The histostereological hepato-teratogenic effects of varied doses of ethanol in albino rats (*Rattus novegicus*)

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Abstract: Background: **Ethanol has been shown to interfere with the normal development of the fetal liver when exposed prenatally. Although data exist on the teratogenic effects of ethanol to the fetal viscera, data on the histostereological effects of ethanol on the fetal liver when administered in varying doses and at different gestational periods is lacking.**

Method: **A static-group experimental study design was adopted. In this study, 30 female albino rat dams weighing between 200 - 230gm from a pure colony breed were used as the experimental model. These 30 rats were broadly organized into four main study groups based on the ethanol dose namely: - Control group, low ethanol group (LEG), medium ethanol group (MEG) and high ethanol group (HEG). Each ethanol group was further sub grouped per the trimesters namely: - trimester one (GD1-GD20), trimester two (GD7-GD20), and trimester three subgroup (GD14-GD20) each comprised of 3 rats. Groups I served as expectant control that received food and water *ad libitum* only while Groups II (LEG), III (MEG) and IV (HEG) received 2g/kgbwt, 3.5g/kgbwt and 5g/kgbwt of ethanol respectively daily via oral gavage. All the rats were humanly sacrificed on the 20th day of gestation. A total of 90 fetuses had their liver harvested, fixed in 10% formaldehyde and processed for stereological analysis. Data was collected using structured datasheets and photomicrographs. The data was then analyzed using STEPnizer software and SPSS version 23. One-way Analysis of Variance (ANOVA), followed by Tukey's post hoc multiple comparison tests were done. The results were expressed as mean ± standard error of the mean (SEM) for all values. The findings were then presented in form of tables.**

Results: **A significant increase (P=0.001) in the total liver volume and volume densities of the portal triad, sinusoids and hepatocytes were observed. In conclusion, the present study established that ethanol consumption during pregnancy has varied histostereological hepato-teratogenic effects in time and dose dependent manner, therefore expectant mothers need to abstain from ethanol consumption any time during pregnancy.**

Key Word: **Stereology, Intrauterine, Prenatal, Fetal, Liver.**

I. INTRODUCTION

Ethanol, is an amphiphile, low molecular compound that is known to be teratogenic (1,2). For this reason, ethanol consumption during pregnancy has been shown to disrupt the normal fetal hepatogenesis (3) . This is coupled with the low molecular weight of 46.069g/mol and amphiphile properties that enhances ethanol to cross the blood placental barrier in to the fetal circulation (1). Consequently, alcohol may disrupts the normal development of the fetal liver, and this has been associated with the generation of oxygen free radicles (OFR) during the oxidative metabolism of alcohol (4). This OFR further causes oxidative stress to the cellular component of the liver, as a result, the normal functioning of the liver is interfered with (5).

Ethanol is commonly consumed orally and its absorption takes place in the stomach and the small intestine into the maternal circulation and finally into the fetal circulation via the blood placental barrier (6). This fetal blood ethanol concentration is highly dependent on the maternal blood ethanol concentration and a counter current flow of maternal and fetal blood exist (7). Due to this ,the fetal and maternal blood ethanol concentrations attains an equilibrium and in this the fetal organs are highly exposed to ethanol (8).

Globally, the prevalence of ethanol related liver disorders that includes alcoholic hepatitis, liver cirrhosis, steatosis, steato-hepatitis among others during pregnancy has been estimated to be 10% and between 3.4%-20.5% in East Africa (3,9). Studies have also projected that with the current rates of maternal ethanol consumption, cases of liver failure may continue to be on rise globally. Though studies have shown that ethanol has detrimental effects to the developing fetal liver, data is lacking on the histoquantitative effect when exposed prenatally at varied doses and at different gestational periods.

II. MATERIAL AND METHODS

Healthy female albino rats weighing between 200 - 230 grams were obtained from Small Animal Facility for Research and Innovation (SAFARI) of Jomo Kenyatta University of Agriculture and Technology for the experimentation. These animals were kept in cages and were *a*cclimatization to

laboratory conditions for a period of one week before mating and administration of ethanol. The animals were also fed on rodent pellets and water *ad libitum.*

Mating and pregnancy determination: Two female rats were introduced into a cage with one male rat at 2:00 P.M. to 09:00 a.m. At 09:20 a.m. To confirm pregnancy, a vaginal swab was taken, smeared on histological glass slide, smear fixed with absolute ethanol and stained with giemsa stain. The glass slide was then mounted on a BP Olympus light microscopy, and observation of large cornified cells, many neutrophils and scattered epithelial cells confirmed pregnancy. The animals were then assigned to either the control or experimental study groups.

