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Neuroprotective Effects of *Kolaviron* on the Hippocampus of Foetal Wistar Rats in-Utero using Biochemical Markers

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ABSTRACT

Pregnancy spans from conception till birth. Drugs and substances administered during pregnancy have the potential to reach the brain by crossing the blood brain barrier (BBB). *Kolaviron* has been reported to have antioxidant and anti-inflammatory properties. This study aimed to investigate the effects of *kolaviron* on the biochemical and histological parameters of the hippocampus of pregnant Wistar rats. Forty-five rats (thirty females and fifteen males) were used for the purpose of this study. Vaginal smear was performed on the female rats to ascertain their estrous cycle before introducing male Wistar rats for sexual activity. Mating was confirmed following presence of sperm cells; the female animals were grouped into 5: A (distilled water), B (200 mg/kg BW of *kolaviron*) and C (0.6 mls of corn oil) in the 2nd and 3rd week of gestation. Administration was done orally with the use of an oral cannula on days 8- 10 and days 15- 18 of gestation respectively. Pregnant animals were sacrificed on day 20 of gestation while their brains were excised and homogenized in 0.25 M of sucrose solution for biochemical analysis. There was no significant statistical difference in the body weight, brain and hippocampal weight changes across the groups of the pregnant animals. Although there was also no statistical significant difference in the levels of antioxidants (SOD, GPX), enzymes of carbohydrate metabolism (G-6-PDH and LDH) and hormones (progesterone and estrogen) across the groups, there was physical reduction in antioxidants in the *kolaviron* groups compared to the control groups. It was also observed that the levels of antioxidants and hormones were higher in the 3rd week of gestation when compared to those observed during the 2nd week. These changes though not statistically significant were found to be augmented in the 3rd week of gestation than during the 2nd week mostly the antioxidants and hormones. This might be attributed to the stress associated with pregnancy particularly during the 2nd week of gestation, the period when development of the hippocampus takes place.

Key words: pregnancy, kolaviron, blood brain barrier, hippocampus, hormones

INTRODUCTION

Pregnancy involves a series of immense physical and emotional changes for women. Deficits in learning and memory amongst pregnant women have been reported[1]. Some other symptoms that accompany pregnancy are headaches, aggressiveness, sleeplessness, loss of appetite, excessive eating, constipation, vomiting, nausea (morning sickness), excessive spitting, mood swings and tastelessness of the mouth. Nausea affects between about 50% to 90% of pregnant women and vomiting affects about 25% to 50% of pregnant women[2,3] . Little is known of the etiology and possible function of these common and incapacitating conditions[4]. It has been observed however that genetic, cultural and physiological components such as emotional and hormonal alterations are contributing factors to these conditions[5,6].

The roles of progesterone and estrogen during pregnancy cannot be overemphasized. The corpus luteum is responsible for the production of these pregnancy hormones until about the tenth week of gestation when the placenta takes over production[7]. Progesterone plays an important role in suppressing maternal immunologic response to the foreign bodies: the fetal antigens while estrogen serves as catalyst for chemical changes at the cellular level that are necessary for foetal growth, development and energy[8].

It has been documented that both gestational and infant stress predisposes individuals to a variety of maladaptive behaviours and psychopathologies in relation to the hippocampus such as attention deficit hyperactivity disease (ADHD), schizophrenia, drug addiction and depression[9]. Studies using rodents have shown that perinatal stress produces behavioural abnormalities which includes impaired learning and memory, deficits in attention, altered exploratory behaviour and prolonged stress response[10]. The most active component of *Garcinia kola* is kolaviron which is rich in biflavonoids: kolaflavonone, GB1 and GB2[11]. Kolaviron is a defatted ethanolic extract from *Garcinia kola.* It is a biflavonoid having strong and natural antioxidant properties[12,13,] as well as antibiotic and anti-

inflammatory properties[14]. Other studies have elucidated the biological activities of flavonoids which include action against allergies, inflammation, free radicals and hepatoxins[15]. Despite these numerous benefits and findings, no studies to the best of my knowledge has sought to investigate effects of kolaviron on pregnant female Wistar rats to explore its effects as it relates to hippocampal studies. Hence this research focuses on the possible effects of kolaviron on wistar rats during pregnancy, exploring its effects as it relates to hippocampal studies. This study is restricted to the effects of kolaviron via oral administration in a weekly-dependent manner (likened to trimester-dependent manner) on the morphology and biochemical components of the hippocampus of maternal Wistar rats.

