Timed Prenatal Ethanol Exposure and Postnatal Cortical Oligodendrocyte and White Matter Changes in Young Wistar Rats

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ABSTRACT

BACKGROUND: The study evaluated postnatal white matter changes following prenatal ethanol exposure in the first, second and third trimesters in Wistar rats sacrificed at the third and sixth postnatal week. This was with a view to providing information on the state of oligodendrocyte and subcortical white matter in prenatal alcohol exposure.

METHODS: Twenty (20) mature female Wistar rats were time-mated and randomly divided into 4 groups (n=5). Group 1 (control) received distilled water (2 mL/kg), Groups 2, 3 and 4 were administered 2.5 mL/kg of 20% ethanol orally on the 4th, 11th and 18th days of gestation respectively. All rats were allowed to litter and pups were weighed weekly. The brain was harvested at 3 and 6 postnatal weeks and fixed in 10% neutral buffered formalin. One mm thick coronal brain slice was at the optic chiasma plane were processed. Morphology and density of oligodendrocytes was studied using Haematoxylin and Eosin stain, while Luxol Fast Blue (LFB) stain was used for white matter morphometry. Data was analyzed using Student t-test. P value < 0.05 was considered statistically significant.

RESULTS: At the third week of postnatal life there was significant reduction in the subcortical white matter thickness which became reversible at the sixth postnatal week while there was no significant difference in the cortical oligodendrocyte density at both the third and sixth postnatal week, however regional comparison showed some differences at the two time lines.

CONCLUSION: The study concluded that prenatal ethanol exposure causes white matter reduction at the third week of postnatal life in Wistar rats with reversal at the sixth week.

KEYWORDS Prenatal; Alcohol; Oligodendrocyte; White matter

INTRODUCTION

A compacted myelin sheath around the axon is derived from the oligodendrocyte plasma membrane (Gnaedinger *et al.,* 1984). Myelin sheath is required for rapid propagation of action potentials in vertebrates (Marina, 2014). The saltatory conduction is energy conserving and faster. Oligodendrocyte precursor cells (OPC) are produced in limited areas of the brain and subsequently migrate to populate the entire brain during their maturation. Some OPCs are found in the mature brain where they may be involved in adult myelinogenic processes (Marina, 2014). White matter defect is a major consequence of alcohol during development resulting in reduced volume and sometimes a total disruption of major white matter tract formation following intrauterine alcohol exposure (Riley *et al.,* 1995; Sowell *et al.,* 2008). Alcohol is known to slow down the process of myelination and result in myelin ultrastructure disruption (Lancaster, 1994; Pinazo-Duran *et al.,* 1997). Recent studies have suggested a specific target of the brain white matter in alcohol teratogenesis (Fan *et al.,* 2015).

A wide range of white matter anomalies have been attributed to prenatal alcohol exposure, these include delay in myelination, reduction in myelin thickness, ultrastructural myelin anomalies and alteration in the biochemistry of myelin. It is also known to result in a reduction in the differentiation and migration of oligodendrocytes as well as expressions of myelin basic protein (MBP) and myelin associated glycoprotein (MAG) (Marina, 2014; Fan *et al.,* 2015).

The first 10 postnatal days is the most crucial for myelin and oligodendrocyte health in alcohol exposure in animal models (Marina, 2014). Derangements of oligodendrocyte morphology and myelin structure have been attributed to exposure to alcohol in the 3rd trimester in sheep (Dalitz *et al.,* 2008). Expression of MBP is delayed and reduced in the cerebellum of PND 15 rats following prenatal alcohol exposure (Zoeller *et al.,* 1994). Similar reduced expression of MBP was noted in cultured oligodendrocytes following alcohol exposure (Bichenkov and Ellingson 2009) while widespread apoptosis of oligodendrocytes was noted in Monkeys following prenatal alcohol exposure (Creeley et al., 2013).

While extensive studies have been done on the effect of postnatal alcohol exposure on oligodendrocytes and the process of myelination, the precise effect of intrauterine alcohol exposure on oligodendrocyte and subcortical white matter at specific periods during pregnancy equivalent to the three trimesters has not enjoyed as much research attention. Therefore, the aim of this study is to provide information on the state of oligodendrocytes and subcortical white matter following ethanol exposure in the first, second and third trimesters of Wistar rats.