Animal grouping: Upon confirmation of pregnancy**,** the animals were assigned into ten subgroups (the control group, low dose ethanol group (LEG) at trimester I, II, III, medium ethanol group (MEG) at trimester I, II, III and high ethanol group (HEG) at trimester I, II, III comprising of three rats in each of the ten subgroups. The first subgroup (control) was give tap water while the LEG, MEG and HEG received 2g/kg, 3.5g/kg and 5g/kg body weight of 30% ethanol respectively once daily via gavage needle at different gestational periods. All trimester I animals received ethanol from GD1-GD20, trimester II received ethanol from GD7-GD20 while trimester III animals received ethanol from GD 14-GD20. On their 20^{th} day of gestation the rats were humanely sacrificed, fetuses were harvested weighed and their weights were recorded.

Three fetuses that is the fetus with the least weight, highest weight and median weight from each rat were selected making a total of 90 fetuses. The 90 fetuses had their liver harvested and weighed. This was followed by the determination of the fetal liver volumes through the water immersion method (Archimedes principle) (11,12). The harvested livers were each immersing in isotonic saline in a calibrated cylinder and the volume displaced was determined. All the livers were fixed in 10% formaldehyde for 24 hours.

Procedure for stereology tissue processing:

After fixing the liver in formaldehyde solution for 24 hours, the 90 fetal livers were each placed in a tissue cassette, dehydrated in an ascending grades of alcohol (50%, 70%, 80%, 90%, 95% and 100%(absolute). In each grade of alcohol, the tissues were allowed to stay for a duration of one hour followed by clearing with xylene for 30 minutes. The tissues were then infiltrated with paraplast wax for 12 hours and embedded in paraffin wax. Leitz sledge microtome was used to cut longitudinal thin liver sections which were floated in water at 37^0 then stuck onto glass slides using egg albumin, applied as thin film with a micro-dropper. The researcher was blinded by coding the tissue slides differently to avoid researcher biasness. In each subgroup 20-25 slides were selected through systematic uniform random sampling, dried in an oven at 37^0 for 24 hours then stained with haematoxylin and eosin (13) to demonstrate the general features of fetal liver components.

Procedure for estimation of the liver volume using cavalieri principal method

To estimate the total liver volume for each foetus, between 20-25 sections of 5um were sampled from each longitudinal liver section, through systematic uniform random sampling (4). The stained liver tissue (on glass slides) were 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 liver volume was determined by combining the Cavalieri method of segmentation with point-counting on evenly spaced organ slices (Figure 1) (4,14). The following steps were followed: i) Preparation of liver Cavalieri sections ii) Selection of the spacing for the point probe iii) The point probe was tossed randomly onto each section iv) The points that hit the region of interest were counted using STEPnizer stereology tool v) All sections were processed keeping a tally of counts per section vi) The total liver volume was finally determined by applying the Cavalieri formula: - $_{est}\mathbf{V} = \sum^{\mathbf{m}}{}_{\mathbf{i-1}}\mathbf{P.\ a/\ p.\ ts\ /M^2}$. Where: $_{est}\mathbf{V}$= was the estimation of the volume of the liver, $\sum\mathbf{P}$= was the sum of the number of points landing within the various components of the fetal liver profiles, **a/p**= the area associated with each point, **t** = the distance between sections, **M**= represented the magnification (x40) and $\mathbf{m}_{\mathbf{i-1}}$ **=** all points in the fetal liver sections from the first to the last.

Following tissue processing, tissue shrinkage is likely to occur therefore the liver volume shrinkage was then calculated by subtracting the volume after from the volume before divided by the volume before (10;11). Volume before were the liver volume which were determined through the water immersion method, while volume after were the volumes determined through the cavalieri method (11).

The water immersion method was used to determine the initial liver volumes while cavalieri method was used to determine the terminal liver volume after tissue processing. The water immersion method was compared to the cavalieri method and the mean and standard error of mean (Mean ± SEM) of the measurements were determined.

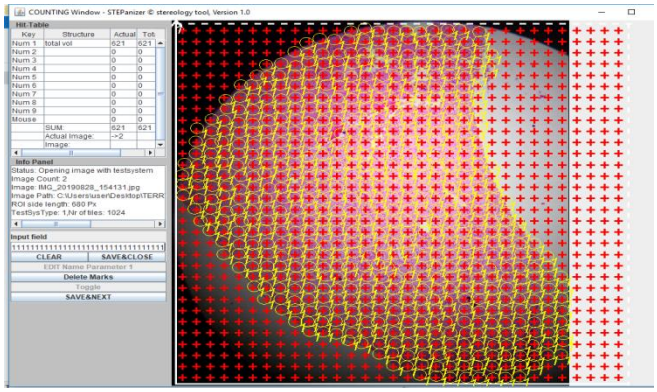

Figure 1: Photomicrograph of Light microscopic liver slice image with superimposed equidistant point grid.

