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Stereological Effects of Alcohol on Fetal Brain in Experimental Rat Model

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ABSTRACT: Heavy alcohol consumption has been associated with brain atrophy, neuronal loss, and poorer white matter fiber integrity. However, there is inadequate data on the stereological effects of alcohol on the fetal brain. Thus the objective of the present study was to perform histological and stereological examination of alcohol induced changes in the brain of albino rats. This study included 30 female albino rats weighing between 200 to 230gm from a pure colony. The rats were divided into two main groups: alcohol treated (n=27) and control (n=3) groups. The alcohol treated group was further subdividing into low, medium and high dose alcohol groups. Each of this three groups were further grouped in to three based on the trimesters summing up to nine alcohol treated groups of three animals each. Alcohol was administered at a dose of 2g/kgbwt, 3.5 g/kgbwt and 5 g/kgbwt for the low, medium and high alcohol group respectively for a period of 21, 14 and 7days. The control group received deionized water for the same time period. At the end of the experiment, their fetal brains were harvested, weighed, fixed in 10% formaldehydeand processed for histological and stereological analysis. The modified Cavalieri method was used for stereological measurement. Data was collected using structured datasheets and photomicrographs and was then analyzed using STEPnizer software and SPSS version 23 where One-way Analysis of Variance (ANOVA), followed by Tukey's post hoc multiple comparison tests. The results were expressed as mean ± standard error of the mean (SEM) and the findings were then presented in form of tables. The statistical comparison of the groups revealed that the ethanol group had a significant reduction in the brain weight and volume compared to the control group ($p < 0.017$, $p < 0.022$, respectively). In conclusion, our findings indicate that alcohol has toxic effects that promotes a reduction in fetal brain weight and volume.

KEYWORDS: Animal model, ethanol, brain, stereology

I. INTRODUCTION

Alcohol (ethanol) is a known teratogen that has been shown to perturb the normal development of the fetal brain among other organs (Treit et al., 2016). At the same time, maternal alcohol consumption is gradually increasing globally and alcohol-related complications pose a serious public health threat more so when exposed during the fetal (Alhowail, 2022; Gautam et al., 2014; Lees et al., 2020).Depending on the quantity and duration of maternal alcohol ingestion, various fetal organs, tissues, and systems are injured (Lees et al., 2020). Evidence from experimental animal and human studies has shown the susceptibility of the brain to alcohol effects and that exposure to alcohol during brain development can cause irreversible defects in fetal brain cytoarchitecture(Donald et al., 2015).

Alcohol is a well-known brain toxin that affects the central nervous system in various ways (Caputo et al., 2016). In addition to its direct toxic effects on neurons, it may injure the fetal brain through its metabolic products (acetaldehyde, etc.), nutritional deficits secondary to heavy maternal alcohol intake, and hepatic disorders (Caputo et al., 2016; Pervin & Stephen, 2021). The occurrence mechanism of alcohol-induced neuropathology has not been fully explained. Generally, it is accepted that the widespread pathway involved in neuronal injury is mitochondrial injury as well as imbalance of extracellular and intracellular-free radical homeostasis (Chung et al., 2021; Michaelis & Michaelis, 1994). Some parts of the brain appear to be more sensitive to alcohol's metabolic products than others (Kaminen-Ahola, 2020). Alcohol injurious effects on brain are generally in different forms that may include changes in brain size, volume, loss in the white and gray matter architecture among others (Chung et al., 2021; Kaminen-Ahola, 2020; Lees et al., 2020; Treit et

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al., 2016). These alterations have been observed in different parts of the brain like the frontal lobes, parietal and medial temporal cortexes, cerebellum, and subcortical/thalamus, pons regions (Caputo et al., 2016).