METHODS

Ethical approval was sought and obtained from Health Research and Ethics Committee of Institute of Public Health, Obafemi Awolowo University Ile-Ife, Nigeria. The animals received humane care according to the guidelines of IACUC.

Rat Breeding and Grouping

Wistar rats (*Rattus novergicus*) were raised in the research animal facility of the Department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife, Nigeria to ascertain their developmental history. Twenty mature female Wistar rats were used for this study. They were housed in clean plastic cages in a clean environment at natural humidity, temperature and light/dark cycle for the study. Rats in all groups were fed with standard laboratory rat chow and allowed access to water *ad libitum*. The rats were randomly assigned into 4 groups (n=5). Pro-estrous female rats were mated overnight with male rat in separate cages. Vaginal plug presence indicated day 0 of gestation. Group 1 (Control) was administered distilled water (2 mL/kg). Group 2, 3 and 4 were administered 2.5 mL/kg of 20% ethanol on days 4, 11 and 18 respectively. Ethanol and distilled water were administered by the use of oral cannula. Following ethanol administration at the above stated time, all pregnant rats were allowed to litter. Rat pups were allowed to grow naturally with the mother and weighed weekly.

Brain Harvest and Histology

Five young rats per group were sacrificed at the end of the 3rd and 6th postnatal week. Sacrifice was by cervical dislocation. The skull was opened carefully to avoid brain tissue damage with the use of a forceps. The brain was harvested using spatula, blotted dry, weighed and fixed in 10% neutral buffered formalin by immersion. A coronal brain slice of 1mm thickness was obtained at the optic chiasma plane and processed for routine histology via paraffin embedding. Five micron thick sections were obtained with the use of a Leica rotary microtome and stained accordingly. Haematoxylin and Eosin staining was used to demonstrate the general histo-architecture of the dorsolateral cortex while Luxol Fast Blue was used to demonstrate white matter. Oligodendrocytes density was estimated with the Hematoxylin and Eosin sections. Sections were captured with a Leica DM750 microscope interfaced with Leica ICC50 camera and digital photomicrographs were taken and archived. Each micrograph was viewed with Image J analysis software superimposed with a grid of predetermined area. The dorsolateral cortex that corresponds with the CA 2 subfield of the hippocampus was chosen as the area of interest. Oligodendrocytes in the alternate squares within the grid were counted. Double counting was avoided and number of squares was noted to calculate the area covered. The thickness of the subcortical white matter was measured using the CA 2 subfield as landmark, while corpus callosum thickness was measured at the midline. Data is presented as mean ± standard error of the mean (mean ± SEM). Data was analyzed using Student t-test for intra-group comparison. P value of less than 0.05 ($p<0.05$) was taken to be statistically significant.

RESULTS

Oligodendrocyte density

The study showed no significant difference in oligodendrocyte density at the third postnatal week when group 1 was compared with group 2 ($p=0.8319$), group 3 ($p=0.5617$) and group 4 ($p=0.4551$). The study also showed no significant difference in oligodendrocyte density when group 2 was compared with 3 ($p=0.5105$) and group 4 ($p=0.5966$). The difference between group 3 and 4 ($p=0.4551$) was not significant (Fig 1). However, regional analysis of oligodendrocyte density revealed a significant decrease in group 4 compared with group 1 in the upper cortical region ($p = 0.0122$). In the middle cortical region, an increase in the oligodendrocyte density was noted in group 3 when compared with other groups, however the increase was

only significant when compared with group 2 (p =0.0295) (Table 1).

The study showed no significant difference in oligodendrocyte density at the sixth week when group 1 was compared with group 2 (p=0.3270), group 3 (p=0.2053) and group 4 (p=0.7653). There was no significant difference when group 2 was compared with group 3 (p=0.3761) and group 4 (p=0.8531). Differences between groups 3 and 4 (p=0.4675) was also not significant. (Fig 2). However, regional analysis of oligodendrocyte density revealed a significant decrease in group 3 when compared with group 2 in the upper cortical region (p =0.0499) (Table 2).