Estimation of liver volume densities

To estimate the mean volume density (Vv), of the hepatocytes, sinusoids, portal triad and central vein on each sampled section, the STEPnizer tool was used. Using the STEPnizer tool, a transparent grid was then superimposed on the digital images projected on the computer screen on the liver hepatocytes, central vein among other structures. The points hitting these areas of interest were counted at a magnification of x10 in all fields of view and the volume determined by using the Cavalieri method. Then estimates of their volume density, (Vv) in the reference space were obtained using the formula *estVv = P (part)/P (ref) .Where P (part)* and *P (ref)* were the number of test points falling in all structure profiles and in the reference space, respectively (14)

III. RESULT

3.1 The influence of Ethanol on the total fetal liver volume

The reference and calculated mean total fetal liver volume as determined by use of water immersion method (WIM) which is the goal standard method and cavalieri method was found to depict a direct dose response relationship in that when the dose of exposure to ethanol increased, the mean total liver volume had a corresponding increase and *vice versa*, (Table 1). On the other hand, when the total liver volume was compared with the time of exposure, it depicted a direct response relationship to the time of ethanol exposure in that when ethanol treatment was administered at trimesters one, two and three (TM_1, TM_2 TM_3), the liver volumes increased directly with the time of exposure. For instance when the ethanol treatment was done at TM_1 the highest mean increase in the total liver volume was noted in TM_1 at 0.766±0.006, followed by TM_2 at 0.666±0.010 mm^3 and lastly TM_3 at 0.526±0.011mm^3. All the intra and intergroup comparisons were also found to be statistically significant ($p<0.05$) when compared with the control group. (Table 1).

Table 1: The TM_1, TM_2 and TM_3 comparative reference, calculated total mean fetal liver volume using WIM and cavalieri method in the control and Ethanol treated groups (LEG, MEG and HEG).

Animal group	Ethanol treatment period	WIM volume in mm^3	Cavalieri volume in mm^3
control	none	0.548±0.007[a]	0.511±0.007[a]
Low Ethanol group(LEG)	TM1	0.816±0.201[c]	0.766±0.006c
	TM2	0.706±0.0103[b]	0.666±0.010[b]
	TM3	0.551±0.012[a]	0.526±0.011[a]
Medium Ethanol group(MEG)	TM1	0.858±0.008[d]	0.796±0.013[d]
	TM2	0.742±0.009[c]	0.708±0.011[c]
	TM3	0.608±0.006[b]	0.572±0.008[b]
High Ethanol group(HEG)	TM1	0.930±0.010[d]	0.882±0.008[d]
	TM2	0.787±0.006[c]	0.757±0.005[c]
	TM3	0.652±0.010[b]	0.612±0.007[b]

Key: The means, followed by the same letter in a column are not statistically different at (P<0.05) using one way ANOVA with Tukey test on post-hoc t-tests.

3.2. The comparative volume densities of the liver hepatocytes, sinusoids, portal vein and central vein in the LEG, MEG and HEG against the control

When the mean volume densities of the fetal liver hepatocytes, sinusoids, portal triad and central vein in the first trimester at varying doses was compared with the control it was observed that the volume densities were significantly increased ($p< 0.05$) in dose dependent manner when administered at TM_1 and TM2 except in TM3 which was found not to be statistically significant ($P>0.05$). The highest mean volume density were observed in the HEG followed by MEG and lastly the LEG when compared to the control. Further, the Post hoc results for the mean volume densities also revealed that the MEG was significantly different from HEG, but not significantly different from the LEG (Table 2).

Table 2: The comparative mean volume densities of the liver hepatocytes, sinusoids, portal vein and central vein in the LEG, MEG & HEG against the control at TM_1, TM_2 and TM_3

Animal group	Ethanol treatment period	Volume densities in mm^3			
		Hepatocytes	Sinusoids	Portal triad	Central vein
control	none	0.2811±0.0039[a]	0.1278±0.0018[a]	0.7667±0.0010[a]	0.0256±0.0004[a]
LEG	TM1	0.4210±0.0036[b]	0.1914±0.0016[b]	0.1148±0.000[b]	0.0382±0.0003[b]
	TM2	0.3660±0.0054[b]	0.1663±0.0025[b]	0.998±0.0015[b]	0.0331±0.0005[b]
	TM3	0.2890±0.0062[a]	0.1314±0.0028[a]	0.7883±0.001[a]	0.0263±0.0006[a]
MEG	TM1	0.4401±0.0085[b]	0.2000±0.0038[b]	0.1200±0.002[b]	0.4000±0.0008[b]
	TM2	0.3892±0.006[c]	0.1769±0.0028[c]	0.1061±0.001[c]	0.353±0.0006[c]
	TM3	0.3166±0.0053[b]	0.1439±0.0023[b]	0.863±0.0014[b]	0.0288±0.0005[b]
HEG	TM1	0.4852±0.0044[c]	0.2206±0.0020[c]	0.1323±0.001[c]	0.4411±0.0004[c]
	TM2	0.4161±0.0029[d]	0.1892±0.0013[d]	0.1135±0.000[d]	0.378±0.0003[d]
	TM3	0.3367±0.0040[c]	0.1531±0.0018[c]	0.918±0.0011[c]	0.0306±0.0004[c]

KEY: The means, followed by the same letter in a column are not statistically different at (P<0.05) using one way ANOVA with Tukey test on post-hoc t-tests.

