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Research Paper

Calcium Supplementation Ameliorates Cerebellar Oxidative Stress in Lactational Aluminum-induced Neurotoxicity in Rats

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ABSTRACT

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

Methods: Four groups of juvenile rats were exposed via lactation to distilled water (control group), aluminum (40 mg/kg/d), calcium supplement (50 mg/kg/d), and a combination of both aluminum and calcium from postnatal day 4 to day 28. The cerebella of the animals were excised to access the levels of antioxidant enzymes (superoxide dismutase [SOD], glutathione peroxidase [GPx]), lipid peroxidation (malondialdehyde), histomorphological alterations (hematoxylin and eosin staining), Nissl profile (cresyl fast violet staining), and glial activation (glial fibrillary acidic protein immunohistochemistry).

Results: Lactational aluminum 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, aluminum-induced chromatolysis changes in the Purkinje cell layer, which was counteracted by the antioxidant propensities of calcium supplementation.

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

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Highlights

- Lactational aluminium depressed cerebellar oxidative markers;
- Aluminium chloride caused significant cerebellar astrogliosis;
- Calcium supplements enhanced endogenous oxidative status.

Plain Language Summary

The study attempted to mimic events occurring in normal humans exposed to high levels of aluminium. Infants can be exposed to aluminium through consumption of contaminated infant milk formula or breast milk of exposed mother. This could accumulate in various parts of the brain, including the cerebellum, thereby adversely affecting its functions. The cerebellum is susceptible to metabolic, medicinal and environmental insults, arising from exposure to chemical substances. Results from this study showed that exposure through contaminated breast milk led to increased oxidative damage to the brain (cerebellum) which was corrected to a good extent by the administration of calcium supplements to the rats. It was inferred that administration of calcium supplements could enhance brain antioxidant mechanism and protect it against oxidative stress.

1. Introduction

Aluminum 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 is usually found in compounds such as aluminum oxide, aluminum hydroxide, and potassium aluminum sulfate, some of which are present to varying extents in rocks, vegetation, and animals (Gandara, 2013).

Products manufactured from aluminum 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, aluminum is used to produce vaccine adjuvants, antacids, antiperspirants, and other skincare products. Aluminum salts have been used as coagulants in water treatment to reduce organic matter, color, turbidity, and microbial levels (Sperczyńska et al., 2016). Despite the numerous benefits of aluminum, it could also constitute a hazard to health.

Exposure to toxic levels of aluminum could occur through ingesting contaminated food or water (Singh and Goel, 2015; Niu, 2018). Breastfeeding infants are exposed to aluminum through contaminated infant milk formula or breast milk of exposed mothers (Niu, 2018). Aluminum crosses the blood-brain barrier using transferrin-mediated transport (Singh and Goel, 2015) and accumulates in various brain parts (Kumar and Gill, 2014). Exposure to aluminum has been associated with cognitive impairment and neurodegenerative disorders (Niu, 2018).

The cerebellum contributes to motor coordination and balance and also plays a role in processing signals for perception, cognition, and emotion (Manto & Marmolino, 2009; Pandolfo and Manto, 2013; Reeber et 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 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 the diet and absorbed through the gastrointestinal tract (Thomas & Weisman, 2006). During pregnancy and lactation, calcium intake supports fetal and neonatal growth and development (Thomas & Weisman, 2006). Despite the beneficial role of calcium in growth and development, certain adverse effects, especially in the elderly, have been reported, including an 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 exposed to aluminum chloride during lactation.

2. Materials and Methods

Chemicals and reagents

Aluminum chloride was purchased from Integrated Sunaf (Nig.) Ilorin, Nigeria. The 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.

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).

Animal mating

A vaginal smear test was carried out between 8:00 AM and 9:00 AM daily before mating to know the phase of the estrous cycle of female rats before introducing the male rats. Female rats in their proestrus 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 0 of pregnancy (Marcondes et al., 2002; Omotoso et al., 2018).

