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Volume 13 (2024) (<https://rescon.jssuni.edu.in/ijhas/vol13/>)

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Volume 12 (2023) (<https://rescon.jssuni.edu.in/ijhas/vol12/>)

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Volume 11 (2022) (<https://rescon.jssuni.edu.in/ijhas/vol11/>)

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Kolaviron ameliorates toxic effects of aluminum chloride on the hippocampus of fetal Wistar rats in utero: Biochemical and ultrastructural observations

Susan Folashade Lewu, Bernard Ufuoma Enaibe

Abstract:

BACKGROUND: This study was designed to investigate the biochemical and ultrastructural effects of kolaviron (Kv) on the hippocampus of fetal Wistar rats exposed to aluminum chloride (AlCl_3) toxicity *in utero*.

MATERIALS AND METHODS: Fifty female Wistar rats were selected at random and mated. Following confirmation of mating, pregnant rats were assigned into five groups ($n = 5$). Controls: Group A received distilled water; Group B: 0.6 ml of corn oil; Group C: 200 mg/kg of Kv; Group D: 100 mg/kg of AlCl_3 and Group E 100 mg/kg of AlCl_3 and 200 mg/kg of Kv. Administration was done from days 8-10 and 15-17 of gestation during the 2nd and 3rd weeks respectively. Biochemical analyses were investigated to assess oxidative stress levels, while transmission electron microscopy (TEM) examined ultrastructural changes. Pregnant animals were sacrificed on day 20 of gestation; fetuses, their brains, and hippocampi were excised, respectively. Hippocampal tissues of fetuses were homogenized in 0.25 M of sucrose solution for biochemical assay while some were fixed in 2.5% phosphate-buffered saline-based glutaraldehyde for TEM.

RESULTS: Elevated levels of Al, malondialdehyde, nitric oxide, and interleukin 6 were observed in the hippocampi of fetuses whose mothers received AlCl_3 . TEM revealed loss of nuclear membrane and increased condensation of chromatin materials in the same group. However, significant reduction of these enzymes including improved ultrastructural alterations were observed in the fetal hippocampus of the AlCl_3 + Kv-treated group.

CONCLUSION: This study showed that Kv significantly reduced neurodegenerative effects induced by AlCl_3 in the hippocampi of fetal Wistar rats *in utero* probably owing to its antioxidant and anti-inflammatory properties.

Keywords:

Aluminum chloride, fetus, free radicals, hippocampus, kolaviron

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Received: 15-05-2020

Revised: 26-07-2020

Accepted: 11-10-2020

Published: 18-05-2021

Introduction

Demographic changes seen in neurodegenerative disorders in the medical and public health sector will become a major challenge in the coming future.^[1] This could be attributed to human and animal exposure to environmental impurities^[2]

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such as excipient, genotoxic, stereoisomer, and elemental impurities. Aluminum (Al), an example of elemental impurity and associated with neurodegenerative diseases, is the third most abundant element in the earth's crust (8.13%) after oxygen (49.5%) and silicon (26%).^[3] It is released into the environment through mining activities and wants for industrial use.^[4] Al can also

How to cite this article: Lewu SF, Enaibe BU. Kolaviron ameliorates toxic effects of aluminum chloride on the hippocampus of fetal Wistar rats in utero: Biochemical and ultrastructural observations. *Int J Health Allied Sci* 2021;10:115-22.

be found in foods such as yellow cheese, salt, herbs, spices, corn, and drinking water and has been established in its use as component of some drugs, for example, antacids.^[5-8]

Al salts (Al chloride [AlCl_3]) induced experimental encephalopathy resulted in Alzheimer's disease with neurological features such as neurofibrillary tangles.^[5] Studies involving the kidney, liver, testes, bone, and heart were associated with Al toxicity. It was able to cross the blood-brain barrier by binding to transferrin receptors to exert its effects on the nervous system, the most vulnerable to its toxicity particularly the hippocampus and neocortex in the brain where cognitive deficiency and dementia were observed.^[3,4] Exposure of the fetus (a vulnerable population) to Al toxicity during pregnancy was reported to be the probable cause of miscarriages which could be as a result of maternal influence through environmental exposure, diet, and Al-containing medications.^[9] Al was also found to facilitate the development of neural tube defects in humans.^[10]

