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Role of Eugenol in Aluminium-Induced Histochemical and Histomorphological Changes within the Purkinje Cells of the Cerebellum of Wistar Rats.

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ABSTRACT

Build-up of Aluminium within the brain can occur via different routes. Accumulation of aluminium in different forms have been known to trigger neuronal excitotoxicity and hence triggering apoptosis among neuronal cells. The aim of this study was to study the protective effects of eugenol on aluminium induced neurotoxicity on the cerebellum of adult Wistar rats. Twenty (20) Wistar rats was divided into Four (4) groups namely: CTRL (2 ml/kg distilled water), AC (100 mg/kg aluminium chloride), EG (150 mg/kg eugenol) and EG+AC (150 mg/kg eugenol + 100 mg/kg aluminium chloride). All rats were treated for a duration of 21 days and on day 22 (24 hrs after last administration), they were sacrificed with 0.8 ml/kg of ketamine as anesthetizing agent. Administration of aluminium chloride resulted in cytoarchitectural distortion of purkinje cells, reduction in staining intensity of Nissl substance within purkinje cells using cresyl fast violet. However treatment with eugenol resulted in improved staining intensity using cresyl fast violet, hence preserved Nissl substance. The use of Congo red however did not however reveal any amyloidal plaques at the dose and duration of administration. Administration of aluminium chloride also resulted in a significant ($p<0.05$) increase in purkinje cell area when compared to the control and treatment with eugenol resulted in significant ($p<0.05$) reduction in purkinje cell area. The use of Eugenol offers a promising prospect in the management of toxicity associated with aluminium chloride and neurodegenerative diseases.

Keywords: Eugenol, Nissl substance, Cresyl fast violet, Excitotoxicity, Purkinje cell.

INTRODUCTION

Aluminium is highly reactive in the presence of carbon and oxygen which are among the leading elements necessary to maintain life within our ecosystem. Due to this reason, the availability of aluminium have a diverse implications towards the health of humans and animals. Evidence shows that aluminium is toxic to all forms of life on earth and where it appears in terrestrial biochemistry[1]. Aluminium has a very diverse use and hence finds its way to almost every aspect of our daily lives and its use includes but not limited to cans, cookware, aluminium foil, housing materials, and components of electrical devices, airplanes, boats, cars and numerous hardware items of all descriptions[2,3]. With regards to bioavailability of aluminium, it can also be found in drinking water (owning to its action as a flocculant). In processed foods, aluminium is used commonly as an additive, cosmetics and increasingly within pharmaceutical products[3]. Human exposure to aluminium from any source can have very severe deleterious effect on both adult and developing nervous system[4,5]. Aluminium as a metal is unable to cross the blood brain barrier (BBB) and the BBB thus acts as a natural filter for the brain. Aluminium compounds such as chlorides, fluorides and sulphides possess the ability to cross the BBB[6]. Eugenol is commonly used in dentistry, anaesthetics, analgesics, anti-inflammatory and flavouring agents[7]. Eugenol also has antioxidant properties with the ability to mop up reactive oxygen species associated with aluminium toxication[8]. This study aimed at investigating neuroprotective effects of eugenol against Histochemical and morphological changes associated with aluminium induced cerebellar neurotoxicity in adult Wistar rats.

MATERIALS AND METHODS

All protocols and treatment procedures were carried out according to the Ahmadu Bello University Research Committee (ABURC) guidelines, and approved by the Department of Anatomy Post Graduate Research and Ethics Committee. A total of 20 adult Wistar rats of average weight 140- 160 g were randomly grouped into four groups with five animals in each group. These rats were obtained from K-Vom National

Research Institute, Jos, Nigeria, and allowed to acclimatize for two weeks before the commencement of this study, and were fed standard pelletized feed (Grand Cereals and Oil Mills Limited, Jos, Nigeria) and clean water ad libitum. Rat groupings and dosage is shown in Table 1. Route of administration was via oral route except otherwise stated.

