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The Intra-Uterine Stereological Tratogenic Effects of Phenytoin on Fetal Kidneys in Albino Rats (*Rattus Norvegicus*)

Jennifer Segut[1], Joseph Kweri[2], Ann Mwangi[3], Caroline Sigei[4], James Kanyoni[5], Peris Macharia[6], Walter Rono[7], Cyrus Kamau[8], Anne Njoki[9], Cynthia Chebii[10], Jane Kuria[11], Joseph Wachira[12], Christopher Mramba[13], Jane Karanja[14]

[1-13](Department of Human Anatomy, School of Medicine (SOMED), College of Health Sciences (COHES) Jomo Kenyatta University of Agriculture and Technology (JKUAT) Kenya.
[14](School of Nursing (SON), College of Health Sciences (COHES) Jomo Kenyatta University of Agriculture and Technology (JKUAT) Kenya.

Abstract:
Background*: The teratogenic stereological effects on the fetal kidney development after exposing to differing doses of phenytoin remain poorly elucidated. This study, therefore, set to evaluate the intrauterine stereological teratogenic effects of varied doses of phenytoin on fetal kidneys in albino rats when prescribed at different gestational period in albino rats as the experimental model.*
Materials and methods*: In conducting this study, a post-test only control experimental study design was used. A resource equation for One-way Analysis of Variance (ANOVA) was applied to determine the sample size and therefore a sample size of thirty Albino rats (Rattus norvegicus) weights ranging from 150-250 mg were used in this study. These 30 albino rat were hence obtained from the school of biomedical sciences of Jomo Kenyatta University of Agriculture and Technology (JKUAT) at the Small Animal Facility for Research and Innovation (SAFARI). This sample size of 30 albino rats were randomly allocated into two wide study categories of 27 rats experimental and the 3 rats control group. To assess the intrauterine stereological teratogenic effect of phenytoin when administered in differing doses, in experimental group, the twenty seven rats were still split into 3 study categories consisting of 9 rats each depending on the three study doses of low, medium, and high phenytoin doses applied in the study hence; 9 rats for the high phenytoin group -that received 124 mg/kg/BW; 9 rats for the medium phenytoin group whereby 62 mg/kg/BW was prescribed and lastly 9 rats for the low phenytoin group that received 31mg/kg/BW. To gauge the intrauterine teratogenic stereological end results of phenytoin when administered on varying incubation periods, the nine rats in each of the 3 study dose categories were still split into 3 small groups of 3 rats depending on the trimester when they received treatment as follows; 3 rats that received the treatment from Trimester I; 3rats that received treatment from trimester II and 3 rats that received treatment from trimester III 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 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 parametric data that included fetal kidneys weights, total kidney volume, medullary volume and corical volume density of the fetal kidney structure which was obtained by using carvalieri technique of point counting and water immersion method (WIM). 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) to analyze. 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.*
Results: *The results of this research has shown that there was statistical significant increase ($P<0.05$) in fetal kidney stereological parameters especially during the first trimester. Phenytoin administered prenatally had a time and dose dependent impact on fetal parameters in that effects were more with (HPTG)-124 mg/kg, and during the first trimester (TM_1) when compared with control.*
Conclusion: *Therefore more studies needs to be done on higher primates to ascertain its teratogenicity prenatally.*
Key words*: stereological, teratogenic, albino rats, phenytoin.*

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I. Introduction.

Phenytoin is a hydantoin derivative (Patocka et al., 2020) used as an anticonvulsant (Mathews et al. 2019)*).* It is considered first-line therapy for some types of seizures despite the risk of dose-related toxicity, (Al-Quteimat, 2016). It is commonly used to treat partial and tonic-clonic seizures, epilepsy, complex partial seizures, status epilepticus *(Abou-Khalil, 2016)* (Gupta *et al* 2021), and eclampsia (Ozcan et al., 2015) chronic renal failure has been considered a major public health issue worldwide and is becoming one of the leading causes of mortality and disability *(*Hasan *et al.,* 2018). A number of patients with chronic kidney disease worldwide is markedly increasing, *(Warady & Chadha, 2007)* Studies have shown that the most frequent cause of end-stage kidney disease in adolescents and children is a congenital malfunction of the urinary tract and kidney *(*Hattori *et al.,* 2015*).* Though all anticonvulsants are known to have teratogenic effects on the fetus(Pennell, 2016), Phenytoin is commonly prescribed anticonvulsant(used to manage some conditions in pregnancy like eclampsia, epilepsy (Al-Quteimat, 2016). However, the teratogenic safety of phenytoin during pregnancy has been controversial because of its unclear stereological teratogenic effects on fetal kidneys, making it difficult to prescribe(Ashtarinezhad *et al.,* 2015). There is insufficient data on its stereological teratogenic effects when given in different dosages and at varrying incubation periods in fetal kidney weights, total kidney volume, cortical volume and medullary volume density of the fetal kidney structures. This study aims to create data that can help researchers carry out further studies on non-human primates that have a closer biological relationship to humans in order to guide the clinicians in prescribing phenytoin prenatally.

