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Effects of in-utero exposure to varied doses of alcohol on fetal growth and development in albino rats (*rattus norvegicus*)

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Abstract: *In-utero exposure to alcohol has been shown to disturb the normal development of various fetal organs. This is due to the fact that expectant mothers continue to use alcohol as a social drink and this has been shown to have injurious effects on the developing fetal organs. Though this data exist, data on whether this effects are dose or time dependent is lacking .This injurious effects on the developing fetal organs can help elucidate some of the behavioral and functional disorders observed in adulthood that are of unknown cause.The broad objective of this study was to determine the growth and developmental parameters upon administration of varying doses of ethanol, therefore to determine this injurious effects, 30 expectant rats weighing between 200 to 230 g were used. Simple random sampling was used to assign them into four study groups A –D (n=9 and 3 for each treat groupand control respectively).Groups A served as expectant control group and received food and water ad libitum only while groups B (low alcohol group), C (medium alcohol group) andD (high alcohol group), wereeach further sub divided into first, second and third trimester ethanol treatment group (n=3) and received 2g/kg, 3.5g/kg and 5g/kg body weight of alcohol respectively once daily via oral gavage.The expectant rats were sacrificed on the 20th day of gestation upon euthanasia with carbon dioxide and the abdomen was excised to expose the uterine horns. The fetal anthropometric body parameters including birth weights, fetal length and head circumference among others wererecorded. The number of implantation sites and resorptions were counted and recorded.Data was then entered in excel sheet, analyzedthrough SPSS version 23 and statistically tested using one way analysis of variance (ANOVA) and p-values of less than 0.05 were taken to be significant. The findings of the study showed that there was a statistical significant decrease I fetal weight, crown rump length as well as the head circumference.Results also showed a statistically significant dose and time-dependent decrease in fetal birth weight, fetallength and head circumference especially in treatment groups C and D (p<0.05). In conclusion, the present study revealed that alcohol consumption during pregnancy has negative effects on growth and development parameters. This effects are time and dose dependent therefore more emphasis on complete abstinence from alcohol consumption anytime during pregnancyshould be done on the expectant mothers.*
Hence forth the above study showed that alcohol causes detrimental effects that are time and dose dependent and range from intrauterine growth retardation manifested by decreased fetal birth weight, head circumference, and fetal length.

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I. Introduction

Alcohol, a social drink that continue to be abused by expectant mothers as well as all age groups, has been shown to disturb the normal morphogenesis of the fetal organs when used during intrauterine life(1–3). Globally, alcohol abuse among expectant mothers continues to be on rise(4), and this is related to a huge health burden including morbidity, mortality and disability(5). Despite that, alcohol effectson growth and development have widely been studied, there is paucity of data on the fetal anthropometric measures and morphogenesis of specific fetal organs following in-utero exposure to alcohol. In addition, data on whether this effects are time or dose dependent has not been well established hence the present study was conducted to observe if the effects are time or dose dependent by using rats as the experimental model.Global prevalence of alcohol consumption during pregnancy has been estimated to be 10%(6,7), which is associated with a wide range of fetal congenital anomalies broadly known as fetal alcohol syndrome(FAS),characterized by Malformations, intrauterine death, growth deficiency, CNS abnormalities, GIT abnormalities and behavioral deficits among others(1).Some studies have associated alcohol injurious effects on the fetal and maternal genetic makeup , while other studies have associated alcohol teratogenic effects on environmental conditions including maternal malnutrition (4,8).These effects could be used to explain some of the structural, neurodevelopmental and behavioral conditions like autism including attention deficit hyperactivity disorder that exist of unknown causes.

