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Cortical Neurons and Astrocytes in Intrauterine Ethanol Exposure: The Role of Exposure Time and Postnatal Timeline

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ABSTRACT

Background: The state of cortical neurons and astrocytes are pointers to the health of the brain. These cells are morphologically distorted by alcohol exposure. Intrauterine alcohol exposure remains a challenge with perinatal consequences. The role of exposure time and postnatal timeline on the degree of cortical cell derangement remains a subject of controversy till date. This study therefore examines alcohol exposure and postnatal changes on brain weight, cortical neurons and astrocytes at different developmental periods.

Methods: Twenty mature female Wistar rats were time-mated and grouped into 4 groups. 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. Rats produced litters and pups' brains were harvested and processed for H&E and Golgi Cox stains at the 3rd and 6th postnatal week. Neuronal and astrocytic densities in the cerebral cortex were evaluated.

Results and Conclusion: There was statistically significant increase ($p<0.05$) in the density of degenerating neurons at the third postnatal week and sixth postnatal weeks in the experimental groups when compared with the control. There was also statistically significant increase ($p<0.05$) in astrocytic density in groups 2 and 4 at the 3rd postnatal week. There was no statistically significant difference ($p>0.05$) in the astrocytic densities across groups at the sixth postnatal week. It was concluded that intrauterine alcohol exposure in any of the developmental periods resulted in postnatal neuronal degeneration which persisted till the 6th week. However, increased astrocytic densities is a feature of 1st and 3rd trimester alcohol exposure noted in the 3rd but absent in the 6th postnatal week.

Keywords: Intrauterine, Ethanol, Astrocytic densities, Neuronal degeneration

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INTRODUCTION

Alcohol consumption during pregnancy can interfere with both embryonic and fetal development, giving rise to a wide range of cellular and structural anomalies (1) resulting in a wide range of cognitive defects (2). Fetal alcohol spectrum disorders (FASD) neuropathology includes reduction in the volume of the corpus callosum, cerebral cortex, cerebellum, hippocampus, basal ganglia, amygdala, and thalamus (3). Post-mortem and magnetic resonance imaging (MRI) studies have demonstrated ventriculomegaly, neuronal atrophy and shrinkage of frontal cortex, cerebellum and white matter following alcohol exposure (4,5).

The developing brain is vulnerable to ethanol toxicity, and prenatal alcohol exposure causes alcohol-related neurodevelopmental birth defects, which manifest as long-lasting structural and functional brain abnormalities including reduced cortical gyration and brain size (6,2,7). A marked reduction of the surface area of the cingulate cortex have been reported in adolescents following heavy prenatal alcohol exposure (8), demonstrating the long-term effect of such exposure. It has been reported that ethanol affects every step of brain development such as proliferation, differentiation, and migration of neural cells (2). Fetal alcohol exposure is known to cause neuro-inflammation and or neurodegeneration which are suspected to result in several anomalies (9), spanning the various developmental stages of brain formation (10). Such ethanol-induced neurodegeneration appears to be linked with glial activation and neuro-inflammation (11). Considering the long-lasting damage to the developing brain triggered by prenatal ethanol exposure and the close relationship between ethanol consumption and glial activation/ neuro-inflammation, it can be deduced that glial activation may be linked to neurodegeneration and play an important role in the structural and cellular consequences of FASD (12).

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Prenatal alcohol exposure is known to result in disruption of cortical neuronal migration through inhibition of radial glia development alongside attenuated expression of PAX 6 in the developing rat brain (13,14,15). It also causes significant loss of pyramidal neurons in CA1 and CA3 and granule neurons in the dentate gyrus (16) as well as reduction in the size and population of neurons in all regions of the hippocampus (17).

Acute astrogliosis has been demonstrated in the cerebral cortex following binge-like alcohol exposure in neonatal rats (18,19). Additionally, premature transformation of radial glia was demonstrated after prenatal exposure to alcohol (20). Low-dose, chronic prenatal alcohol exposure produced astroglial hypertrophy and increased GFAP and S-100β immunoreactivity in the prefrontal cortex which lasted more than three weeks after birth (21). Persistent neocortical astrogliosis was observed in adult rats following prenatal alcohol exposure (22). Ethanol-induced neurodegeneration has been associated with glial activation (23) and elevation of pro- and anti-inflammatory cytokines (24) resulting in long-lasting cellular, structural and functional deficits (25).