A wide range of drugs produced from some plants have been implicated in having medicinal values.^[11] Kolaviron (Kv) which is rich in natural bioflavonoids, namely: kolaflavonone, *Garcinia* bi-flavonoid I, and *Garcinia* bi-flavonoid II, is an ethanolic extract of *Garcinia kola* plant generally known as bitter or male kola.^[12] Metabolites of Kv enable its ability to cross the blood-brain barrier through which it exhibits cholinergic, antioxidative, anti-inflammatory, antinociceptive, antigenotoxic, and chelating properties.^[13-17] Kv has been implicated in neuroprotective activities against methamphetamine-induced toxicity through the inhibition of acetylcholinesterase enzyme activity.^[18] It was also found to prevent oxidative damage by boosting antioxidant indices in gamma radiation-induced toxicity.^[19]

The neuroprotective and ameliorative effects of Kv have been widely researched at 200 mg/kg body weight in adult Wistar rats; however, there is a dearth of literature on the relationship between Al toxicity and the possible effects of Kv on the hippocampus of fetal Wistar rats *in utero*.

Materials and Methods

Chemicals

AlCl_3 and corn oil were purchased from Sigma-Aldrich (USA). Phosphate-buffered saline (pH 7.4) was freshly prepared. Al, malondialdehyde (MDA), and nitric oxide (NO) assay kits were obtained from Abcam®, USA. Petroleum ether, acetone, and ethyl acetate were of analytical grade and purchased from Sigma Aldrich (USA). Other materials and chemicals were of analytical grade and were sourced from within our laboratory.

Kolaviron extract

Seeds of fresh *Garcinia kola* (*G.kola*) were obtained locally in Ilorin, Nigeria and certified by the curator in the Department of Botany, Faculty of Life Sciences, University of Ilorin, Ilorin where a voucher specimen is available in the herbarium of the same institution (Specimen voucher number: UILH/001/1217). About 4 kg of the peeled seeds of *G. kola* were cut into pieces and air-dried for 2 weeks at room temperature (28°C–30°C). The dried seeds were pounded to fine powder with a mortar and pestle. The powdered seeds were defatted using light petroleum ether (boiling point: 40°C–60°C) for 48 h in the Laboratory of the Anatomy Department. The defatted dried marc was further extracted with acetone (boiling point: 56°C–60°C) in a soxhlet extractor for 24 h. The yield was then concentrated and diluted twice its volume with distilled water and then extracted with ethyl acetate which yielded a golden yellow solid known as Kv. This procedure was carried out according to the method of Iwu as modified by Farombi *et al.* and Olajide *et al.*^[12,20,21]

Purification and validation of Kv were determined by subjecting it to thin-layer chromatography in the laboratory of Prof. E. O. Farombi at the Drug Metabolism Unit, Faculty of Basic Medical Sciences, University of Ibadan, Nigeria. It was achieved through the use of silica gel GF 254-coated plates and solvent mixture of methanol and chloroform in a ratio 1:4 v/v. The separation revealed the presence of three bands which were viewed under UV light at a wavelength of 254 nm with RF values of 0.48, 0.71 and 0.76 respectively. The extract was kept at a room temperature (23°C–25.5°C) before and after each use.

