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Neuroprotective Effects of Kolaviron on the Hippocampus of Foetal Wistar Rats Induced with Aluminium Chloride *In-utero*

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ABSTRACT

Exposure to toxicants *in-utero* could lead to teratogenic malformation including neurodegeneration. Aluminium, a neurotoxin causes the release of free radicals which results in disrupted homeostasis however, its mechanism of action is not fully established. The present study was designed to investigate the effects of kolaviron on the hippocampus of foetal Wistar rats exposed to aluminium chloride *In-utero*. Fifty (50) female rats were introduced to twenty-five (25) male rats for mating in a ratio of 3:2 once their estrus phase was confirmed through vaginal smear. Female rats were assigned into five (5) members per group once mating was confirmed following presence of sperm cells through vaginal smear the day after male introduction into the female's compartments. They were administered the following: Group A: distilled water, Group B: 0.6 mls of corn oil, Group C: 200mg/kg BW of kolaviron, Group D: 100mg/kg BW of $AlCl_3$ and Group E: 100mg/kg BW of $AlCl_3$ + 200mg/kg BW of kolaviron. Administration was done orally with the use of an oral cannula during the 2nd and 3rd week of gestation on days 8- 10 and days 15- 18 respectively. Animal sacrifice was carried out on day 20 of gestation and feotuses were carefully removed. Both body and brain weights of the fetuses were evaluated. Foetal hippocampii were further excised from the brains and homogenized in 0.25 M of sucrose solution for biochemical analysis. There was a reduction in both body and brain weights of the fetuses whose mothers received $AlCl_3$ in both 2nd and 3rd weeks of gestation when compared to those treated with $AlCl_{3+}$ kolaviron respectively. Significant increase in glucose metabolism through increase in G-6-PDH levels in the hippocampus of rats in group E was observed when compared to those in group D. Expression of cyt-c was found to be significantly reduced in the hippocampus of foetal rats in group D when compared to those in group E. This study concluded that contact with AlCl3 during the 2nd and 3rd weeks of gestation affected neurodevelopment. The intervention of kolaviron minimized AlCl3-induced toxicity in foetal hippocampus by boosting antioxidative status. Hence this study recommends that kolaviron could be considered as an agent in targeting Al-induced neurodegeneration.

Key words: kolaviron, aluminium chloride, foetus, hippocampus, glucose metabolism

INTRODUCTION

Aluminium (Al) is a toxic metal whose ability to form complexes with organic matter and exist in trivalent forms such as oxides and hydroxides has made its unavoidable nature safe to say we are living in the 'aluminium age'[1,2]. Its ubiquitous nature has increased its use in chemical compounds such as aluminium chloride, aluminium hydroxide, aluminium potassium and aluminium silicate which are highly associated with human activities[3,4,5]. It is a naturally occurring free ion which is highly biologically active and is able to hamper normal cellular activities[6]. Humans and animals can be exposed to Al through dietary supplements, use of foils and kitchen utensils[7]. Other means of exposure are through mining activities associated with ore and recycling of aluminum containing products such as scrap metals[5]. Accumulation of Al in the body can be through ingestion or inhalation and this could result in neurotoxicity over time[8].

Aluminium toxicity has been associated with gradual progressive loss of neurons which is an important feature of neurodegenerative diseases[9,10,11]. Although the etiology of this condition is yet to be fully understood[12,13], the brain and nervous system are thought to undergo free radical processes from Al toxicity resulting in oxidative damage[14]. This could lead to mitochondrial dysfunction, impaired deoxyribonucleic acid (DNA) repair and cellular damage[15]. These conditions are characterized by disorganized movement control and disordered sensory transmission of information[13], loss of memory and cognitive functions as seen in Alzheimer's Disease (AD)[16,12] and Parkinson's Disease (PD)[17,18,19].