II. Material and Methods

Healthy female nulliparous Albino rats (dams) weighing between 200 and 230 grams were used for the experiment and were obtained from the Safari Animal house of JKUAT. All the animals *were a*cclimatization to laboratory conditions for a period of one week before treatment. All the animals were kept in spacious plastic cages with shredded papers in the animal house and received food (rodent pellets) from UNGA meals and water *ad libitum.*

Study Design: Laboratory based experimental study

Study Location: All experiments including breeding, handling, weighing and alcohol administration was done at the Safari Animal house in the School of biomedical Sciences of JKUAT while processing of specimens for light microscopy and stereology were done at the department of human anatomy histology labs in COHES complex of JKUAT

Study Duration: The study was carried out from November 2018 to January 2019

Sample size: 90 fetuses were used in the study.

Sample size calculation: Resource equation was used to determine the sample size since the standard deviation was not available from previous studies(9).
The measured value 'E' which is the degree of freedom of analysis of variance (ANOVA) based on a decided sample size value ('E') should lie between 10 and 20 animals according to this equation. Therefore, a value less than 10 necessitates adding more animals which increases the chance of getting significant results while a value more than 20 has been shown to increase the cost of the study without increasing the significance of the results(9).
E=Total number of animals-Total number of groups
Total number of groups=10
Total number of animals=30
E=30-10
Since, every adult female rat is assumed to have a minimum average of six (6) fetuses per pregnancy making a total of **180 rats (6x 30**)(10,11). All fetuses were weighted and three fetuses with the least, median and highest weights per rat were taken for study making a total of **90 rats (3x30).**

Grouping of animals: once pregnancy was confirmed, animals were randomly assigned to either the control or the experimental category (i.e. 3 rats as control group and 27 rats as experimental). The 27 rats in the experimental category were further divided into three study groups of 9 rats each assigned :- low alcohol group (LAG), Medium alcohol group (MAG) and High alcohol group (HAG) .Each of the three study groups were further sub-grouped into first (TM1), second(TM2) and third(TM3) trimesters subgroups comprising of 3 rats each.

Determination of the alcohol doses for the experiment:

A lethal dose of ethanol is 7.1 g/kg/Bwt has been shown to be the LD_{50} for adult rats(12). For purposes of this study this dose will be taken as the reference dose. [$N=LD_{50}$ (i.e.the dose that would cause disturbance to the development of the fetal organs to 50% of the animal population].Given the molecular nature of ethanol and the physiological counter current flow of maternal and fetal blood, a blood ethanol concentration equilibrium between the two will be always achieved at any given time. The weight in grams of the ethanol for the reference dose (LD_{50}) of 7.1g/kg/BW for an average weight dam of 200g will be calculated as:-
Weight of rat= 200g
Lethal dose LD50= 7.1g/kg/BW

Therefore: 1 rat =200g x $\frac{7.1g}{1000g}$ = **1.42g absolute ethanol**

Calculation and administration of the doses*:*

Absolute alcohol obtained from Chemo quip chemistry, Nairobi (batch number 1614105017, country of origin, Netherlands) was used to make the reconstitutions. Absolute alcohol was diluted in deionized water to make 30% alcohol. Since alcohol concentration more than 30% causes gastric ulcerations in rats, a concentration of 30% was used**.** Therefore 30mls of ethanol was withdrawn and diluted with 70 mls of distilled water (diluent) to make 30% ethanol i.e. 30g in 100mls of solvent.

How to determine the mls to be administered

1.42 x 100mls = 4.7mls

30gms

- All trimester ones (TM_1) animals:- (LAG,MAG,HAG) categories received alcohol from gestation day GD1-GD20
- All trimester twos (TM_2) animals:- (LAG,MAG,HAG) categories received alcohol doses from gestation day GD7-GD20
- All trimester three (TM_3) animals:- (LAG,MAG,HAG) categories received alcohol doses from gestation day GD14-GD20

Determination of the critical dose of alcohol

Animal groupings was done as follows; in each of the groups (LAG, MAG, HAG), the 9 dams were randomly sub divided in three sub-groups the Trimester 1(TM_1) = 3dams, Trimester 2 (TM_2) = 3dams and trimester 3 TM_3=3 dams

NB> the gestation period of a rat is 21 days, therefore trimester one was between gestational day GD1 to GD7, while trimester 2 was between GD7-GD14 and third trimester GD14-20.