Prenatal alcohol consumption is also associated with changes in brain connectivity due to the injurious effects on the brain white and grey matter (Kaminen-Ahola, 2020; Michaelis & Michaelis, 1994). Further, neuroimaging studies have shown that chronic prenatal alcohol consumption (3 or more drinks for women on any day) is associated with widespread patterns of macrostructural and microstructural changes of the prefrontal cortex, insula, superior temporal gyrus, striatum, thalamus, and hippocampus compared to healthy controls (Caputo et al., 2016; Olson, 1994). Neuroimaging studies have shown white matter degeneration of the corpus callosum in animal studies (Gautam et al., 2014; Long & Lebel, 2022). However, the effects alcohol on white matter microstructure, as evidenced by decreased fractional anisotropy and increased mean diffusivity are not limited to the corpus callosum but are also seen in the internal and external capsules, fornix, frontal forceps and cingulate gyri in dose dependent manner (Long & Lebel, 2022; Wang et al., 2020). Although alcohol consumption can produce global and regional brain changes, data on the stereological effects of alcohol on the developing fetal brain is lacking. Thus, this study was aimed at performing stereological examination of the changes in the fetal brain at different gestational periods.

II. EXPERIMENTATION

Our study was approved by the animal ethical and research committee of JKUAT (2019, ref no JKU/ 2/4/896A). It included a total of 30 female Albino rats aged 60 days and weighing 200 to 230gm. The rats were divided into two groups: alcohol (n=27) and control (n=3) groups. The alcohol group was administered alcohol via gavage needle at varying doses of 2g/kgbwt, 3.5 g/kgbwt and 5 g/kgbwt diluted in deionizes water for 21, 14 and 7 days for trimester I, II and III respectively. The control group received deionized water for the same time period. The rats were kept in a cage with normal light–dark cycle (12-h/12-h) at an ambient temperature of 21 + 2C and were fed standard feed ad libitum. The animals were humanly sacrificed on the 21st day of gestation and a total of 90 fetuses chosen through systematic random sampling had their skull opened, brain harvested, weighed and brain width and length determined. This was followed by fixation of the fetal brains in 10% formaldehyde over 24 hours. The brains were processed for histological and stereological analysis. All experimental protocols conducted on the laboratory animals were performed in accordance with the guidelines for the care of laboratory animals.

Histological staining

The fetal brains were dehydrated in graded concentrations of ethanol, cleared with xylene, infiltrated with paraplast wax for 12 hours and embedded in paraffin wax. For light microscopy, 5um longitudinal sections were obtained using Leitz sledge. The sections were then stained with hematoxylin and eosin and viewed under light microscope (Optika microscope, Italy) and photographed.

Stereological analysis

To estimate the total brain volume of the fetuses, archimedes principle (Water emersion method) was applied by immersing the whole fetal brain into a graduated beakers containing normal saline and the fluid displacement was determined (Mohazzab, 2017). The fluid that was displaced by the fetal brains represented the actual brain volume which were used as the reference volumes for this study (Mohazzab, 2017; Musa et al., 2021). This was followed by immersing the brain tissue in 10% formalin for fixation over 24 hours. The fetal brain volume determined via this method was compared to the fetal brain volume determined via the cavalieri method by determination of the mean and standard error of mean (Mean ± SEM) of the measurements. After the processing of the brains, systematic random sampling was utilized to obtain quantitative and unbiased information. To estimate the total brain volume for each fetus, between 20-25 sections of 5um were sampled from each longitudinal brain section, through systematic uniform random sampling (Mohazzab, 2017; Musa et al., 2021). The stained brain tissue was mounted on the BH2-Olympus light microscope in the Department of Human Anatomy. Digital images were taken for all fields of view in the sampled sections at a magnification of x4. The brain volume was determined by combining the Cavalieri method of segmentation with point-counting on evenly spaced organ slices (Mohazzab, 2017) by applying the Cavalieri formula below:-

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$$_{est}V = \frac{\sum_{i-1}^{m} P.\ a/\ p.\ ts}{M^2}$$

Where: $_{est}V$= was the estimation of the volume of the brain,
$\sum P$= was the sum of the number of points landing within the various
components of the brain profiles,
a/p= the area associated with each point,
t = the distance between sections and
M= represented the magnification (x40)
m_{i-1} = all points in the brain sections from the first to the last

III. RESULTS

Influence of alcohol on the fetal brain weight, length and width

At the end of the experiment, we found changes in fetal brain weight, length and width in the alcohol treated groups as compared to the control group (Table 1). The comparative gross appearance of the fetal brain from the alcohol treated groups looked relatively small in size with poorly defined brain lobes when compared with the control group. Likewise, when the fetal brain weight, length and width comparisons were done for the alcohol treated groups, there was a marked variance between and within them in time and dose depended manner.