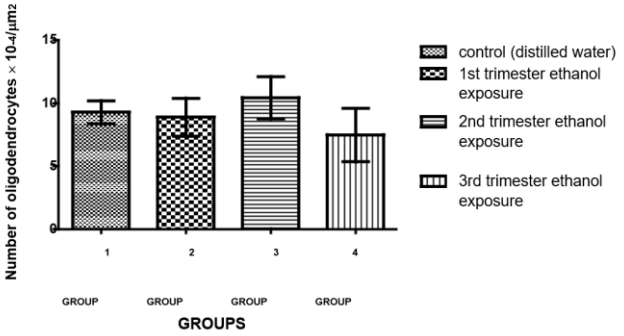

FIG 1. Bar chart showing the oligodendrocyte density across groups sacrificed at the third postnatal week. Value are expressed as mean ± SEM. Differences between groups not significant

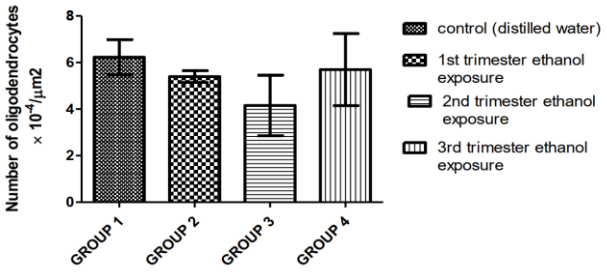

FIG 2. Bar chart showing the oligodendrocyte density across groups sacrificed at sixth postnatal week. Value are expressed as mean ± SEM. Differences between groups not significant.

TABLE I. Regional Density of Oligodendrocyte (/µm2) at the third postnatal week

GROUPS	UPPER CORTICAL REGION (×10⁻⁴)	MIDDLE CORTICAL REGION (×10⁻⁴)	LOWER CORTICAL REGION (×10⁻⁴)
GROUP 1	2.58±0.32	2.69±0.60	4.00±0.41
GROUP 2	2.51±0.62	2.26±0.40	4.11±0.64
GROUP 3	1.96±0.42	3.91±0.48$^{\beta}$	4.55±0.90
GROUP 4	0.85±0.43$^{\alpha}$	2.46±0.44	4.16±1.36

Values are expressed as mean ± SEM
– significantly different from group
1 β – significantly different from group 2
γ – significantly different from group 3

TABLE 2. Regional Density of Oligodendrocyte (/µm2) at the sixth postnatal week

GROUPS	UPPER CORTICAL REGION (×10⁻⁴)	MIDDLE CORTICAL REGION (×10⁻⁴)	LOWER CORTICAL REGION (×10⁻⁴)
GROUP 1	1.86±0.41	1.86±0.25	2.50±0.50
GROUP 2	1.71±0.29	1.48±0.32	2.20±0.28
GROUP 3	0.80±0.27$^{\beta}$	1.80±0.31	2.11±0.44
GROUP 4	1.51±0.57	1.75±0.51	2.43±0.67

Values are expressed as mean ± SEM
– significantly different from group
1 β – significantly different from group 2
γ – significantly different from group 3

White matter Thickness

After 3 post-natal weeks there was significant decrease in the thickness of the subcortical white matter of group 2 (p = 0.0232), group 3 (p=0.0346) and group 4 (p=0.0018) when compared with group 1. However, the study showed no significant difference in the subcortical white matter thickness at 3 post-natal weeks when group 2 was compared with group 3 (p=0.7265) and group 4 (p=0.0597). Significant reduction of the thickness of the subcortical white matter was noted in group 4 was compared with group 3 (p=0.0281) (Fig 3).

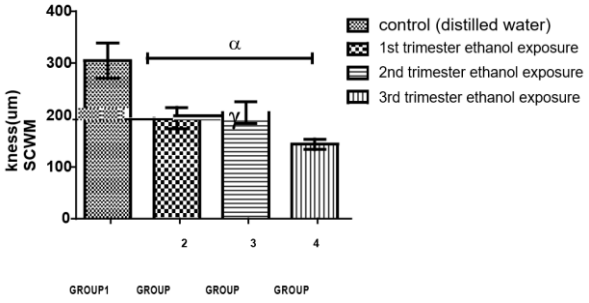

FIG 3. Bar chart showing the sub-cortical white matter (SCWM) thickness at the level of CA2, at 3 postnatal weeks in Wistar rats. Values are expressed as mean ± SEM

α- Significantly different from group 1
γ- Significantly different from group 3

There was no significant difference in the subcortical white matter thickness at 6 post-natal weeks when control (group1) was compared with group 2 (p = 0.7737), group 3 (p=0.5020) and group 4 (p=0.6004). Same pattern was noted at 6 weeks when group 2 was compared with group 3 (p=0.6376) and group 4 (p=0.7901) and when group 3 was compared with group 4 (p=0.7908) (Fig 4).