Determination of fetal growth parameters

The expectant mothers were sacrificed on day 20 of gestation and the fetal weights were determined using digital weighing scale, (scout pro model from Japan, serial no.B519923500) as shown in figure 2. The machine was calibrated to zero before measuring each fetus, while fetal length (crown rump length) was measured from the snout to the side of anus. The animal was placed lying supine on a dissecting board without stretching its body. One pin was placed on the tip of the nose and another at the side of the anus followed by removal of the animal from the dissecting board, and using a ruler the distance between the two pins was determined and recorded as shown in figure 3. On the other hand, the fetal head circumference was determined using a thread extending from the forehead, passing anterior to the pinna all round to the occiput. This was transferred to a ruler to determine the circumference as shown in figure 4(13).

Statistical analysis

Data was expressed and analyzed using SPSS version 23.0 (SPSS Inc., Chicago, IL). One-way Analysis of Variance (ANOVA) followed by Tukeypost hoc for multiple comparison tests was done and results were expressed as mean ± standard error of mean (SEM) for all values.. The results were considered to be significant at $P<0.05$.

Ethical clearance

The ethical clearance was sought from JKUAT Animal Ethical Committee (AEC) before initiation of the study.

III. Result

The study sought to examine the effect ethanolon fetus growth and development upon sacrifice of the animals on day 20 of gestation .This was determined through the measurement of the fetal weight, fetal length and head circumference upon administration varied doses of ethanol. The data was analyzed using SPSS and Excel statistical software and was expressed as mean ± standard error (SEM). The study compared how the threedose levels (Low, medium and high) and control in the three trimesters (T1, T2 and T3), affected the different growth parameters. This parameters were: fetal weight, Crown Lump length and Head Circumference. To determine the significance, one way analysis of variance with Tukey post hoc test was used and 5% significance level (α = 0.05) was assumed. The results were considered to be significant whenever the probability value is less than 0.05 ($p<0.05$). The results were presented below:

Table 1: intra group comparison of fetal weight, body length (crown rump length) and head circumference in the control, low, medium and high dosage groups in the first Trimester

Parameter	Control	Low	Medium	High	F	P-value
Fetal Weight	6.27±0.033a	4.18±0.117b	4.07±0.056b	3.63±0.088c	216.23	0.0001*
Crown rump Length	4.65±0.074a	3.20±0.058b	3.083±0.0441b	2.93±0.067b	167.24	0.0001*
Head Circumference	3.73±0145a	2.37±0.033b	2.03±0.033bc	1.633±0.133c	80.9	0.0001*

*Notes: The means, followed by the same letter in a row are not statistically different at ($P<0.05$) using one way ANOVA with Tukey test on post-hoc t-tests. * indicates significance ($p<0.05$).*

Table 1 above revealed that fetal weight in the control group (6.27±0.033) was found to be significantly higher than that in the low dose group (4.18±0.117), medium (4.07±0.056) and high dose (3.63±0.088), F (3, 8) = 216.23, p = 0.0001. The weight in low dose and the medium dose was not statistically different but was found to be lower in the high dose group. Crown rump length in the control group (4.65±0.074) was found to be significantly higher than that in the low dose group (3.20±0.058), medium (3.083±0.0441) and high dose (2.93±0.067), F (3, 8) = 167.24, p = 0.0001. Crown rump length in medium and high dose were not significantly different. The head circumference in the control group (3.73±0145a) was found to be significantly higher than that in the low dose group (2.37±0.033), medium (2.03±0.033) and high dose (1.633±0.133), F (3, 8) = 80.9, p = 0.0001. The head circumference in low dose and the medium dose was not statistically different but was found to be lower in the high dose group.