MATERIALS AND METHODS

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

Seeds of Garcinia Kola were obtained locally in Ilorin, Nigeria and certified by the curator in the Department of Plant Biology, 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 freshly peeled seeds were weighed using an electronic weighing scale, then cut into pieces and air-dried for 2 weeks at room temperature (28-30˚C). The dried seeds were pounded to fine powder using a mortar and pestle. Extraction was done in the Laboratory of the Anatomy Department using light petroleum ether (boiling point: 40-60˚C) for 48hrs. The defatted dried marc was repacked and extracted with acetone (boiling point: 56-60˚C) in the water bath. 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 kolaviron. This procedure was carried out according to the method [16] and modified[17]. Purification and validation of kolaviron was determined by subjecting it 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. This 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 yield of kolaviron in this study was 6.3% and was kept at a room temperature of 4 ˚ □[1] before and after each use.

Animal Model: Thirty (30) adult females and fifteen (15) adult male Wistar rats weighing from 150g to 250g were purchased from Department of Zoology, Faculty of Life Sciences, University of Ilorin, Ilorin. The animals were acclimatized for two weeks 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. During the whole period of experiment, all animals received standard laboratory animal's pallete (from Ogo-Oluwa Feeds at Sango, Ilorin) and tap water *ad-libitum*.

Determination of the Estrus Phase Via Vaginal Smaearing: Vaginal smear test was performed first on daily basis prior to mating in order to observe the estrous cycle of the female rats[18]. The vaginal smear was done 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. The unstained vaginal smears were then observed under the light microscope, without the use of condenser lens, 4x and 10x objective lenses. Three types of cells could be recognized; the round and nucleated cells are the epithelial cells, irregular ones without nucleus are cornified cells and the little round ones are leukocytic cells. The proportion among them was used to determine the estrus phases[19,20]. The proestrus smear consist of nucleated epithelial cells, the estrous smear consists of a mixture of about 75% nucleated cells and about 25% cornified cells, metestrus smear consist of equal distribution among leukocytes, cornified and nucleated epithelial cells, and the diestrus smear primarily consist of a predominance of leukocytic cells, in most cases 100% leucocytic [19,20].

MATING: Mating was done using Marconde *et al.,* (2002) method[18]. After the estrous phases have been determined, males of reproductive ages were introduced into the female's cages that contain Wistar rats confirmed to be in their proestrous and estrous phases. This was done to encourage acceptance of the male rats by the female rats for mating purposes. The presence of sperm plug ascertained that mating has taken place, hence the day following the day of male introduction into the female's cage was taken as day 1 of pregnancy[21].

Grouping of Animals / Mode of Administration: Thirty (30) rats were carefully divided into 2 groups representing the 2nd and 3rd weeks of gestation respectively. This was done based on the number of animals that were found to be in the proestrous and estrous phase, then further into 3 sub-groups of 5 rats each group A: Control which were given distilled water (DW), group B: which were given corn oil as vehicle for kolaviron (CO) and group C: the treatment group which were given kolaviron (KV).

GROUPS	CONTROL (1ml)	VEHICLE Corn oil (0.6ml)	TREATMENT (200mg/kg Kv + vehicle)	DURATION (DAYS)	TOTAL No. OF ANIMALS
2ND WEEK (A)	Distilled water (5)	Corn oil (5)	kolaviron (5)	8 – 10	15
3RD WEEK (B)	Distilled water (5)	Corn oil (5)	kolaviron (5)	15 - 17	15

Animal Sacrifice and Tissue Extraction: The pregnant adult female Wistar rats were sacrificed on day 20 of gestation while their braind and were excised and weighed. Some of these samples were then fixed in 4% paraformaldehyde for histological processing. Their hippocampii were also excised from their brain tissues, weighed then meshed using porcelain mortar and pestle in 2mls of 0.25M sucrose solution before being centrifuged (in tubes padded with ice) at 5000 r/m for 15 minutes using Gallenkomp centrifuge. The supernatant was then decanted and kept in the freezer.