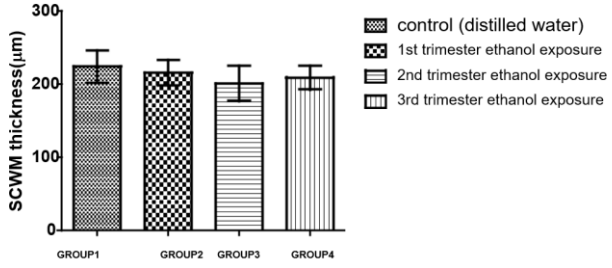

FIG 4. Bar chart showing the sub-cortical white matter thickness at the level of CA2, at 6 weeks after birth in Wistar rats. Values are expressed as mean ± SEM. Differences between groups not significant.

At the 3rd postnatal week, there was significant decrease in the corpus callosum thickness when group 2 (p = 0.0431), group 3 (p=0.023) and group 4 (p=0.0005) were compared with group 1. However, there was no significant difference in the corpus callosum thickness when group 2 was compared with group 3 (p=0.2514) and group 4 (p=0.2077). The difference in corpus callosum thickness between group 3 and group 4 was not statistically significant (p=0.9243) (Fig 5).

At the 6th postnatal week, the differences in the thickness of the corpus callosum between groups were not statistically significant (Fig 6).

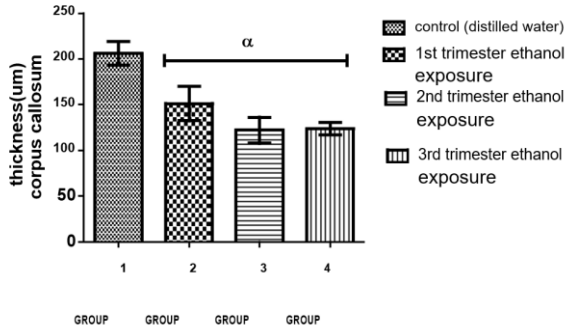

FIG 5. Bar chart showing the corpus callosum thickness at the midline, at 3 weeks postnatal in Wistar rats. Values are expressed as mean ± SEM. α- Significantly different from group 1

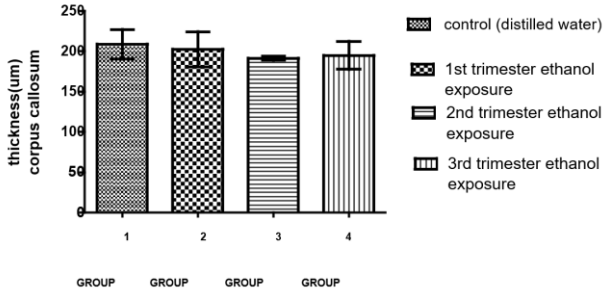

FIG 6. Bar chart showing the corpus callosum thickness at the midline at 6 weeks postnatal in Wistar rats. Values are expressed as mean ± SEM. Differences between groups not significant.

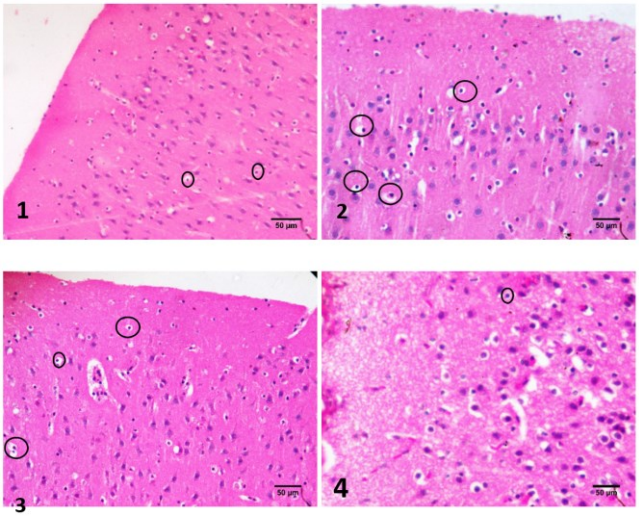

PLATE 1. Photomicrograph of the upper cortical region (consisting of layers I and II) subjected to H&E stain at 3 weeks (Groups 1 to 4- from upper to lower panel). Black circle - oligodendrocytes. Scale bars-50µm