Table 2: Intra group comparison of fetal weight, body length (crown rump length) and head circumference in the control, low, medium and high dosage groups in the second Trimeste

Parameter	Control	Low	Medium	High	F	P-value
Fetal Weight	6.27±0.033a	5.17±0.067b	4097±0.033b	4.87±0.088c	115.4	0.0001*
Crown Lump Length	4.65±0.0742a	3.933±0.088b	3.6±0.0577c	3.33±0.067c	61.9	0.0001*
Head Circumference	3.73±.0145a	3±0.00b	2.67±0.088bc	2.33±0.033c	47.8	0.0001*

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

From table 2 above, fetal weight in the control group (6.27±0.033) was found to be significantly higher than that in the low dose group (4.18±0.117), medium (4097±0.033) and high dose (4.87±0.088), F (3, 8) = 115.4, p =0.0001. The weight in low dose and the medium dose was not statistically different but was found to be lower in the high dose group. Crown rump length in the control group (4.65±0.0742a) was found to be significantly higher than that in the low dose group (3.933±0.088), medium (3.6±0.0577) and high dose (3.33±0.067), F (3, 8) = 61.9, p =0.0001. However crown rump length in medium and high dose were not significantly different. The head circumference in the control group (3.73±.0145) was found to be significantly higher than that in the low dose group (3±0.00), medium (2.67±0.088) and high dose (2.33±0.033), F (3, 8) = 47.8, p =0.0001. The head circumference in low dose and the medium dose as well as medium and high dose was not statistically different but was found to be lower in the high dose group.

Table 3: Intra group comparison of fetal weight, body length (crown rump length) and head circumference in the control, low, medium and high dosage groups in the third Trimester

Parameter	Control	Low	Medium	High	F	P-value
Fetal Weight	6.27±0.033a	6.10±0.058a	6.13±0.03ab	5.8±0.12c	8.22	0.008*
Crown Lump Length	4.62±0.099a	4.533±0.0333a	4.27±.0145ab	4.03±0.033b	8.6	0.007*
Head Circumference	3.73±0.1453a	3.5±0.00ab	3.23±0.088b	3.2±.0100b	6.4	0.02*

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

Table 3 above showed that fetal weight in the control group (6.27±0.033) was not found to be significantly higher than that in the low dose group (6.10±0.058), and medium (6.13±0.03) but was found to be significantly lower in the high dose (5.8±0.12), F (3, 8) = 8.22, p =0.05. The weight in low dose and the medium dose was not statistically different but was found to be lower in the high dose group. Crown rumplength in the control group (4.62±0.099) was found to be significantly higher than that in the high dose (4.03±0.033) but no significant difference was observed in the low dose group (4.533±0.0333), and medium (4.27±.0145) and F (3, 8) = 8.6, p = 0.05. However crown rump length in medium and high dose were not significantly different. The head circumference in the control group (3.73±.0145) was found to be significantly higher than that in the medium (3.23±0.088b)and high dose (3.2±.0100), but not statistically significant in the low dose group (3.5±0.00), F (3, 8) = 6.4, p = < 0.05. The head circumference in low dose, medium dose and high dose was not statistically different but was found to be lower in the high dose group.

Table 4: Comparison of Low alcoholgroup (LAG) across the trimester levels and the control

Parameter	Control	T1	T2	T3	F (3,8)	P-value
Fetal Weight	6.27±0.033a	4.18±0.117b	5.17±0.067c	6.10±0.058a	164.29	0.000*
Crown rump Length	4.65±0.074a	3.20±0.058b	3.933±0.088c	4.533±0.0333a	100.01	0.000*
Head Circumference	3.73±0145a	2.37±0.033b	3±0.00c	3.5±0.00a	65.93	0.000*

*Notes: The means, followed by the same letter in a row are not statistically different at (P <0.05) using one way ANOVA.withTukey test on post-hoc t-tests. * indicates significance (p<0.05).*