IV. DISCUSSION

In the current study, ethanol was shown to trigger hepatomegaly in the albino fetuses when exposed prenatally at different gestational period and doses. This is due to the fact that the liver, is the primary site for ethanol metabolism is more prone to ethanol-related injury (15). For this reason, Ethanol intake puts a great metabolic overload on the hepatocytes (16), and the response to this overload could lead to liver enlargement. Further, the liver mostly prefers ethanol as an energy source and due to this it stops the use of fats which accumulates in the hepatocytes leading to fatty liver (17) which could have been the cause of hepatomegaly in the current study. In addition, the hydrogen produced as a by-

product of ethanol metabolism is converted to more fat for the synthesis of cholesterol and lipoproteins which further accumulates as fat droplets in the liver (6,17-18). All this could be responsible for the hepatomegaly observed in the HEG, MEG and LEG when ethanol was administered in the first, second and third trimester.

On the other hand, the rats that were exposed to ethanol in the entire gestational period presented with increased liver weight than those that received ethanol for a short duration (Table 1). This difference may be associated with the toxic effects of ethanol and its toxic byproduct (acetaldehyde) which have been shown to cause varying degree of cytoarchitectural distortion of the hepatocytes. The current study concurs with a study which was done to establish the effects of ethanol on cytoskeletal of the fetal liver where it was shown that ethanol distorted liver proteins which ends up causing hepatomegaly in rats (19).

Following administration of varying doses of ethanol, there was evident increase in the total liver volume and volume densities among the ethanol treated groups as compared to control (Table 1 & 2). The liver volume were significantly increased ($p=0.001$) in the high, medium and low ethanol treated groups in the first as well as second trimester when compared to the control group. This concurs with a study in which rats were administered 5% ethanol prenatally and an increase in the fetal liver hepatocyte cytoplasm in addition to the nucleus volume were established (20-21). A dose dependent increase in the liver volume was also reported following administration of fatty diet and ethanol concurrently (22). In the present study, the increase in liver volume might have been due to the vascular dilatation, inflammation of the liver, recruitment of inflammatory cells, and liver fatty change upon ethanol administration. A study done by Altunkaynak, *et al*, (2017), established an increase in total liver parenchyma upon administration of ethanol and a fat diet to rats which concurs with the current study. The presumed reason for hepatic volume increase would be the ballooning of hepatocytes along with increased vascular dilatation.

In this study, the fetal mean volume densities of the hepatocytes, sinusoids, portal triad and the central vein were shown to increase in a time and dose dependent manner in the treatment groups when compared to the control group. This agrees with a study which was done by Gabriela et al, (1998) which shown interferences in fetal development following prenatal ethanol consumption. This developmental interferences not only occurred directly through the adverse effects exerted by ethanol upon crossing the blood placenta barrier into the fetal circulation, but also indirectly, by disturbing the functions and interactions of maternal and fetal hormones (22). Ethanol exposure was shown to impair the functioning of hypothalamic-pituitary-thyroid axis, which regulates the metabolism of almost all tissues. This impairment promotes histo-cytoarchitecture distortion of the liver parenchyma which is involved in the metabolic processes (22). The parenchymal distortion may consequently increase the liver weight which could have been the case in the current study. *In-utero* exposure to ethanol was also shown to increase both liver weight and total protein content in the Golgi complex and this altered its morphological and functional properties (19). This was based on the fact that ethanol perturbed the developing fetal liver by inducing retention of proteins in hepatocytes thereby resulting into alteration of the liver histo-cytoarchitare (19).

V. CONCLUSION

Ethanol consumption during pregnancy is associated with varied histostereological hepato-teratogenic effects throughout the gestational periods in dose dependent manner. Expectant mothers therefore, need to abstain from ethanol consumption any time during pregnancy

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ETHICAL APPROVAL

Author hereby affirms that the experimental protocol was approved by the Jomo Kenyatta University of Agriculture and Technology Animal ethical Committee (JKUAT AEC). The animals were only used once. They were all sacrificed using humane end points at the end of the study. The protocol followed to the letter the Guidelines for Care and Use of Laboratory Animals in Biomedical Research.

CONFLICT OF INTEREST

None

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