Pregnant women are also caught up in the accidental or deliberate exposure to Al toxicity. This brings to focus the condition and well-being of the foetuses who unfortunately, are also exposed to Al through their mother's contact with aluminium-associated products such as antacids, toothpaste, cosmetics and food additives during gestation[20,21]. As a result of the foetuses' vulnerability during gestation, an important stage of development, they are left to their fate owing to their mother's conscious or unconscious exposure to this toxic substance. Evident to these are results from studies that have documented passage of Al through the placenta prenatally and through breast feeding after birth[22]. The placenta is a foetomaternal organ which functionally and structurally serves as communication between the foetus and the uterine wall of the mother during pregnancy [23]. Another study observed that Al was able to cross the blood brain barrier to reach the foetal brain[24]. Although significantly high amounts of Al was found in the liver of fetuses whose mothers were exposed to Al, copper, zinc and iron deficits were documented with copper having the highest concentration in the brain[25]. Deficit in normal neurobehavioral patterns and neurochemical abnormalities were reported in a study where 200 mg/kg of Al sulphate was administered to pregnant mice on day 10 to 13 of gestation[26,27]. These observations were observed in newly born mice and the findings persevered into adulthood. While another study discovered that excess intake of Al led to permanent neurobehavioral patterns in both weaning rats and mice[28].

Studies have shown that some plants have been patronized in recent times owing to their efficacious pharmacological activities, low cost and minimal toxicity[29,30,31]. Other qualities attributed to this interest are their biological and antioxidative properties[32]. Hence, consumption of plant products with strong antioxidative properties could be responsible for the safeguarding cells against toxic substances[33]. Those in the less developed countries whose population make up above 80% of the world's population highly rely on medicines based on local plants to cater for their health needs[34]. Free radicals are scavenged by antioxidants and phenolic compounds extracted from different components of these plants such as their leaves, fruits, seeds, roots and stem barks[35,36,37,38,39,40]. They contain flavanoids that can produce other antioxidants and prevent formation of free radicals that are produced through Fenton-type reactions[41]. *Garcinia kola* is one of such plants; it is of the *Guttiferae* Family and widely known for both its traditional hospitality and medicinal values in Africa [42,43,44,45,46].

It has a resinous, bitter and mordant taste attributed to its characteristic qualities[32]. Kolaviron is an ethanolic extract of *Garcinia kola,* made up of bioactive flavonoid complex[47]. Protection of neurons against toxic damage and oxidative stress induced by gamma radiation in rat brain was reported by Adramoye[48]. Morphological, Pathophysiological and biochemical changes due to ischemia in rat brains were observed to have been remarkably improved by kolaviron[49,50,47]. Kolaviron was shown to exhibit chemopreventive properties through metal chelating, anti-inflammatory and antioxidant properties while also exhibiting scavenging properties against free radicals[51,52].

The significance of this study was born out of the exposure of vulnerable fetuses either accidentally or consciously to aluminium toxicity *in-utero* and the significance of the intervention of kolaviron particularly on the hippocampus to ensure quality future health.

Scope of Study: This study is restricted to the effects of kolaviron on aluminium chloride via oral administration in the 2nd and 3rd week of gestation considering morphological and biochemical assessments in the hippocampus of foetal Wistar rats *in-utero*.

MATERIALS AND METHODS

Ethical Clarance Clearance: Ethical approval for the study was obtained from the University Ethical Review Committee, University of Ilorin with approval number; UERC/ASN/2016/361.

Preparation of Extract: *Garcinia kola* seeds were obtained from Pata Market in Ilorin, Kwara State, Nigeria. The seeds were certified in the Department of Plant Biology, Faculty of Life Sciences, University of Ilorin, Ilorin by the curator with the specimen voucher number: UILH/001/1217. Four (4) kg of peeled *Garcinia kola* seeds were weighed with an electronic weighing scale. The seed were then cut into thin sliced and air-dried at room temperature (28-30˚C) for 2 weeks. The dried seeds were pounded to fine powder using a mortar and pestle. Light petroleum ether (boiling point: 40-60˚C) was used for fats extraction for 48hrs in the Laboratory of the Anatomy Department. Further extraction was done on the defatted dried marc with acetone with boiling point 56-60˚C in a water bath. A golden yellow solid known as kolaviron was the yield of the further extraction with ethyl acetate.