In spite of the studies on the effect of fetal alcohol exposure, the postnatal consequences of this seemingly common condition on cortical neurons and astrocytes is yet to receive deserving research attention. This study therefore is aimed at unravelling the state of cortical neurons and astrocytes following fetal alcohol exposure in the first, second and third trimester in Wistar rats.

MATERIALS AND METHOD

Ethical clearance was obtained from the Health Research and Ethics Committee of the Institute of Public Health, Obafemi Awolowo Ile-Ife (HREC, IPH OAU) with clearance number **IPHOAU/12/1023**. The animals received humane care according to the guidelines of Institutional Animal Care and Use Committee (IACUC).

Animal preparation and groupings

Wistar rats (*Rattus novergicus*) were bred in the animal holding of the Department of Anatomy and Cell Biology, Obafemi Awolowo

University to monitor their developmental history. Twenty pro-estrous mature female Wistar rats were time-mated and used for this study. The presence of mucous copulatory plug indicated day 0 of gestation. The rats were randomly assigned into 4 groups (n=5). Rats were housed in clean plastic cages in a clean environment at room temperature. Rats in all groups were fed with standard laboratory rat chow and allowed access to water *ad libitum*. Group 1 (Control) rats administered distilled water (2 mL/kg) while Groups 2, 3 and 4 rats were administered orally 2.5 mL/kg of 20% ethanol once on gestational days 4, 11 and 18 respectively. Days 4, 11 and 18 corresponds to the first, second and third trimester of pregnancy as reported by (26,27) Pregnant Wistar rats were allowed to produce litters and nurse their pups. At the end of the 3rd and 6th postnatal week, rats were sacrificed by cervical dislocation.

Brain Harvest and Histology

Following sacrifice, the skull was quickly and carefully opened to avoid brain tissue damage. The brain was excised using bone forceps, blotted dry, weighed and fixed in 10% neutral buffered formalin. A coronal section of 1mm thickness brain slice was obtained at the level of the optic chiasma and processed for routine paraffin embedding. Routine Hematoxylin and eosin (H&E) staining was used to demonstrate the general architecture of neurons while Golgi-Cox staining technique was used to demonstrate astrocytes. The Golgi stained slides were prepared by immersing sections in already prepared Golgi-cox solution and allowed to impregnate in complete darkness for 10 days. The slides were further transferred into 1% potassium dichromate. After drying in a humid chamber, the sections were transferred into ammonium hydroxide in distilled water for 30 minutes, followed by a 5-minute rinse in tap water. The slides were dehydrated by immersing in ascending grades (50%, 70%, 90%, absolute I and absolute II) of alcohol solutions, cleared in xylene, coverslipped in Dystrene plasticizer xylene (DPX) mountant.

Photomicrography and Image Analysis

Stained sections were viewed under a Leica DM750 microscope interfaced with Leica ICC50 camera and digital photomicrographs were taken and archived**.** Image Analysis and Processing for Java (Image J) software, was used to analyze the neuronal and astrocytic density of each layer of the dorsolateral cortex with the aid of Image J cell counter tool. Archived images were imported on to the Image J software for cellular count and density determination with a superimposed grid of area 10,000μm^2 on the photomicrographs obtained from the upper, middle and lower cortical regions. The superior and inferior landmarks were the pial surface and the subcortical white matter respectively. The upper, middle and lower cortical regions are layers I&II, III&IV, V&VI of the cortex respectively. The superior land mark being the pial surface and inferior landmark the white matter below the cortical region (subcortical white matter). Taking this into consideration the upper cortical region is the molecular layer lying under the pial surface and external granular layer, the middle is the external pyramidal and internal granular, while the lower cortical region is the internal pyramidal layer and the multiform layer. These regions were identified under the microscope and are represented in the figures.

Statistics

Graph pad prism software (version 6.1) was used to analyze data from the study. 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 less than 0.05 ($p<0.05$) was taken to be statistically significant.

Results

Brain Weight

At the third postnatal week, there was no significant difference in brain weight when group 1 was compared with group 2 ($p = 0.0783$). However, there was significant decrease in the brain weight when group 1 was compared with groups 3 ($p=0.0165$) and 4 ($p=0.0005$). There was no significant difference in brain weight at 3 weeks when group 2 was compared with group 3 ($p=0.2309$). However, there was significant decrease when group 2 was compared with group 4 ($p=0.0056$). Differences between groups 3 and 4 was however not significant ($p=0.1467$) (Fig 1a).

