THE HISTOSTEREOLOGICAL EFFECTS OF VARIED DOSES OF PHENYTOIN ON THE DEVELOPMENT OF FETAL HEART WALLS, INTER-ATRIAL AND INTERVENTRICULAR SEPTUM IN FETUSES OF ALBINO RATS (RATTUS NORVEGICUS)

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IJASR 2021
VOLUME 4
ISSUE 3 MAY – JUNE **ISSN: 2581-7876**

Abstract: Maternal use of phenytoin during pregnancy has been shown to disturb the development of the fetal heart structures hence increasing the risks of structural cardiovascular defects during childhood. This may also be a contributor to the increasing cases of adult CVS diseases such as cardiac dysfunction, coronary heart diseases, myocardial infarction among others. Though data exist on possible teratogenic effects of phenytoin to the developing heart structures, there is paucity of data on anatomical histo-quantitative effects of phenytoin on the developing fetal heart structures. In carrying out the study a total of 30 female albino rats' dams weighing between 150-250grams were used as the experimental model whereby they were randomly assigned into two broad study groups of 3 controls and 27 experimental group. The 27 rats in the experimental group were further subdivided into three study groups of 9 rats each into low, mediam and high phenytoin groups where the low phenytoin groups received 31mgs/kg/bw, medium (62mg/kg/bw) and high phenytoin group(124mg/kg/bw)]. In order to determine the effects of phenytoin when exposed on different gestational periods, the 9 rats in each of the three study groups of low, medium and high phenytoin groups were further subdivided into three sub- groups of 3 rats each as per the three trimesters of study; trimester one (T1) trimester two (T2), and trimester three (T3). All rats both experimental and the control group received standard rodent pellets from Unga limited and water adlibitum while all rats in the experimental groups further recieved varying doses of phenytoin based on their study category and as per the trimester of exposure. All the pregnant rats were humanely sacrificed on the 20th day of gestation and the fetuses were then harvested and weighed. A total of 90 fetuses, three fetuses with low, medium and high weights objectively obtained from each dam were hence used in this study. The three fetuses in each group were sacrificed and their hearts harvested and processed for histo-stereological analysis. Data was then recorded in the data capture sheets, entered into Microsoft excel and analyzed using statistical packages for social science version 23 (SPSS VERSION23) for windows. The intergroup and intragroup comparisons were statistically analyzed using one way analysis of variance (ANOVA) and all P-values less than 0.05 were taken to be statistically significant. The mean total fetal heart volume decreased with increasing phenytoin dose when administered particularly in TM1 and TM2 when compared to the controls.

Keywords: Phenytoin, Anticonvulsant, Teratogenic, histostereology, fetal heart.

1.1 Introduction

Phenytoin also called Dilantin is an anti-seizure medication and it is used when preventing partial and tonic chronic seizures and it has a molar mass of 252.268 g/ml with a formula C_{15} H_{12} N_2 O_2(Patocka *et al.*, 2020). Phenytoin exerts its anticonvulsant effects by binding to specific site on voltage dependent sodium channel suppressing neuronal firing through inhibition of sodium flux through these voltage dependent channels (Nelson *et al.*, 2015) hence it protects the sodium pump in the brain and the heart by stabilizing membranes hence minimizing maximal convulsive activity (Bittigau *et al.*, 2002).

Phenytoin administration to epileptic pregnant mothers alone or in combination with other anticonvulsants increase the risk of delivering a child with congenital defects by 2-3 times (Lander *et al.*, 2008) through accumulation of its metabolites that cause fetal hypoxia, vascular disruption and necrosis of existing and developing structures hence affecting fetal cardiovascular system histo-morphogenesis and cell cytodifferentiation (Webster *et al.*, 1997).

