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The Teratogenic Effects Of Prenatal Exposure To Phenobarbital On Maternal Outcome In Albino Rats (Rattus Norvegicus)

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Abstract:
The intrauterine teratogenic effects of phenobarbital on maternal outcome when prescribed in varied doses remain poorly understood. This study, therefore, set to evaluate the prenatal teratogenic effects of varied doses of phenobarbital on maternal outcome in albino rats when prescribed at different gestational period in albino rats. In conducting this study, a 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 therefore a sample size of 30 Albino rats (Rattus norvegicus) weighing between 150-250 mg were used in this study. These 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). This sample size of 30 albino rats were randomly assigned into two broad study groups of 27 rats experimental and the three rats control group. To determine the intrauterine teratogenic effect of phenobarbital on maternal outcome when administered in varied doses, the 27 rats in the experimental group were further subdivided into three study groups of nine rats each according to the three study doses as follows; nine rats for the high phenobarbital group -that received 41.5 mg/kg/bw; nine rats for the medium phenobarbital group that received 19.2 mg/kg/BW and lastly nine rats for the low phenobarbital group that received 3.1mg/kg/BW. To evaluate the intrauterine teratogenic effects of phenobarbital when administered on differing gestation periods, the nine rats in each of the three study dose categories were further sub-divided into three sub-groups of three rats according to the trimester when they received treatment as follows; three rats that received the treatment from Trimester I (TM_1); three rats that received treatment from trimester II (TM_2) and three rats that received treatment from trimester III (TM_3)respectively. At gestation day 20, all the rats were humanely sacrificed and three fetuses from each rat were selected based on their weights as follows; the first one with the highest weight, another one with the median weight, and the last one with the lowest weight. The parameters evaluated and reported in this study included measurements on the mean terminal placental weights, the mean terminal weight, mean total weight gain, the number of resorbed glands, Dead fetuses and the Litter size. 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 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 increase ($P<0.05$) in the mean terminal placental weights, the mean terminal weight, mean total weight gain parameters 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 during the first trimester (TM_1) when compared with control. Therefore, more studies needs to be done on higher primates to ascertain its teratogenicity.

Keywords: *Albino rats, dead fetuses, litter size, resorbed glands, teratogenic, phenobarbital, resorbed glands.*

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I. Introduction

Phenobarbital is considered first-line therapy for some types of seizures despite the risk of teratogenicity(Holbrook, 2004). It is first generation anticonvulsant drug classified under category D as per Food and Drug Administration (FDA) (Koo & Zavras, 2013). It is commonly used to treat tonic-clonic and partial seizures (Kim et al., 2020) and because of its relatively low cost and favorable cost-efficacy ratio, which is lower than that of any other antiepileptic drug in current use, it most commonly prescribed anticonvulsants medicines (Grinspan et al., 2018) makes the drug affordable and suitable for use in low and middle-income

countries, where cost-effectiveness often supersedes other priorities (Ilangaratne et al., 2012). Although phenobarbital is classified as class-D medicine (i.e. they should not be used during pregnancy) they cannot be withdrawn abruptly because of withdrawal syndrome. Its mode of action is that it increases the amount of time chloride channels are open by binding to GABA receptors consequently depressing the central nervous system. Studies have shown that prenatal exposure of phenobarbital is associated with *maternal outcome* among children born of mother on phenobarbital during pregnancy (Marsot et al., 2014) Its mode of teratogenicity is that it crosses the maternal placental blood barrier (MPBB) because of the fluctuating levels of drug metabolizing enzymes- cytochrome P450 (Whelehan & Delanty, 2019) in pregnancy, together with their low molecular weights Phenobarbital 232.24 g mol-1 (Patocka *et al.,* 2020*). It* also upregulation of cytochrome P450 enzymes leading to formation of 8-oxodeoxyguanine resulting in G.C to T.A transversion. Affects the developing fetal tissue morphogenesis and differentiation with resultant structural malformations *-(Tomson & Battino, 2012*.However, the teratogenic safety of phenobarbital during pregnancy has been controversial because of its unclear teratogenic effects on *maternal outcome,* making it difficult to prescribe (Mifsud, 2010). There is limited data on its teratogenic effects when administered in varied doses and at different window periods on *maternal outcome*. 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 pregnancy.

II. Material And Methods

Study area: 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: In conducting the study a post-test-only control experimental study design was adopted.

Study sample: A pure colony of 30 nulliparous Albino rat dams of the *Rattus norvegicus* species were used as the study model. The choose 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 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 gestational day 20(GD_{20}), and 3 rats for trimester three that started receiving 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 185 mg/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 6.7 mg/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 (100mg) 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 20th between 0900 hours and 1100 hours by use of concentrated carbon dioxide. The sacrificing of the rats on day 20th was to prevent the mothers from devouring any malformed offspring (Rai & Kaushik, 2018).