Quantitative Histochemistry

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 brain tissue were stored in the refrigerator and used for Superoxide dismutase (SOD) and *Glutathione Peroxidase (GPx) and analysis*.

Glutathione peroxidase (GPx) Assay[22]: The activity of glutathione peroxidase was assayed according to the method of Principle: In the presence of the hydrogen donor, peroxidase coverts hydrogen peroxide to water and oxygen. The oxidation of peroxidase substrate to a coloured product can be followed spectrophotometrically at 430 nm.

Procedure:

To 3ml of Glutathione peroxidase substrate solution, 0.1ml of the enzyme extract was added and the spectrophotometer was adjusted to zero at 430nm. To the test cuveette, 0.5ml of hydrogen peroxide was added and mixed. The change in absorbance was recorded every 30 seconds up to 3 minutes in a spectrophotometer. One unit of peroxidase is defined as the change in absorbance per minute at 430nm using the coefficient of $0.00622 Nm^{-1}cm^{-1}$.

Calculation of Glutathione Peroxidase Activity

The rate of change in absorbance for both blank and homogenate test was calculated by subtracting values obtained from the blank from that of the homogenate to arrive at the actual rate of change in absorbance for the GPx (ΔA) and GPx activity can be calculated with this formula:

$$Gpx = \frac{\Delta \text{Absorbance} \times E}{0.1 \times 12}$$

Where E= 3.6

Superoxide Dismutase (SOD) Assay[23]: The procedure for SOD assay is described below: Superoxide dismutase activity was determined as described by (Sun and Zigma, 1978). The reaction mixture 3ml contained 2.95ml carbonate buffer 0.02ml of brain homogenate and 0.03ml of 2mM SOD substrate in 0.005N HCL was used initiate the reaction. The reference cuvette contained 2.95ml buffer, 0.03ml of substrate and 0.02ml of water. The absorbance were read at regular interval of 1 minute for 5 minutes at 480 nm.

Activity of SOD was calculated with this formula= $\Delta A/\text{min} \times TV/£ \times SV$

Where

ΔA= change in absorbance.
TV= Total volume.
SV= sample volume.
£= molar extinction.

Statistical Analysis: Outcomes were analyzed using One-way ANOVA followed by Turkey's post hoc test for multiple comparison on IBM SPSS (Version 20). Graphical values were represented as mean ± standard error of mean (SEM) and plotted using the GraphPad Prism® software (version 6). Level of significance was taken as $P < 0.05$.

RESULTS

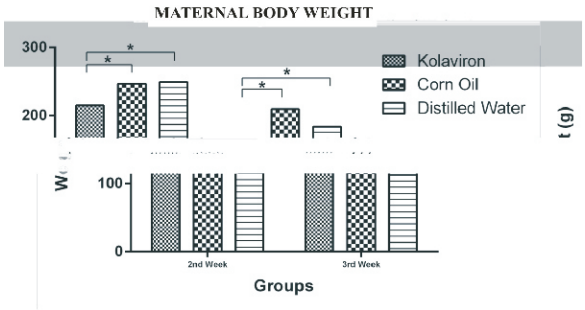

Figure 2: Graph showing the weight gain observed in dams during gestation. P<0.05 is considered significant with * meaning a statistically significant difference between experimental groups being compared. Values are represented as mean ± standard error of mean.

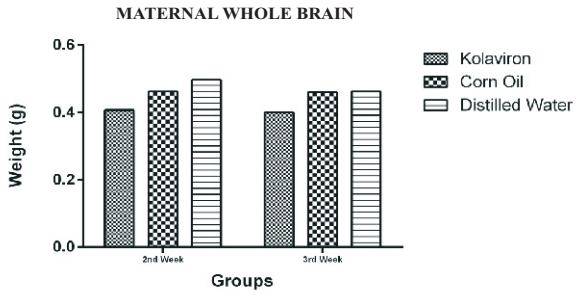

Figure 3: Comparison of the whole brain weight of adult animals reveals a stepwise increase across the experimental groups, Kolaviron, corn oil, and distilled water respectively. P<0.05 is considered significant. Differences across the group are however insignificant. Values are represented as mean ± standard error of mean.