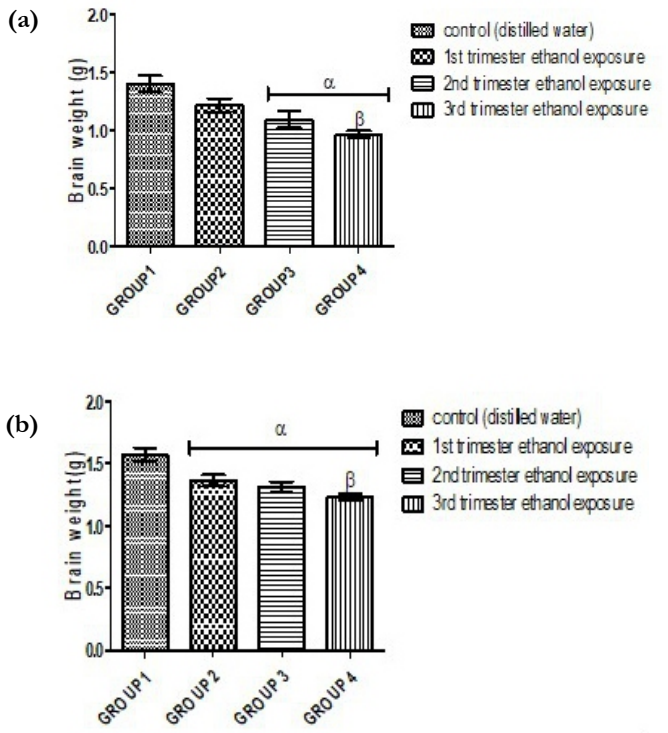


Figure 1: Bar chart showing the effect of prenatal ethanol exposure on brain weight at 3 weeks (a) and 6 weeks (b) postnatally in Wistar rats. Values are expressed as mean ± SEM α- significantly different from group 1, β- significantly different from group 2.

Histological and Histomorphometric assessment

At 3 weeks (Fig. 2a), the result revealed that group 4 showed significant decrease in the neuronal density of normal cortical neurons when compared with group 1 ($p=0.0144$). However, there was no significant difference in the neuronal density of normal neurons when group 1 was compared with group 2 ($p=0.1355$) and group 3 ($p=0.0548$). No significant difference was also observed in the neuronal density of normal neurons when group 2 was compared with 3 ($p=0.6448$) and group 4 ($p=0.2079$), likewise when group 3 was compared with group 4 ($p=0.3621$).

However, the density of neurons showing degenerative features (Fig. 2b) was significantly increased in group 4 ($p<0.0001$) when compared with group 1. There was also significant increase in neurons showing degenerative features when group 1 was compared with group 2 ($p=0.0003$) and group 3 ($p=0.0006$). No significant difference was observed in neurons showing degenerative features when group 2 was compared with group 3 (0.4925), but there was significant increase in neurons showing degenerative features when group 2 was compared with group 4 ($p=0.0012$), there was also significant increase in neurons showing degenerative features when group 3 was compared with group 4 ($p=0.0005$).