Dysmorphogenesis of the fetal heart is key predictor to postnatal cardiovascular anomalies called congenital heart diseases which are the most common birth defects in human that result in spectrum of heart problems like coronary artery disease, congestive heart problems and pulmonary hypertension among others (Ksoo*et al.*, 2017). These defects have been attributed to genetic and environmental factors. However, some of the medications such as phenytoin that have been shown to affect heart development hence predisposing children to higher risk of having congenital heart disease (Shah, 2010). Congenital heart defects are common in infants born to women with epilepsy taking antiepileptic drugs and the most implicated drug was phenytoin (Waltman, 2003).

1.2 Study Objectives

1. To establish the histostereological teratogenic effects of phenytoin on the fetal heart wall.

2. To establish the histostereological teratogenic effects of phenytoin on the fetal inter-atrial and interventricular septum

3. To establish whether the histostereological teratogenic effects of phenytoin on the fetal heart wall and septum are dose and time dependent

1.3 Hypothesis (H_0)

Prenatal exposures to phenytoindo not have effectson the fetal heart wall and septal histostereology.

1.4 The study assumptions

In carrying out this study it was assumed that the albino rat (*RattusNorvegicus)* model would replicate the actual teratogenic induction scenario that would occur in humans due to the known close association of this kind of rat species with human biological and functional outcomes when exposed.

2.0 Materials and Methods

2.1 Study site/Location

All experiments that included breeding, handling, weighing, phenytoin administration and measurements of fetal parameters was done at the Small Animal Facility for Research and Innovation (SAFARI) situated in Jomo Kenyatta University of Agriculture and Technology (JKUAT).

2.2 Study Design

A static group laboratory based experimental study design was adopted

2.3 Description of Albino rats used in the study

Female albino dams used in the study were of the 3rd series breed and weighed between 200-250g.They were used because of the following known scientific facts; (i)They have a large litter size,(ii)Low incidence of spontaneously occurring congenital defects, (iii)a relatively short gestational span,(iv)low cost of maintaining the animals and, (v)considerable amount of the reproductive data on the rat is already available (Bailey *et al.,* 2014; Pritchett & Corning, 2016).

2.4. Acquisition and feeding of the dams

The albino rats were purchased from the Small animal facility for research and innovation (SAFARI) animal house, located in Jomo Kenyatta University of Agriculture and Technology (JKUAT) main campus. They were fed on a standard diet as determined by American institute of nutrition (2011) that included rodent pellets and water *adlibitum*. They were kept in spacious polycarbonate plastic cages in the animal house as determined by (Allen *et al.,* 2016).

2.5Sample size calculation

In calculation of the sample size, resource equation was applied to get 30 albino ratsas determined by (Arifin*et al.*, 2017). The formula states that the measured value 'E' which is the degree of freedom of analysis of variance (ANOVA) based on a decided sample size value ('E') should lie between 10 and 20 animals according to this equation. Therefore, a value less than 10 necessitates adding more animals which increases the chance of getting significant results while a value more than 20 has been shown to increase the cost of the study without increasing the significance of the results.Therefore, total number of groups=10 while the total number of animal sis 30. E=Total number of Animals-Total number of groups. E is therefore is 30-10 which is 20

2.6 Grouping of animals

A total of 30 nulliparous albino rat dams of the species Rattusnorvegicus weighing between 150-250g were derived from a pure colony were used as the animal experimental model in this study. The 30 dams were calculated using the resource equation method (E=TA-TG), (Charan& Biswas, 2013) since the standard deviation from previous studies was not available as well as the effect size.

2.7 Determination of phenytoin doses

A simple guide for conversion of human to animal dosages was used as determined by (Nair & Jacob, 2016)formula as follows; The correction factor (Km) is estimated by dividing the average body weight (kg) of species to its body surface area (m2). For example, the average human body weight is 60 kg, and the body surface area is 1.62 m2. Therefore, the Km factor for human is calculated by dividing 60 by 1.62, which is 37. The Km factor values of a rat is used to estimate the HED as: HED mg / kg = Rat dose mg / kg Animal K /Human K Eq. As the Km factor for each species is constant, the Km ratio is used to simplify calculations. Hence, Equation is modified as: HED mg / kg = Animal dose mg / kg K ratio Eq. The Km ratio values are already provided and are obtained by dividing human Km factor by animal Km factor or vice versa.Phenytoin administration was done using an oral gavage needle gauge 16.

