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The Intra-Uterine Effects of Phenobarbital on Fetal Growth and Development in Albino Rats (Rattus Norvegicus)

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ABSTRACT:
Background:
The intrauterine developmental consequences of phenobarbital when administered in varied doses on the fetal growth and development remains poorly understood. This study therefore set to evaluate the intrauterine effects of varied doses of phenobarbital when administered at different gestational period on fetal growth and development in albino rats as the experimental model. In carrying out this study, post-test only control experimental study design was adopted. A resource equation for One-way Analysis of Variance (ANOVA) was used to determine the sample size and as such a sample size of 30 Albino rats (Rattus norvegicus) weighing between 150-250 mg were used in this study. These 30 albino rat were hence obtained from the Small Animal Facility for Research and Innovation (SAFARI) in the school of biomedical sciences of Jomo Kenyatta University of Agriculture and Technology (JKUAT). This sample size of 30 albino rats were randomly assigned into two broad study groups of 3 rats control and 27 rats the experimental group. To evaluate the intrauterine effect of phenobarbital when administered in varied doses, the 27 rats in the experimental group were further subdivided in to three study groups of 9 rats each according to the three study doses of low, medium and high phenobarbital doses applied in the study as follows; 9 rats for the high phenobarbital group (HPBG)-that received 41.5 mg/kg/bw; 9 rats for the medium phenobarbital group (MPBG) that received 19.2mg/kg/bw and lastly 9 rats for the low phenobarbital group (LPBG) that received 3.1mg/kg/bw. To evaluate the intrauterine effects of phenobarbital when administered on differing gestation periods, the 9 rats in each of the three study dose categories were further sub-divided into three sub-groups of 3 rats according to the trimester when they received treatment as follows; 3 rats that received the treatment from Trimester I-(TM_1); 3rats that received treatment from trimester II -(TM_2)and 3 rats that received treatment from trimester III-(TM_3)-respectively. At gestation day 20, all the rats were humanely sacrificed and 3 fetuses from each rat were selected based on their weights as follows; the one first with highest weight, another one with the median weight and the last one with lowest weight. The fetal growth and developmental parameters evaluated in this study included the fetal weight (FW), crown lump length (CRL), and bi-parietal diameters (BPD). The data was collected using a structured a check list, then entered into the computer using an excel spreadsheet for windows version 10, the data in the excel spreadsheets was then exported to the Statistical Package for the Social Scientist (SPSS) version 25 for analysis. To determine the causal effects and interaction effects the statistical significance was determined by use of Turkey's post hoc multiple comparison tests and All values whose $P<0.05$ were considered to be significant. The finding of the study shown that there was statistical significant reduction ($P<0.05$) in fetal weight and bi-parietal diameter especially during the first and second trimester. Phenobarbital administered prenatally had a dose and time dependent influence on fetal parameters in that effects were more with (HPBG)-41.5 mg/kg, and

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during the first trimester (TM_1) when compared with control. Therefore more studies needs to be done on higher primates to ascertain its teratogenic safety in pregnancy.

I. INTRODUCTION.

Phenobarbital, a first line anticonvulsant is commonly prescribed in management of wide a range of conditions such as insomnia, neuralgic pains, epilepsy, among other convulsive disorders (Sahadevan et al., 2017). Though all anticonvulsants are known to have teratogenic effects on the fetus the phenobarbital usage during pregnancy is particularly gaining preference in management of maternal epilepsy pre-eclampsia and eclampsias, among other conditions(Pennell, 2016). Phenobarbital is commonly prescribed because of its low cost, its pharmacotherapeutic effectiveness in management of neuralgic pains as well as its easy accessibility in the local pharmacies (Li et al., 2019). However, the teratogenic safety of phenobarbital during pregnancy has been controversial because of its unclear teratogenic effects on growth and development of fetus, making it difficult to prescribe(Ashtarinezhad et al., 2015). There is limited data on its teratogenic effects when administered in varied doses and at different window periods on fetal growth parameters including fetal weights(FWs), crown lump length(CRL), biparietal diameters (BD), and the fetal head circumference(FHC). This study aims to generate data that can help scientist carry further studies to non-human primates that have a closer genetic relationship to humans with a view to guiding the clinicians in prescribing phenobarbital during pregnacy.