Kolaviron was subjected to thin layer chromatography (TLC) in the laboratory of Prof. E. O. Farombi at the Drug Metabolism Unit, Faculty of Basic Medical Sciences, University of Ibadan, Nigeria. Hence purification and validation of kolaviron was done through the use of silica gel GF 254-coated plates and solvent mixture of methanol and chloroform in a ratio 1:4 v/v. This process led to deposition of three bands under UV light at a wavelength of 254 nm with RF values of 0.48, 0.71 and 0.76 respectively. The yield of kolaviron in this study was 6.3% and was kept at a room temperature of 4 ˚ before and after each use. The procedure was carried out according to the method of Iwu[53] as modified by Farombi *et al.,*[54] and Olajide *et al.,*[55].

Animal Model: For the purpose of this study, fifty (50) adult females and twenty-five (25) adult male Wistar rats weighing from 200g to 220g were purchased. The animals were allowed to acclimatize and were fed with standard laboratory animal's pallete and tap water *ad-libitum* in the Animal holdings of the Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin, Ilorin under light and dark cycle at room temperature (28-30˚C) for two weeks.

Determination of the Estrus Phase via Vaginal Smaearing: The vaginal smear test was carried out according to Marconde *et al.,*[56] . Briefly, the smear was carried out by introducing a micro-pipette containing 0.5mls normal saline into the vagina of the female rats then withdrawn and placed on a microscope slide for viewing under a light microscope. The estrus cycle consist of the proestrus phase (nucleated epithelial cells) the estrous phase (a mixture of about 75% nucleated cells and about 25% cornified cells), metestrus phase (equal distribution among leukocytes, cornified and nucleated epithelial cells) and the diestrus phase (predominance of leukocytic cells)[57,58]. The proportion of cells was used to determine the estrus phases[57,58].

Mating: Mating was done according to Maconde *et al.,*[56] method. The male rats were introduced into the female's compartments which already houses 2 Wistar rats confirmed to be in their proestrous phase. This phase was observed to be the stage when female rats show acceptance to the male rats hence encourage mating activities. Hence the day following the day of male introduction into the female's cage was taken as day 1 of pregnancy following the presence of active sperm cells confirmed on vaginal smear[59].

Animal Grouping, Administration and Sacrifice: Fifty (50) female rats were carefully assigned into 2 groups representing the 2nd and 3rd weeks of gestation respectively. They were then further assigned into 5 sub-groups of 5 rats each as follows: Group A: Control given distilled water, Group B: Corn oil (CO), Group C: 200 mg/Kg kolaviron (KV), Group D: 100 mg/Kg $AlCl_3$, E: 100 mg/Kg AlCl + 200 mg/Kg kolaviron. This was done once mating was confirmed.

The pregnant Wistar rats were sacrificed on day 20 of gestation. Their fetuses were excised including their brains and then hippocampii as well. Both body and brain weights were however documented for observation. Hippocampal samples were meshed and homogenized in 2 mls of 0.25 M sucrose solution before being centrifuged in tubes padded with ice at 5000 r/m for 15 minutes using Gallenkomp centrifuge. The supernatant was decanted while activities of G-6-PDH and cyt-c oxidase were assessed using ELISA assay kits.

Preparation of Brain Homogenate for Biochemical analysis: Hippocampal homogenate preparation is described below:

- Ratio 4:1 of 0.25 M sucrose solution to hippocampal tissue was prepared to be homogenated.
- This mixture was carefully meshed in homogenate plate with its pestle.
- Homogenate tissues were centrifuged at 5000 rpm for 15minutes and supernatant were decanted.
- Frozen section of supernatant hippocampal tissue were stored at -20 ºC for G-6-PDH and cyt-c oxidase *analysis*.