2.8 Administration of phenytoin

All rats in first trimester (TM_1) group in Low, Medium and High dose categories received phenytoin doses from gestation day GD_1-GD_{20}while the rats in second trimester (TM_2) group in Low, Medium and High dose categories received phenytoin from gestation day GD_7-GD_{20}. Rats in third trimester (TM_3) group in Low, Medium and High dose categories received phenytoin from gestation day GD_{14}-GD_{20}

2.9 Harvesting of fetal hearts

On day 21, the dams were sacrifice and the fetuses were obtained.Thefetuses were also humanely sacrificed using carbondioxide, their thorax was opened and the hearts were carefully removed after transverse dissection proximally at the level of the great vessels that is before bifurcation of the pulmonary trunk and at the level of aortic arch then the hearts were kept immersed in formaldehyde.

2.10 Processing of heart tissues for light microscoscopy

When the fetal hearts were harvested, trimmed, weighed then volumes determined using the water emersion method, then fixed in formaldehyde solution for 24 hours then dehydrated in an ascending grade of alcohol (50%, 60%, 70%, 80%, 90%, 95% and 100% (absolute) each for one hour. The hearts were then cleared with xylene, infiltrated with paraplastwax for 12 hours at 56^0c, embedded in paraffin wax on the wooden blocks then excess wax was trimmed-off till the entire length of the heart tissue is exposed,5µm thick sections was cut entirely with Leitzsledge rotary microtome, The cut sections were then floated in water at 37^0 to spread the tissue,The sections were stacked onto glass slides, applied as thin film with a micro-dropper. The slides were then dried in an oven at 37^0 for 24 hours. They were stained with Hematoxylin and eosin (H&E) (Ahmed., 2016).50 slides in each subgroup were selected for light microscopy processing using systematic random sampling so that the entire heart is represented by slides with every level of interest, 6 slides from each group were selected for observation (1 from each 10 was selected using simple random sampling)

2.11 Procedure for histo- stereological analysis

The Archimedes principle was used to obtain an independent heart volume by inserting the whole heart tissue into graduated beakers containing normal saline and the displacement was measured. The normal saline displaced by the heart represented the actual heart volume (Stephen *et al.*,2005)

Total heart volume was also determined using cavalieri method of point counting using stereoinvestigator system (Altukaynak*et al.*, 2005). In order to calculate the volume of the heart wall and septum using cavalieri method, examination of prepared cross-sections was done using a light microscope under (mag x40) and the heart image was shown on the screen then the point probe was tossed randomly onto each section then the points that hit the region of interest (septum and the wall) was counted using stepanizer tool. All counts per slice were done for the number of the slices that were chosen using systematic simple random sampling for each animal. Then the total volume of the heart ventricular wall and the septum was determined using the following formula:

$_{est}V = \sum^{m}_{i-1} P.\ a/\ p.\ t$ / M^2Where: $_{est}\mathbf{V}$= was the estimation of the volume of the heart, $\sum^{m}_{i-1} P$= was the sum of the number of points landing within the various components of the fetal heart profiles, from the first to the last point A/p= was the area associated with each point (434µm) , **t** = was the distance between sections (5µm thick) M= was the magnification (x40) (Welniack- Kaminska*et al.,* 2019)

2.12 Histo-quantititaive Statistical analysis

The study examined the histoquantitative teratogenic effects of phenytoin on the fetal heart in albino rats.
The data was analyzed using SPSS version 21 and was expressed as mean ± standard error (SEM). The study compared the heart histoquantive outcomes when the three dose levels of phenytoin (Low, medium and high) were administered in different gestational periods (T1, T2 and T3) of the experimental groups and control. The histoquantitative parameters included the total fetal heart volume, the wall and septal volume densities. The results are presented below.