II. MATERIAL AND METHODS

Study location : The animal experimentation that included animal feeding, drug administration, maternal weights, fetal weights, the fetal growth and developmental parameters and sacrificing the mothers was carried out in the Small Animal Facility for Research and Innovation (SAFARI) of Jomo Kenyatta University of Agriculture and Technology (JKUAT).

Study Design: A post test only control experimental study design was adopted in conducting the study.

Sample study. A pure colony of 30 nulliparous Albino rat dams of the Rattus norvegicus species were used as the study model. The choos to use this species was based on the following known facts on albino rats;(i) they have Low prevalence of spontaneously occurring congenital malformation in their fetuses, (ii) they usually have large litter size of between 1-16, (iii) Their gestation period is relatively short compared with other experimental animals as its is 21 days. (Ferreira et al., 2019)

Acquisition of the rats: The 30 albino rat were obtained from the Small Animal Facility for Research and Innovation (SAFARI) in the school of biomedical sciences of Jomo Kenyatta University of Agriculture and Technology (JKUAT).

Determination of sample size: In determining the sample size, the resource equation by Arifin & Zahiruddin, 2017, whose formula is n=DF/k + 1 was used where in this study:- **n** represented the total number of rat dams that formed my sample size. **DF** was the degree of freedom while **k** represented the total number of subgroups. Based on this research equation, the acceptable range of degrees of freedom (DF) was taken to be between 10 to 20. However since a value less than ten may not yield actual significant results and in this case DF of 20 was taken therefore a total number of 30 animals was obtained. This number of animals was considered adequate because, a value of more than 20 has been shown in previous studies to increase the cost of the study without increasing the significance of the results. To effectively evaluate the effects of phenobarbital in terms of the trimester of exposure as well as effects as per varied doses of exposure, the study model had therefore a total of 10 sub-groups of three rats each namely:- Control group, Low dose TM_1, Low dose TM_2, Low dose TM_3, Medium dose TM_1, Medium dose TM_2,Medium dose TM_3 and High dose TM_1, High dose TM_2 and High dose TM_3.

Hence **n** =20/10 +1 = 3 (subjects per group).

Therefore 10 groups x 3 subjects per group = **30 dams.**

Grouping of rats in to study groups: The 30 rats were first randomly assigned into two broad study groups of 3 rats (control) and 27 rats (experimental). To evaluate the intrauterine effect of phenobarbital when administered in varied doses, the 27 rats in the experimental group were subdivided in to three broad study groups of 9 rats each according doses as follows:- ; 9 rats for the high phenobarbital group (HPBG)- that received 41.5 mg/kg/bw; 9 rats for the medium phenobarbital group (MPBG) that received 19.2mg/kg/bw and lastly 9 rats for the low phenobarbital group (LPBG) that received 3.1mg/kg/bw.. To further evaluate the intrauterine effects of phenobarbital when administered on

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differing gestation periods, the 9 rats in each of the three dose groups, the nine rats were further sub-divided into three sub-groups of 3 rats each according to the trimester when they received the phenobarbital treatment as follows; 3 rats for trimester one that received phenobarbital treatment from the gestational day one (GD_1) all the way to gestational day 20(GD_{20}); three rats for trimester two that started receiving phenobarbital treatment from gestational day 7 GD_7 all the way to gestaional day 20(GD_{20}), and 3 rats for trimester three that started receiveing phenobarbital treatment from gestational day 14 (GD_{14}) all the way to gestational day 20(GD_{20}) respectively.

