidant system of the cell, and further damages the cell organelles. Administration of calcium through breast milk was able to reduce lipid peroxidation in the current study, as measured by the reduced concentration of MDA in the juvenile rats administered with calcium supplement during lactation. The activity of calcium as seen in this study was responsible for the remarkable mitigation of ROS generation and ROS-associated damage on the cerebellum of postnatally exposed rats.

Previous investigations have reported the occurrence of marked changes in brain histomorphology and chemistry following aluminium cytotoxicity (Ghorbel et al., 2016). As also observed in the current study, lactational exposure of rats in early life to aluminium chloride resulted in marked alterations in the cerebellar cortex of rats. The pattern of structural damage to the cerebellum included histoarchitectural distortion, degeneration of cells and poor characterisation of Purkinje cells. Peripheral chromatolysis was also present and Nissl staining was poor. These changes could be due to the altered cerebellum redox state that followed aluminium-induced oxidative stress.

Furthermore, as a response to CNS injury and as part of the brain defence mechanism, astrocytes undergo a certain transformation in their molecular expression and morphology (called reactive astrogliosis), the complexity of which depends on the severity of the injury (Sofroniew, 2009). Reactive astrogliosis is a common feature of CNS pathologies (Hamby and Sofroniew, 2010). As seen in the current study, aluminium-induced oxidative stress was responsible for the cerebellar damage, and this injury triggered reactive astrogliosis. However, simultaneous supplementation with calcium prevented astrocytosis in the cerebellum of aluminium-exposed rats.

Cumulatively, the neurotoxic mechanism of aluminium in this study is through exacerbation of reactive oxygen species production which resulted in depletion of the intrinsic antioxidant
defense system as marked by the reduced levels of SOD and GPx (Campbell et al., 1999; Greger et al., 1997). This uncleared ROS then initiated a cascade of chemical events that aggravated lipid peroxidation as marked by the increase in the level of MDA (Chao et al., 2014; Olajide et al., 2017a). The unchecked lipid peroxidation helped the compromised structural integrity of the granule and Purkinje cells of the cerebellum, breaking down the lipid bilayer of the cell and nuclear membrane, thereby perturbing the normal process of protein synthesis and resultanty promoting chromatolysis. The glial cell in the extracellular milieu of the cerebellar neurons then became activated to fix or clear out cells with compromised cellular and nuclear integrity. Lactation calcium supplementation conferred a significant degree of protection against aluminum-induced neurotoxicity. Arguably, the mechanism of calcium neuroprotection in this study is through enhancing and sustaining the integrity of the intrinsic cerebellar antioxidant defence system. This finding agrees with a previous study that reported that calcium supplementation protects lead-induced disturbances in antioxidant enzymes and lipid peroxidation in developing cerebellum (Prasanthi et al., 2010). In the aforementioned study, it was gathered that the toxic effects of Pb in the developing brain could be accredited to oxidative stress which was greatly reduced when supplemented with calcium (Prasanthi et al., 2010).

In line with the findings from our study, calcium has been reported to exhibit antioxidant proclivities in cadmium-induced toxicity (Valko et al. 2005; Ahmad et al. 2016), which has been attributed to the physiological role that calcium plays in downstream signal transduction that culminates in the enhancement of gene expression of antioxidant enzymes (Elsner et al. 1994). The central nervous system requires a steady supply of oxygen to maintain its physiological integrity at a subcellular level (Lahiri et al. 2006). Once this supply of cellular oxygen is compromised by excessive generation of reactive singlet and triplet oxygen species, as seen induced by aluminium in this study, an increase in intracellular calcium levels is a primary coping mechanism (Duffy and MacVicar 1996). Intracellularly, calcium mediates the phosphorylation of the essential kinases, such as tyrosine kinase which drives the molecular cascades that results in enhanced antioxidant defence system (Gonzalez-Fernandez et al. 2013; Macías-García et al. 2016). Calcium-mediated kinase phosphorylation kickstarts the activation of hypoxia-inducible factor (HIF), as a response to elevated levels of ROS (Berchner-Pfannschmidt et al. 2004; Lee and Lee 2013). HIF, in turn, activates multiple signalling pathways such as the phosphatidylinositol 3-kinase/Akt pathway, cyclic AMP pathway and MAP kinase pathway (Fan et al. 2009). These pathways converge to drive gene
transcription in a bid to cope with the elevated level of oxidative stress (Fan et al. 2009; Lee and Lee 2013). Suggestively, calcium supplementation in this study counterbalanced aluminium-induced oxidative stress by modulating intracellular signal transduction, kinase phosphorylation, and ultimately, gene expression.