The results in Table 4 revealed that fetal weight in the trimester one group (4.18±0.117) and trimester two group (5.17±0.067) was found to be significantly different from that in the control group (6.27±0.033), F (3, 8) = 164.29, p= 0.000. However, fetal weight in the third trimester was not significantly different from the control group. This meant that ethanol administered in low dose affects fetal weight across the different trimesters with trimester one and two being significantly different from the control group at 5% significance level. The two trimesters were also significantly different from each other. On the other hand, crown rump length in the trimester one group (3.20±0.058) and trimester two group (3.933±0.088) was found to be significantly different from that in the control group (4.65±0.074), F (3, 8) = 100.01, p= 0.000. However, fetal length in the third trimester was not significantly different from the control group. This meant that ethanol administered in low dose affects fetal length across the different trimesters with trimester one and two being significantly different from the control group at 5% significance level.Fetal head circumference in the trimester one group (2.37±0.033) and trimester two group (3±0.00) was found to be significantly different from that in the control group (3.73±0145), F (3, 8) = 65.93, p= 0.000. However, fetal head circumference in the third trimester was not significantly different from the control group. This meant that ethanol administered in low dose affects fetal head circumference across the different trimesters with trimester one and two being significantly different from the control group at 5% significance level.

Table 5: Comparison of Medium alcohol group (MAG) across the trimester levels and the control

Parameter	Control	T1	T2	T3	F	P-value
Fetal Weight	6.27±0.033a	4.07±0.056b	4097±0.033c	6.13±0.03a	671.27	0.000*
Crown rump Length	4.65±0.074a	3.083±0.0441b	3.6±0.0577c	4.27±.0145a	60.98	0.000*
Head Circumference	3.73±0145a	2.03±0.033b	2.67±0.088c	3.23±0.088d	56.82	0.000*

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

The results in Table 5 showed that fetal weight in the trimester one group (4.07±0.056) and trimester two group (4097±0.033) was found to be significantly different from that in the control group (6.27±0.033), F (3, 8) = 671.27, p= 0.000. However, fetal weight in the third trimester was not significantly different from the control group. This meant that ethanol administered in medium dose affects fetal weight across the different trimesters with trimester one and two being significantly different from the control group at 5% significance level. The two trimesters were also significantly different from each other. In addition, crown rump length in the trimester one group (3.083±0.0441) and trimester two group (3.6±0.0577) was found to be significantly different from that in the control group (4.65±0.074), F (3, 8) = 60.98, p= 0.000. However, fetal length in the third trimester was not significantly different from the control group. Fetal head circumference in the trimester one group (2.03±0.033), trimester two group (2.67±0.088) and trimester three (3.23±0.088) was found to be significantly different from that in the control group (3.73±0145), F (3, 8) = 56.82, p= 0.000. This meant that ethanol administered in medium dose affects fetal head circumference across the different trimesters being significantly different from the control group at 5% significance level.

Table 6: Comparison of High alcohol group (HAG) across the trimester levels and the control

Parameter	Control	T1	T2	T3	F	P-value
Fetal Weight	6.27±0.033a	3.63±0.088b	4.87±0.088c	5.8±0.12d	179.99	0.000*
Crown rump Length	4.65±0.074a	2.93±0.067b	3.33±0.067c	4.03±0.033d	149.25	0.000*
Head Circumference	3.73±0145a	1.633±0.133b	2.33±0.033c	3.2±.0100d	69.0	0.000*

*Notes: The means, followed by the same letter in a row are not statistically different at (P <0.05) using one way ANOVA.withTurkey test on post-hoc t-tests. * indicates significance (p<0.05).*

The results in Table 6 revealed that fetal weight in the trimester one group (3.63±0.088), trimester two group (4.87±0.088) and trimester three (5.8±0.12) was found to be significantly different from that in the control

group (6.27±0.033), F (3, 8) = 179.99, p= 0.000.This meant that ethanol administered in high dose affects fetal weight across the different being significantly different from the control group at 5% significance level. In addition, crown rump length in the trimester one group (2.93±0.067) and trimester two group (3.33±0.067) and trimester three (4.03±0.033) was found to be significantly different from that in the control group (4.65±0.074), F (3, 8) = 149.25, p= 0.000. Fetal head circumference in the trimester one group (1.633±0.133), trimester two group (2.33±0.033) and trimester three (3.2±.0100) was found to be significantly different from that in the control group (3.73±0145), F (3, 8) = 69.0, p= 0.000. This meant that ethanol administered in high dose affects fetal head circumference across the different trimesters being significantly different from the control group at 5% significance level.