Mating of the rats and determination of their pregnancy: The mating process was done by introducing one male albino rat from third series breed of a pure colony in to the standard cage mating cages with two female rats at 1530 hours (+/-30 minutes).Then the male rats were removed the following morning at 0930 hours (+/- 30 minutes) and returned to their separate cage. The confirmation of pregnancy was done by taking vaginal wash from the mated rats after 24 hours , the presence of polyhedral epithelial cells on the swab was used to denote estrous changes, that marked the first day of gestation (GD_1), (Telendo et al., 2019)

The feeding of the rats:

All rats were fed on standard rodent pellets obtained from Unga feed Limited situated in Thika town that contained weight (g/100g):- 68% starch, 4% cellulose, 5% lipid (corn oil) and 20% protein) and by calories:- 20% proteins, 72% carbohydrates,12% lipids, and 54mg/kg zinc and they also received water ad libitum that was given via rat water bottle every morning at 0830 hours as outlined by (Curfs et al. (2011),

Determination of the phenobarbital doses used in the study.

Phenobarbital tablets obtained from Hikma Pharmaceuticals in USA batch number NSC 9848 bought from government chemist in Nairobi. **A** simple guide for converting animal dosages from human dosages by (A. Nair et al., 2018,Nair & Jacob, (2016) was applied, which states that dose is equally related to body weight. The minimum dose of Phenobarbital in human is 30 mg/day, the medium dose is 185mg/day, and the maximum dose is 400 mg/day. To determine human equivalent dose (HED) for the phenobarbital, average body weight of a human being that is 60 kg was used. These doses were divided by 60kg to obtain HED and 0.5 mg/kg/bw, 3.1 mg/kg/bw and 6.7mg/kg/bw were obtained for low, medium and dose respectively.

After obtaining the human equivalent dose HED, animal equivalent dose (AED) was arrived at by multiplying human equivalent dose (HED) by Km factor which is 6.2 which is equivalent to 3.1mg/kg/bw for the low phenobarbital dose group, 19.2mg/kg/bw for the medium phenobarbital dose group and 41.5mg/kg/bw for high phenobarbital dose. Since the study used low, medium and high dosages, these dosages were arrived at by multiplying the weights of each rats with animal equivalent dose calculated for each category, that is 3.1mg/kg/bw, 19.2mg/kg/bw and 41.5mg/kg/bw respectively.

Reconstituting the doses: Phenobarbital which was obtained in form of tablet (30mg) were dissolved in 10 millimeters of distilled water. The dissolved phenobarbital was then administered to the rats guided by their weights and specific dosage.

Drug administration: all experimental animals received phenobarbital treatment and the phenobarbital treatment was administered as follows:- For all rats that were to receive phenobarbital treatment in trimester one ($TM_{1);}$ treatment was done from gestational day GD_1 all through to gestational day 20(GD_{20}) while those that were to receive the treatment in trimester two ($TM_{2);}$ treatment was done from gestational day GD_7 all through to gestational day 20(GD_{20}) and those that were to receive the treatment in trimester three ($TM_{3);}$ treatment was done from gestational day GD_{14} all through to gestational day 20(GD_{20})

Sacrificing the animals: All the pregnant rats were humanely sacrificed on the gestation day 20^{th} between 0900 hours and 1100 hours by use of concentrated carbon dioxide. The sacrificing of the rats on day 20^{th} was to prevent the mothers from devouring any malformed offspring (Rai & Kaushik, 2018).

Statistical analysis: The parametric data that included fetal weight, crown lump length, head circumference and bi-parietal diameter parameters was collected using a structured a check list. It was then entered into the computer using an excel spreadsheet for windows version 10, this data in the excel spreadsheets was then exported to the Statistical Package for the Social Scientist (SPSS) version 25 for statistical analysis. To determine the teratogenic effects of phenobarbital through comparing these parametric data across and within groups, the multivariate analysis of variance