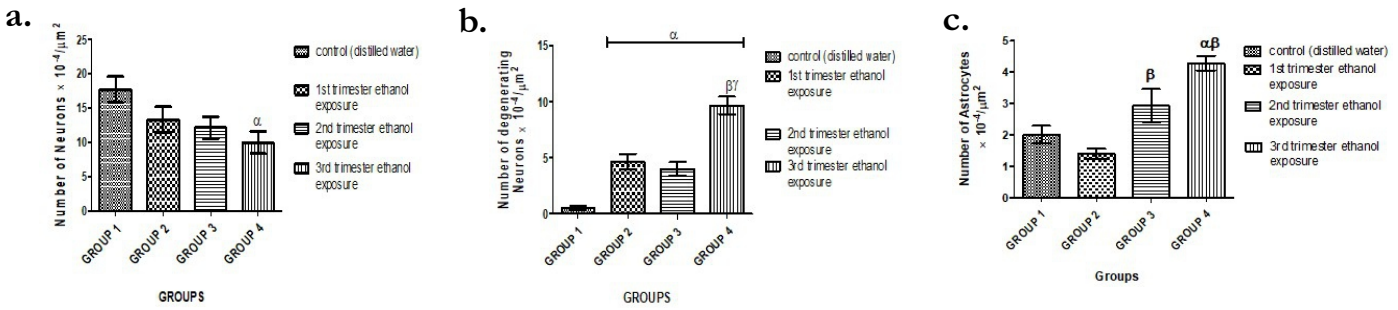


Figure 2: Bar chart showing the effects of prenatal ethanol exposure on cortical normal neuronal density (a), degenerating neuronal density (b) and cortical astrocytic density (c) at 3 weeks. Values are expressed as mean ± SEM. α- significantly different from group 1, β- significantly different from group 2, γ- significantly different from group 3.

This study showed no significant increase in astrocytic density (Fig. 2c) when group 1 was compared with group 2 (p=0.0994) and group 3 (p=0.1593). Although, significant difference exists when group 1 was compared with group 4 (p=0.0003) and also when group 2 was compared with group 3 (p=0.0236) and group 4 (p<0.0001).

At 6 weeks, results from this study revealed that group 2 (p=0.0003), group 3 (p<0.0001) and group 4 (p=0.0006) showed significant decrease (Fig. 3a) in the density of normal neurons present in the cerebral cortex when compared with group 1. However, there was no significant difference in the density of normal neurons when group 2 was compared with group 3 (p=0.1820), and group 4 (p=0.4149). No significant difference was also observed when group 3 was compared with group 4 (p=0.9872).

However, the density of neurons showing degenerative features was significantly increased (Fig. 3b) when group 1 was compared with group 2 (p=0.0006), group 3 (p=0.0005) and group 4 (p=0.0001). No significant difference was observed when group 2 was compared with group 3 (p=0.4364) and group 4 (p=0.7381), there was also no significant difference when group 3 was compared with group 4 (p=0.5492).

This study showed no significant difference in astrocytic density (Fig. 3c) when group 1 was compared with groups 2 (p=0.4303), group 3 (p=0.3019) and group 4 (p=0.8952) and when group 2 was compared with group 3 (p=0.7434) and group 4 (p=0.3736).

DISCUSSION

This study was aimed at evaluating the histomorphology of cortical neurons and astrocytes following alcohol exposure at different developmental period of alcohol exposure (1st, 2nd and 3rd trimester) in Wistar rats with focus also on the postnatal consequences at the third and sixth postnatal weeks. A significant decrease in brain weight at the third week in groups 3 and 4 when compared with the control group which persisted after 6 postnatal weeks across the 3 experimental groups when compared with the control indicated a perturbation of the cortical components to a degree that was not reversible within the first 6 postnatal weeks. While the reduction in brain weight in the group 2 rats compared with the control was not significant at the end of the 3rd week, the reduction became significant at the end of the 6th postnatal week. This indicated that the anomalies occasioned by intrauterine alcohol exposure in Wistar rats have postnatal consequences. The developing nervous system is known to respond to the same neurotoxic insult at different degrees depending on the time of exposure, making the time of exposure a critical factor in determining the morphological consequences of intrauterine alcohol exposure (28). Ethanol metabolism is a function of alcohol dehydrogenase (ADH) enzyme which is a cytosolic enzyme in a reversible reaction into acetaldehyde and which is also further converted to acetate through the action of acetaldehyde dehydrogenase (ALDH). Acetaldehyde, a metabolite of ethanol is known to be toxic, and can accumulate in the fetal brain after prenatal alcohol exposure (29). The delayed effect of intrauterine alcohol exposure in the group 2 rats might be due to the effect of the accumulated metabolites of alcohol. However, the restriction of such delay to the rats exposed to alcohol in the first semester alone revealed a differential handling of intrauterine alcohol and or its metabolites across the different periods of pregnancy. Accumulation of alcohol metabolites is expected to cause inflammation which results in some form of fluid movement into the brain tissue (edema) resulting in an increase in brain volume and weight. However, in this study, a persistent reduction in brain weight as time of alcohol exposure increased did not point to an inflammatory process as a means of explaining the brain weight loss. Since the early period of development is characterized by cortical cell proliferation, while maturation and migration are latter events, the precise process that is susceptible to the toxic effect of alcohol and or its metabolites should be a focus of subsequent research work. Results from this study on brain weight is also in tandem with studies attributing alcohol-related neurodevelopmental disorders to reduction in the overall size of the brain (6).