Glucose-6-Phosphate Dehydrogenase (G-6-PDH) Assay: Principle: Glucose-6-phosphate dehydrogenase (G6PDH) catalyses the oxidation of glucose-6-phosphate to 6-phosphogluconate with a concurrent conversion of NADPH. The enzyme activity was determined by measurement of the rate of increase in NADPH Concentration. The rate of increase in absorbance at 365 nm was used to determine enzyme activity.the procedure involved addition of 1.0 ml of G6PDH buffer to 0.5ml of the enzyme extract which was rocked gently. 0.05 ml of NADP and 0.025 ml G-6-PDH substrate solution was added to mixture of buffer and enzyme extract and rocked again before setting it to rest for a minute before recording the change in absorbance every 1 minute up to 3 minutes in a spectrophotometer.

One unit of G-6-PDH is defined as the change in absorbance per minute at 365nm using the coefficient of 0.00622 $Nm^{-1}cm^{-1}$.

Calculation of Glucose-6- Phosphate dehydrogenase Activity

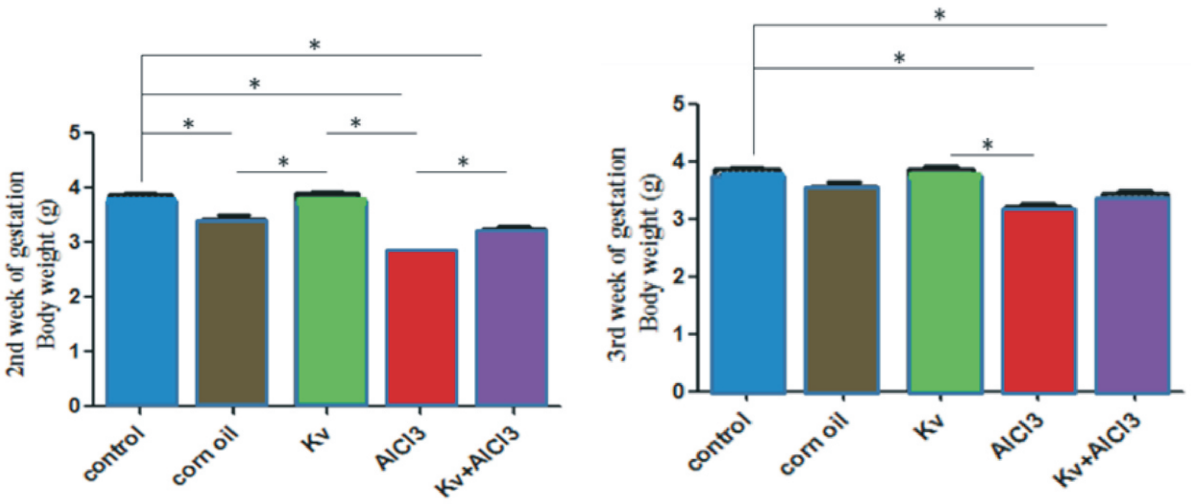
G-6-PDH = x E

Where E= 9($\frac{\Delta Absorbance}{3.0}$

Cytochrome-c oxidase (CYT-C) Assay: Samples stored at -20 ºC were prepared following standard instructions. 100 µl standard and hippocampus tissue sample was added into each well, covered with adhesive strip and then incubated for 2 hours at 37 ºC. The liquid was then removed from each well without washing. After that 100 µl of Biotin-antibody 1x was added to each well, covered with a new adhesive strip and incubated for 37 ºC. as a result of the cloudy appearance of Biotin-antibody 1x, the solution was warmed up to room temperature and mixed gently until the solution appears uniform. The solution was aspirated from each well by filling each well with 200 µl of Wash Buffer. This process was repeated twice in 3 washes. Wash buffer was removed by decantation, the plate was inverted and blot against paper towels. Next, 100 µl HRP-avidin (1x) was added to each well then covered with a new adhesive and left to incubate for 1 hour at 37 ºC. The aspiration process was repeated 5 times then 90 µl of TMB substrate was added to each well and incubated for 15-30 minutes at 37 ºC. It was important that this samples were kept away from light. 50 µl of **stop solution** to each well. The optical density of each well was determined within 5 minutes using a micropipette reader set to 450 nm.