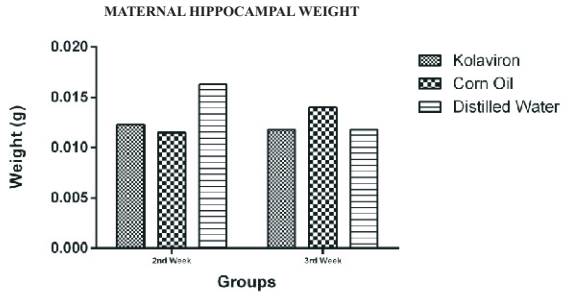

Figure 4: Showing the mean weight of adult (maternal) hippocampus. Differences observed across groups are not significant statistically. P<0.05 is considered significant. Values are represented as mean ± standard error of mean.

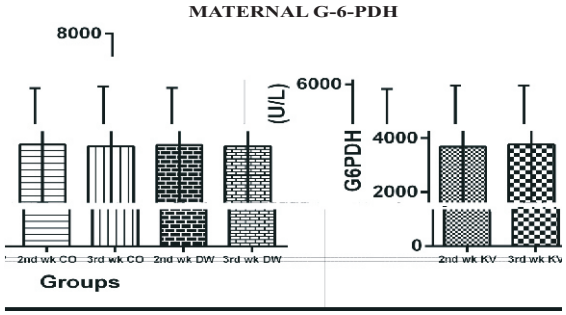

Figure 5: Analysis of glucose-6-phosphate dehydrogenase in tissue lysate of dams. KV = Kolaviron, CO = Corn Oil, DW = Distilled Water, wk = week. No significant level of difference is observed between the experimental groups. P<0.05 is considered significant. Values are represented as mean ± standard error of mean.

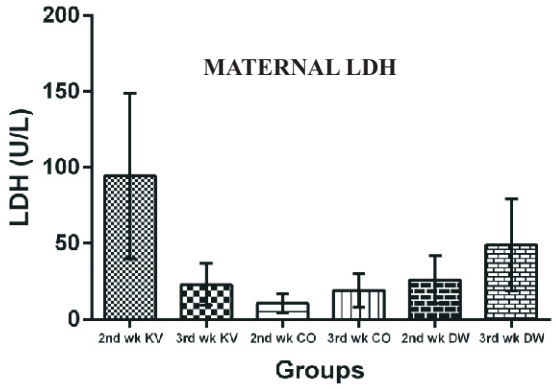

Figure 6: Graph showing the activity of lactate dehydrogenase (LDH) in dams. KV = Kolaviron, CO = Corn Oil, DW = Distilled Water, wk = week. The levels of LDH in the dams which received Kolaviron in the 2nd week of gestation seem to be overexpressed when compared to the levels in other experimental groups. This increment is however, insignificant statistically. P<0.05 is considered significant. Values are represented as mean ± standard error of mean.

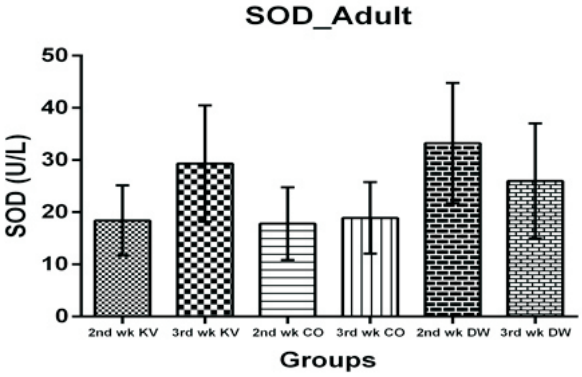

Figure 7: Graph showing the profiles of superoxide dismutase in the experimental dams. KV = Kolaviron, CO = Corn Oil, DW = Distilled Water, wk = week. Visual increases and decreases are observable, but these are not statistically significant when put through between groups comparisons. P<0.05 is considered significant. Values are represented as mean ± standard error of mean.