Animal grouping and drug administration

Immediately after delivery, the pups were randomly divided into four groups of 6 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 aluminum chloride (40 mg/kg bodyweight/d) (Olajide et al., 2017b) via oral gavage; group C received calcium supplement (50 mg/kg body weight/d) (Weingarten et al., 2008) via oral gavage. Finally, group D was co-administered with aluminum chloride and calcium at the abovementioned doses. The pups were allowed to remain with their mothers for breastfeeding up to postnatal day 28, while treatment commenced from postnatal day 4 to 28 (for 25 consecutive days).

Preparation of brain samples

At the end of the experiment, the young rats were sacrificed into two categories for each group. Rats in the first category were anesthetized with ketamine and perfused transcardially with a flush of phosphate buffer saline (PBS, pH=7.0), 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 hematoxylin and eosin (H&E) and using Cresyl fast violet (CFV) staining techniques. PFA- fixed tissues were also processed to immunohistochemically characterize reactive astrocytes using an 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 (12000 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.

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 were represented in bar charts with error bars to show the mean and standard error of the mean, respectively.

3. Results

Calcium supplementation and aluminum-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 aluminum-induced rats when compared to the control ($P<0.01$) and other treated groups ($P<0.05$)

(Figure 1). The rats that received only calcium supplement (group C) had an increase in SOD level compared with the aluminum-induced rats (P<0.05). However, the SOD level in calcium-treated rats was still lower than that in the control and the rats co-treated with aluminum and calcium, though not statistically significant. Although the tissue level of SOD appeared higher in animals co-treated with aluminum and calcium compared to those that received only calcium, the difference was not statistically significant (P>0.05). Aluminum 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) (Figure 2). On the other hand, rats that received only calcium supplementation had the highest level of cerebellar GPx. However, a significant difference only existed between the former and the aluminum-induced rats (P<0.05) and not with the control or rats co-treated with calcium and aluminum. Administration of calcium during aluminum-induced neurotoxicity increased the level of GPx significantly compared to rats exposed to only aluminum (P<0.05). Although the level was higher than the control, it was not statistically significant (Figure 2).

Calcium supplementation and aluminum-Induced lipid peroxidation

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

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) (Figure 4A and 4C) presented

with a typical cerebellar cortical array of cells characterized by a proper delineation of the molecular layer from the granular layer as separated by the Purkinje cell layer. The granular layer comprises dense clusters of tiny granule cell soma, while the molecular layer comprises fewer cells. The adjoining single-cell layer comprised 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 (Figure 4E, 4I) and C (Figure 4G, 4K) showed proper characterization of the Nissl substance with no chromatolytic changes. However, the histological manifestation of the cerebellar cortex of animals postnatally exposed to aluminum (Group B: Figure 4B) showed perturbed histoarchitectural delineation, 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: Figure 4F, 4J). Animals co-treated with calcium and aluminum chloride showed fine arrays of cells across the cerebellar cortex similar to those of the control groups (Group D: Figure 4H, 4L). 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 aluminum-induced neurotoxicity.

Effects of lactation aluminum and calcium exposure on astrocyte activation

To check the roles of aluminum and calcium supplementation on glial activation, reactive astrocytes were immunohistochemically labeled using a GFAP antibody. Glial expression in the control group appears characteristically normal. Increased GFAP immunopositive cells were observed in aluminum-treated groups, with increased reactive astroglia within the granule cell layer (Figure 5). In rats co-treated with aluminum 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 aluminum, its exposure to the body could affect brain morphology, biochemistry, and behavior (Kumar & Gill, 2014). Oxidative stress stands out as one of the mechanisms of neurotoxicity associated with aluminum exposure (Andrade et al.,