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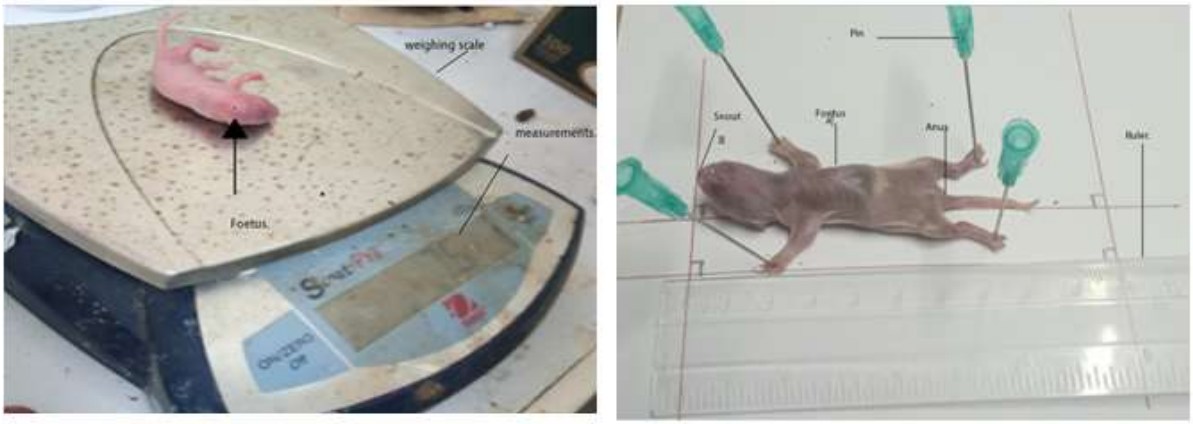
(MANOVA) was applied. To determine the causal and interaction effects Turkey's post hoc multiple comparison tests was applied and all values whose P<0.05 were considered to be statistically significant.

The fetal pregnancy outcome Parameters: Fetal Weight (FW), Crown Lump Length (CRL), Head Circumference (HC), Bi-parietal Diameter (BPD)



A B

Figure 2.1 Showing (A) how the Fetal Weight (FW), were taken using electronic weighing scale (using Scout pro model SPU 4001),(B) how the Crown Lump Length(CRL)from the snout to the base of the tail using a ruler.

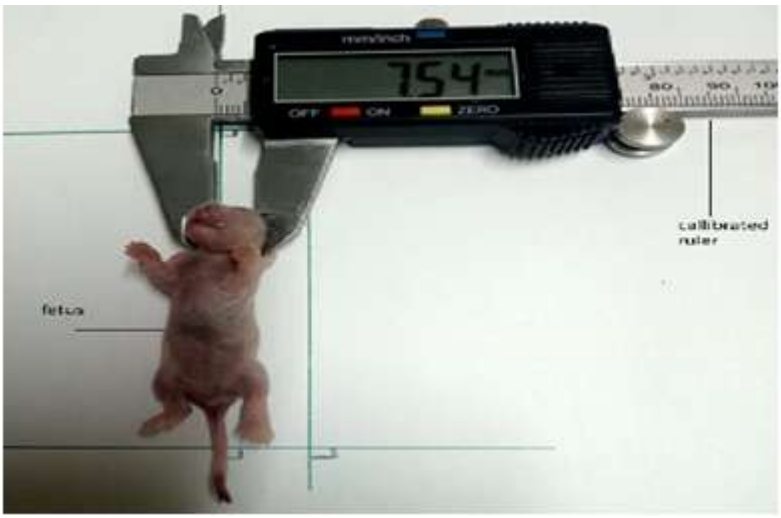


Figure 2.3. Showing picture of how the bi-parietal diameter (BPD) were taken from left parietal region to the right parietal region using a digital vernier caliper from Hercules- sealing product Japan Model 1.13.2017).

III. RESULTS.

The effects of phenobarbital on fetal weight (FW), crown lump length (CRL), head circumference (HC), bi-parietal diameter (BPD).