Animal care and ethical approval

Fifty female Wistar rats weighing 200–220 g were procured from an authorized vendor (MCTEMMEY Laboratory Animals Concept, Oshogbo, Nigeria) for the purpose of this study. Twenty-five male rats of the same strain were also procured for mating purposes. Animals were housed in the animal holding of the Faculty of Basic Medical Science, College of Health Sciences, University of Ilorin, under a 12 h light and 12 h dark cycle at the room temperature in a well-ventilated environment. They were fed with standard animal pellet (Ogo-Oluwa Feeds at Sango, Ilorin Kwara State, Nigeria) and water *ad libitum*. The study design and protocol were approved while ethical clearance was obtained from the University of Ilorin Ethical and Review Committee with the approval number UERC/ASN/2016/361.

Vaginal smear and estrous cycle

Vaginal smear test was done by introducing a micropipette containing 0.5 ml of normal saline into the vagina of the nonpregnant rats and then withdrawn and placed on a

clean glass slide for viewing under a light microscope.^[22] Female animals in their pro-estrous phase (represented by the presence of about 75% cornified cells and about 25% nucleated cells under a light microscope) were then randomly assigned in a ratio of 2 females to 1 male for mating to occur. Mating was confirmed by the presence of sperm cells the day following the day of male introduction into the female rat's compartments.^[23] Hence, animals were again grouped based on this observation which was recorded as day 1 of gestation.

Experimental design

Pregnant animals in their 2nd and 3rd weeks of gestation were randomly assigned into five groups of five animals each ($n = 5$). Group A which served as the control group received distilled water; Group B (cornoil group) received 0.6 ml of corn oil which served as vehicle for Kv; Group C (Kv group) received 200 mg/kg body weight of Kv; Group D (AlCl_3 group) received 100 mg/Kg body weight of AlCl_3 and Group E (AlCl_3 + Kv group) received 100 mg/kg body weight + 200 mg/kg body weight of Kv, respectively. Chemicals and extracts were administered through oral route with the aid of an orogastric cannula during the 2nd week of gestation (from day 8 to 10) and 3rd week of gestation (from day 15 to 17) respectively.

Preparation of brain sample

Once administration was completed, pregnant Wistar rats were sacrificed on day 20 of gestation by cervical dislocation from each group. Five fetuses were excised from the mother rats. Their brains were excised and post fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Hippocampi were excised on ice plate, homogenized in 0.25 M of sucrose solution and centrifuged at 5000 rpm for 5 min in a centrifuge under ice cold conditions.

Procedure for enzymatic assays

Activities of Al, MDA, NO, and interleukin (IL)-6 were assessed directly from the hippocampal tissue of fetal Wistar rats using the spectrophotometric technique. The fetal brains were excised, and their hippocampi were again carefully excised from the rats across the groups. They were homogenized in 0.25 M sucrose solution with a homogenizer and were centrifuged at 5000 for 5 min. The supernatant obtained were aspirated into plain labeled bottles and placed in ice to assay for enzymes based on the manufacturer's guidelines documented on the assay kits.

Transmission electron microscopy

Hippocampal samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4°C. Samples were post fixed in 1% aqueous osmium tetroxide for 2 h. The rest of the procedure was carried out according to Spurr 1969.^[24] It was incubated 80°C

for 48 h then ultra-thin (60 nm) sections were made with a glass knife on ultra-microtome (Leica Ultra Cut UCT-GA-D/E-1/00). It was then mounted on copper Grids, stained with saturated aqueous uranyl acetate^[25] and counter stained with Reynolds lead citrate and then viewed under the transmission electron microscope (TEM) (Model: Hitachi, H-7500 from Japan).

Statistical analysis

GraphPad Prism software (Version 5: Graphpad Inc., USA) was used for the quantitative analysis which was expressed as mean $\pm$ standard error of the mean $n = 5$ using the one-way analysis of variance followed by Tukey's *post hoc* test. P value set at $P < 0.05^*$, $P < 0.01^{**}$ and $P < 0.005^{***}$ were considered statistically significant.

