Journal of Anatomical Sciences

Email:anatomicaljournal@gmail.com

J Anat Sci 11 (1)

Neuronal Degeneration in Kaolin-Induced Hydrocephalus in Wistar Rat; Kolaviron to The Rescue?

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ABSTRACT
Hydrocephalus is known to result in neuronal degeneration which have been attributed to cognitive and motor deficit associated with the disease. Delay in surgical intervention owing to poor infrastructure, inadequate manpower and a state of denial necessitated the search for neuroprotective plant materials. Kolaviron is a defatted ethanol extract from the seeds of *Garcinia kola* with anti-inflammatory and anti-oxidative properties. This study is aimed at investigating the possible neuroprotective role of Kolaviron in Kaolin-induced hydrocephalic rats. Ninety Wistar rats aged three weeks old were randomly assigned into three groups (n=30) sacrificed at 1st, 2nd and 3rd week post-kaolin induction respectively. Each group was further divided into six sub-groups (n=5) (Control, Hydrocephalus only, Kolaviron 100 mg/kg, Kolaviron 200 mg/kg, Hydrocephalus+Kolaviron 100 mg/kg, Hydrocephalus+Kolaviron 200 mg/kg). Hydrocephalus was induced by intra-cisternal injection of 0.04 ml of kaolin suspension (200 mg/ml). Neurobehavioral assessment of anxiety and locomotion was done 2 hours before sacrifice. Brain coronal slices at optic chiasma level were fixed in 10 % neutral buffered formalin. Paraffin sections (5µm) were stained with hematoxylin and eosin for neuronal density assessment. Data were analyzed using descriptive statistics and ANOVA at $p< 0.05$. Compared with their corresponding controls, brain weight and neuronal density were significantly reduced in hydrocephalic rats. Neuronal degeneration was observed in all hydrocephalic groups. Degree of neurodegeneration was reduced in those that received 200 mg/kg of Kolaviron when compared with the 100 mg/kg of Kolaviron group. The study concluded that Kolaviron is neuroprotective at 200 mg/kg but not at 100 mg/kg.

Keywords: Hydrocephalus, neurons, neuro-degeneration, Kolaviron, Garcinia kola.

INTRODUCTION
Hydrocephalus is a common neurological condition in children, usually resulting from an obstruction of Cerebrospinal fluid (CSF) in the ventricular system[1,2]or from the inadequate absorption of CSF resulting in ventricular dilation[3]. Although hydrocephalus is common in children, it is not limited to this age group as it also affects adults[4]. For the brain to function properly, the integrity, connectivity, and function of neurons must be preserved[5]. Events such as, gradual physical stretching and compression of the brain, ischemia with calcium-mediated axo-skeletal damages, and possible accumulation of metabolic waste products result from ventriculomegaly[1,6,7]. Many events known to involve oxidative stress (infection, hemorrhage, brain trauma) are associated with hydrocephalus[8], these have been implicated in different degrees of neuronal damage. The findings of Owen- Lynch *et al.,* revealed the presence of some factors in the cerebrospinal fluid of hydrocephalic Texas (H-Tx) rats which are capable of inhibiting neuronal proliferation by arresting them at the S-phase of the cell cycle in both in-vivo and in vitro models laying credence to the precarious state of neurons in hydrocephalus[9].

Kolaviron is a defatted ethanol extract from the seeds of *Garcinia kola.* It is a mixture of three compounds- Garcina biflavonoid GB_1, GB_2 and kola flavonone in ratio 2:2:1[10].Flavonoids have protective effects including anti-inflammatory, anti-oxidant, antiviral, and anti-carcinogenic properties. They are found in a variety of foods, such as oranges, tangerines, berries, apples and onions[11]. It has a scavenging ability that is specifically important in reducing oxidative tissue damage in neural tissue after an induced oxidative stress[12,13]. Studies have shown that Kolaviron has the ability to reduce oxidative stress and tissue damage by slowing down the rate of lipid peroxidation and oxygen radical production in vivo and in vitro[14,15].

Ijomone *et al*., reported its neuro-protective effects in the treatment of Methamorphine-induced neurotoxicity when administered at 200mg/kg[16], Adaramoye also reported the protective effect of kolaviron at 250mg/kg on the neurons in the brain of rats against oxidative stress induced by gamma radiation[17]. In a study carried out by Olajide *et al.,*[15] it was reported that kolaviron at 200mg/kg had neuroprotective properties against

sodium azide (NaN_3) induced oxidative damage in the prefrontal cortex of adult Wistar rats.