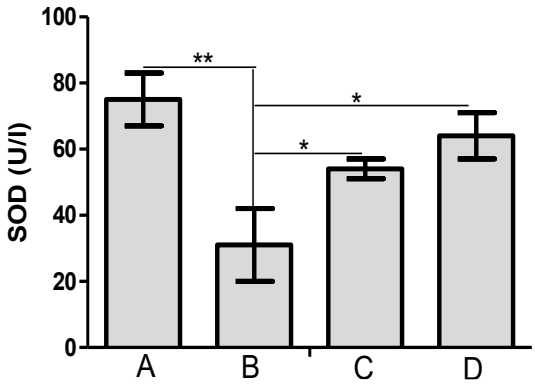

Figure 1. Superoxide dismutase activity

The activity of superoxide dismutase (SOD) in the cerebellum of juvenile rats shows a significant reduction in SOD level in group B compared to other groups, while group C shows an increase in SOD level compared to group B (P<0.05).

A, control group; B, aluminum chloride group; C, calcium supplementation group; D, aluminum chloride + calcium group.

* and ** are significant values at P<0.05 and P<0.01, respectively.

2017). Utilizing this pathway, the current study explored the role of calcium supplementation in protecting the cerebellum against aluminum-induced oxidative stress.

The current study exposed the inability of the antioxidant capacity of the cell to cope with the free radicals generated as a result of aluminum 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 from generated free radicals has been linked to the development of some diseases in the body, including neurodegenerative disorders (Phaniendra et al., 2015).

The body's antioxidant system, 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

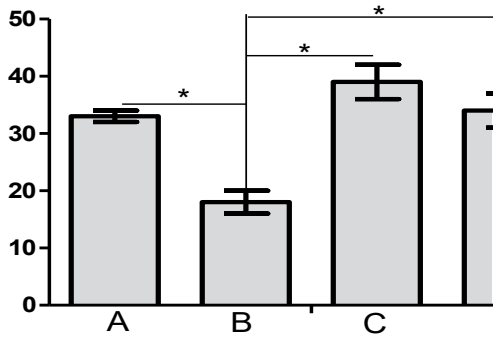

Figure 2. Glutathione peroxidise activity

The cerebellar level of glutathione peroxidase (GPx) enzyme in juvenile rats.

A, control group; B, aluminum chloride group; C, calcium group; D, aluminum 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 the 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.

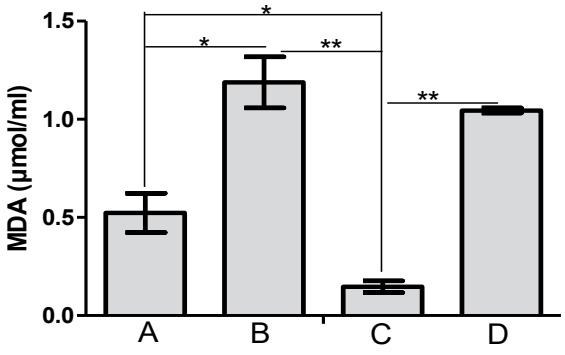

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Figure 3. Lipid peroxidation

Cerebellar levels of lipid peroxidation in rats as shown by the level of malondialdehyde (MDA).

A, control group; B, aluminum chloride group; C, calcium group; D, aluminum 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 the 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.

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 aluminum-induced cytotoxicity (Ghorbel et al., 2016). SOD is a metal enzyme found in prokaryotic and eukaryotic cells and is the first line of defense against ROS (El- Bahr, 2013). Hence, to evaluate the molecular mechanisms underlying cerebellar damage in aluminum neurotoxicity and calcium protective mechanisms,