Reagent Preparation and Treatment Regimen: Aluminium chloride (AlCl3, #7446-70-0; Guandong Guanghua Sci-Tech Co. Ltd., Shantou, Guandong, China) was prepared at a dose of 100 mg/kg bwt[8,9]. Eugenol (#58-23-4; Yueyang Jiazhiyuan Biological Co Ltd, Hunan China) of 99.9% purity level and prepared at a dose of 150 mg/kg bwt respectively (5 % LD50 of 3000 mg/kg). Rats were divided into four groups and were designated as groups' I-IV. Group I was administered 2 ml/kg distilled water (CTRL); Group II was administered 100 mg/kg of Aluminium chloride (AC); Group III was administered 150 mg/kg Eugenol (EG); Group IV was administered 150 mg/kg Eugenol and 100 mg/kg AlCl3 (EG + AC).

Table 1: Rat Grouping and Dosage

GROUP	DOSE
Control (CTRL)	2 ml/kg
Aluminium chloride (AC)	100 mg/kg
Eugenol (EG)	150 mg/kg
Eugenol + Aluminium Chloride (EG+AC)	150 mg/kg + 100 mg/kg

N=5, Eugenol (EG), Control (CTRL), Aluminium Chloride (AC)

Animal Sacrifice and Tissue Processing: Twenty-four hours following the termination of the treatment, the animals were weighed and then sacrificed using ketamine (0.8 mg/kg)[8] as the anaesthetic agent, intraperitoneally. They were then decapitated and the skull carefully opened with a surgical blade and a pair of scissors. Paraffin embedded tissues were sectioned at a thickness of 5µm and processed histochemically for Cresyl fast violet and golgi stain respectively as summarized below

Cresyl Fast Violet Staining: Tissues were fixed at 10% formalin and paraffin embedded tissues sectioned at 5µm. Deparaffinized sections in xylene 2 or 3 changes at 10 min each. This is followed by Hydration in 100% alcohol two changes and 5 min each, 95% alcohol for 3 min, 70% for 3 minutes. Rinse in tap water and then in distilled water. Insufficient deparaffinization will cause uneven staining. **S**tain in 0.1% cresyl violet solution for 3-10 min. Notes: Staining in warmed cresyl violet solution (warm up in 37-50 ºC oven) can improve penetration and enhance even staining. It is particularly beneficial for thicker (20-50 um) sections. Rinse quickly in distilled water and differentiate in 95% ethyl alcohol for 2-30 min and check microscopically for best result. Dehydrate in 100% alcohol twice with 5 min each. Clear in xylene twice with 5 min. Mount with permanent mounting medium.[10]

Congo Red: Tissues were fixed at 10% formalin and paraffin sectioned at 5µm. Deparaffinize and hydrate sections to distilled water. Stain in Congo red solution for 30-60 min. Rinse in distilled water and differentiate rapidly (5-10 dips) in alkaline alcohol solution. Rinse in running tap water for 5 min. Counterstain in Gill's hematoxylin for 30 seconds. Rinse in tap water for 1 minute. Dip in ammonia water (add a few drops of ammonium hydroxide to tap water and mix well) for 30 seconds or until sections turn blue. Rinse in tap water for 5 min. Dehydrate through 95% alcohol, 100% alcohol Clear in xylene and mount with resinous mounting medium[11].

Morphometric Studies: Twenty four hours after the last administration (Day 22), rats were sacrificed and brain was removed and cleaned of any adhering structures, embedded in paraffin sections and sectioned at a thickness of 5µm stained with cresyl fast violet. Cresyl fast violet micrographs (digital microscopic images) were used for histometry. Three micrographs were randomly captured[12,13] with emphasis on the purkinje cells of the cerebellum and 5–20 cells were randomly selected, measured and averaged in three micrographs[14,15] .

Procedure: This is briefly described below:
Using a light microscope with a x25 objective (x 250 magnification) and computer running image processing software (Amscope MT *version* 3.0.0.5, USA) for microscopy, digital microscopic images were captured.

Histometry and calibration (Using the Amscope Microscope Cameras; Psi Wave function, 2009): Firstly a micrometer slide (microscope slide with scale on it), is captured at the same magnification and quality (pixels) the micrograph was photographed

- A line tool was selected and this was used to carefully measure from start of one major line (of the captured microscope slide) to the start of the next, or end to end.
- Recorded length in pixels; in this study was 408 pixels/ 100µm or 4.08 pixel/µm.
- Next use a polygon tool to measure the area and perimeter of desired cells. Values are computed and presented automatically by the software.