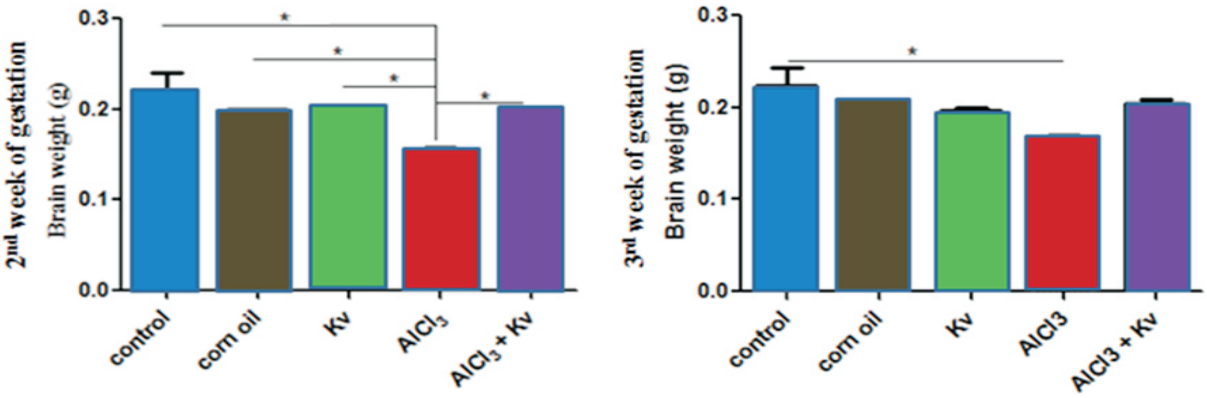
Statistical Analysis: One-way ANOVA followed by Turkey's post hoc test for multiple comparison on IBM SPSS (Version 20) was used to analyze the data in this study. Graphical values were plotted using the GraphPad Prism® software (version 6) and represented as mean ± standard error of mean (SEM). Level of significance was taken as $P < 0.05$ at 95 % Confidence Interval.

RESULTS



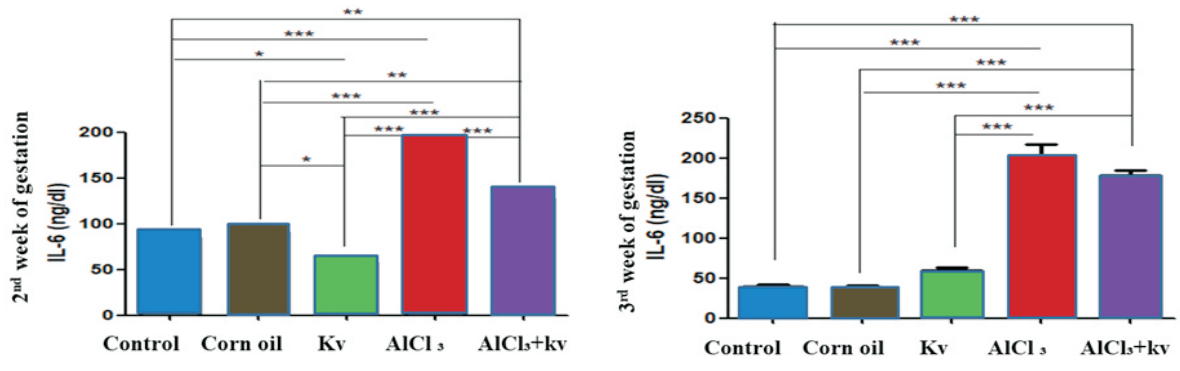
A B

Figure 1: Bar chat showing changes in body weight of foetal Wistar rats. Control, Corn Oil, KV = Kolaviron, $AlCl_3$ = Aluminium chloride, A= Second week of gestation, B= Third week of gestation. There was significant statistical difference between body weight changes in foetal rats of the $AlCl_3$ group compared to the control group and Kv group in both A and B. Also, significant statistical difference between foetal rats of the $AlCl_3$+ kv group were observed compared to the control group. ($p<0.05$).