The state of neurons in the cortex of Wistar rats might shed some light on the brain weight across the groups. that group 4 rats had significantly less normal neuronal density in the cerebral cortex

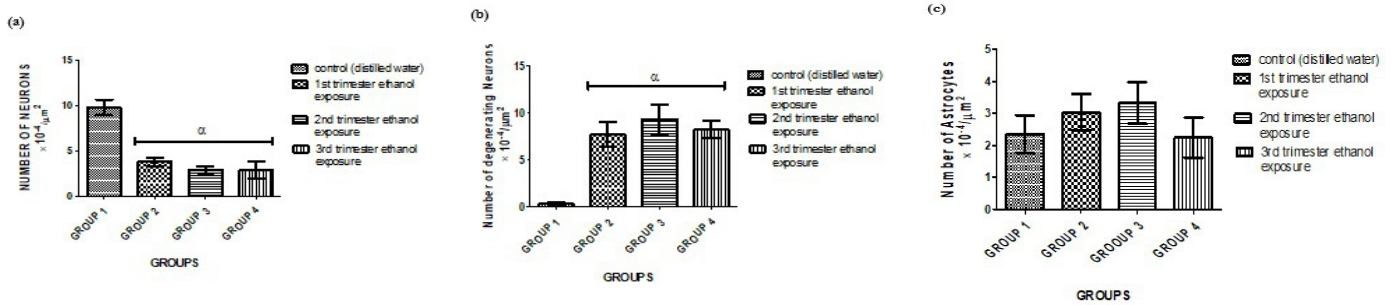


Figure 3: Bar chart showing the effects of prenatal ethanol exposure on cortical normal neuronal density (a), degenerating neuronal density (b) and cortical astrocytic density (c) at 6 weeks. Value are expressed as mean ± SEM. α- significantly different from group 1.

when compared with group 1 after the 3rd postnatal week while the difference between the control rats (Group 1) and the experimental rats (Groups 2, 3 and 4) in the density of normal neurons in the cerebral cortex became significant by the 6th postnatal week across the groups. This delayed effect of intrauterine alcohol exposure on the morphological state of the neurons is in tandem with the brain weight picture discussed above. While the effect of alcohol may not be inflammatory, the delayed effect points to the fact that the metabolites of alcohol are probably as (or more) dangerous as alcohol itself on the developing cerebral cortex when encountered in the intrauterine period. In this study widespread neuronal degenerating features such as pyknotic, eosinophilic and karryohectic neurons were observed. Some of the known mechanisms of perturbation by intrauterine ethanol are apoptosis and oxidative stress. Ethanol intake is known to elevate reactive oxygen species (ROS), a situation that is detrimental to the health of neurons (30) in either the developing or developed brain. Factors that induce apoptosis in central nervous system (CNS) cells include vasoconstriction, mitochondrial dysfunction and disruption of calcium homeostasis (31). Generation of ROS and or apoptosis are processes that might take some time to manifest which might explain delayed effect noted in this study. However, the determination of the exact trigger of the oxidative stress and or apoptosis between alcohol and or its metabolites will help to further explain the effect of intrauterine alcohol exposure in this study. A possible mechanism for the marked delayed neuronal density in this study might be a disruption of calcium homeostasis which is a known consequence of mitochondrial membrane derangement resulting in neuronal degeneration (32). It is however intriguing to note that neuronal degeneration was widespread as shown by the significant increase in the density of the degenerating neurons in the experimental groups compared with the control at the 3rd and 6th postnatal weeks. The implication of this finding is that the factors responsible for neuronal degeneration might be different from those that impede neuronal formation both in mechanism and time of action. While the effect of intrauterine alcohol was seen early on density of degenerating neurons, it was delayed on the density of normal neuron. In addition, while the neuronal degeneration was noted in the group 4 rats at the 3rd postnatal week, it was only by the 6th postnatal week that it was noted in the groups 2 and 3 rats. Since development of the nervous system is an early event, such differential effect of intrauterine alcohol exposure based on the period of exposure is in tandem with the general principles of teratology. This indicate a possible time-dependent expression of chemical components and or environmental factors that will facilitate the neuronal degeneration. There is a possibility of such chemical and or environmental condition being optimized in the latter phase of intrauterine life or early postnatal life.