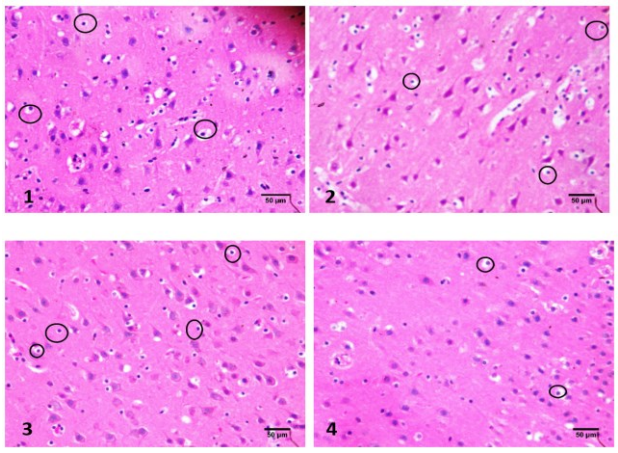

PLATE 2. Photomicrograph of the mid-cortical region (consisting of layers III and IV) subjected to H&E stain at 3 weeks (Groups 1 to 4- from upper to lower panel). Black circle - oligodendrocytes. Scale bars-50µm

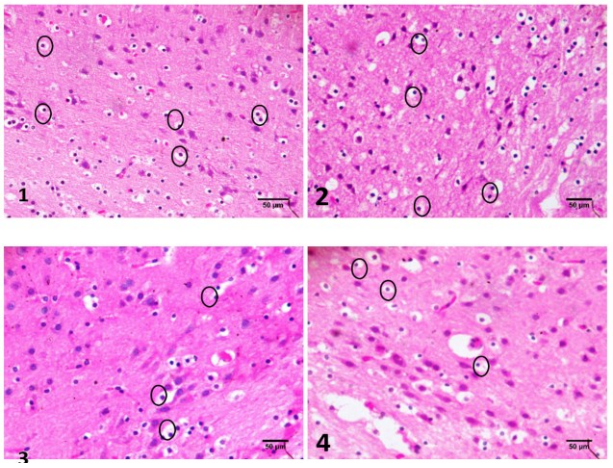

PLATE 3. Photomicrograph of the lower cortical region (consisting of layers V and VI) subjected to H&E stain at 3 weeks (Groups 1 to 4- from upper to lower panel). Black circle - oligodendrocytes. Scale bars-50µm

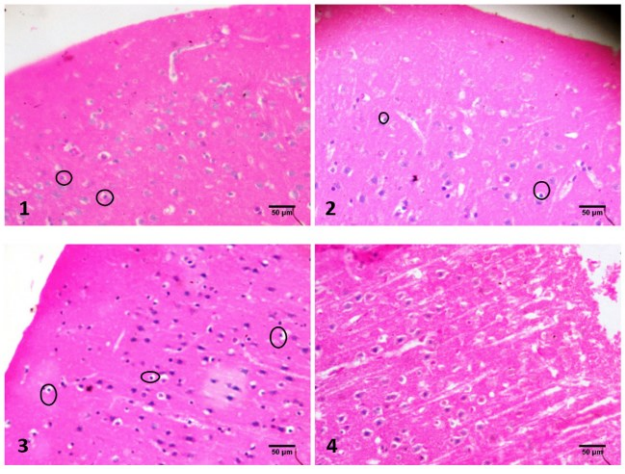

PLATE 4. Photomicrograph of the upper cortical region (consisting of layers I and II) subjected to H&E stain at 3 weeks (Groups 1 to 4- from upper to lower panel). Black circle - oligodendrocytes. Scale bars-50µm