It was observed that mean fetal weights, mean crown rump length, mean head circumference and mean bi-parietal diameter showed an inverse dose relationship at the same time depicting a direct dose response relationship with the time of exposure. A statistical significant difference ($p<0.05$) was observed when the drugs were given during TM_1 and TM_2 across all phenobarbital treated group (LPBG, MPBG, HPBG). When compared with the control, those who were given the drug during the third trimester did not show statistical significant difference ($P>0.05$) in the LPBG and MPBG except in mean

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phenobarbital fetal weight whereby it was statistically significant

Table 3.1 The intra and inter group comparative means of the fetal body weight, , CRL and bi-parietal diameter of LDGG, MDGG and the HDGG in (TM_1, TM_2 and TM_3) against the control (C).

Study groups	Time of exposure to phenobarbital treatment	Mean phenobarbital fetal BPD (cm)	Mean phenobarbital fetal CRL (cm)	Mean phenobarbital fetal weight (g)	Mean head circumference (cm)
Control	--------	1.4552 ±1.328984	4.347619±0.059809	6.353175±0.064628	4.205925±0.080717
LD Phenobarbital group	TM_1 TM_2 TM_3	0.795185±0.032750* 1.087222±0.023498* 1.442424±0.010926	3.620648±0.046997* 3.798519±0.049464* 4.248485±0.021852	4.651204±0.086878* 5.612963±0.039847* 6.045455±0.024052*	3.492593±0.107650* 3.557618±0.083378 4.187879±0.047625
Md phenobarbital group	TM_1 TM_2 TM_3	0.679167±0.015023* 0.933333±0.016667* 1.3251520.003573*	3.270833±0.006365* 3.584722±0.034219* 4.116061±0.006970	4.034167±0.018276* 5.095833±0.023199* 5.913030±0.008881*	3.212857±0.089834* 3.452976±0.054907* 3.994377±0.015175*
HD phenobarbital group	TM_1 TM_2 TM_3	0.526667±0.039299* 0.850317±0.031999* 1.276323±0.032675*	3.020000±0.035119* 3.462698±0.054578* 3.847090±0.025103*	3.310000±0.095394* 4.665397±0.038921* 5.646032±0.030656*	2.9250000.114564* 3.282778±0.050464* 3.840476±0.021162*

Key: using one way ANOVA with Tukey test on post-hoc t-tests. * indicates significance ($p<0.05$)

Pearson correlation co-efficient (rho p)on fetal parameters in phenobarbital treated groups.

When a Pearson correlation analysis was done within and across the phenobarbital treated groups on the fetal outcomes to establish the correlation significant levels, the strength and the direction of linear relationship on fetal pregnancy outcome namely :- mean fetal weight, mean crown lump length, mean head circumference, mean bi-parietal diameter, it showed a strong linear relationship between all these variables when phenobarbital was administered at TM_1 and TM_2 variables across and within groups.

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Table 3.2 Pearson correlation co-efficient (rho p)on mean fetal weight, mean crown lump length, mean head circumference, mean bi-parietal diameter in phenobarbital treated groups.

		AV.	TM1HC	TM2HC	TM3HC	TM1FW	TM2FW	TM3FW	TM1BD	TM2BD	TM3BD
AV.	r	1	-.930**	-.885**	-.873**	-.965**	-.987**	-.969**	-.909**	-.937**	-.885**
	P		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
TM1HC	r	-.930**	1	.894**	.829**	.978**	.941**	.950**	.972**	.929**	.831**
	p	0.000		0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.001
TM2HC	r	-.885**	.894**	1	.727**	.944**	.945**	.927**	.950**	.982**	.730**
	p	0.000	0.000		0.007	0.000	0.000	0.000	0.000	0.000	0.007
TM3HC	r	-.873**	.829**	.727**	1	.807**	.857**	.900**	.727**	.777**	.909**
	p	0.000	0.001	0.007		0.002	0.000	0.000	0.007	0.003	0.000
TM1FW	r	-.965**	.978**	.944**	.807**	1	.981**	.969**	.986**	.975**	.814**
	p	0.000	0.000	0.000	0.002		0.000	0.000	0.000	0.000	0.001
TM2FW	r	-.987**	.941**	.945**	.857**	.981**	1	.978**	.944**	.979**	.866**
	p	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000
TM3FW	r	-.969**	.950**	.927**	.900**	.969**	.978**	1	.931**	.948**	.849**
	p	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000
TM1BD	r	-.909**	.972**	.950**	.727**	.986**	.944**	.931**	1	.967**	.748**
	p	0.000	0.000	0.000	0.007	0.000	0.000	0.000		0.000	0.005
TM2BD	r	-.937**	.929**	.982**	.777**	.975**	.979**	.948**	.967**	1	.792**
	p	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000		0.002
TM3BD	r	-.885**	.831**	.730**	.909**	.814**	.866**	.849**	.748**	.792**	1
	p	0.000	0.001	0.007	0.000	0.001	0.000	0.000	0.005	0.002	