In poor nations of the world with poor healthcare infrastructure, the waiting time for hydrocephalic patients for surgical intervention is very long. This, in addition to the prevalent culture driven-denial state in such patient make the cortical cells such as neurons very vulnerable to degenerative processes which has been found to be heightened by prolonged duration of ventriculomegaly[18]. Such possible neuroprotective ability of Kolaviron may be very critical in attenuating the neuronal consequences of hydrocephalus especially in sub-saharan Africa where delay in surgical intervention is common.

MATERIALS AND METHODS

Chemicals and Drugs: Kaolin Powder was procured from Hopkins and Williams (England). Ketamine hydrochloride injection USP was procured from ROTEXMEDICA (Trittau.Germany). Diazepam was procured from F. Hoffmann-La Roche Ltd, Basel (Switzerland), *Kolaviron capsules (40mg) was obtained from CTC Bio America Inc*. Other reagents used were of analytical grade.

Rat Care and Management: A total of 90 three weeks old Wistar rats of both sexes were used for the study. Rats were housed in plastic cages. Rats were fed with standard laboratory rat chow (ACE feed, Osogbo, Nigeria) and they had access to clean drinking water *ad libitum*. Ethical clearance was obtained from the Health Research and Ethics Committee (HREC) of the Institute of Public Health, Obafemi Awolowo University, Ile-Ife. The rats received humane care according to the guidelines for the institutional use of animals.

Experimental design: Rats were randomly divided into three groups based on their time of sacrifice post-induction (n=30 per group) (1st, 2nd and 3rd week post-kaolin induction). Each group was further divided into six sub-groups (n=5) (A {Control}, B {Hydrocephalus only}, C {Kolaviron 100 mg/kg}, D {Kolaviron 200 mg/kg}, E {Hydrocephalus + Kolaviron 100 mg/kg}and F {Hydrocephalus + Kolaviron 200 mg/kg}). Hydrocephalus was induced in Groups B, E and F by intra-cisternal injection of 0.04 ml of 200 mg/ml of kaolin suspension.

Kolaviron administration: Groups A rats received distilled water, B was only induced with hydrocephalus but received no treatment, C received 100 mg/kg of Kolaviron, D received 200 mg/kg, E was induced with hydrocephalus and was treated with 100 mg/kg of Kolaviron, F was induced with hydrocephalus and treated with 200 mg/kg of Kolaviron. Contents of the kolaviron capsules were dissolved in distilled water, the suspension was administered orally using an oral cannula.

Neurobehavioural assessment: Neurobehavioral assessment was done using the open field maze two hours prior to sacrifice. The assessment was done in a quiet environment. The maze floor was wiped with ethanol in between assessments. Rats were introduced to the maze to acclimatize for a few minutes. Each rat was subsequently tested for 2 minutes and a video recording of the activities of the rats in the maze was taken. The number of lines crossed by each rat was noted.

Animal Sacrifice and Histology: Following neurobehavioral assessment, rats were sacrificed under intramuscular ketamine anesthesia (90 mg/kg). Rats were decapitated and the brain was excised using bone forceps, was blotted dry and weighed with AB204 Mettler Toledo weighing balance. The brains were fixed in 10% Neutral Buffered Formalin (NBF) by immersion. One mm thick coronal brain slice was obtained subsequently at the level of the optic chiasma and processed for routine paraffin embedding. Sections were stained with H&E to demonstrate cortical histoarchitecture and neuronal morphology.

Photomicrograph of stained sections was imported on to Image-J software for histomorphometric analysis. Neuronal density was done by counting the normal and degenerating neuron per unit area with a grid superimposed on the cortical micrographs. Only neurons whose more than 50% of its cell bodies are located within the grid are counted to avoid double counting. Pyknotic, karyorrhetic and eosinophilic neurons were all counted as degenerating neuron while those with clear cut nucleus and identifiable cell body with or without extensions (axons and dendrites) are counted as normal neurons.

Statistical analysis: Data are presented as mean ± SEM and was analyzed using one-way analysis of variance (ANOVA) for intergroup comparisons, and unpaired t test for two-group comparisons. P-values<0.05 will be considered statistically significant.

RESULTS

General Observation: The experimental and control rats recovered from anesthesia within 12 to 24 hours post-induction. The experimental rats exhibited signs of lethargy and motor weakness in the first 24 hours. After recovery from anesthesia, experimental rats remained slow and tended to fall and stagger. Food consumption and general activity was reduced in experimental rats. There was normal food consumption in control rats. Varying degrees of unsteady movements, weight loss, hind-limb weakness, lethargic pace, scruffy fur, gritting of teeth was observed in experimental rats as early as day 4 in some rat and at the end of week 1 in others. This signs continued in this group of rats throughout the duration of the experiment. There was progressive enlargement of head and urinary incontinence (showed by persisting wetness of the fur around the genital) in hydrocephalic rats that showed the above stated signs early. The non-hydrocephalic rats did not exhibit any of the

aforementioned signs.