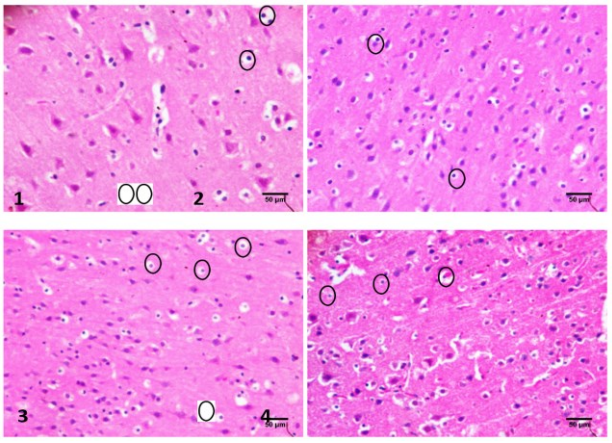

PLATE 5. Photomicrograph of the mid-cortical region (consisting of layers III and IV) subjected to H&E stain at 3 weeks (Groups 1 to 4- from upper to lower panel). Black circle - oligodendrocytes. Scale bars-50µm

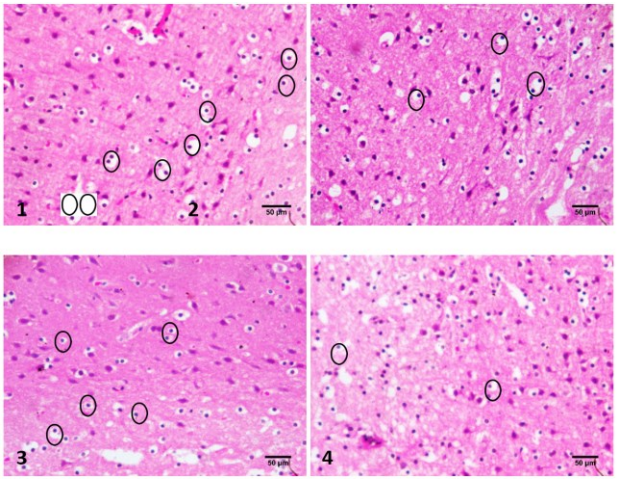

PLATE 6. Photomicrograph of the lower cortical region (consisting of layers V and VI) subjected to H&E stain at 3 weeks (Groups 1 to 4- from upper to lower panel). Black circle - oligodendrocytes. Scale bars-50µm

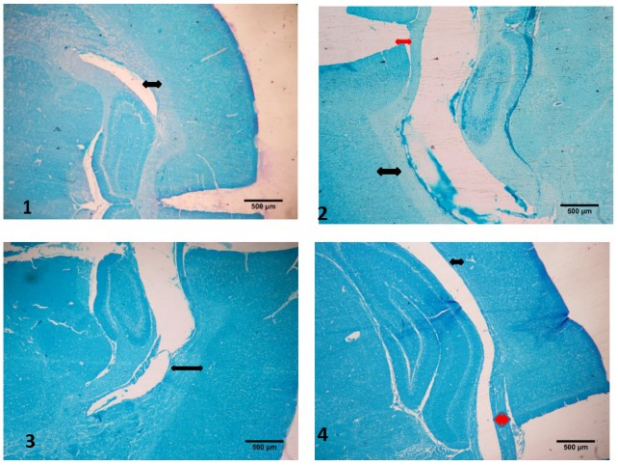

PLATE 7. Photomicrograph of the dorsolateral cortex of Wistar rats at 3 postnatal week showing the subcortical white matter (Black double head arrow) and the corpus callosum (red double head arrow) Scale bar; 500µm. Stain- Luxol fast blue.