Results

Effects of aluminum chloride and kolaviron on aluminum levels

In the present study, Figure 1 showed significant increase in Al levels in the hippocampi of fetuses whose mothers were exposed to AlCl_3 when compared to the control group. However, the group treated with AlCl_3 + Kv showed a significant decline in the level of Al when compared to the control and AlCl_3 -only treated groups. These were observed in both 2nd and 3rd weeks of gestation; although, the reduction in the AlCl_3 + Kv was seen to be significantly reduced in the 2nd week when compared to the 3rd week of gestation.

Effects of aluminum chloride and kolaviron on some enzymes of oxidative stress

The AlCl_3 treated groups all showed an increase in the MDA, NO, and IL-6 activities in the hippocampal tissues of fetuses whose mothers received AlCl_3 when compared to the control group. However, AlCl_3 + Kv group revealed a significant decrease in MDA, NO, and IL-6 activities when compared to the AlCl_3 group. Similar changes were seen in both 2nd and 3rd weeks of gestation; however, there were decreased levels observed in the 2nd week when compared to the 3rd week of gestation [Figures 2-4].

Ultrastructural changes of aluminum chloride and kolaviron

Figures 5 and 6 showed TEM photomicrographs of foetal hippocampi whose mothers were exposed to treatments during the 2nd and 3rd weeks of gestation. Ultrastructural changes in the fetuses of groups that received AlCl_3 in the 2nd [Figure 5A4] and 3rd [Figure 6A4] weeks of gestation revealed marked degenerative hippocampal changes which included mitochondrial damage, chromatin condensation, vacuolation, and compromise of the integrity of the nuclear membrane when compared to the control group. These observations were seen to be better improved in the fetal hippocampi

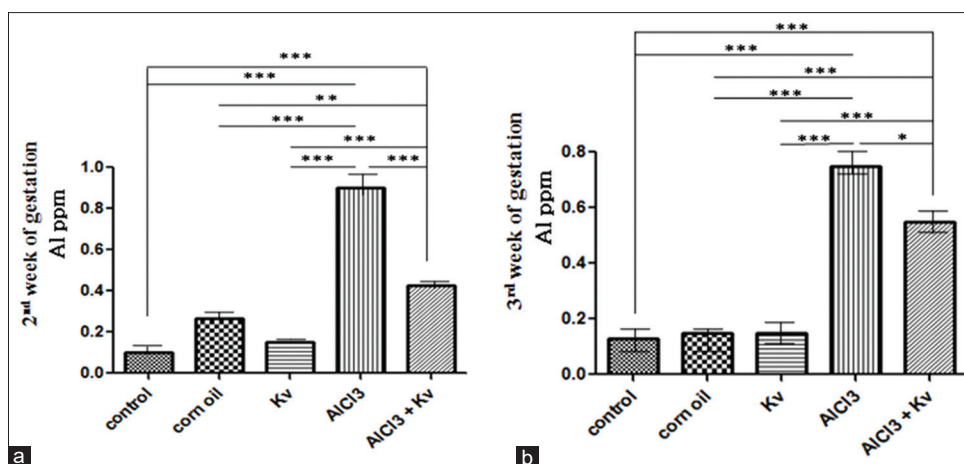


Figure 1: Analysis of changes in Al profiles in foetal hippocampus. (a) 2nd gestation week, (b) 3rd gestation week. Groups that received AlCl₃ showed significantly elevated levels of Al compared to the control ($P < 0.005$) in groups (a and b). Intervention with Kv significantly down-regulated levels of Al in the AlCl₃ + Kv group compared to the AlCl₃ group ($P < 0.005$ and $P < 0.01$) in the 2nd and 3rd weeks respectively. Values are represented as mean $\pm$ SEM (****Are significant level of difference at $P < 0.05$, $P < 0.01$ and $P < 0.005$ respectively). SEM = Standard error of mean, Kv = Kolaviron, AlCl₃ = Aluminium chloride