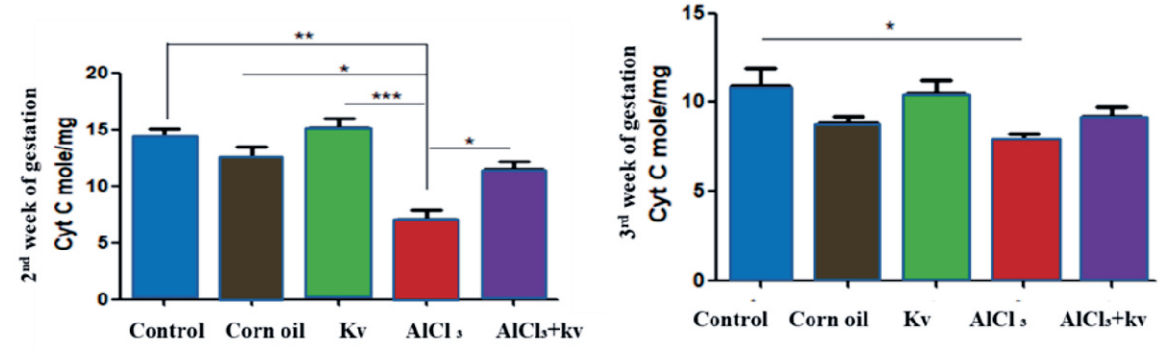
A B

Figure 2: Bar chat showing changes in brain weight of foetal Wistar rats. Control, Corn Oil, KV = Kolaviron, AlCl $_3$= Aluminium chloride, A= Second week of gestation, B= Third week of gestation. There was significant statistical difference between levels of brain weight changes in foetal rats of the AlCl $_3$group compared to the control group and all other groups in group A however, this change was found to be statistically significant in the AlCl $_3$group when compared to the control in group B. ($p<0.05$).



A **B**

Figure 3: Bar chat showing analysis of changes in G-6-PDH profiles in foetal Wistar rats. Control, Corn Oil, KV = Kolaviron, AlCl $_3$= Aluminium chloride, A= Second week of gestation, B= Third week of gestation. There was significant statistical difference between levels of G-6-PDH in foetal rats of the control group and and all other experimental groups both in A and B. Significant statistical difference was also observed between the AlCl $_3$+ kv group and all other groups both in A and B. ($p<0.05$).



A B

Figure 4: Bar chat showing analysis of changes in **cyt-c** oxidase concentration in foetal Wistar rats. Control, Corn Oil, KV = Kolaviron, AlCl $_3$= Aluminium chloride, A= Second week of gestation, B= Third week of gestation. There was significant statistical difference between levels of cyt-c oxidase concentration in foetal rats of the AlCl $_3$group and all other groups in A, however, tthere was significant statistical difference seen only in the AlCl $_3$compared to the control group. There was significant reduction in the level of cyt-c in A when compared to B. ($p<0.05$).

