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The Intra-Uterine Stereological Tratogenic Effects Of Phenobarbital On Fetal Kidneys In Albino Rats (Rattus Norvegicus)

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Abstract:
The stereological teratogenic effects of phenobarbital on the developing fetal kidneys when prescribed in varied doses remain poorly understood. This study, therefore, set to evaluate the prenatal stereological teratogenic effects of varied doses of phenobarbital on fetal kidneys 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 stereological teratogenic effect of phenobarbital 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 stereological 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. Their kidneys were then harvested for stereological analysis. The stereological parameters evaluated and reported in this study included measurements on fetal kidneys weights, kidney volume and volume densities of both cortex and medulla of the fetal kidney structures. 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 fetal kidney stereological 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, cavalieri volume, medullary density, teratogenic, total kidney volume, phenobarbital*

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I. Introduction

Phenobarbital (PB) 1st generation anticonvulsant medicines category D as per FDA (Ilangaratne et al., 2012). It most commonly prescribed anticonvulsants medicines (Ornoy, 2006) because of its relatively low cost and favorable cost-efficacy ratio, which is lower than that of any other antiepileptic drug in current use, 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. 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 kidney tissue morphogenesis and differentiation with resultant structural malformations *-(Tomson & Battino, 2012).* Its histomorphological and histostereological teratogenic effects to the kidney in dose and time dependency is not clear hence prompt this research (Al-bakri *et al.,* 2016). Globally, Kidney diseases are currently on the increase and are among the leading cause of death *(*Hasan *et al.,* 2018, *Warady & Chadha, 2007)* - WHO report of 2021 - 11-13% *(*Masalskienė *et al.,* 2021), Sub Saharan Africa and reported an overall prevalence of 15·8% (Matsha & Erasmus, 2019) with 3.7% (2.7–5.1%) in Kenya (Muiru et al., 2020). In particular, studies have shown that prenatal exposure of phenobarbital is associated with *fetal* renal dysgenesis among children born of mother on phenobarbital during pregnancy (Al-bakri *et al.,* 2016). Though, phenobarbital is known to have teratogenic effects to the fetal kidneys, there is paucity of data on their effects on the histomorphological and histostereological differentiation of the fetal kidneys at different gestational period and in varied doses.

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 stereological parametric data that included total kidney volume by use of both water immersion method (WIM) and calvarieli method of point counting, volume densities of both cortex and medulla of the fetal kidney structures 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.

The stereological parametric data: total kidney volume, volume densities of both cortex and medulla of the fetal kidney.

Stereological analysis.

Estimation of total kidney volume using Archimedes principle.

After removing the kidneys from both the control and the experimental group, Archimedes principle was applied to determine the total kidney volumes (Archimedes volume). This was done by inserting the whole kidney tissue into graduated beakers containing normal saline, and the amount of fluid displacement upward was measured to determine the Archimedes kidney volumes. The normal saline displaced by the kidney represented the (Archimedes volume) actual kidney volume as described by a study by (Hughes, 2005). When determining the total volume, cavalieri stereological method was applied and the volumes determined using Archimedes as the reference volumes.

Determination of stereological total kidney volume and volume densities using cavalieri and point counting methods.

Combination of both the cavalieri and point-counting method were employed to determine stereological total kidney volume and the estimation of the volume densities of both the cortical and medullary layers of the kidney structures. The following steps were followed a) Preparation of kidney cavalieri sections of 5µm thick. b) Selection of the spacing for the point probe. c) The point probe was then tossed randomly onto each section. d) STEPnizer stereology tool was used to count the number of points that hit the region of interest for example when determining the cortical density, the researcher was interested on counting the points that hit only at the cortex (region of interest) e) All sections were processed keeping a tally of counts per section. f) The volume was then calculated. Twenty sections of 5µm thickness from each longitudinal kidney section were selected by systematic uniform random sampling, (Bural *et al.,* 2015). The researcher then used the microscope's stage Vernier to view the images at magnification of X40 and X100. The volume of the kidney was then obtained by multiplying the number of points that hit the region of interest (kidney) X the area per point and the slice thickness (5 micrometers).

Volume = no of points x area per point x slice thickness.