Statistical Analysis: Results obtained from this

research were analyzed with statistical, package for social sciences (IBM SPSS version 21.0, SPSS Inc.) and Microsoft office Excel 2007 for charts. These results were expressed as mean ± Standard error of mean (S.E.M) and significant differences among means of the groups was determined with one way analysis of variance (ANOVA) with Turkey's *post -hoc test* for significance. Paired 6sample t-test was used for comparison of mean. Values were considered significant when $p \leq 0.05$.

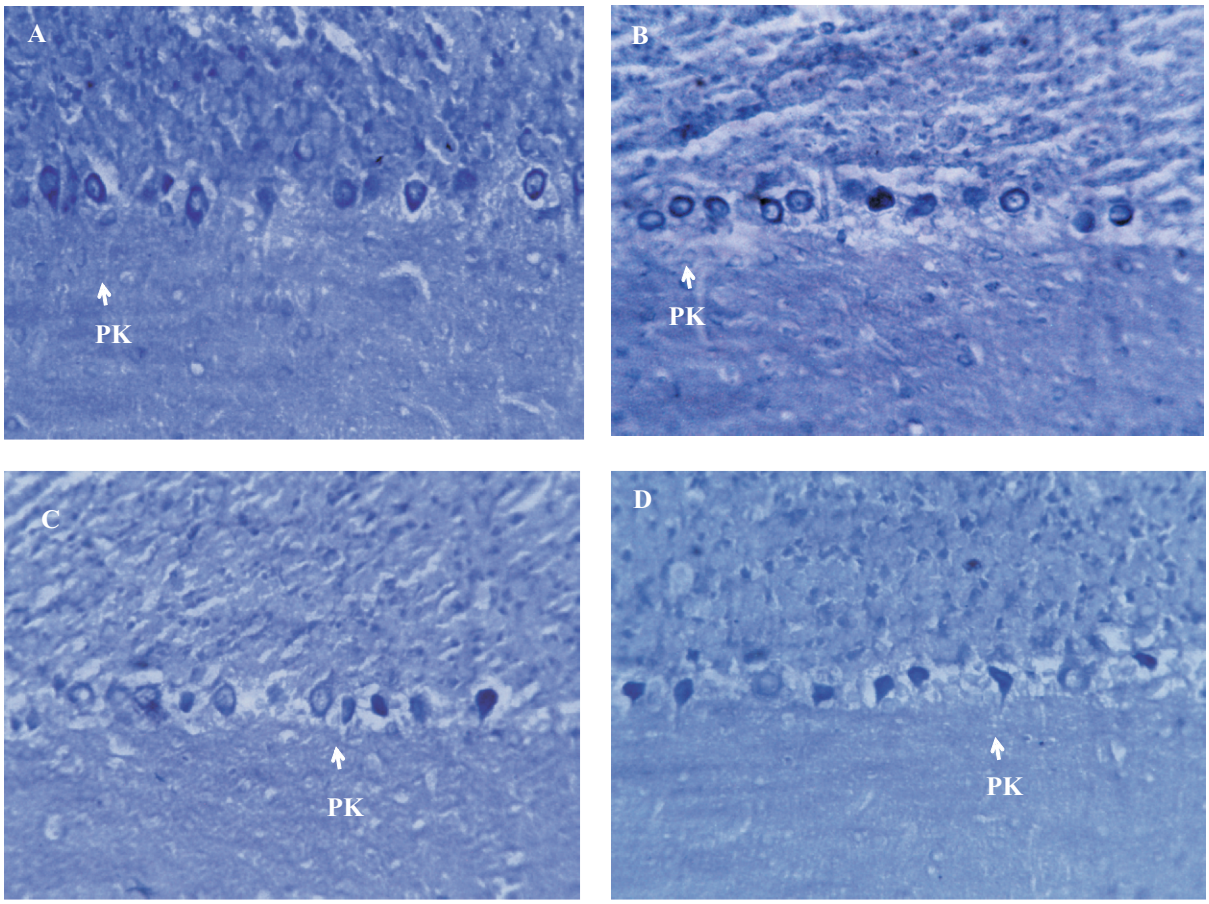
RESULTS

Histometric analysis of soma area (μm^2) and perimeter (μm) of purkinje neuronal cells revealed a significant ($p<0.05$) increase in area of soma purkinje cells among the group administered 100 mg/kg aluminium chloride (AC) when compared to the control and a significant ($p<0.05$) reduction in Purkinje soma perimeter when aluminium chloride (AC). Administration of eugenol with aluminium chloride (EG+AC) however resulted in a significant ($p<0.05$) reduction in purkinje soma perimeter when compared to the AC treated group. The group administered 150 mg/kg eugenol (EG) revealed a non-significant ($p>0.05$) in soma area and perimeter of purkinje cells when compared to control but significant when compared to AC treated group.

Table 2: Histometric analysis of the effect of Eugenol on cortical neurons of the cerebellum (Purkinje cells) Wistar rats

Groups	Area (μm^2)	Perimeter (μm)
CTRL	1.79 ± 0.14	5.28± 0.13
AC	2.50 ± 0.11*	3.38±0.61*
EG	1.84 ± 0.12$^+$	5.20±0.16$^+$
EG+AC	1.78 ± 0.19$^+$	5.21±0.22$^+$

n = 5; mean ± SEM One way ANOVA *LSD post hoc test*: *= $p<0.05$ when compared to the control (CTRL) group; + = $p<0.05$; when compared to the aluminium chloride (AC).