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 performed 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 selected.

Study sample: A pure colony of thirty non-parturient 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 Jomo Kenyatta University of Agriculture and Technology school of biomedical sciences (JKUAT) at Small Animal Facility for Research and Innovation (SAFARI) 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: 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. Depending 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 which is greater than 20proved in previous studies to elevate the study cost while the result's significance was not increased. To effectively evaluate the effects of phenytoin 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 2 wide study groups that is:- control group (3 rats) and experimental group (27 rats). To determine the intrauterine effect of phenytoin when administered in varied doses, the twenty seven rats from the experimental category were split in to 3 wide study groups consisting of nine rats in every group depending on the amount of phenytoin that is ;- nine rats for the high phenytoin group (HPTG)- that received 124 mg/kg/bw; 9 rats for the medium phenytoin group (MPTG) where 62mg/kg/bw was prescribed and lastly 9 rats for the low phenytoin group (LPTG) that received 31mg/kg/bw. To additionally determine the intrauterine sequel of phenytoin when administered on differing incubation periods, nine rats in each of the 3 dose categories, the nine rats were still split into 3 small groups of 3 rats each depending on the trimester when they received the phenytoin treatment as follows; 3 rats for trimester whereby phenytoin was administered from the gestational day one (GD_1) all the way to 20^{th} day of gestation (GD_{20}); three rats for trimester two that started receiving phenytoin drug from 7^{th} day of gestation GD_7 all the way to gestational day 20(GD_{20}), and 3 rats for trimester three that started receiving phenytoin treatment from 14^{th} day of gestation (GD_{14}) all the way to 20^{th} day of gestational (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 2 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. Vaginal waash was performed to confirm the pregnancy 24 hours after mating and the polyhedral epithelial cells present on the swab was used to denote estrous changes marking day 1 of conception (Telendo *et al*., 2019)

The feeding of the rats:

The standard rodent pellets collected from Unga feed Limited loc in Thika town was used to feed rats and were also given water without restriction (Curfs *et al*. (2011).

Determination of the phenytoin doses used in the study.

Phenytoin tablets obtained from Aurobindo Pharma - Milpharm Ltd in india batch number AUST R 297268 bought from government chemist in Nairobi. The minimum dose of Phenytoin in human is 300 mg/day, the medium dose is 600 mg/day, and the maximum dose is 1200 mg/day. To determine human equivalent dose (HED) for the Phenytoin, average body weight of a human being that is 60 kg was used. These doses were divided by 60kg to obtain HED and 5 mg/kg/bw, 10 mg/kg/bw and 20 mg/kg/bw were obtained for low, medium and dose respectively.

The researcher first calculate the human equivalent dose (HED), then animal equivalent dose (AED) was arrived at by multiplying human equivalent dose (HED) by Km factor which is 6.2 which is equivalent to 31mg/kg/bw for the low phenytoin dose category, 62mg/kg/bw for the medium Phenytoin dose category and 124mg/kg/bw for high phenytoin 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 31mg/kg/bw, 62mg/kg/bw and 124mg/kg/bw respectively.

Reconstituting the doses: Phenytoin which was obtained in form of tablet (100mg) were dissolved in 10 millimeters of distilled water. The dissolved phenytoin was then administered to the rats guided by their weights and specific dosage.

Drug administration: all experimental animals received phenytoin treatment and the phenytoin treatment was administered as follows:- For all rats that were to receive phenytoin treatment in trimester one ($TM_{1);}$ treatment was done from the first day of conception all the way to 20^{th} day while those that were to receive the treatment in 2n trimester ($TM_{2);}$ treatment was done from 7^{th} day of conception all through to 20^{th} day and those that were to receive the treatment in 3^{rd} trimester ($TM_{3);}$ treatment was done from 14^{th} day of conception to 20^{th} day.