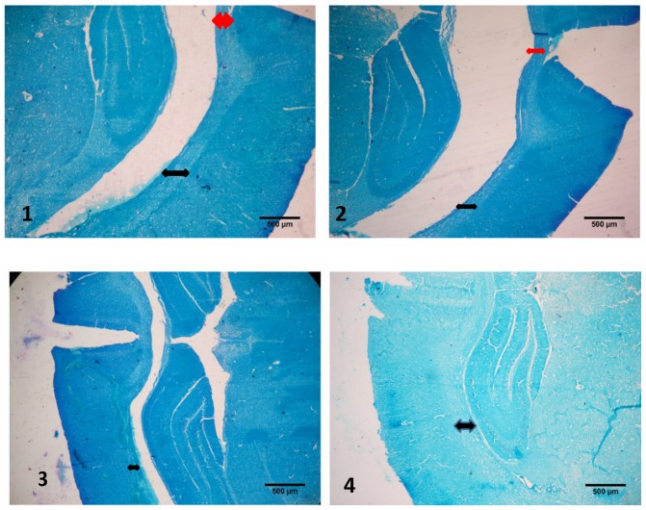

PLATE 8. Photomicrograph of the dorsolateral cortex of Wistar rats at 6 postnatal week showing the subcortical white matter (Black double head arrow) and the corpus callosum (red double head arrow) Scale bar; 500µm. Stain- Luxol fast blue.

DISCUSSION

The deleterious effect of continuous use of alcohol on myelin sheath of the central nervous system has been well established (Konrad *et al.,* 2012; Samantaray *et al.,* 2015) resulting in impairment of cognition and emotion, however there is need for more research effort around the state of white matter in prenatal alcohol exposure. The effect of specifically timed intrauterine alcohol exposure on the subcortical white matter and oligodendrocyte remains under-investigated. The density of cortical oligodendrocyte after first, second and third trimester intrauterine alcohol exposure did not differ significantly across the groups in Wistar rats by the 3rd and 6th post-natal weeks. However, regional cortical assessment revealed a significant reduction in the oligodendrocyte density in the rats exposed to alcohol in the 3rd trimester when compared with the control in the upper cortical region by the 3rd postnatal week, as well as a significant reduction in oligodendrocyte density in rats exposed to intrauterine alcohol in the 2nd trimester when compared with those exposed in the 1st trimester. In mice, the upper cortical layer does not contain as much oligodendrocyte as the deeper layers (Tan *et al.,* 2009) indicating that the response of cortical oligodendrocytes to perturbations such as intrauterine alcohol exposure may be different from one cortical layer to another.

This finding indicates the need for precision and specificity in brain research. While the density of oligodendrocyte in the whole cerebral cortex did not present any difference, layer by layer assessment showed some significant differences. The cerebral cortex consists of six layers characterized by specific mixture of cortical cells with unique densities. The findings on the density of oligodendrocyte in the upper cortical region showed that the oligodendrocytes in the different layers respond differently to insults such as intrauterine alcohol. The density of the oligodendrocytes after 3 and 6 postnatal week appeared to increase from the pial end to the ventricular end of the cerebral cortex. The exemption to this general picture is a sharp drop in the density of oligodendrocyte in the middle cortical region at 3 and 6 postnatal weeks in the rats that were exposed to alcohol in the first trimester of gestation. The consistency of such finding at 3 and 6 postnatal week indicated that intrauterine alcohol appeared to affect the mid-cortical oligodendrocyte adversely thereby reducing the density of oligodendrocyte. Another possibility is alcohol related cortical edema specifically in the mid-cortical region sparing the other regions. After 6 postnatal weeks, oligodendrocyte density across the whole cortex was not significantly disrupted (Table 2). However, there was a significant drop in oligodendrocyte density in the upper cortical region in the rats exposed to alcohol in the second trimester when compared with the control and those exposed in the first trimester. This finding is similar to the finding in the mid-cortical region after 3 postnatal weeks. Whatever morphological feature or chemical environment that made oligodendrocytes in these region vulnerable to intrauterine alcohol and or its metabolites at those specific times is worthy of research focus. This could also be possible as a result of the dose, pattern and timing of ethanol exposure.