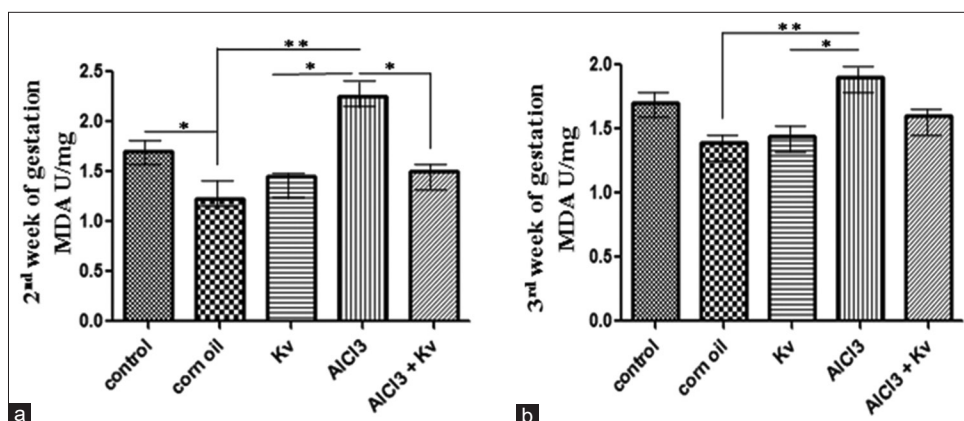


Figure 2: Changes in MDA profiles in foetal hippocampus. (a) 2nd gestation week, (b) 3rd gestation week. AlCl₃ group showed up-regulation in MDA compared to the control. This increase was not statistically significant in both 2nd and 3rd weeks. Kv significantly reduced MDA production in the AlCl₃ + Kv group compared to the AlCl₃ group ($P < 0.005$) in the 2nd week. Although, a reduction was observed in the 3rd week, it was not statistically significant. Values are represented as mean $\pm$ SEM (****Are significant level of difference at $P < 0.05$, $P < 0.01$). SEM = Standard error of mean, Kv = Kolaviron, AlCl₃ = Aluminium chloride, MDA = Malondialdehyde

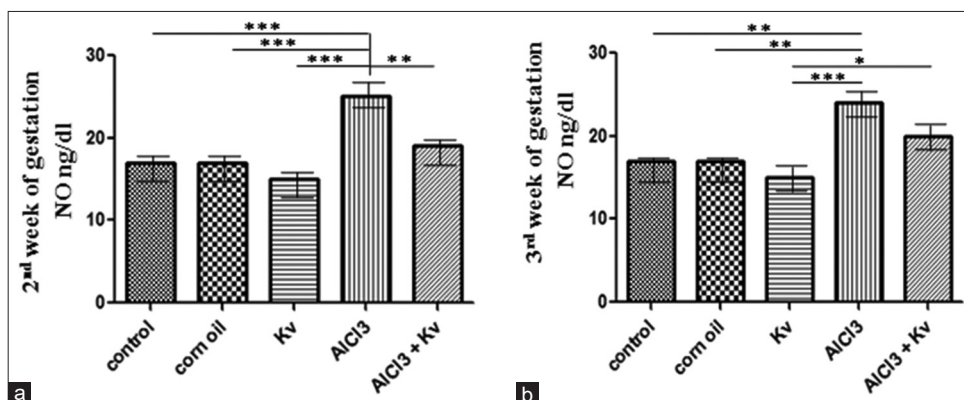


Figure 3: Changes in NO profiles in foetal hippocampus. (a) 2nd week of gestation, (b) 3rd week of gestation. The AlCl₃ group revealed a significant expression of NO compared to the control in the 2nd ($P < 0.005$) and 3rd ($P < 0.01$) week. The AlCl₃ + Kv group revealed a significant decrease in NO ($P < 0.01$) when compared to the AlCl₃ group in the 2nd week. This decrease was not statistically significant in the 3rd week. Values are represented as mean $\pm$ SEM (****Are significant level of difference at $P < 0.05$, $P < 0.01$ and $P < 0.005$ respectively). SEM = Standard error of mean, Kv = Kolaviron, AlCl₃ = Aluminium chloride, NO = Nitric oxide

whose mothers received AlCl₃ + Kv (particularly in the 2nd week when compared to those who received only

AlCl₃ [Figure 5A5]. Similar findings were also observed in the 3rd week [Figure 6B].