Statistical analysis: The parametric data that included the mean terminal placental weights, the mean terminal weight, mean total weight gain, the number of **resorbed glands, Dead fetuses and the Litter size** 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 (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.

III. Results

The Effects on How the phenobarbital Influenced the maternal outcome.

In evaluating the maternal pregnancy outcomes, the following parameters were evaluated; (i) Terminal weight (ii) maternal weight gain (iii) placental weight (iv) resorbed endometrial glands, (v) placental weights, (vi) litter size, (vii) number of dead fetuses. This was done by doing the analysis by use of ANOVA. The overall global effects of phenobarbital on maternal pregnancy outcome was assessed by the univariate and bivariate analysis using ANOVA. This study established that following the administration of varied doses of phenobarbital during pregnancy, the mean terminal placental weights, the mean terminal weight, mean total weight gain depicted an inverse dose response relationship in that when the dose of exposure to phenobarbital increased, those parameters also decreased and vice versa

This study found out that there was statistical significant difference in the mean terminal placental weights, the mean terminal weight, mean total weight gain ($p<0.05$) when phenobarbital was administered in high doses (HPBG) in trimester one (TM_1) and trimester two TM_2 compared with that of the control at ($p<0.05$).

In medium phenobarbital dose group (MPBG), it was observed that there was a statistical significant difference ($p<0.05$) in the mean terminal placental weights, the mean terminal weight, mean total weight gain when administered in trimester one (TM_1) compared with that of control. However, there was no significance difference in the mean terminal placental weights, the mean terminal weight, mean total weight gain when phenobarbital was administered in medium doses at TM_2 and TM_3. When phenobarbital was administered at low dose there was no statistical significance difference on those parameters also across all the trimesters compared with that of the control.

It was further noted that, when the mean terminal placental weights, the mean terminal weight, mean total weight gain was compared with the time of exposure, it depicted a higher effect during TM_1, followed by TM_2 then lastly TM_3 (***Table 3.1)***.

Table 3.1: The ANOVA Comparative Findings On How The Phenobarbital Affected The Terminal Placental Weights, The Mean Terminal Weight, Mean Total Weight Gain Between The Treatment Groups Compared With The Controls.

The Study Groups	**Study Groups And Dosage Levels.**	**The Time Of Exposure**	**The Comparative Mean Terminal Weight, Terminal Maternal Weight Gain And Placental Weight For Various Study Groups**		
			Mean Terminal Weight (G)+ SD)	**Mean Maternal Weight Gain(G) + SD)**	**Mean Placental Weight (G) + SD)**
Control.	Control (C) No Treatment	None.	292.1923±. .0287	98.000 ±.0007	0.4378±.0003
The Phenobarbital Treatment Groups	Low Dose Treatment Group (LPB)-[3.1 Mg/Kg/Bw)	TM_1 TM_2 TM_3	247.1205± .0215 264.2559± .0938 293.0472± .0033	51.2717±.0033 57.2645±.0048 87.2643±.0019	0.2906±0.0028 0.3410 ±0.0036 0.3760 ±0.0135
	Medium Dose Treatment Group(MPB)-[19.2mg/Kg/Bw)	TM_1 TM_2 TM_3	**243.2458±.0868*** 249.2110± .0646 260.1454± .0312	**45.3050±.00326*** 55.2890±.0012 69.2835±.00165	**0.2959 ±0.0032*** 0.3239 ±0.0046 0.3691 ±0.0007
	High Dose Treatment Group (HPB) (41.5 Mg/Kg/Bw)	TM_1 TM_2 TM_3	**243.1873± .0513*** **244.1703± .7589*** 267.1454± .0312	**37.3350±.0011*** **50.3262±.0005*** 59.3126±.0008	**0.2318±0.0012*** **0.2777 ±0.0034*** 0.3165 ±0.0012
			F(18,38)= 13.639 P= 0.042	F(18,38)= 33.963 P= 0.049	F(18,38)=156.082 P= 0.001

*Key: The Means Followed 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).*

In evaluating the dose response relationship the phenobarbital treated groups against control on the number of resorbed glands and dead fetuses to assess their level of toxicity in utero, it was observed that the teratogenic effects on the maternal resorbed endometrial glands and the number of dead fetuses depicted a direct doses response relationship across the trimesters in that when the doses were increased, the number of resorbed glands and the number of dead fetuses also increased ***(Figure3.1).*** The mean litter sizes on the other hand was observed to depict an inverse dose relationship in that when the dose was increased the number of litter size decreased. More effects were observed in the high treatment groups in that they had the least number of litter size, highest numbers of both dead fetuses, and the number of resorbed glands. To the contrary these teratogenic outcomes were observed to be least at low doses and particularly lowest when treated at trimester three (TM_3) ***(Figure 3.1*)**.