DISCUSSION

Effects of kolaviron following aluminium chloride toxicity on body and brain weight changes of foetal Wistar Rtas: The most common means through which aluminium enters into the body is via oral route where small amounts are absorbed and accumulate in the body. Absorption occurs in the small intestine where it binds to transferrin receptors in the blood through which it is transported to the brain crossing the blood brain barrier. The present study revealed that rats treated with $AlCl_3$ showed decrease in both body and brain weights in the 2nd and 3rd week of gestation. It was suggested the relationship between Al and phosphate groups was responsible for disruption of protein synthesis via RNA and DNA synthesis which eventually interfered with normal cell proliferation during development [21,24] hence resulting in decrease in the weight parameters. Outcomes relating to body weight in this study was supported by Samar et al., [21] who reported a decrease in all growth parameters which included crown rump length, biparietal diameter, head length, liver weight and body weight. Findings in this study were also in accordance with Olajide et al., [46] who reported a decrease in percentage body weight in rats associated with $AlCl_3$ administration for 15 days however, their study was carried out on male rats. Their study suggested that the reduction in body weight could have been as a result of abnormal glucose tolerance associated with poorly defined mechanisms. This could also be as a result of loss of appetite in the mother rats which resulted in reduced food intake that could have affected the normal growth of the foetuses thereby affecting the body weight changes observed.

Rats treated with $AlCl_3$ showed marked reduction in brain weight when compared to both control and the kolaviron treated group in both the 2nd and 3rd weeks of gestation. This might be due to the vulnerability of the polyunsaturated fatty acid contained in the cell membrane to the free radicals produced by Al which led to lipid peroxidation affecting both the structure and function of the neuronal cells[60]. There was also significant statistical difference in the group administered $AlCl_3$ when compared to the both kolaviron and the kolaviron- treated group.

However, the present study revealed more decline in both body and brain weight gain during the second week of gestation compared to the 3rd week, this observation during the 2nd week of gestation might be as a result of the commencement of brain developmental activities experienced by the foetus on gestational day 10.5-11 in rats[61]. Reduced body and brain weights of mice offsprings exposed to a toxic substance (Fluorine) was observed by Chen et al.,[62] likely resulting from the effect of the toxic substance affecting the growth and development of the young brains[62].

On the other hand, the present study showed that kolaviron was able to reverse the decline in both body and brain weight loss resulting from $AlCl_3$ toxicity in the 2nd week of gestation. Although, it was observed that there were no significant difference between the body and brain weight changes of the $AlCl_3$ groups compared to the kolaviron-treated group in the 3rd week of gestation, there was a physical increase in the weight change. This is in concurrence with the study of Adaramoye et al.,[63] who showed that the bodyweight gains were not significantly different among the groups investigated when they administered 0.26 g/kg of a hypolipidemic drug along with 100 and 200 mg/kg kolaviron to male rats orally.

Effects of kolaviron on the hippocampus of foetal Wistar rats following aluminium chloride toxicity via assessment of glucose metabolic enzymes: The present study also assessed the significance of glucose-6-phosphate dehydrogenase (G-6-PDH) on the effects of kolaviron on aluminum chloride toxicity in the foetal hippocampus in-utero. During development, the foetal brain is vulnerable and highly sensitive to exposure to toxic agents[64] which makes glucose metabolism a vital tool through which energy is provided to support these cellular activities while serving as an indicator for cell death[65]. This establishes the important role played by G-6-PDH during cellular response to oxidative stress[66,67]. The pentose phosphate pathway as a means through which energy is produced is needed for the cell to carry out metabolic activities. This reaction is catalysed by the glucose-6- phosphate (G-6-PDH) enzyme to allow supply of nicotinamide adenine dinucleotide phosphate (NADPH) which takes part in the biosynthesis of fatty acids[68,69]. Researchers have reported the role of G-6-PDH in protecting the growing foetus from oxidative DNA damage and embryonic pathologies[70,34]. Mitochondrial function has a huge impact on the survival and tasks associated with the neuron[71]. Hence abnormalities which involves imbalance of ATP/ADP ratio could lead to high production of ROS, severe energy deficiency, loss of adequate energy and neuronal death[72,73,71].