Discussion

The ubiquitous nature and profitable industrial use of Al in recent times has raised some concerns as it is associated with neurodegenerative diseases.^[26] The hippocampus, important in learning and memory is vulnerable to oxidative stress as a result of increase in the production of free radicals. In the current study, there was significant level of Al in the hippocampus of fetuses whose mothers received AlCl_3 during the 2nd week of gestation when

compared to the 3rd week. This might be attributed to the vulnerability of the blood–brain barrier of the fetus to this toxic substance at the very early stage of brain development (2nd week equivalent to gestational day 9–9.5^[27]) compared to its vulnerability in the 3rd week when it has already commenced formation. This significant increase of Al levels in both groups could be attributed to the ability of Al to bind to transferrin receptors through which they were able to cross blood–brain barrier.^[28,29] Researches involving the chemistry of Al have shown

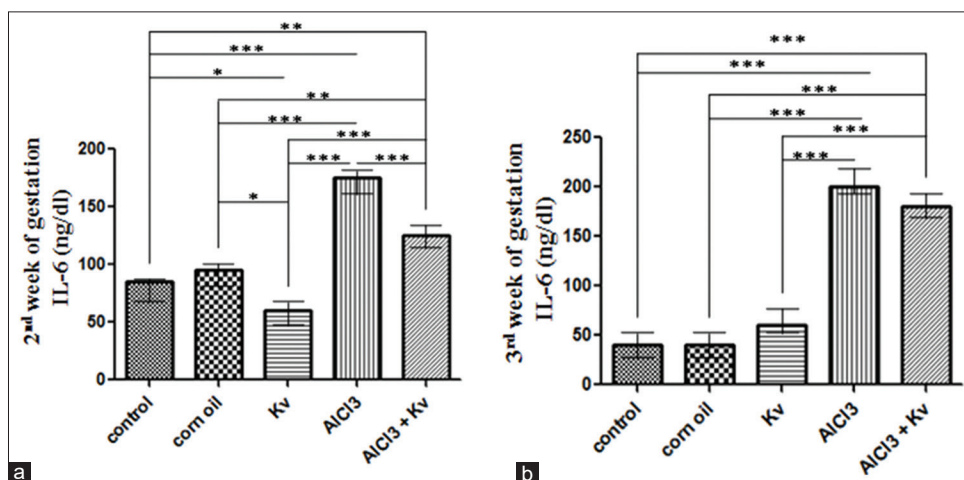


Figure 4: Changes in IL-6 profiles in foetal hippocampus. (a) 2nd gestation week, (b) 3rd gestation week. The AlCl_3 group showed significant expression of IL-6 in the 2nd ($P < 0.01$) and 3rd ($P < 0.05$) weeks compared to the control. The $\text{AlCl}_3 + \text{Kv}$ group revealed a significant decrease ($P < 0.005$) in IL-6 from the AlCl_3 group in the 2nd week. This decrease was observed in the 3rd week but not significant. Values are represented as mean $\pm$ SEM (****Are significant level of difference at $P < 0.05$, $P < 0.01$ and $P < 0.005$ respectively). SEM = Standard error of mean, Kv = Kolaviron, AlCl_3 = Aluminium chloride, IL-6 = Interleukin-6