NB: r is the Pearson's correlation coefficient, P is the p-value, ** indicate significant difference i.e. $p<0.05$

IV. DISCUSSION

This study has established that all the fetal growth parameters that included:- fetal weight, bi-parietal diameters, head lengths, head circumferences, crown-rump length had an inverse dose dependent relationship in that as the phenobarbital dose was increased, those parameters decreased. It further established that all those parameters had a direct time dependent relationship in that when phenobarbital treatment was administered at TM_1, TM_2 and TM_3, these parameters increased directly with time of exposure.

Upon administration of phenobarbital, it was observed that the mean fetal weight significantly decreased with increasing dose of phenobarbital among the treatment groups particularly when given during TM_1 at high dose (3.310000±0.095394) as compared with the control (6.353175±0.064628) ($p < .05$.) (Table 3.1). Mean crown lump length mean was also found to be statistically significantly low in high dose group when phenobarbital was administered during trimester one ($TM_{1)}$ and two ($TM_{2)}$ (3.020000±0.035119, 3.270833±0.006365) respectively compared to that of the control (4.347619±0.059809) (Table 3.1). This current study results are in agreement with those of a study done by (Hamdi et al., 2016) which showed that in valproic acid treated group, an anticonvulsant in the same generation with phenobarbital, there was significant reduction in crown lump length and fetal weight in comparison to the control group. Another study also done by (El-Gaafarawi & Abouel-Magd, 2015) showed that upon administration of anticonvulsants like carbamazepine, which is in the same generation with phenobarbital, there was decreased crown-rump length and fetal body weight in carbamazepine treated groups when it was compared to the control group.

This study also found out that there was statistical significant reduction in head circumference ($p<0.05$) when phenobarbital was administered during the first trimester (TM_1) and at

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high dose (HPBG) 41.5mg/kg/Bw (2.9250000.114564) compared to control group (4.205925±0.080717) (Table 3.1). This findings are in agreement with results of previous study done by (Margulis et al., 2019) which showed that there was reduction in head circumference when anticonvulsants including carbamazepine and valproic acid which are in the same generation with phenobarbital.

V. CONCLUSION

In conclusion, the study established that, Phenobarbital administered during pregnancy have a dose and time dependent influence on fetal weight, bi-parietal diameters, head lengths and crown-rump length. The doses that have been established to have more teratogenic effects are high dose of 41.5 mg/kg/bw (HPBG) and medium dose 19.2 mg/kg/bw (MPBG) especially when administered during first trimester (**TM1**) and second trimester (**TM2**), for all the doses low medium and high doses. Its teratogenic effect to the developing fetus when administered in trimester three has no significant outcomes except when administered in high doses. The most teratogenic dose was however established to be 41.5 mg/kg/bw (HPBG) while most vulnerable gestation period for phenobarbital teratogenicity was the first trimester (**TM1**).

VI. RECOMMENDATIONS

The study recommends that;

1. phenobarbital was found to negatively influence fetal growth and development in rats hence more studies needs to be done on the higher primates to ascertain it's safety in pregnancy in order to curb cases of congenital anomalies which may be associated with it.

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