Generation of adenosine triphosphate (ATP) is very important for biochemical and metabolic activities concerning the cell which can be achieved via glucose metabolism[74,75]. Cytochrome-c is a basic water- soluble heme-containing protein which functions as a terminal enzyme in Complex IV of the electron transport containing tightly bound cardiolipin molecules[76,77,78]. This study showed significant decrease in levels of glucose-6-phosphate dehydrogenase (G-6-PDH) and cytochrome-c oxidase (cyt-c oxidase) in the AlCl3 group when compared with the control. This observation was made both in the 2nd and 3rd weeks of gestation. Cyt-c oxidase, an important enzyme which oxidizes cytochrome-c molecules by producing water via transfer of electrons to oxygen also produces a transmembrane proton electrochemical potential by binding four protons to produce ATP in complex IV of the electron transport chain [79,80,81]. Aluminium was able to facilitate the release of cytochrome-c (cyt-c) from the mitochondria through

the production of reactive oxygen species (ROS). This might be through peroxidation of cardiolipin, a mitochondrial phospholipid that binds cyt-c to the inner mitochondrial membrane through hydrophobic and electrostatic connections. Reduction in cyt-c oxidase activity in the hippocampal tissue could be as a result of alterations in the cardiolipin contents as a result of oxidative attack on the phospholipid by reactive oxygen species.

Cyt-c oxidase as the most important constituent of the electron transport chain showed reduced activities in heart, liver and brain mitochondria in rats exposed to Al[12]. A decrease in activities of cyt- c oxidase in the electron transport chain was seen in the hippocampus (49 %), corpus striatum (51 %) and cerebral cortex (58 %) of rats exposed to Al for 12 weeks. This decrease was found to be lowest in the hippocampus compared to other parts of the brain [12]. They also recorded deficits in ATP production in the brain of rats which disrupted mitochondrial energy metabolism following Al exposure. In the present study, decrease in ATP synthesis could be attributed to increased rate of ATP hydrolysis perhaps as a result of sudden energy demand, possibly owing to increased ROS formation exacerbated by Al toxicity[12]. Atlante et al.,[82] reported release of cyt-c from mitochondria as a result of accumulation of free radicals via glutamate neurotoxicity where its activity in the biosynthetic pathway functions as an electron career in the mitochondrial electron transport chain. Hence, this shows that production of ATP; the principal role of the mitochondria is sequel to the functionality, availability and location of cardiolipin and its close interaction with cytochrome-c oxidase, the terminal enzyme complex of the electron transport chain.

The present study revealed an upregulation in both G-6-PDH and cyt-c oxidase activities in the group administered $AlCl_3$ + Kv compared to the group whose mothers received $AlCl_3$ only. Kolaviron was able to exhibit its antioxidative characteristics by scavenging for free radicals that were generated by Al thereby allowing transfer of electrons in and out of the cell which translates into production of energy trough generation of ATP. Kolaviron prevented. An upregulation of neuronal G-6-PDH was observed in the group of male Wistar rats administered 2.4 g/kg body weight of aqueous extract of Garcinia kola for 21 days compared to the control group. This was attributed to the ability of Garcinia kola which contains kolaviron as its active component to stimulate increase in energy and ribose production in their brain tissues[69].

In order to meet up with regular active metabolic activities such as cell growth and development, high levels of oxygen consumption is required and this is dependent on relatively high levels of these enzymes [71,81].

Therefore, both enzymes significantly play a vital role in the survival of the neuron via energy production. The mitochondria are known for their role in the generation and storage of energy for use by the neuron. They are also useful in activities that involve neuronal growth and overall development of the brain[81]. Hence once these enzymes are compromised, it could result in inability of the mitochondria to function properly. This could lead to its damage resulting in impairment of the respiratory chain function as a result loss of energy production, neurological disorders and neurodegenerative diseases[83,81].

CONCLUSION

In conclusion, aluminium was found to cause degenerative changes *in-utero* in the hippocampus of developing fetuses both in the 2nd and 3rd week of gestation. These changes were however ameliorated by the intervention of kolaviron through morphological and biochemical assessments. Hence, it is important to understand how daily interaction or consumption of the toxic substance, Al accumulates over time, the harmful effects on the developing foetus and the intervention by kolaviron as a possible antidote agent. This will help improve the quality of life experienced by such individuals in future.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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