Sacrificing the animals: All the gravid rats were humanely slaughtered 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 one so that the mothers will not feed on the malformed fetuses (Rai & Kaushik, 2018).

Statistical analysis: The stereological parametric data that included fetal total kidney volume, kidneys weights, which was obtained by using carvalieri method of point counting and water immersion method (WIM), volume density of medulla and cortex of the fetal kidney was collected using a structured a check list. The data was then fed 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 phenytoin 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: Kidneys weights, total kidney volume, medullary and cortical volume densities.

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). It was done by dipping the entire kidney in to glasses that were graduated filled with normal saline and the Archimedes kidney volumes were arrived at by measuring the amount of fluid displacement upward (Hughes, 2005).

When determining the total volume, cavalieri stereological method was applied and the volumes determined using Archimedes as the reference volumes.

Calculation of stereological volume densities and total kidney volume using point counting and cavalieri technique.

Combination of both the cavalieri and point-counting method were employed estimate the medullary density, cortical volume density and determination of total kidney volume a) cavalieri sections measuring 5µm thick prepared b) spacing selected for the probe, c) tossing of the point probe randomly on to 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 (area of interest) e) total counts per section done after processing all sections. f) Calculation of volumes. Systematic uniform random sampling was used to 20 sections from each kidney each section measuring 5µm thickness whreby the kidneys were cut longitudinally (Bural *et al.,* 2015). The images were the viewed using microscope stage vernier at magnification of X4, 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. **(figure 2.1)**

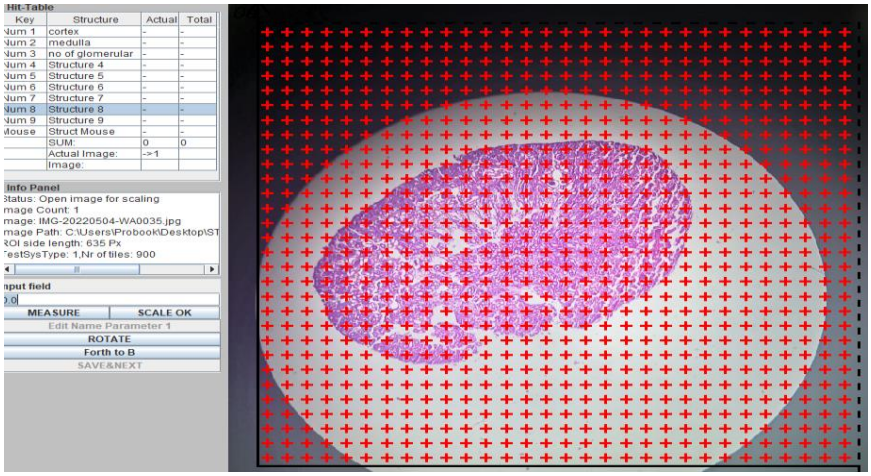

Figure 2.1: showing equidistant point counting grid laid over a cavalieri 5µm section image of a 20 day old fetal kidney from control group (mag X 40) (The points that hit the renal tissue are demonstrated by purple in the region marked red).

III. Results.

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

The effects of phenytoin **on fetal kidney size and weight.**

This study depicted a significance difference between phenytoin treated groups (within and between the groups) comparison of the fetal kidney weight and size compared with the control. It was detected that the mean fetal kidney weights and mean fetal kidney size depicted a direct dose response relationship.

In high phenytoin group (HPTG), it was noted that there was a statistical significant difference ($p<0.05$) in mean fetal kidney weights in trimester one (TM_1) (0.041421±0.002509) and trimester two TM_2 (0.038257±0.000555) compared with that of the control (0.020556±0.001111). There was also significance difference ($p<0.05$) between fetal kidney weight of medium dose phenytoin group (MPTG) when administered in trimester one (TM_1) (0.033889±0.001111b) compared with that of control. However there was no significance difference in fetal kidney weight when phenytoin was administered in medium doses at TM_2 (0.031211±0.000675) and TM_3 (0.031667±0.000000) and also across all the trimesters when phenytoin was administered at low dose (LPTG) compared with that of the control. In addition, when the total kidney weight was compared depending on the exposure time, it delineated a higher effect during TM_1 (0.366586±0.001497), followed by TM2(0.355322±0.001923) then lastly TM3(0.344591±0.002212).