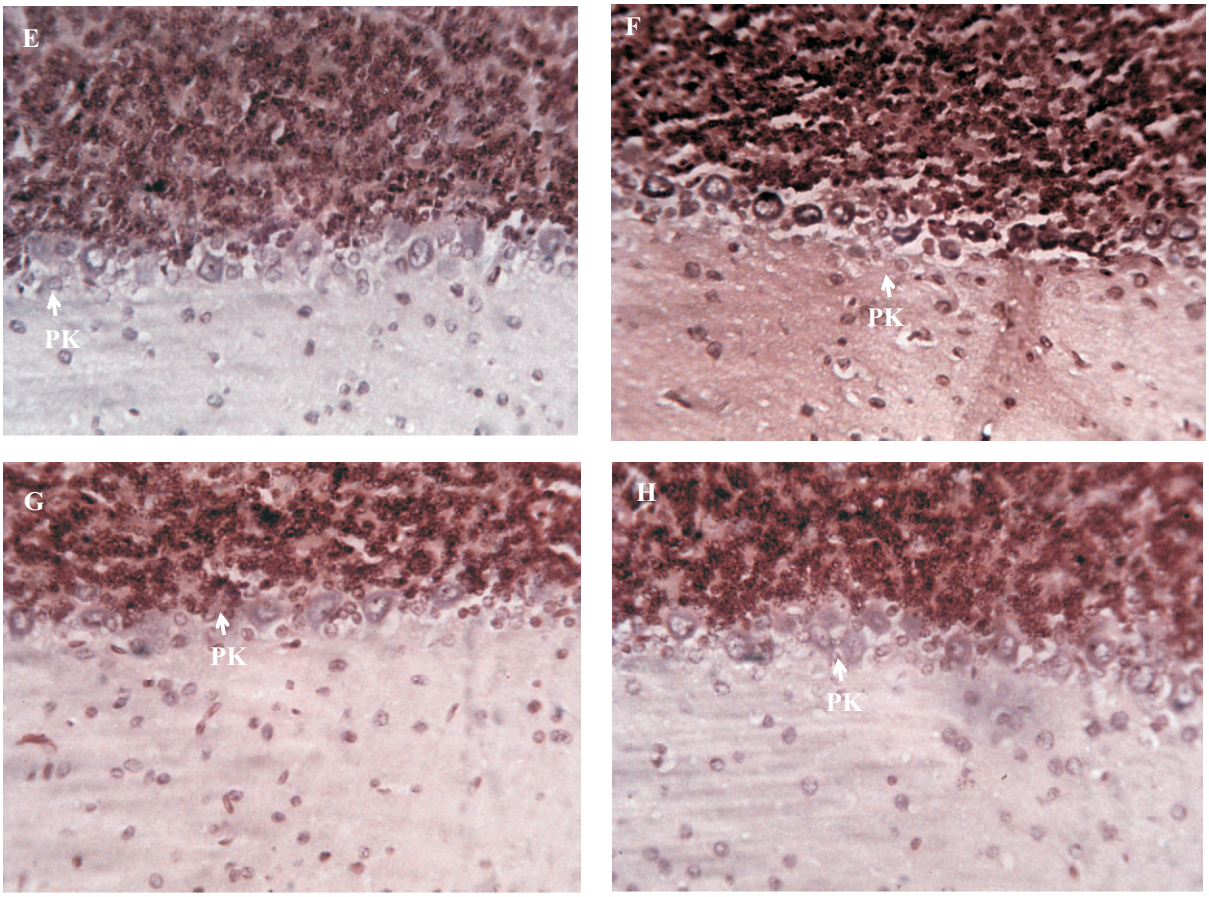


Figure 1: A = Section of Wistar rat cerebellar cortex of the control (CTRL) untreated group showing normal cytoarchitecture and normal staining intensity of nissil substance of Purkinje cells of the cerebellar cortex. Purkinje cell (PK) (Mag x 250 CFV).

B = Section of Wistar of cerebellar cortex of the aluminium treated group (AC) (100mg/kg of aluminium chloride) showing distortion in the cytoarchitecture and reduced staining intensity of nissl substance of Purkinje cell of the cerebellar cortex. Purkinje cell (PK); (Mag x 250 CFV).

C = Section of Wistar rat Cerebellar cortex of Group EG (150mg/kg of Eugenol) revealing a normal cytoarchitecture and normal nissl stain intensity of purkinje cells of the cerebellar cortex when compared to the control. Purkinje cell (PK) (Mag x 250 CFV).

D = Section of Wistar rat cerebellar cortex of Group EG+AC (150mg/kg of Eugenol and 100mg/kg of aluminium chloride) revealing a mild distortion in the cytoarchitecture and reduced stain intensity of nissl substance of Purkinje cells of the cerebellar cortex with perineuronal vacoulations. Purkinje layer (PK); (Mag x 250 CFV).

E = Section of Wistar rat cerebellar cortex of the control (CTRL) untreated group showing normal cytoarchitecture of the cerebellar cortex. Purkinje cell (PK) (Mag x 250 Congo red)

F = Section of Wistar rat cerebellar cortex of Group the aluminium treated (AC) group (AC) (100mg/kg of aluminium chloride), showing distortion the cytoarchitecture of the cerebellar cortex but no visible amyloid plaques. Purkinje cell (PK); (Mag x 250 Congo).

G = Section of Wistar rat Cerebellar cortex of Group EGL (150mg/kg of Eugenol) revealing a normal cytoarchitecture of the cerebellar cortex and no visible amyloid plaques. Purkinje cell (PK) (Mag x 250 Congo red).