Table 1: Showing a comparative means fetal brain weight, brain length, and width for LAG, MAG and the HAG treated at TM1, TM2 and TM3 against the control.

Study groups	Period of alcohol treatment	Mean brain weight(g) + SEM	Mean brain length(mm) + SEM	Mean brain width(mm) + SEM
Control group	----------	0.397±0.004	1.295±0.005	1.097±0.002
Low dose alcohol group (LAG, 2g/kgbwt)	Trimester one (TM1)	0.297±0.0021 bc *	1.197±0.0021 b *	0.997±0.0021 b *
	Trimester two (TM2)	0.347±0.0059 bc *	1.247±0.0021 bc *	1.0711±0.0066 b *
	Trimester three (TM3)	0.394±0.0051	1.304±0.136	1.09±0.009
Medium dose alcohol group (MAG, 3.5g/kgbwt)	Trimester one (TM1)	0.216±0.007 bc *	1.12±0.007b *	0.916±0.0078 b *
	Trimester two (TM2)	0.303±0.001 bc *	1.2000±0.001bc *	1.023±0.012 b *
	Trimester three (TM3)	0.370±0.008	1.31±0.0024	1.073±0.009
High dose alcohol group(HAG, 5g/kgbwt)	Trimester one (TM1)	0.14±0.012 b *	1.02±0.012 bc *	0.813±0.007 b *
	Trimester two (TM2)	0.248±0.0071 bc *	1.285±0.1421	0.948±0.0026 b *
	Trimester three (TM3)	0.33±0.0029	1.287±0.0017	1.02±0.00684 b *

Key: All value that bear (*) as a superscript indicates that they depict statistical significance differences ($p<0.05$) when compared with the control. Values with (b) &(c) superscripts have a statistical significance difference ($p<0.05$) within and between comparison groups respectively using one-way ANOVA with Turkey post hoc t-tests

The influence of alcohol on the total fetal brain volume

The reference and calculated mean total fetal brain volume as determined through the goal standard method (Archimedes principle) and cavalieri method was found to depict a direct dose response relationship in that when the dose of exposure to alcohol increased, the mean total brain volume had a corresponding increase and vice versa (Table 2). On the other hand, when the total brain volume was compared with the time of exposure, it depicted a direct response relationship to the time of alcohol exposure. A statistical significant difference ($p <0.05$) between and within the groups was found when compared with the control group.

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Table 2:The TM1, TM2 and TM3 comparative reference, calculated total mean fetal brain volume via WIM and cavalieri method in the control and alcohol treated groups (LAG, MAG and HAG).

Study groups	Period of alcohol treatment	Mean total fetal brain volume (WIM) (mm3) + SEM	Mean total fetal brain volume (Cavalieri method) (mm3) + SEM
Control group	none	0.249±0.003	0.24±0.001
Low dose alcohol group (LAG, 5g/kgbwt)	Trimester one (TM1)	0.235±0.001 b*	0.234±0.002 b*
	Trimester two (TM2)	0.242±0.003 b*	0.237±0.002 b*
	Trimester three (TM3)	0.246±0.003	0.244±0.001 b*
Medium dose alcohol group (MAG, 5g/kgbwt)	Trimester one (TM1)	0.234±0.001 b*	0.231±0.002 b*
	Trimester two (TM2)	0.238±0.002 b*	0.234±0.002 b*
	Trimester three (TM3)	0.244±0.0001c*	0.240±0.001 b*
High dose alcohol group(HAG, 5g/kgbwt)	Trimester one (TM1)	0.230±0.001 b*	0.228±0.001 bc*
	Trimester two (TM2)	0.235±0.002 b*	0.232±0.002 b*
	Trimester three (TM3)	0.241±0.001 c*	0.238±0.002 bc*