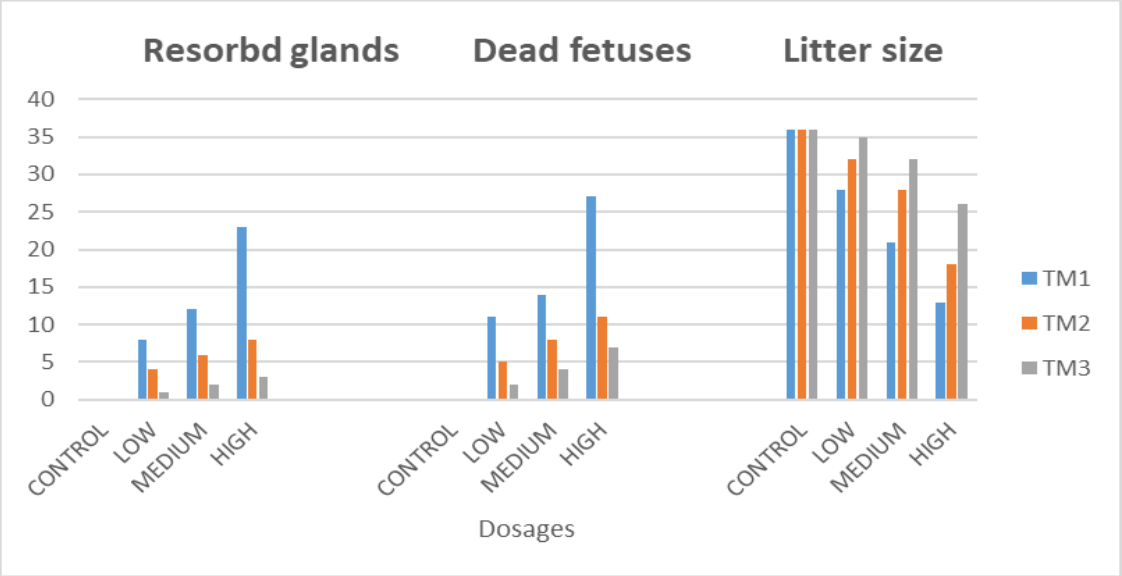

Figure 3.1: The Bar Graphs Showing Litter Size, Dead Fetuses and Resorbed Glands

IV. Discussion

In abeyance to the principles of teratogenesis where the *in-utero* environmental toxicity is a predictive pointer to the fetal teratogenic perturbations that can interfere with the normal fetal growth and development *in-utero*, an evaluation of the maternal and fetal pregnancy outcomes was carried out. The maternal pregnancy outcome parameters evaluated included; (i) Terminal weight (ii) maternal weight gain (iii) placental weight (iv) resorbed endometrial glands, (v) placental weights, (vi) litter size, (vii) number of dead fetuses. This study established that terminal weight, maternal weight gain and placental weight were significantly worse ($P<0.05$) as compared with those of the control (***Table 3.1)***. The observed reduction in maternal weight gain treads and placental weights could be attributed to the interference of the phenobarbital to the maternal nutritional status that could be occasioned by the gastrointestinal irritations, or Nutrients mal-absorptions in the intestines while the reduction in litter sizes, increased number of dead fetuses, as well as the increase in the number of the resorbed endometrial glands (***Figure 3.1***) could be attributed to inhibitory effects of the phenobarbital in the closure of the phenobarbital plus their metabolites in the blood placental barriers. These findings are in line with (Ritchie *et al*., 2019) who made similar observations. These findings could as well be due to several side effects of the phenobarbital like the maternal catalepsy or anorexia that are some of the most commonly reported side-effects of anticonvulsant therapy as reported by (Venâncio *et al*., 2014;(Harden et al., 2009)(Harden et al., 2009) Kaplan & Demir, 2021). Similarly, a study by (Gupta, 2016) observed that maternal food intake was reduced up to 14.61 % in gabapentin and 22.29 % in valproic acid treatment while maternal body weight deficit was 34.55 % in gabapentin and 38.73 % in valproic acid exposed subjects (both drugs with the same mode of action with phenobarbital) when administered at higher doses respectively.

V. Conclusion

In conclusion, the study established that, phenobarbital administered during pregnancy have a dose and time dependent influence on maternal outcome. The doses that have been established to have more teratogenic effects are high dose (HPBG) especially when administered during first trimester (**TM1**) for all the doses low medium and high doses. Its teratogenic effect to the developing fetus when administered in second and third trimester has no significant outcomes except when administered in high doses. The most teratogenic dose was however established to be (HPBG) while most vulnerable gestation period for phenobarbital teratogenicity was the first trimester (**TM_1**).

VI. Recommendations

The study recommends that;

a. Phenobarbital was found to have effects on maternal outcome 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.
b. For mothers who got pregnancy while on medium and high doses of phenobarbital, the doses should be adjusted to low dose as other safer drugs are introduced.

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