Calcium is an imperative mineral essential for structural and functional development of the brain during critical period. Despite not being produced in the body, regular oral intake of calcium serves numerous advantages (Lote and Saunders, 1991; Thomas and Weisman, 2006).

5. Conclusion

From the foregoing, it could be stated that calcium supplementation tends to up-regulate antioxidant mechanisms in order to fight ROS being generated as a result of lactational aluminium exposure.
References


Legends

**Figure 1: Superoxide dismutase activity**

Figure 1: Activity of superoxide dismutase in the cerebellum of juvenile rats, showing significant reduction in SOD level in group B compared to other groups, while group C had a significant increase in SOD level compared to group B (p<0.05). A = Control group, B = Aluminium chloride group, C = Calcium supplementation group and D = Aluminium chloride + Calcium group. * and ** are significant values at p<0.05 and p<0.01 respectively.

**Figure 2: Glutathione peroxidise activity**

Figure 2: The cerebellar level of glutathione peroxidase enzyme in juvenile rats. A = Control group, B = Aluminium chloride group, C = Calcium group and D = Aluminium chloride + Calcium group. GPx activity was significantly reduced in group B relative to other experimental groups (p<0.05), with the highest level of GPx seen in calcium-treated group (C); the difference was significant between groups C and B (p<0.05), and between groups C and A (p<0.05), but not group D. * is the significant value at p<0.05.

**Figure 3: Lipid peroxidation**

Figure 3: Cerebellar levels of lipid peroxidation in rats as shown by the level of malondialdehyde (MDA). A = Control group, B = Aluminium chloride group, C = Calcium group and D = Aluminium chloride + Calcium group. MDA level was highest in group B with significant differences compared to the Control and other treated groups, while the level was least in calcium-treated group (C) with significant differences compared with the Control and other treated groups. * and ** are significant values at p<0.05 and p<0.01 respectively.

**Figure 4: Histology and Nissl profile of the cerebellar cortex**

Figure 4: Representative photomicrographs of the general histology (H&E: A-D) and Nissl staining (CFV: E-L) of the cerebellar cortex, showing the molecular layer (ML), Purkinje cell (yellow circles), Purkinje cell layer (PCL) and Granule layer (GL). A, E, I: Control; B, F, J: AlCl₃-induced; C, G, K: Calcium supplementation group; D, H, L: AlCl₃+Calcium group.
Figure 5: Immunoreactivity of astrocytes

Figure 5: Immunohistochemical demonstration of astroglia using anti-rat-GFAP across the layers of the cerebellar cortex in rats. The molecular cell layer (ML), Purkinje cell layer (black arrows), and granule cell layer (GL) were demonstrated across the study groups. GFAP immunopositive cells (red arrows) in control (A) appeared sparse around neurons and between layers, with regular processes, distribution and sizes within the neuropil. However, increased astrocytic densities with reactive astroglia within granule cell layer and hypertrophic cells appeared within the cerebellar layers in Aluminium-treated rats (B). The cerebellar cortex of rats co-treated with Aluminium and calcium (D) had close similarities with those in control (A) and calcium supplementation (C) groups. In both groups A and C, astrocytic processes, cellular distribution and size appeared normal (GFAP x400).

Figure 6: Immunoreactive astrocytes count

Figure 6: Immunopositivity for anti-GFAP. A = Control group, B = Aluminium chloride group, C = Calcium supplementation group and D = Aluminium chloride + Calcium group ** is significant value at p<0.01.
Figures

Figure 1

Figure 2
Figure 3

Figure 4
Figure 5

Figure 6