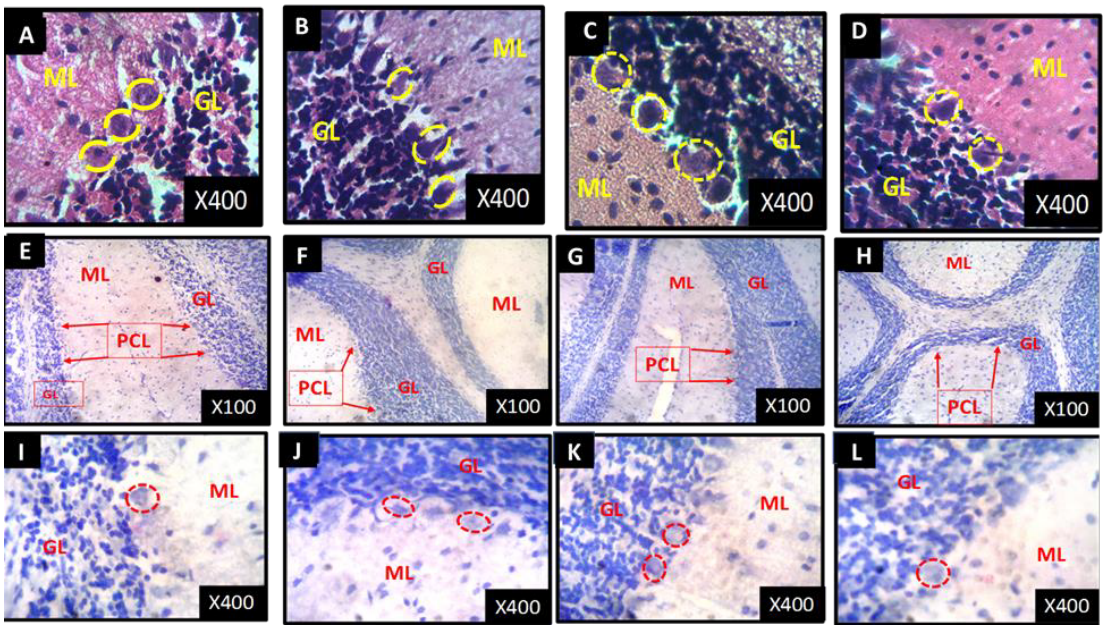

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Figure 4. Histology and nissl profile of the cerebellar cortex

Representative photomicrographs of the general histology (H&E: A-D) and Nissl staining (CFV: E-L) of the cerebellar cortex show the molecular layer (ML), Purkinje cell (yellow circles), Purkinje cell layer (PCL) and Granule layer (GL).

A, E, and I, control; B, F, and J, AlCl3-induced; C, G, and K, calcium supplementation group; D, H, and L: AlCl3+calcium group.

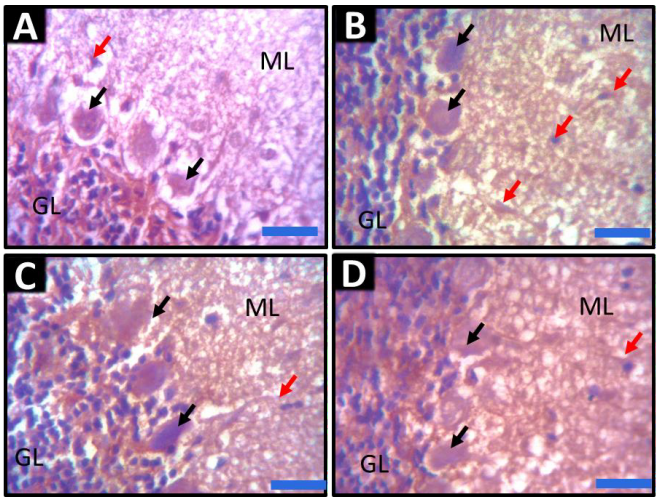

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Figure 5. Immunoreactivity of astrocytes

Immunohistochemical demonstration of astroglia using anti-rat-glial fibrillary acidic protein (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 the 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 the granule cell layer and hypertrophic cells appeared within the cerebellar layers in aluminum-treated rats (B). The cerebellar cortex of rats co-treated with aluminum and calcium (D) had close similarities with those in the control (A) and calcium supplementation (C) groups. In groups A and C, astrocytic processes, cellular distribution, and size appeared normal (GFAP x400).