H = Section of Wistar rat cerebellar cortex of Group EG+AC (150mg/kg of Eugenol and 100mg/kg of aluminium chloride) with mild distortion in the histology of the cerebellar cortex but no visible amyloid plaques. Purkinje cell (PK); (Mag x 250 Congo red).

Histochemical Findings: Thin sections of the cerebellum were studied qualitatively using Cresyl fast violet (CFV) and Congo red to characterize histochemical and histopathological changes associated with aluminium chloride intoxication. Purkinje cells within the cerebellum revealed a reduction in staining intensity with nissl substance in the group administered aluminium chloride (AC) when compared to the control group. Congo red displayed some cytoarchitectural distortion such as perineuronal vacoulations but no visible amyloid deposition. Treatment with eugenol (EG+AC) however revealed an increase the staining intensity of nissl substance within the purkinje cells and reduced perineuronal vacoulations. Congo red stain showed reduced cytoarchitectural distortion and no visible amyloid plaques.

DISCUSSION

In the present study, we investigated the role eugenol on aluminium induced histochemical and histomorphological changes within the purkinje cells of the cerebellum.

Histological observations can be quantified with histometry and hence these provides a sound basis for comparison of these observation. Histometry confers objectivity and as a result increasing precision compared with direct visual appraisal and it makes statistical analysis easier. It improves assessment of certain histological changes, which can be observable by eye, are accurately graded and these changes are better appreciated and quantified[16,17,18].