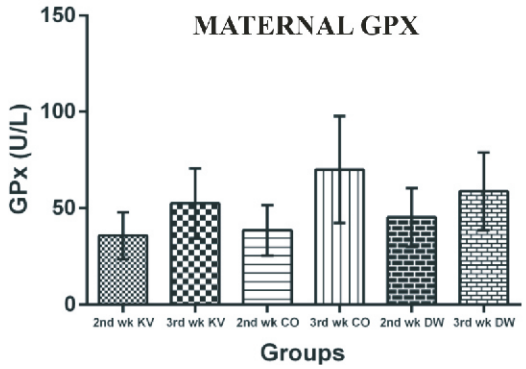

Figure 8: Graph showing the levels of glutathione peroxidase (GPx) in dams. KV = Kolaviron, CO = Corn Oil, DW = Distilled Water, wk = week. Decreases and increases observed are visual and thus, not statistically significant. P<0.05 is considered significant. Values are represented as mean ± standard error of mean.

HORMONAL ASSAY RESULTS

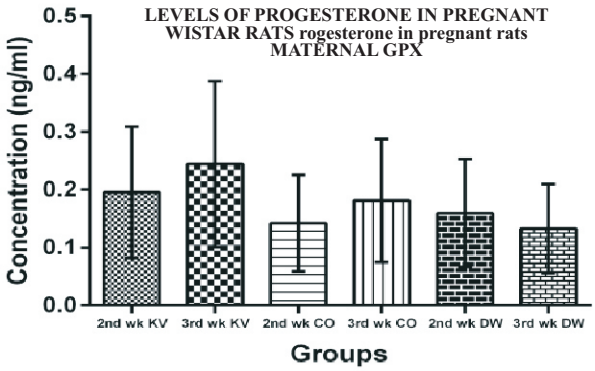

Figure 9: Graph showing the representative concentration of progesterone in the different experimental groups. KV = Kolaviron, CO = Corn Oil, DW = Distilled Water, wk = week. No statistically significant difference was observed between groups. P<0.05 is considered significant. Values are represented as mean ± standard error of mean.

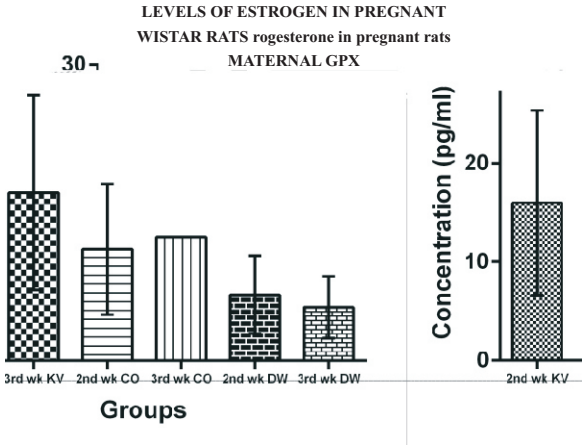

Figure 10: Graph showing the representative concentration of estrogen in dams of the different experimental groups. KV = Kolaviron, CO = Corn Oil, DW = Distilled Water, wk = week. Kolaviron groups had increases in the levels of estrogen when compared to the corn oil and distilled water groups. This observed increase was insignificant statistically. P<0.05 is considered significant. Values are represented as mean ± standard error of mean

DISCUSSION

Weight Parameters: Observations on the body weights of the pregnant rats show significant statistical difference when comparing the kolaviron group to the control group (Figure 2). This study supports[24,25] who recorded significant statistical difference in body weights of kolaviron treated male rats compared to their control groups. This reduction in body weight was suggested to be as a result of mal-absorption in the small intestine similar to effects possessed by flavonoids and tannin containing substances just as seen in kolaviron. however, an increase in body weight of animals administered 400 mg/kg BW kolaviron treatment with cyclophosphamide for 14 days was reported[26], though in this study male animals were used and an ameliorative substance. Maternal brain weights showed successive increase in the 2nd week and 3rd week of gestation with the kolaviron group recording the least however, differences observed across these groups were not statistically significant (Figure 3 and 4). This study is in support with the findings of[24] however, he reported loss of brain weight in male Wistar rats and this was observed in the groups administered 400 mg/kg BW and 800mg/ kg BW respectively.