Gross anatomical observation: At 1st week post-induction, there was a significant decrease in the mean brain weight in Group B when compared with C (p=0.0460) but there was no significant difference in the brain weight of Group B when compared with Groups A (p= 0.0736), D (p=0.0736) and F (p=0.7404). There was a significant decrease in the brain weight of Group E when compared with Groups A (p=0.0294), C (p=0.0216) and D (p=0.0294) but not significant when compared with Group F (p=0.4714). There was no significant difference in the brain weight of Group F when compared with A (p=0.0955), C (p=0.0578) and D (p=0.0955). At 2nd week post-induction, there was no significant difference in the brain weight of all groups when compared with each other (p=0.6145). At 3rd week post-induction, there was no significant difference in the brain weight (p=0.8493) (Table 1).

Table 1: Brain weight (g) across groups and timeline

Groups	1st Week (n=5)	2nd Week (n=5)	3rd Week (n=5)
A		1.320±0.200	1.420±0.03742
B	1.200±0.04472[c]	1.280±0.03742	1.320±0.06633
C	1.360±0.05099	1.360±0.5099	1.340±0.05099
D	1.320±0.03742	1.320±0.3742	1.360±0.05099
E	1.180±0.03742[acd]	1.294±0.5776	1.320±0.06633
F	1.220±0.03742	1.314±0.03105	1.376±0.08442

Behavioral assessment: At 1st week post-induction, there was a significant decrease in number of line crossed in group E when compared with groups A (p<0.001), C (p<0.001) and D (p<0.001) and also in Group F when compared with A (p<0.001), C (p<0.001) and D (p=0.0006). Number of lines crossed was also decreased in Group B when compared with C (p< 0.0001). At the 2nd week post-induction, there was significant decrease in number of lines crossed in Group E when compared with group A (p=0.0249) and C (p= 0.0489) and in Group F when compared with Groups A (p=0.0057), C (p=0.0131) and D (p=0.0014). At the 3rd post-induction week, there was significant decrease in number of lines crossed when Group B was compared with Groups C (p=0.0312) and D (p=0.0115), in Group E when compared with Group D (p=0.0141) and Group F when compared with D (p=0.0203) (Fig. 1).