In addition, significant increase in oligodendrocyte density in group 3 rats was noted when compared with those in group 2 in the mid-cortical region. While the global cortical picture showed no differences in oligodendrocyte density, the regional differences confirmed the differences in the consequences of intrauterine alcohol on oligodendrocyte based on the location. This is a pointer to the morphogenic property of the alcohol and or its metabolites in effecting the oligodendrocyte density changes in the dorsolateral cortex. The other possibility is that oligodendrocytes in the different parts of the dorsolateral cortex might have different properties thereby leading to differences in their response to teratogen. While the former is a proven property of teratogen, the latter hasn't been suggested by scientific literature. Ethanol has been documented to induce oligodendrocyte apoptosis in fetal macaque brain and cause a marked reduction of differentiated oligodendrocytes and its progenitors in the corpus callosum by the early postnatal life in mice (Creeley *et al.,* 2013). This finding partly confirms the regional differences in population and state of oligodendrocyte following prenatal alcohol exposure. Timed alcohol exposure appeared to provide more critical information on the effect of intrauterine alcohol exposure. The reduced oligodendrocyte density is in consonance with the findings of the present study in the upper cortical region after exposure at the 3rd trimester. This revealed that the effect of intrauterine alcohol exposure on cortical oligodendrocytes is dependent on the time of exposure and the specific region of interest. It is known that oligodendrocyte differentiation is largely post-natal, it is therefore imperative to assess whether prenatal alcohol effect

on oligodendrocyte precursor cells is what is reflected at the post-natal stage or that the effect of prenatal alcohol and or its metabolites is sustained long enough to affect the latter formed oligodendrocyte.

At the 6th postnatal week, the global picture of the cortical oligodendrocyte density remains the same as that of the 3rd week. However, a regional assessment of the oligodendrocyte density revealed a significant reduction in the oligodendrocyte density in the upper cortical region in rats exposed to alcohol at the 2nd trimester when compared with those exposed at the first trimester and control. This showed an interplay between the timing of exposure and specific area of the dorsolateral cortex as major determinant of the effect of intrauterine alcohol exposure on oligodendrocyte precursor cells and or oligodendrocytes. The consequences of intrauterine alcohol exposure are known to be long lasting (Guerri *et al.,* 2009) thereby making the oligodendrocytes that are differentiated postnatally to be susceptible to the direct effect of the alcohol and or its metabolites.

In addition to the differentiation of oligodendrocytes, myelination is also a postnatal event. Formation of white matter which is secondary to the process of myelination appeared to be adversely affected by the intrauterine alcohol exposure. A significant decrease of the subcortical white matter thickness was noted in the groups 2, 3 and 4 rats when compared with those of group 1 at the 3rd postnatal week. In addition, a significant reduction of the subcortical white matter was also noted in the group 4 rats when compared with group 3 (Fig. 3). While a reduction in thickness of subcortical white matter appeared to be a global consequence of prenatal alcohol exposure, the degree of white matter atrophy was dependent on the timing of the teratogenic insult.

Ventriculomegaly is a known effect of maternal alcohol consumption (Narendran *et al.*, 2013), such ventricular enlargement might result in a mechanical reduction of the contiguous subcortical white matter. Though ventricular size assessment is beyond the scope of this study, it might explain the reduction in the thickness of the subcortical white matter. However, the varying degree of the ventriculomegaly resulting in varying degree of subcortical white matter thickness should be further investigated to unravel the basis of such phenomenon. An impairment of the process of myelination may also be able to explain the significant reduction in white matter thickness. This is possible because of the known sustained effect of intrauterine exposure even during postnatal period. The significant presence of lipid in the myelin sheath may account for its vulnerability to some metabolites of alcohol which may result in oxidative stress. This is because oxidative stress has been implicated in lipid peroxidation process (Ayala *et al.,* 2014). The reduction in the thickness of subcortical white matter may be restricted to structural anomaly in which case functional integrity of the myelin sheath will be retained. However, a disruption of the functional integrity of the atrophied subcortical white matter will result in a slower and more energy consuming conduction of electrical impulse.

At 6 postnatal week there was no significant difference in the subcortical white matter thickness when groups 2, 3 and 4 were compared with group 1. This is suggestive of a transient nature of the effect of intrauterine alcohol on the subcortical white matter. This is not in tandem with the widely held view that the effect of intrauterine alcohol on white matter is irreversible.

There was significant reduction in the corpus callosum thickness at the third week postnatal life when the experimental group was compared with control which became reversible at the sixth postnatal week. Reduction in corpus callosum volume is a consequence of PAE (Astley *et al.,* 2009).

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Nothing declared

CONTRIBUTORS

We confirm that this manuscript contains original work that has not been previously published and is not submitted for publication else-where. All authors have agreed to the content and submission and have no conflicts of interest.

CONFLICT OF INTEREST

No conflict declared

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