Figure 1: showing varied sizes of fetuses from the control, LAG, MAG and HAG group

The figure above shows different fetal sizes following administration of varied doses of ethanol .The control fetus is bigger in size and healthier as compared to the MAG, LAG and HAG fetus

Figure 2: showing varied fetal weights of control, LAG, MAG and HAG

The figure above shows the varied weights of fetuses upon administration of different ethanol doses. The control has high weight as compared to the treatment group fetuses with the high dose group fetuses having the least weight a clear indicator of the injurious effects of alcohol

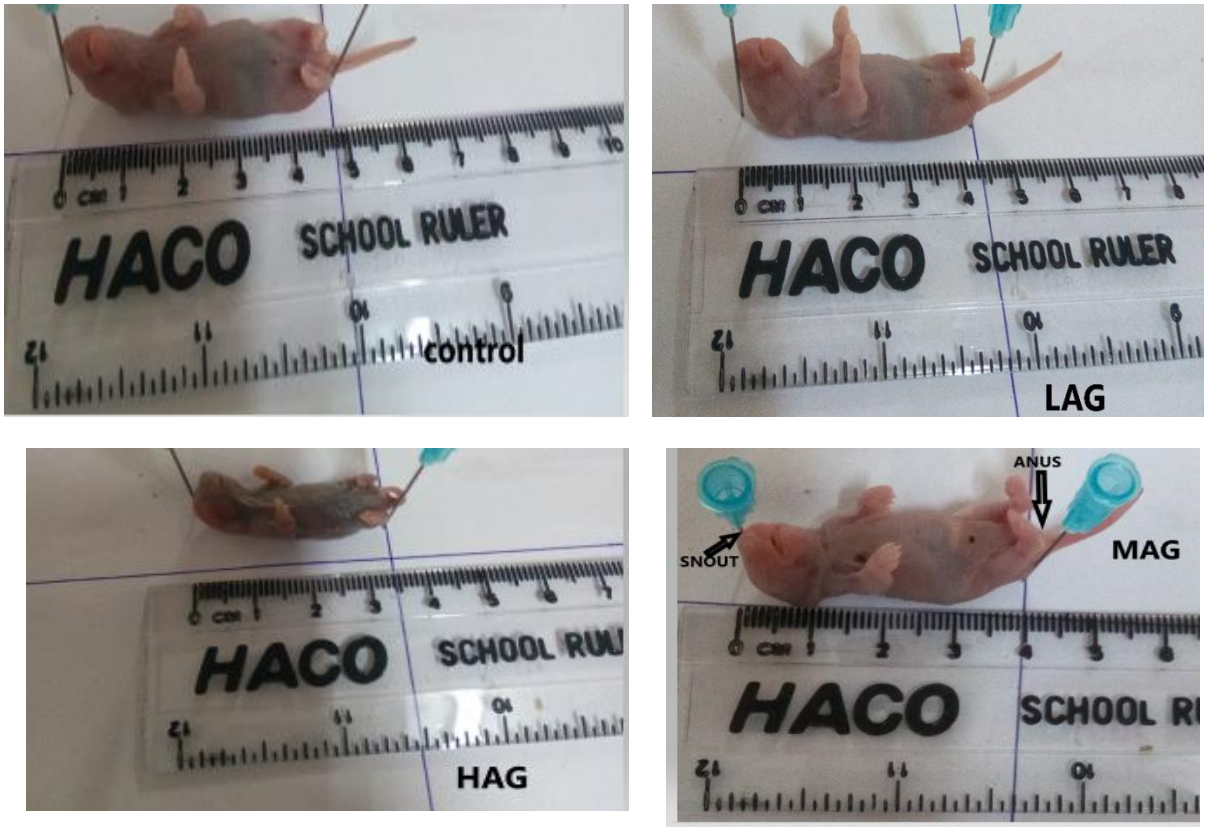

Figure 3: showing the length of the fetus at varied alcohol doses

Figure 3above shows the different length of fetuses upon administration of alcohol an indicator that alcohol affects the fetal growth, manifested by the different fetal body lengths.