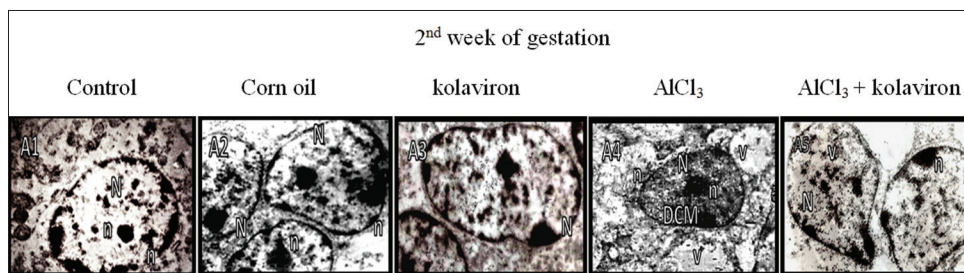


Figure 5: TEM photomicrographs. (A) Fetal hippocampus of maternal administration. A1: Distilled water. A2: Corn oil. A3: Received 200 mg/kg of kolaviron. A1, A2, and A3 showed normal membrane, N and n. A4: Received 100 mg/kg of AlCl_3 and showed a thin membrane, shrunken N, V and increased DCM. A5: 100 mg/kg of $\text{AlCl}_3 + 200$ mg/kg of kolaviron. There was decreased V, DCM and normal N and membrane integrity compared to the AlCl_3 group. Scale bar = 2.0 μm . DCM = Dense chromatin material, N = Nucleus, n = Nucleoli, V = Vacuolation, TEM = Transmission electron microscopy

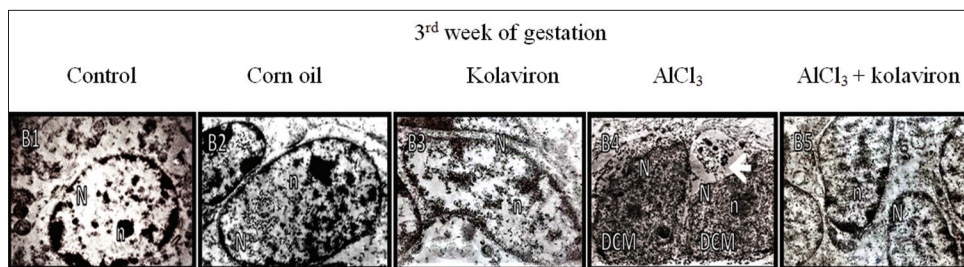


Figure 6: TEM photomicrographs. (B) Fetal hippocampus of maternal administration. B1: Distilled water. B2: Corn oil. B3: Received 200 mg/kg of kolaviron. B1, B2, and B3 showed normal membrane, N and n. B4: 100 mg/kg of AlCl_3 and showed compromised membrane, leaked out nuclear content (arrow), enlarged N, V and DCM. B5: 100 mg/kg of $\text{AlCl}_3 + 200$ mg/kg of kolaviron. There was decreased V, DCM and normal N and membrane integrity compared to the AlCl_3 group. Scale bar = 2.0 μm . DCM = Dense chromatin material, N = Nucleus, n = Nucleoli, V = Vacuolation, TEM = Transmission electron microscopy

that it does not constitute any biological importance. Hence, there is a need for Al to be excreted from the body as its accumulation over time has been found to be associated with neurodegenerative diseases such as Alzheimer's disease.^[30,31] Significant levels of Al were found in the cerebrospinal fluid of patients with neurodegenerative diseases.^[26] However, in the present study, it was observed that the Kv-treated groups showed a significant decrease in Al levels when compared with the control group. This change was observed more in the 3rd week of gestation when compared to the 2nd week. The decrease in Al levels might be attributed to the chelating properties contained in the flavonoids which make up Kv. It could also be attributed to their ability to form multiple bonds with Al³⁺ through their hydroxyl group to enable excretion from the body through urine.

The present study revealed significant increase in MDA, NO, and IL-6 levels in fetal hippocampi whose mothers received 100 mg/kg body weight of AlCl₃ both in the 2nd and 3rd weeks of gestation respectively. However, these increases were observed to be more in the 2nd week when compared to the 3rd week.