In contrast to the widely held view that early intrauterine alcohol exposure in pregnant women tend to have more deleterious effect than late exposure (33), this study showed that 3rd trimester exposure to alcohol resulted in neuronal degeneration earlier than the 1st and 2nd trimester exposure. This shows the need for a possible review of the workings of alcohol as a teratogen and further studies on its teratogenic effects should be undertaken. The progressive neurodegenerating effects of intrauterine alcohol up till the 6th postnatal week is in consonance with the view that neuro-inflammation in animal models of FASD results in long-lasting developmental consequences in the brain (34,35). However, whether or not an inflammatory process precedes the widespread neurodegeneration and whether this can help explain the delayed neurodegeneration in groups 2 and 3 rats will be addressed in a further study. The early neurodegeneration observed in the group 4 rats might be as a result of earlier documented increased synaptogenesis in the 3rd trimester thereby making the neurons vulnerable to toxins such as alcohol (36).

The supporting role of astrocyte in the health and function of neuron has been documented (37, 38). In this study, the astrocytic densities in groups 3 and 4 was significantly increased when compared with the control group at the 3rd postnatal week. The densities of astrocyte were however, not significantly altered across the different groups by the 6th postnatal week. Since the 3rd postnatal week also witnessed intense neurodegeneration, this study therefore corroborated the possible link between the health of neurons and the presence and activation of astrocyte. Though the astrogliosis was limited to the 3rd postnatal week, neuro-degeneration continued into the 6th postnatal week. This showed that, even if increase in astrocyte density is causal to the neuro-degeneration, it cannot explain the neurodegeneration of the 6th postnatal week. This means nthat astrocyte might be an initiator of the process of neurodegeneration or that there are other neurodegenerating factors beyond the 3rd postnatal week. Astrocytes are known to get activated following brain pathology; therefore, the intrauterine alcohol-induced neurodegeneration might be the required trigger for the astrogliosis noted by the 3rd postnatal week, however, the trigger does not appear to last for a long time because of the resolution of the astrogliosis by the

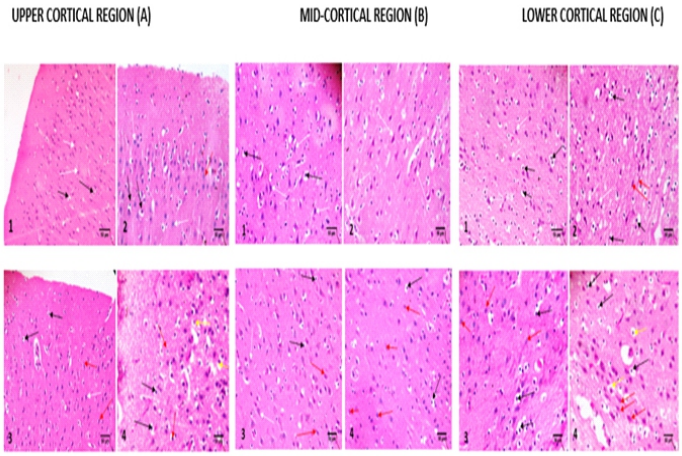


PLATE 1: Representative light photomicrographs of the upper, mid and lower cortical regions {A, B and C} subjected to H&E stain at 3 weeks. Upper to lower panel {Groups 1-4}. Neurons with preserved morphology are represented with white arrows, while oligodendrocytes are represented with black arrow. Neurons exhibiting degenerating features {red arrow- points to pyknotic neuron, yellow arrow-intensely stained eosinophilic neuron}. Scale bar: *50 μm.*