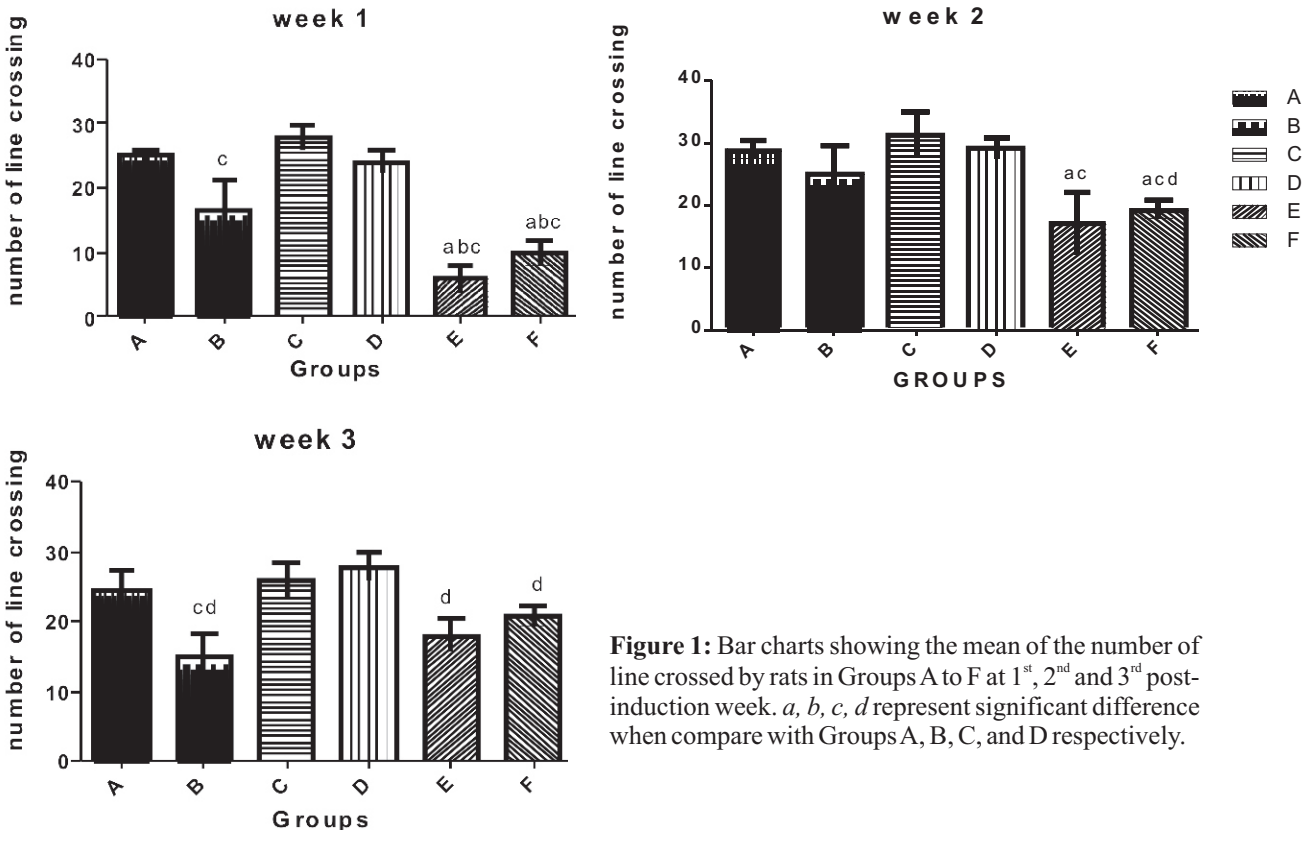

Figure 1: Bar charts showing the mean of the number of line crossed by rats in Groups A to F at 1st, 2nd and 3rd post-induction week. *a, b, c, d* represent significant difference when compare with Groups A, B, C, and D respectively.

Histological and Histomorphometry Assessment: At 1st week post-induction, the density of normal neuron in Group B was significantly reduced when compared with Groups A (p=0.0025), C (p=0.0010) and D (p=0.0012). There was a significant decrease in the normal neuronal density in Group E when compared with Groups A (p<0.0001), C (p<0.0001), D (p<0.0001). There was significant increase in the density of degenerating neurons in Group E when compared to B (p=0.0012). At 2nd week post-induction, the normal neuronal density of Group B was not significantly different when compared with Groups A (p=0.0633), C (p=0.0812), D (p=0.8833), E (p=0.0567) and F (p=0.6347), density of normal neuron in Group E was significantly reduced when compared with Group A (p=0.0002), C (p=0.0003), D (p=0.0465) and F (p= 0.0144). At 3rd week post-induction, the normal neuronal density in Group B was significantly reduced when compared with Groups A (p=0.0005), C (p=0.0035), D (p=0.0018), E (p=0.0234) and F (p=0.0030). The normal neuronal density in Group E was significantly reduced when compared with Groups A (p=0.0053) and D (p= 0.0018). There was significant increase in degenerating neurons only in Group B when compared to Group E (p=0.0235). There was no significant difference in the density of degenerating neuron when Groups B, E and F were compared (Table 2).

Table 2: Showing the density of normal and degenerating cortical neuron across the groups in the three timelines. Superscript indicate significant difference compared to indicate group.

	GROUP A	GROUP B	GROUP C	GROUP D	GROUP E	GROUP F
NORMAL NEURON (Wk. 1)	6.120 ± 0.2177	4.360 ± 0.3556^{acd}	6.840 ± 0.3614	6.300 ± 0.1612	3.080 ± 0.2396^{abcd}	5.560 ± 0.3970^{c}
DEGENERATING NEURON (Wk. 1)	-	0.284 ± 0.1319	-	-	2.108 ± 0.3500	2.010 ± 1.1000
NORMAL NEURON (Wk. 2)	5.020 ± 0.2245	3.580 ± 0.6151	4.920 ± 0.2035	3.800 ± 0.5941	2.160 ± 0.4007^{acdf}	4.080 ± 0.4620
DEGENERATING NEURON (Wk. 2)	-	0.5720 ± 0.2296	-	-	1.582 ± 0.2524	1.078 ± 0.3117
NORMAL NEURON (Wk. 3)	5.340 ± 0.2694	3.560 ± 0.4632^{ad}	4.680 ± 0.5073	5.560 ± 0.2112	3.260 ± 0.314^{ad}	4.600 ± 0.4701^{ad}
DEGENERATING NEURON (Wk. 3)	-	0.5720 ± 0.2296	-	-	1.582 ± 0.2524	1.078 ± 0.3117

Different morphological derangement of cortical neurons such as eosinophilic, karryohectic and pyknotic neurons were noted in the upper and lower cortical regions of the group B rats across the 3-week timeline. Similar pattern was noted in the group E and to a lesser extent in group F rats (Fig. 2 and 3).