Figure 4: shows fetal head circumference measurements of the control.

Shows how the fetal head circumference was measured in the control as well as the treatment groups.

IV. Discussion

In-utero exposure to alcohol continue to be on rise and remains a substantial public-health problem as many expectant woman continue to consume alcohol regardless of the clinical recommendation and public health campaigns warning about the risks associated(14,15).In the current study, alcohol administration at high and medium dose was shown to cause injurious effects on fetal growth and development and this effects were manifested by reduced fetal birth weight, reduced fetal length and head circumference among others as shown in figure 1 ,2 and 3. Most of the effects were observed in fetuses who were exposed to alcohol in the first and the second trimester. Alcohol exerts this injurious effects through multiple ways with influence on free radicle formation, cell programing and apoptosis, as well as gene expression, among other mechanisms.

Fetal weight and length

Prenatal exposure to alcohol causes intrauterine growth retardation. In this study, the fetal birth weight and length at gestational day 20 was found to be significantly reduced in the high, medium and low dose ethanol groups when alcohol was administered in the first, secondand thirdtrimester as compared to the control group (table 1, 2 and 3 above) with $p<0.0001$ which was less than 0.05 significance level.The varied fetal body weight and fetal body length are shown in figure 2 and figure 3 aboverespectively and this concurs with a study which was done by **Michele Ramsay,(2010)** who correlates fetal alcohol spectrum disorder with level and nature of alcohol exposure where he observed that clinical severity upon in-utero ethanol exposure were time and dose dependent(15). A similar effects were also described by **Anulika*et al*(2018)**who associated teratogenic effects of alcohol on litter size and fetal birth weight(16). However, it has been demonstrated that newborns who are born to alcoholic mothers are of small size (weight and or length) for a given gestational age possibly because alcohol increases the production of prostaglandins which consequently increase cAMP activity thus reducing cell division(17). In addition, alcohol has been shown to interfere with nutritional supply to the fetal-placental unit consequently disrupting the absorption, transport and storage of iron to the developing fetus (18–20).Another study which was done by**Ertem *et al*,(2006)**contradicts the current study where no significant difference in fetal birth weight was observed upon prenatal maternal exposure to ethanol and this may have been due to the methodology used(21).

Head circumference

In the current study, head circumference was found to be significantly reduced in the high, medium and low alcohol treated groups compared to control group when alcohol was administered in the first, second and third trimester (table 1,2, 3,4,5 and 6). However the head circumference was shown to be more reduced in the high ethanol group as compared to the medium, low dose and the control group when ethanol was administered throughout the gestation as shown in table 6. A study done by **Georges *et al*(2017)** on fetal alcohol exposure observed a statistical significant difference on fetal head circumference which was associated with indirect alcohol injurious effects of fetal placental unit or direct fetal toxicity from ethanol and its toxic metabolite(acetaldehyde)(18).However this confirms another study which was done by **Kartik*et al*(2007)**(19). This is probably due to the fact that prenatal alcohol consumption directly or indirectly compromises nutritional status to the developing fetus as well as causing gene alteration that are involved in the embryological development of

the skeletal systemalong with the brain(15,19). Despite that the current study concurs with above studies, this confirms that alcohol has detrimental effect on fetal growth and development and that the effects are time and dose dependent.

V. Conclusion

Prenatal alcohol exposure is associated with reduced fetal birth weight, fetal body length and head circumference which is a clear indicator of the injurious effects on fetal growth and developmental depending on the dose, duration and developmental stage of the embryo at exposure. Women need to be concealed to completely stop alcohol consumption during their preconception and antenatal visits.

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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(22)

CONFLICT OF INTEREST: None

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