activities of SOD were assayed in the cerebellum. This was significantly reduced in the cerebellum of juvenile rats that were exposed to aluminum during lactation, signifying the occurrence of oxidative stress, which was significantly corrected by lactational calcium supplementation. Aluminum can cross the blood-brain barrier to reach the central nervous system (CNS) to affect its specific action (Yokel, 2012). Calcium has an important role during brain development, and its usefulness in mitigating aluminum 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).

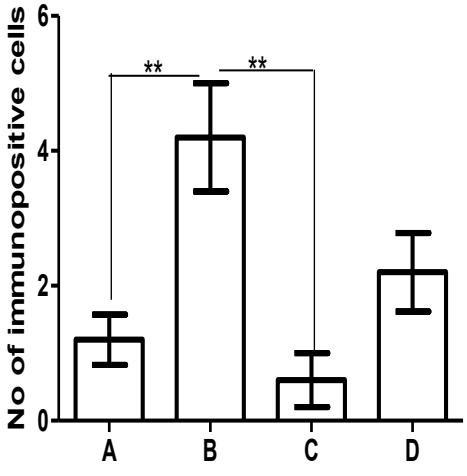

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Figure 6. Immunoreactive astrocytes count

Immunopositivity for anti-glial fibrillary acidic protein (GFAP).

A, Control group; B, Aluminum chloride group; C, Calcium supplementation group; ;D, Aluminum chloride + Calcium group.

** is significant at P<0.01.

The severity of lipid peroxidation is directly proportional to an increase in MDA level, which was the case with aluminum neurotoxicity. Lipid peroxidation further impairs the equilibrium between ROS generation and the antioxidant system of the cell and further damages the cell organelles. Administration of calcium through breast milk could reduce lipid peroxidation in the current study, as measured by the reduced concentration of MDA in the juvenile rats administered with calcium supplements during lactation. Calcium activity, as seen in this study, was responsible for remarkably mitigating ROS generation and ROS-associated damage on the cerebellum of postnatally exposed rats.

Previous investigations have reported marked changes in brain histomorphology and chemistry following aluminum cytotoxicity (Ghorbel et al., 2016). As also observed in the current study, lactational exposure of rats in early life to aluminum chloride resulted in marked alterations in the cerebellar cortex of rats. The structural damage pattern to the cerebellum included histoarchitectural distortion, degeneration of cells, and poor characterization 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 aluminum-induced oxidative stress.

Furthermore, as a response to CNS injury and as part of the brain defense 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, aluminum-induced oxidative stress was responsible for cerebellar damage, and this injury triggered reactive astrogliosis. However, simultaneous supplementation with calcium prevented astrocytosis in the cerebellum of aluminum-exposed rats.

Cumulatively, the neurotoxic mechanism of aluminum in this study is through exacerbation of reactive oxygen species production which resulted in the 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 resultantly 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 enhances and sustains the integrity of the intrinsic cerebellar antioxidant defense system. This finding agrees with a previous study that reported that calcium supplementation protects against lead-induced disturbances in antioxidant enzymes and lipid peroxidation in the developing cerebellum (Prasanthi et al., 2010). The aforementioned study found that the toxic effects of lead 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 aluminum in this study, an increase in intracellular calcium levels is a primary coping mechanism (Duffy and MacVicar, 1996). Intracellularly, calcium mediates the phosphorylation of essential kinases, such as tyrosine kinase, which drives the molecular cascades that result in an enhanced antioxidant defense 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 signaling 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 aluminum-induced oxidative stress by modulating intracellular signal transduction, kinase phosphorylation, and, ultimately, gene expression.

Calcium is an imperative mineral essential for the structural and functional development of the brain during the 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.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declare no conflict of interest.

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