When phenytoin was administered in high doses (HPTG) there was statistical significant difference in mean fetal kidney length in trimester one (0.366586±0.001497) and trimester two (0.355322±0.001923) compared with that of the control (0.344591±0.002212) at ($p<0.05$). In medium phenytoin dose group (MPTG), it was noted that there was a statistical significant difference ($p<0.05$) in mean fetal kidney lengths when administered in trimester one (TM1) (0.033889±0.001111) compared with that of control. However there was no significance difference in fetal kidney length when phenytoin was administered in medium doses at TM_2 (0.031211±0.000675) and TM_3 (0.031667±0.000000). When phenytoin was administered at LPTG there was no significant difference on the mean kidney length compared with that of the control. To add on that, when the total kidney length was compared with exposure time, it was noted that on the mean fetal kidney lengths more effects were observed when phenytoin was administered at TM_1 followed by TM_2 then least effects in TM_3 **(Table 3.1)**

Table 3.1: The intra and inter group comparative means fetal Kidney weight and Kidney length for LPTG, MPTG and the HPTG treated at 1st, 2nd and 3rd trimester against the control.

Study groups	Time of exposure to phenytoin treatment	Mean phenytoin fetal kidney weight (g)	Mean phenytoin fetal kidney length (mm)
Control	--------	0.020556±0.001111 [a]	0.262833±0.001925 [a]
LD phenytoin group	TM_1 TM_2 TM_3	0.028333±0.000000[a] 0.026556±0.000111[a] 0.025556±0.000556[a]	0.312044±0.005803 [a] 0.281667±0.001925 [a] 0.267167±0.001925 [a]
Md phenytoin group	TM_1 TM_2 TM_3	**0.033889±0.001111b** * 0.031211±0.000675 [a] 0.031667±0.000000 [a]	**0.317928±0.003968b*** 0.299378±0.005830 [a] 0.301627±0.002451 [a]
HD phenytoin group	TM_1 TM_2 TM_3	0**.041421±0.002509c *** **0.038257±0.000555b** * 0.037316±0.000551[a]	**0.366586±0.001497c*** **0.355322±0.001923b*** 0.344591±0.002212 [a]

Applying one way analysis of variance (ANOVA), the means that are found in the same column and are followed by the same alphabetical letter are not statistically significant at (P>0.05).
**** designates values that were statistically significant when subjected to Tukey post-hoc t-tests (p<0.05).***

The effects of phenytoin on the total fetal kidney volume

The study findings showed that the mean total kidney volume increased when the amount of exposure to phenytoin was 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 phenytoin was prescribed in high doses (HPTG) in trimester one (TM_1) (**0.345494±0.002654b**) and trimester two TM_2 (0.336121±0.001647b) compared with that of the control (0.262667±0.001926) at ($p<0.05$).

In medium phenytoin dose group (MPTG), it was noted that there was a statistical significant difference ($p<0.05$) in mean mean kidney volume when administered in trimester one (TM1) (0.325267±0.001926) compared with that of control. However there was no significance difference in mean kidney volume when phenytoin was administered in medium doses at TM_2 (0.316017 ± 0.001926) and TM_3 (0.309667±0.001926). When phenytoin was administered at low dose (LPTG) there was no significant difference on the mean kidney volume also across all the trimesters compared with that of the control **(Table 3.2)**

Table 3.2: Table showing total mean fetal kidney volumes in the LPTG, MPTG and the HPTG treated at 1st, 2nd, and 3rd trimester against the control.