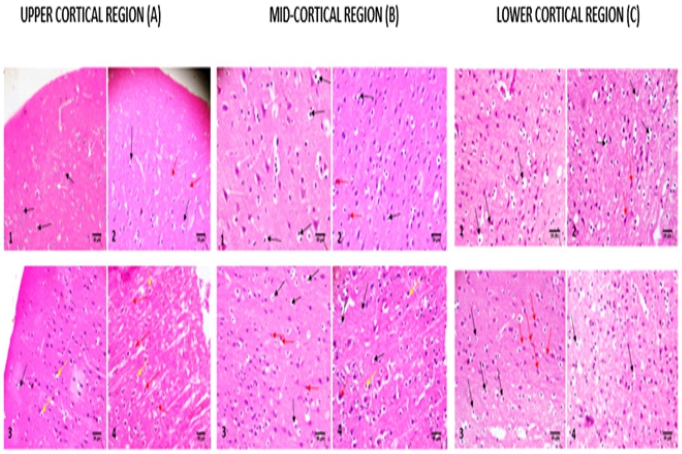


PLATE 2: Representative light photomicrographs of the upper, mid and lower cortical regions {A, B, and C} of rats subjected to H&E stain at 6 weeks. Upper to lower panel {Groups 1-4}. Neurons with preserved morphology are represented with white arrows, while oligodendrocytes are represented with black arrow. Neurons exhibiting degenerating features {red arrow- points to pyknotic neuron, yellow arrow-intensely stained eosinophilic neuron}. Scale bar: *50 μm.*

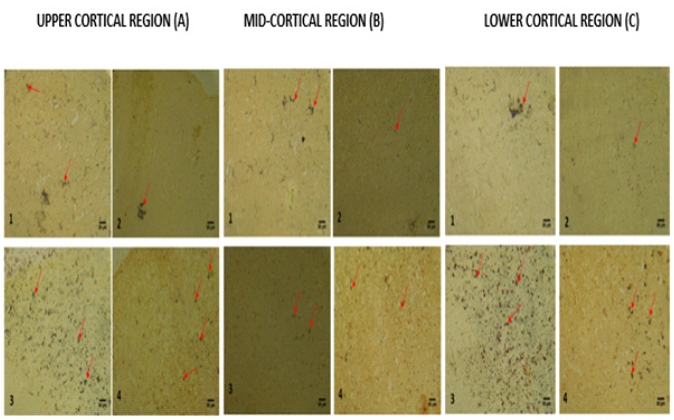


PLATE 3: Representative light photomicrograph of the upper, mid and lower cortical region {A, B, and C} of rats subjected to Golgi stain at 3 weeks post-natal life shows astrocytes with identical morphological pattern. Groups 1-4 {upper to lower panel}. Astrocytes {red arrow}. Scale bar: **50 µm**.

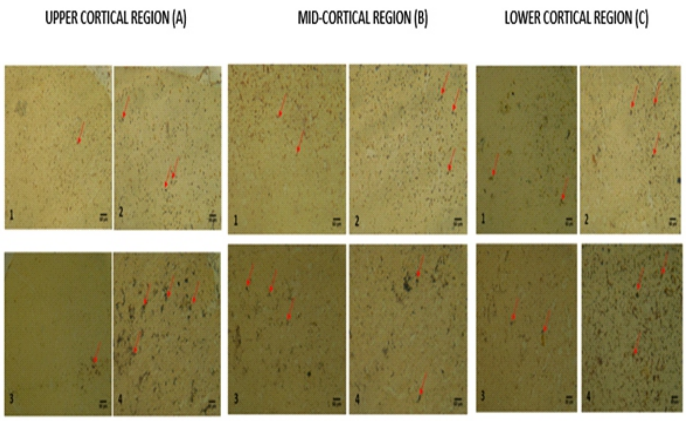


PLATE 4: Representative light photomicrograph of the upper, mid and lower cortical region {A, B and C} of rats subjected to Golgi stain at 6 weeks post-natal life shows astrocytes with identical morphological pattern. Groups 1-4 {upper to lower panel}. Astrocytes {red arrow}. Scale bar: **50 µm**.

6th postnatal week. Astrocyte activation therefore appear to be a feature of the acute phase of alcohol-induced neurodegenerative process. While persistent neocortical astrogliosis was reported in adult rats following prenatal alcohol exposure (22), transient cortical astrogliosis was reportedly induced by ethanol exposure during the neonatal growth spurt in rats (18). This study showed that the intrauterine alcohol-induced astrogliosis occur within a short period of postnatal life.

Conclusion

The study concluded that intrauterine alcohol exposure in any of the trimesters resulted in postnatal neuronal degeneration at both the third and sixth weeks' post-natal life. However, increased density of astrocytes is a feature of the1st and 3rd trimester alcohol exposure in the 3rd but not in the 6th postnatal week.

Conflict of Interest

Authors declare no conflict of interest

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