Key: All value that bear (*) as a superscript indicates that they depict statistical significance differences ($p<0.05$) when compared with the control. Values with (b) &(b) superscripts have a statistical significance difference ($p<0.05$) within and between comparison groups respectively using one-way ANOVA with Turkey post hoc t-tests

IV. DISCUSSION

Alcohol exposure during pregnancy usually injure the central nervous system in various ways (Pervin & Stephen, 2021). This study established that the fetal brains from the alcohol treated groups had a relatively small brain size with poorly defines lobes when compared with the control group. The current study is in agreement with a study done by Wang et al in which fetal alcohol exposure was associated with a range of developmental disorders, including impaired fetal growth and development of multiple organ systems (Wang et al., 2020). Adequate nutrition and a conducive intrauterine environment are essential for healthy fetal development. Nutrient deficiencies resulting from inadequate maternal nutrient ingestion may be compounded by alcohol-induced altered nutrient metabolism, placental clearance, and malabsorption (Long & Lebel, 2022; Wang et al., 2020). Alcohol-induced alteration of the intrauterine environment is the main source of developmental deficits and nutritional insufficiencies can worsen the effects on fetal development which may be associated with the current study. In another study prenatal alcohol exposure was found to exerts specific direct effects on molecules and pathways that usually control fundamental developmental processes of the fetal brain (Naik et al., 2022).

Imaging studies using MRI have revealed several differences between the brains of alcohol-exposed and non-exposed fetuses (Mattson et al., 2001). Consistent with the characteristic small head size, imaging studies have shown a decrease in the overall size of the brain which has been associated with alcohol injurious effects on several brain areas, including the basal ganglia, corpus callosum, cerebellum, and hippocampus (Mattson et al., 2001; Popova et al., 2017)

Similarly, the calculated mean total fetal brain volume was also smaller and the variances in size and volumes were in a dose dependent manner (table 1 & 2). Further, the reduction in total brain volume were seen to depict a direct correlation with the time of exposure (table 2). These findings

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were in agreement with a study by Donald et al and another one by Miles (Donald et al., 2015; Miles et al., 2021; Treit et al., 2016) Neuroimaging studies has demonstrated alcoholism-related abnormalities in white matter macrostructure and microstructure. At the same time, neuroimaging has shown a fetal brain volume reduction related to prenatal alcohol exposure (Donald et al., 2015) which is in agreement with the current study. Prenatal alcohol exposure is usually associated with important volume loss in subcortical and cortical brain structures, including both white and gray substance shrinkage (Alhowail, 2022; Lees et al., 2020; Miles et al., 2021) which might be cause of the reduced brain volume in the current study.

It has been shown that neurons are formed from neuronal stem cells in human brain (Alhowail, 2022) and animal studies have shown that alcohol prevents neuronal stem cell proliferation and reduces neuronal survival in a dose-dependent manner (Alhowail, 2022). Prenatal alcohol exposure causes various effects and clinical symptoms in the central nervous system and probably this might have been the case in the current study. Fetal brain from prenatally alcohol-exposed fetuses demonstrated a decrease in length and width and this is in agreement with previous studies (Donald et al., 2015; Treit et al., 2016). Although the underlying mechanisms of alcohol damage caused by alcohol during the prenatal period are still unclear, different mechanisms have been proposed. These mechanisms suggest that alcohol consumption might induce toxic neuronal damage and induce fetal brain damage by mechanisms involved in neuroinflammation (Costardi et al., 2015; Crews & Nixon, 2009). In agreement with these literature data, our study stereologically demonstrated a reduction in the mean fetal brain volume, in alcohol exposed rats compared to the control group.

V. CONCLUSIONS

In the light of these data, results suggest that prenatal alcohol exposure, even in small amounts, has a measurable effect on fetal brain volume and size. Therefore, more effective alcohol control strategies during pregnancy should be established as alcohol adverse effects cause more serious health effects during those periods.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

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