Al has been found to facilitate the production of free radicals^[32] while exhibiting pro-oxidant characteristics.^[33,34] A free radical captures a hydrogen moiety from an unsaturated carbon to form water leaving an unpaired electron on the fatty acid that is then capable of capturing oxygen forming a peroxy radical which eventually becomes unstable and decompose to produce MDA. The present study suggests that aluminium chloride significantly induced oxidative stress which in turn resulted in the increase in the levels of MDA. Reports show that increased oxidative stress is consequential to the overproduction of active reactive oxygen species (ROS) which has been associated with decrease in antioxidants.^[35-37] Al(NO₃)-induced oxidative stress which resulted in increased levels of both Al and thiobarbituric acid;^[38,39] by design, an increase in MDA production.

The present study showed that there was increase formation of NO as seen in the groups administered AlCl₃ only when compared to the control group. Studies have reported the ability of Al to activate iNOS resulting from the production of free radicals leading to increased production of NO in the brain among other deleterious cascade of events.^[40] However, there was significant reduction in NO production in the AlCl₃ + Kv-treated group when compared to the control and AlCl₃ only group. This could be as a result of Kv's ability to inhibit iNOS enzymes which have been activated by Al in the AlCl₃ group. The production of ROS also sets off an inflammatory cascade through the production of pro-inflammatory cytokines such as IL-6. In the present

study, a significant increase in IL-6 was observed in the group whose mothers received AlCl₃ compared to the control. This could be in response to inflammation due to release of free radicals. The antioxidative properties of Kv were probably able to reduce levels of IL-6 by mopping up free radicals whose presence stimulates the cascade of events that led to the inflammatory response. Free radicals are able to significantly affect cell components by affecting DNA, proteins and lipids.^[41]

This study showed that the group whose mothers received AlCl₃ both in the 2nd and 3rd weeks of gestation-induced toxicity through oxidative damage to components of the cells when compared to the control group. Ultrastructural observations revealed marked degenerative changes such as mitochondrial damage and condensed chromatin material within the nuclei suggestive of DNA fragmentation. Evidence of these findings were in consonance with Mahitha and Sushma^[42] who carried out studies on the cerebral cortex.

Exposure to Al during pregnancy in the 2nd and 3rd weeks of gestation in this study also showed partial loss of cellular integrity which included thin irregular nuclear membrane and vacuolation within the cytoplasm and mitochondria of the groups whose mothers received AlCl₃ only. Its neurotoxic effects led to lipid peroxidation which resulted in increase in the production of MDA responsible for inhibiting the activities of antioxidants and affected the integrity of the nuclear membrane. The structural modifications of the cells could be attributed to the release of ROS which overwhelmed the activities of antioxidants.^[43] However, there were improvements in the ultrastructural appearances of the nuclei of the group treated with Kv. There were reduced vacuolations and less dense chromatin material (suggestive of minimized DNA fragmentation) as observed within the nuclei of the AlCl₃ + Kv group when compared with the control or AlCl₃ group.

Conclusion

The findings in the present study suggest that the intervention of Kv was able to restore hippocampal damages in the hippocampi of fetal Wistar rats by exerting its antioxidant effects on the oxidative properties which led to a cascade of neurotoxic events exhibited by AlCl₃.

Acknowledgments

The authors acknowledge Prof. M. Lakshman Head and officer in charge of Ruska Labs (Electron Microscope Laboratory) Department of Veterinary Pathology College of Veterinary Science, P. V. Narsimha Rao Telengana Veterinary University and Dr. Omotoso, G. O. of the Department of Anatomy, University of Ilorin valuable support and technical guidance.

Financial support and sponsorship

This research was supported by the Staff development fund (SDA), University of Ilorin. The transmission electron microscopy was carried out at Ruska Laboratory and was supported by C. V. Raman International Fellowship Program for African Researchers through the Department of Science and Technology, Government of India and Federation of Indian Chambers of Commerce and Industry.

Conflicts of interest

There are no conflicts of interest.

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