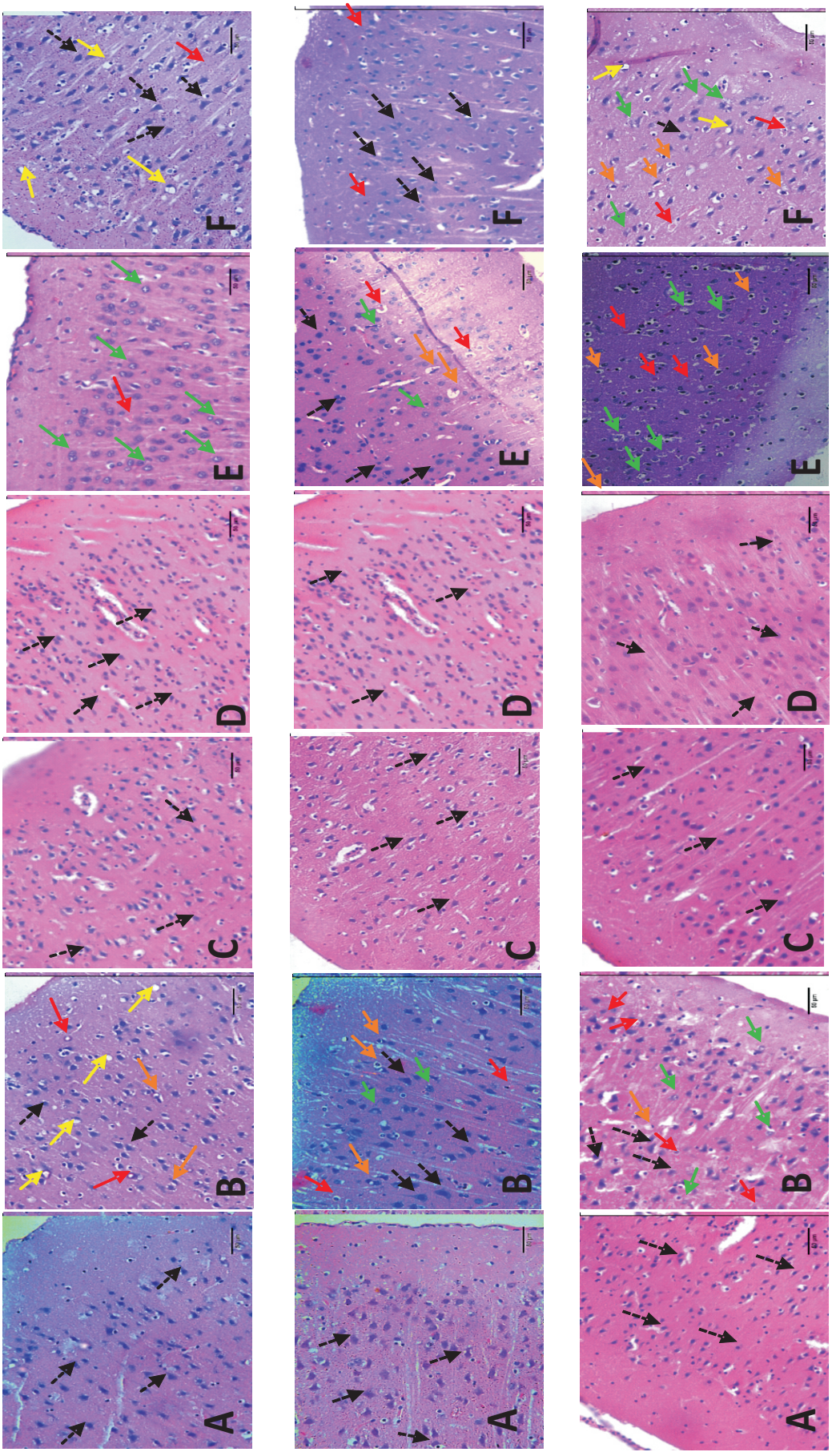

Figure 2: Photomicrographs of the Upper cortical region (Cortical layers I to III) in groups A, B, C, D, E and F at 1 (Upper panel), 2 (Middle panel) and 3 (Lower panel) week post-induction showing eosinophilic neurons (Red arrow), neuropil vacuolation (Yellow arrow), karyorrhettic neuron (Green arrow), pyknotic neuron (Brown arrow) and normal neurons (dashed black arrow). Stain H&E. Scale bar- 50µm.