STEPnizer software was used to do the point counting. The digital images of the kidney tissue were captured using stereological sampling rules with same magnification and saved in the jpeg (joint photograph expert group) file format at adequate resolution. Images taken both for the experimental and control groups were organized appropriately and saved in one folder. A calibrated scale bar was added to one image of a batch to define the real dimensions of the structures under investigation, and placed on left hand side. Where stereological estimation required the use of a guard area it was set and were not be changed in the course of the whole experiment to obtain consistent results **(figure 3.1)**

III. Results

The study findings on the mean total kidney volume, volume densities of both cortex and the medulla are as follows: -

The influence of phenobarbital **on the total fetal kidney volume**

The study findings on fetal kidney volume depicted a direct dose response relationship in that when the dose of exposure to phenobarbital increased, the mean total kidney volume also increased and vice versa (table 3.1)

This study found out that there was statistical significant difference in mean total kidney volume using Calvarieli method) ($p<0.05$) 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 mean fetal kidney lengths when administered in trimester one (TM_1) compared with that of control. However, there was no significance difference in total kidney volume 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 the total kidney volume also across all the trimesters compared with that of the control.

It was further noted that, when the total kidney volume was compared with the time of exposure, it depicted a higher effect during TM_1, followed by TM_2 then lastly TM_3.

On the mean medullary density, it was observed that when phenobarbital was administered in high doses, there was a statistical significant difference ($p<0.05$) was observed during trimester one (TM_1) and trimester two (TM_2) compared with that of control. When it was administered in medium doses, statistical significant difference ($p<0.05$) was noted during trimester one (TM_1) only while in low doses there was no statistical significance difference across all the trimesters compared with that of the control. It was also observed t that, when the mean medullary density 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: A Comparative reference, calculated and percentage shrinkage on total mean fetal kidney volume using (WIM) and cavarieli method in the LPBG, MPBG and the HPBG treated at TM_1, TM_2 and TM_3 against the control.

The study groups	The Study groups and Dosage level	The time of exposure to			
			Mean Medullary density (MD) (mm^3)± SD)	Mean Cortical density (CD) (mm^3) ± SD)	Mean Carvalieli volume (CV) (mm^3) (cm) ±

		treatment			SD
Control	Control (C) No treatment		.1591±.0026	0.0794±0.0014	.2383±.0036
The phenobarbital treatment groups	Low phenobarbital group (PB)- [3.1 mg/kg/bw)	TM_1	.2086±.0140	.0792±.0012	.2958±0032
		TM_2	.1949±.0020	.0732±.0015	.2808±.0033
		TM_3	.1844±.0079	.0658±.0014	.2716±.0034
	Medium phenobarbital group (PB)-[19.2mg/kg/bw)	TM_1	.2221±.0037	.0576±.0016	.3181±.0041*
		TM_2	.2011±.0033	.0545±.0019	.3089±.0044
		TM_3	.1939±.0012	.0519±.001	.3010±.0041
	High phenobarbital group (PB) (41.5 mg/kg/bw)	TM_1	.2444±.0035*	.0486±.0012	.3589±.0007*
		TM_2	.2364±.0039*	.0436±.0017	.3461±.0004*
		TM_3	.2217±.0016	.0405±.0018	.3314±.0009

*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).*

IV. Discussion

In assessing how the phenobarbital affected the medullary density and carvalieli volume, this current study established that the administration of varied doses of phenobarbital during pregnancy caused a significant (P<0.05) increase in medullary density and carvalieli volume when compared with the control. It was further noted that the in phenobarbital treatment groups the medullary density and carvalieli volume were highly affected when the treatments were initiated at TM_1 at high dose and TM_2 compared to control (Table 3.1).

In this study, the increase in fetal kidney volumes noticed in phenobarbital treatment groups could be due to the fact that phenobarbital up-regulates CYP2B (Mifsud, 2010). Oxygen stress could be generated via that pathway (Deutsch *et al*., 2001). Leading to renal damage. Tubular injury causing edema and hydrophic changes hence increase in the kidney volume- especially the medullary density. The cortical density slight reduction is due to the reduction in the number of the glomeruli though there was enlargement of the bowman's space. All these observed changes could result in increased kidney volume. This concurs with previous studies done on sodium valproate which also has the same mode of action as phenobarbital showed some changes in the kidney for example:-, swelling of renal tubules, blood vessels congestion, hydropic changes on the proximal and distal convoluted tubules *(El-Shenawy & Hamza, 2016).* Other past study done by (Mifsud, 2010) also found out that kidney sections acquired from fetuses whose mothers were managed with oxcarbazepine (an anticonvulsant) from day 17 to day 20 of gestation showed changes histologically which included edema between glomeruli and the tubules, bowman's capsular spaced were widened leading to increase in the volumes of the kidneys.

V. Conclusion

In conclusion, the study established that, phenobarbital administered during pregnancy have a dose and time dependent influence, total kidney volume, volume densities of both cortex and medulla of the fetal kidney. 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 teratogenic effects on the fetal kidneys 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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