In this study, histometry of the Soma cell area and perimeter of purkinje cells of the cerebellum were conducted. Histometric analysis revealed a significant ($p<0.05$) reduction in purkinje cell soma area and increase in perimeter of the aluminium chloride treated group relative to control. Aluminium as a well-established neurotoxicant has the ability to result in neuronal shrinkage arising from excitotoxicity which results in neuronal degeneration and death[19].

Neurodegneration is a process involved in both neuropathological conditions and brain ageing[20]. Histoarchitectural distortion of neural tissue manifesting as neuronal degenerative changes are indicative of neurotoxicity in the central nervous system[21,22]. Degenerative changes are observed as cortical neuronal shrinkage and perineuronal vacoulations in sections of the cerebellar regions of aluminium-treated rat compared to the control and these indicates treatment (aluminium) related neurotoxicity. This is in concordance to the findings of previous researchers on aluminium, who reported that this metal have the capacity to induce nervous tissue damage[23,24,25].

Neuronal injury may result in irreversible cell damage or cell death[26]. Cell death has also been reported to result from neuronal degeneration[27]. Cell death may also result from necrosis, a pathologic type of cell death that occurs from extrinsic insults to the cells or after abnormal stress such as toxin, chemical injury, thermal and mechanical factors[28].

Necrosis is an indication of treated related toxicity[28]. Cresyl fast violet (CFV) is a stain for Nissl bodies (essentially rough endoplasmic reticulum RER). Degenerating neurons can stain very poorly (with low staining intensity) with CFV as a result of dissociation of ribosomes from RER which occurs in the early stage of cell degeneration[29].

Treatment of rats with Eugenol showed preserved of cerebellar cortex parenchyma and cytoarchitectural preservation of neuronal cell Nissl substance. This implies that Eugenol has a protective effect against aluminium-induced neurotoxicity. Results from this study are in consistence with the report on the neuroprotective effects of Eugenol against aluminium-induced toxicity[30]. Purkinje and granule cells are the most important targets in cerebellum for toxic substances[31]. Neurodegenerative changes such as perineuronal vacoulations, neuronal cytoplasmic shrinkage, gliosis and swelling of neuronal cytoplasm occur within the purkinje cells of the cerebellum. Findings are in agreement with the studies related to aluminium chloride and enlarged cell bodies[32].

Administration of Eugenol showed a preserved neuronal cell Nissl substance in Wistar rats. This implies that Eugenol has a protective effect against aluminium induced neurotoxocity. Results from this study are in agreement with the report on the neuroprotective of eugenol[30].

In this study administration of aluminium chloride to rats, did not reveal any amyloidal plaques at the administered dose and duration using Congo red stain (to detect amyloidal plaques). This is in agreement with previous studies on Long-term oral intake of aluminium or zinc does not accelerate Alzheimer pathology in transgenic mice[33].

It was observed by[33] that long-term oral intake of Al does not accelerate AD pathology in transgenic mouse models and does not promote the deposition of amyloid plaques in mouse models. Oral ingestion of Aluminium did not accelerate deposition of amyloid plaques throughout the duration of the study, hence no visible amyloid plaques in cerebellum of the experimental animals. Absence of amyloid plaques was reported by[34] following oral administration of aluminium chloride for the duration of a three week study within the cerebral, cerebellar cortex and hippocampus of Wistar rats.

Neuroprotection refers to the relative preservation of

neuronal structure and/or function[35]. Relative preservation of neuronal integrity implies amelioration in the rate of neuronal damage in the presence of a neurotoxicant such as aluminium.

Findings are in consistence with reports related to the neuroprotective activity of plants which also contain eugenol[36,37,38]. Most of this studies attribute the neuroprotective effects to the antioxidant properties of eugeno[39,40].

CONCLUSION

Eugenol administration at a dose of 150 mg/kg was able to ameliorate degenerative changes associated with oral administration of aluminium chloride. These degenerative changes include degeneration of the nissl substance, perneuronal vacoulation and neuronal shrinkage which lead to degeneration of purkinje cells. These degeneration were quantified with histometry and revealed and significant ($p<0.05$) reduction in soma cell area of purkinje.

Eugenol thus offers a promosing prospect in attenuating neurodegenerative changes associated with neirodegenerative diseases.

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