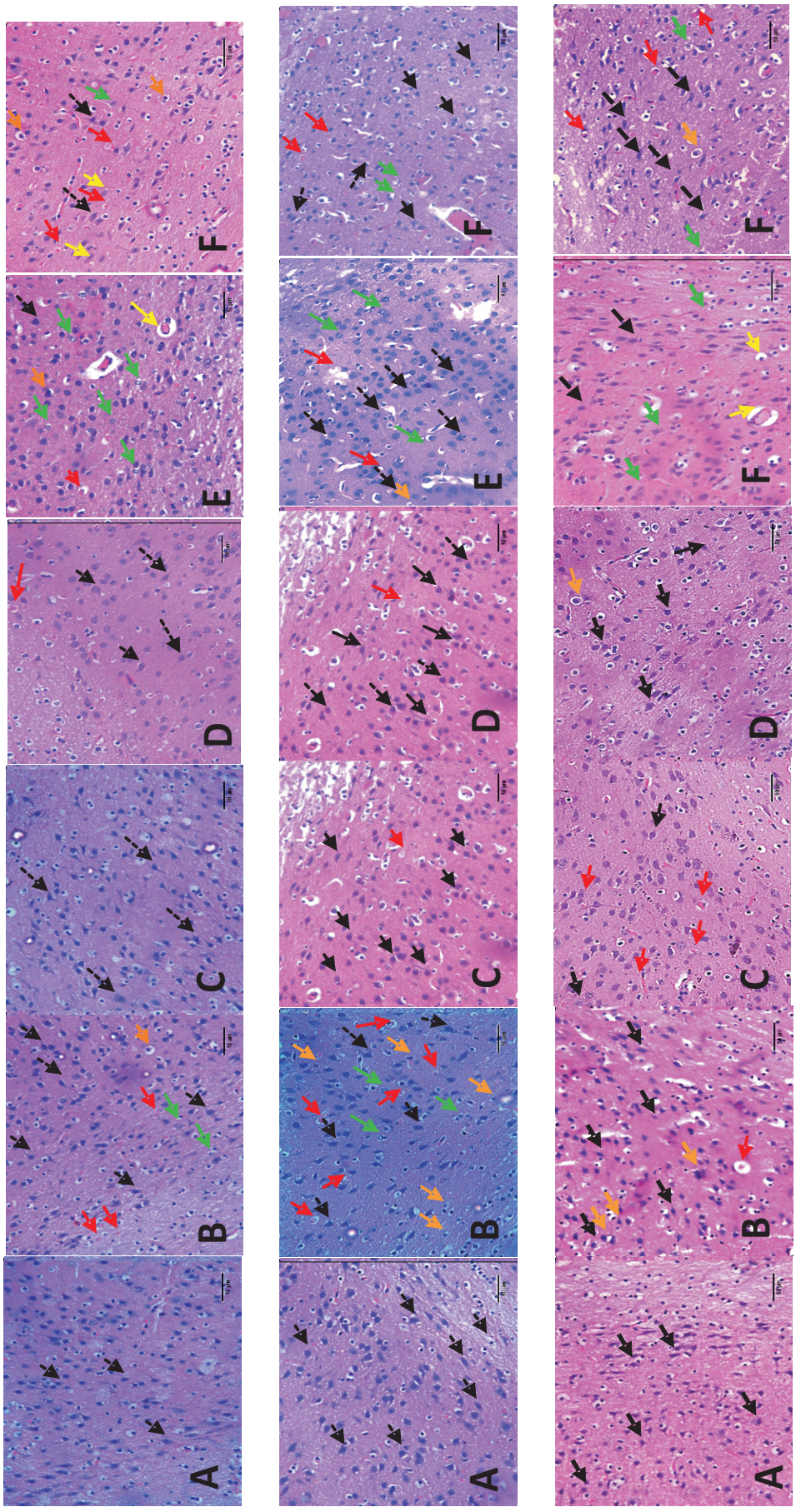

Figure 3: Photomicrographs of the Lower cortical region (Cortical layers IV to VI) in groups A, B, C, D, E and F at 1 (Upper panel), 2 (Middle panel) and 3 (Lower panel) week post-induction showing eosinophilic neurons (Red arrow), neuropil vacuolation (Yellow arrow), karyorrhettic neuron (Green arrow), pyknotic neuron (Brown arrow) and normal neurons (dashed black arrow). Stain H&E. Scale bar- 50µm.