Study groups	Time of exposure to phenytoin treatment	Mean total fetal kidney volume (Calvarieli method) +	Mean total fetal kidney volume (WIM) +SEM	Mean kidney medulla volume density + SEM	Mean kidney cortical volume density + SEM
Control	--------	0.262667±0.001926a	0.238350±0.002077a	0.159052±0.001528a	0.079450±0.000692a
LD phenytoin group	TM_1 TM_2 TM_3	0.302172±0.001926a 0.288017±0.001926a 0.279517±0.001926a	0.295838±0.001899a 0.280767±0.001908a 0.271583±0.001997a	0.197487±0.001511a 0.205970±0.009726a 0.184406±0.004613a	0.098613±0.000633a 0.093589±0.000636a 0.090528±0.000666a
Md phenytoin group	TM_1 TM_2 TM_3	**0.325267±0.001926b*** 0.309667±0.001926a 0.316017±0.001926a	**0.322833±0.001920b*** 0.313400±0.002117a 0.305817±0.001975a	**0.215544±0.001354b*** 0.209092±0.001556a 0.204153±0.001555a	0**.107611±0.000640b*** 0.104467±0.000706a 0.101939±0.000658a
HD phenytoin group	TM_1 TM_2 TM_3	**0.345494±0.002654c*** **0.336121±0.001647b*** 0.331533±0.000520c*	**0.358932±0.000410c*** **0.346055±0.000270b*** 0.331350±0.000548b*	**0.244402±0.002043c*** **0.236423±0.002264b*** 0.221669±0.000922b*	**0.117657±0.000589c*** **0.113921±0.000506b*** 0.110450±0.000183b*

Applying one way analysis of variance (ANOVA), the means, followed by the same letter in a column are not statistically different at (P>0.05).
**** indicates values that were statistically significant (p<0.05) when subjected to Tukey post-hoc t-tests***

IV. Discussion

In this study, it was observed that among the treatment categories, the mean fetal kidney weight significantly increased with increasing amount of phenytoin especially when administered TM_1 at high dose (0.041421±0.002509) as compared with that of the control (0.020556±0.001111) at ($p < .05$.) (Table 3.1).

This may be due to interference of renal development for example; causing changes embryonic blood pressure and blood flow leading to kidney injury causing edema between glomeruli and the tubules, vacuoles in the cytoplasm (Danielsson & Skold, 2001).These results help explain the increase in the total kidney weight due to edema. This is in accordance with results of past study done by (Elshama et al., n.d. (2015) who also found out that when carbamazepine, (anticonvulsant in the same generation with phenytoin) was administered to pregnant mice, it showed that weight of fetal kidneys had a significant increase which was dose dependent.

Mean fetal kidney length was also found to be statistically significantly high in high dose group when phenytoin was prescribedS during TM_1 (0.366586±0.001497) and TM_2 (0.355322±0.001923) respectively compared to that of the control (0.262833±0.001925) (Table 3.1). This findings could be assigned to another study results done by (Katsiki *et al*., 2014) that found out that phenytoin disrupts blood vessels of the developing and develop fetal structures as a result of creation of reactive oxygen species within the embryo that is during re-oxygenation leading to kidney injury as a result of free radicle damage which could lead to increase in kidney size. This is in agreement to study done by*(El-Shenawy & Hamza, 2016)* who also found out that when sodium valproate which is also from first-generation was administered, it showed some changes in the kidney for example:- interstitial hemorrhage, cloudy swelling of renal tubules, proliferation of mesangial cells,

hyper cellular glomeruli, and blood vessels congestion, hydropic changes on the proximal and distal convoluted tubules.

This study also found out that there was statistical significant increase in kidney volume ($p<0.05$) when phenytoin was prescribed during the trimester one (TM_1) and at high dose (HPTG) 41.5mg/kg/Bw (0.345494±0.002654) compared to control group (0.262667±0.001926) (Table 3.2). In this study, the increase in fetal kidney volumes noticed in phenytoin treatment groups could as a result of the fact that phenytoin induces hypoxia to the fetus which lead to disrupted of blood vessels developing structures including the kidney leading to kidney injury*(Danielson et al., 1992).* This is in consensus with results of past study done by *(*Hamdi *et al.,* 2017*)* who 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, vacuoles in the cytoplasm and deterioration of the outer borders of cell lining the tubules.

V. Conclusion.

Finally, the research confirmed that, phenytoin administered to expectant mothers have a time and dose dependent impact on cortical volume density and medullary volume density of the fetal kidney, kidney weights and total kidney volume. The doses that have been established to have more teratogenic effects are high dose (HPTG) especially when administered during first trimester (**TM_1**) especially when the phenytoin was prescribed during the first trimester. The most teratogenic dose was however established to be (HPTG) while most vulnerable gestation period for phenytoin teratogenicity was the first trimester (**TM_1**).

VI. Recommendations

The study recommends that;

a. Phenytoin 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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