A dose-dependent adverse effect on alteration in the estrous cycle, partial blockage of ovulation, foetal development and loss of foetal weight as a result of the consumption of *Garcinia kola,* which has been found to contain kolaviron as its most active component[27].

Enzymes of Carbohydrate Metabolism (Lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G-6-PDH): A remarkable increase in LDH was seen in rats administered KV in the 2nd week (Figure 6) when compared to that observed in rats administered same extract in the 3rd week of gestation. However, there was a decrease in levels of LDH observed in all other groups when compared to the control groups. A decrease in LDH when kolaviron was administered alone, before and after the insult by NaN3 in brain tissue homogenate of the prefrontal cortex of male Wistar rats, whereas an increase was seen in animals administered with NaN_3 only[28].

Glucose -6-phosphate (G-6-PDH) protects the growing foetus from oxidative DNA damage and embryonic pathologies[29]. It is a very important enzyme found within the cytoplasm of cells and accounts for the synthesis of nucleic acid in the hexose monophosphate pathway[29]. Its major physiological role is to supply nicotinamide adenine dinucleotide phosphate (NADPH) by the conversion of G-6-PDH to ribulose 5-phosphate by 6-phosphogluconate dehydrogenase. G-6-PDH showed insignificant statistical difference between pregnant rats in the CO and DW in the 2nd week and 3rd week of gestation. Though insignificant, perhaps kolaviron contributed to the increase in levels of cytoplasmic activity of the enzymes[30] as supported by Russel *et al.,* who reported an increase in neuronal G-6-PDH expression in the hippocampus of Alzheimer's disease patients when compared to age-matched controls. The result in this study however does not support[31] who reported unaltered levels of LDH and G-6-PDH on the effect of antioxidant caffeic acid phenethyl ester (cape) in cisplatin-induced neurotoxicity in rats.

Superoxide Dismutase and Glutathione Peroxidase : SOD and GPx are known to be the first line of defense against free radical injury[32]. The result in this present study showed increased profiles of SOD in the 2nd week compared to the levels in the 3rd week and controls. Superoxide dismutase helps to break down potentially harmful oxygen molecules in cells such as superoxide radical (O_2^-). Hydrogen peroxide (H_2O_2) is one of the main reactive oxygen species (ROS) which results in oxidative stress[33] and is continuously being generated when in short supply of some enzymes such as the glutathione peroxidase (GPX) and superoxide dismutase (SOD) [34]. In line with this study[34] also reported reduced levels of GPX and SOD in the hippocampus and brain tissues of kolaviron treated pregnant Wistar rats. Kolaviron was reported effective as a chemopreventive agent against AFB1-induced genotoxicity and hepatic oxidative stress[17].

Hormonal Changes: Observations show that there was increased levels of progesterone and estrogen in the KV group that was administered during the 3rd week when compared to the 2nd week of pregnant animals. This increase was also observed when compared to animals that were administered CO in both 2nd and 3rd week respectively. The levels of the hormones in the 2nd and 3rd weeks of administration were higher than those of their control groups. Though the statistical difference was insignificant, it could be said that kolaviron could serve as hormonal supplementation during pregnancy to further suppress maternal immunologic response to foreign DNA (foetal antigens) and encourage the foetal growth and development. This is in line with [8] who reported that these hormones are indispensable in creating a suitable endometrial environment for implantation and maintaining pregnancy. It was reported by[35] that hormones affected adult neurogenesis during pregnancy and postpartum however, progesterone and estrogen showed no effect on adult neurogenesis with the exception of prolactin which induced neurogenesis directly in the olfactory bulb of pregnant mice[36]. Progesterone plays an important role in the protection of the endometrium and myometrium, implantation of the embryo and breast development[37].

CONCLUSION

In conclusion, general increased levels in hormones and reduced levels of enzymes that take part in oxidative stress in the kolaviron administered groups especially during the third week of gestation might be attributed to the reduced development activity taking place in the foetusm which in- turn has an effect on the pregnant rat.

RECOMMENDATION

Immunohistochemical and Western blotting investigations should be carried out to further analyze the effect of kolaviron during pregnancy.

No conflict of interest.

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