DISCUSSION

Neuronal damage is common in hydrocephalus and ventriculomegaly poses significant threat to the normal morphology and function of neurons which in turn affects the overall performance of the brain. Disruption of the morphology and function of cortical neurons have deleterious effects on the cognitive and motor activities in the brain. In this study, gait disturbance and hind limb weakness were observed in the hydrocephalic rats as early as the 4thday after induction of hydrocephalus. Gradual compression of the motor cortex and basal ganglia due to the progressing ventriculomegaly could be responsible for the disruption of the cognitive and motor functions in the hydrocephalic rats. The contiguous position of the cerebral cortex and the basal ganglia to the lateral ventricle makes them very susceptible to the effect of the enlarging ventricle.

While the motor cortex has been reputed with the control of muscular movement across the body, mechanical compression of structures contiguous to the lateral ventricle may not fully explain the gait disruption in the acute phase of hydrocephalus. This is because the degree of ventriculomegaly in this model of hydrocephalus peaks much later. This points to neurotoxic environment occasioned by the accumulation of metabolic waste in the cerebral cortex due to stasis secondary to ventriculomegaly as a possible contributory factor to motor dysfunction. Such delay in the clearance of metabolites was reported in a similar model of hydrocephalus in rats[19]. Although, Kolaviron has been reported to have neuro-protective effects, it was not able to reverse the gait impairment that was exhibited by the hydrocephalic rats within the 3-week period of this study. Gait impairment has been noted as one of the earliest signs of normal pressure hydrocephalus in human[20], it is therefore not surprising that it was one of the features of the acute phase of kaolin-induced hydrocephalus in Wistar rats.

In this study, an assessment of anxiety state and locomotion in the rats was done using an open field maze. It was noted that the control groups explored the maze more freely which showed their non-anxious state. However, the hydrocephalic rats showed significant reduction in their exploratory tendencies, demonstrated by a reduction in the number of line crossed in the maze when compared with the non-hydrocephalic rats. This study revealed that administration of the 100mg and 200mg/kg of Kolaviron did not improve the anxiety state of the hydrocephalic rats in the first two weeks post induction. However, by the third week post-induction, the number of lines crossed was more in groups E and F rats in a dose dependent fashion, though this was not significantly different from group B. The gradual increase in the number of lines crossed in the hydrocephalic rats that had Kolaviron over the 3-week period is suggestive of a possible reversal of the reduced exploratory ability of such rats if they were observed for longer than 3 weeks with continuous administration of Kolaviron. Reduced line crossings in the hydrocephalic rats could be from an increased state of anxiety occasioned by the hydrocephalus and or a consequence of impaired mobility.

The change in the dynamics of cortical pressure and metabolic waste stasis could have increased the anxiety state of the hydrocephalic rats. Similar finding was reported in Sprague Dawley rats as early as 3 days of hydrocephalus[21]. Increased intracranial pressure and periventricular axonal damage may also contribute to the reduced exploratory activities in rats in groups B, E and F. The implication of the findings is that within the 3-week period of this study, Kolaviron does not ameliorate the anxiety state of the hydrocephalic rats. In fact, Kolaviron appeared to accentuate the anxiety state of hydrocephalic rats in the first 2 weeks of hydrocephalus. Whether or not the explorative capacity of hydrocephalic rats was masked by the aforementioned impairment of gait and movement will be the focus of another study. Significant reduction of line crossed was not recorded in Groups C and D across the 3-week timeline when compared with control, showing that Kolaviron alone does not have any adverse effect on locomotion and anxiety state in rats.

Data from this study showed a significant reduction in the brain weight of the hydrocephalic rats that had no Kolaviron intervention in the first week post-induction when compared with the control rats (Table 1). The brain weight of the rats that had 100mg/kg body weight of Kolaviron following hydrocephalus induction was significantly reduced compared with those that had the two doses of Kolaviron without hydrocephalus induction and the control rats. All the aforementioned was noted in the first week post-induction, while differences in the brain weight among the groups in the second and third week post-induction were not significant. This showed that the brain weight reducing consequences of hydrocephalus is an acute feature in this model and is amenable to administration of 200mg/kg body weight of Kolaviron, however,100mg/kg of Kolaviron does not appear to offer any protection on the brain weight of juvenile hydrocephalic rats. Although, reduced cortical thickness has been observed in various models of experimental and congenital hydrocephalus[22], the fate of the weight of the brain following hydrocephalus have not been frequently reported. Despite the severally reported seeping of cerebrospinal fluid (CSF) into the cortical tissue secondary to denudation of the ventricular ependymal layer[23,24] which is expected to result in CSF oedema of the cerebral cortex in hydrocephalic rats, the weight of the brain in the hydrocephalic rats of this study were significantly reduced in the first week of hydrocephalus.

Neuronal density of the dorsolateral cortex in all groups at 1st, 2nd and 3rd week post-induction,is shown in Table 2. Significant reduction in normal neuronal density was noted in all hydrocephalic group when compared with

the control rats across the 3-week timeline. This finding confirmed the fact that hydrocephalus has neurodegenerative consequences spanning the acute to the subacute phase. Metabolic stasis in hydrocephalus is a known mechanism of cellular injury[25]. An increase in the concentration of metabolites which is directly proportional to the severity of ventriculomegaly was reported in a cat model of obstructive hydrocephalus[26]. A combination of the mechanical effect of hydrocephalus occasioned by ventriculomegaly and metabolic stasis may result in a cytotoxic environment resulting in significantly reduced density of apparently normal neuron in the hydrocephalic rats. While this study confirmed significant reduction in normal neuronal density in the hydrocephalic rats when compared with control, similar reduction was noted in the hydrocephalic rats that had 100mg/kg of Kolaviron. This showed that Kolaviron at 100mg/kg body weight does not have any protective effect on cortical neurons following hydrocephalus. The hydrocephalic rats that received 200mg/kg body weight showed significant increase in the density of normal neurons when compared with the hydrocephalic only rats in the first to third week post-induction pointing to a neuroprotective capacity of Kolaviron at doses from 200mg/kg body weight. Oxidative stress had been implicated in the hydrocephalus-induced neurodegeneration[8], therefore the cocktail of antioxidant present in Kolaviron are the likely agents responsible for its neuroprotective role in hydrocephalus as earlier reported in other brain injury models[15,16]. Though there was a reduction in normal neuronal density in the hydrocephalic rats that were administered 200mg/kg body weight of Kolaviron when compared with the control rats, the difference was only significant at the third post-induction week. The acute phase of hydrocephalus in this model is associated with inflammation alongside other pathological processes

The acute phase inflammation in hydrocephalus have been implicated in neurodegenerative process, therefore, this study indicated a possible anti-inflammatory role of Kolaviron in hydrocephalus. Such anti-inflammatory capacity of Kolaviron have been reported in other systems. Studies have shown that kolaviron has the ability to reduce oxidative stress and tissue damage by slowing down the rate of lipid peroxidation and oxygen radical production both in vivo and in vitro[14,15,27]. Kolaviron has a scavenging ability that is specifically important in reducing oxidative damage in neural tissue after an induced oxidative stress[13,28]. Kolaviron has been identified as a neuroprotective and ameliorative agent in chemotoxin-induced oxidative stress; mostly through its anti-inflammatory and antioxidant properties. The advantage of kolaviron as a therapeutic target in reducing oxidative tissue damage has been linked to its role in reduction of reactive oxygen species (ROS) production[29,30], scavenging for formed ROS and prevention of lipid peroxidation in cells[15,31].

In this study, degeneration of neurons was observed in the hydrocephalic rats. Morphological features of cell death such as pyknosis, karyolysis, and eosinophilic changes were observed in experimental rats in the 1st to 3rd week post kaolin induction. Neuropil vacuolation was seen in close proximity to the degenerating neurons. The pyknotic and karyorrhetic cells points to an irreversible nuclear changes, while eosinophilic neuronal changes appeared to be an early feature of degeneration. The toxic environment following metabolic stasis in hydrocephalus and the mechanical effect of ventriculomegaly on the cerebral cortex might explain the different morphological derangements of the cortical neurons. Neuronal degeneration has been reported to be secondary to the effect of ischemia and reduced blood flow[3] which are known consequences of ventriculomegaly. Del Bigio and Zhang, (1998) reported the presence of karyohetic cells only within the periventricular white matter of kaolin induced hydrocephalic rats in the 3rd week post-induction, however, this study showed a wider spread of karyorrhetic neuron in the cerebral cortex. This might indicate a myriad of mechanism involved in the neuronal degeneration process in hydrocephalus[2]

CONCLUSION

This study showed that Kolaviron has a dose-dependent neuroprotective ability in kaolin-induced hydrocephalus, however, it does not improve the anxiety state of hydrocephalic Wistar rats.

ACKNOWLEDGEMENT

Author wish to acknowledge the technical support provided by Mr. Ige of the Histology laboratory, Department of Anatomy and Cell Biology, Obafemi Awolowo University, Nigeria.

CONFLICT OF INTEREST

The authors hereby declared that there is no conflict of interest as regards this study.

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