Ethical Approval

All procedures for animal handling, feeding, humane sacrificing and harvesting of organs were performed as per laid down protocols, with approval from Animal Ethics Committee Jomo Kenyatta University of Science and Technology REF: JKU/2/4/896A**).**

RESULTS

Phenytoin when administered in TM1, there was reduction in the total fetal heart volumes in that high doses had the lowest mean total fetal heart volume of 311.40 mm3 followed by MPG (338.99 mm3) and LPG (353.88 mm3) and this was significantly different with that of the control (378.13 mm3) P=0.001. However, post hoc test results showed there was no significance difference between LPG and MPG but there was significance difference between MPG and HPG.

When phenytoin was administered in TM2, it was observed that the total heart volume was lowest in HPG (317.10mm3) followed by MPG (345.59 mm3) which was significantly different from that of the control (378.13 mm3) p=0. 001.The total fetal heart volume of the LPG in TM2 was 371.13 mm3..However, post hoc test results showed there was no significance difference between LPG and MPG as well as between MPG and HPG.

From the study findings it was observed that when phenytoin doses were administered in TM3, the mean total fetal heart volume decreased remarkedly among the high and medium phenytoin dose groups (339.82mm3, 370.51 mm3) respectively and this difference was significant when compared to that of the control (378.13) p= 0.001. The mean total heart volume among the LPG was 371.37 mm3 which was lower than that of the control group, however the difference was not significant. Also post hoc test results showed there was no significance difference between MPG and HPG.

Table 1: The TM_1, TM_2 and TM_3 comparative means of the total fetal heart volumes (mm^3) in LPG, MPG and HPG against the control.

		Control	LPG (31mg/kg)	MPG (62mg/kg)	HPG (124mg/kg)	F-value	P-value
TM_1	WIM	399.53±4.80^a	376.84±4.27^b	351.91±4.79^c	335.33±4.32^c	38.307	<0.001*
	CM	378.13±4.57^a	353.88±3.75^{ab}	338.99±4.36^b	311.40±10.4^c	19.253	<0.001*
TM_2	WIM	399.53±4.80^a	390.90±4.54^a	370.51±4.60^b	357.70±3.52^b	18.771	<0.001*
	CM	378.13±4.57^a	371.37±13.09^a	349.91±4.49^{bc}	339.82±4.14^c	7.388	<0.001*
TM_3	WIM	399.53±4.80^a	390.90±4.54^a	370.51±4.60^b	357.70±3.52^b	18.771	<0.001*
	CM	378.13±4.57^a	371.37±13.09^a	349.91±4.49^{bc}	339.82±4.14^c	7.388	<0.001*

*Notes: The means, followed by the same letter in a row are not statistically different at ($p < .05$) using one-way ANOVA. with Tukey test on post-hoc t-tests. * indicates significance ($p < .05$).*

The histostereological findings on the mean total fetal heart wall and septal volume densities in the LPG, MPG and HPG against the control.

When phenytoin was administered in TM_1, the mean total heart wall volume was decreased in HPG (155.70mm^3) followed by the MPG (169.49 mm^3) while that of the LPG was 176.94 mm^3 when compared with the heart wall and septal volume of the control (189.07 mm^3). There was significance difference between the mean total volume of the fetal heart wall of the high phenytoin group and that of the control p= 0.001. At the same time the mean total heart septal volume was decreased in HPG (52.94±1.77) followed by the MPG (57.63.87±0.74) while that of the LPG was 60.16±0.64 when compared with the heart wall and septal volume of the control (64.28±0.78 mm^3). There was significance difference between the mean total volume of the heart septum of the high phenytoin group and that of the control

According to the study outcomes it was observed that when phenytoin was administered at TM_2 the mean total heart wall volume was found to be (158.55mm^3, 172.79mm^3 ,178.84mm^3) in HPG, MPG and LPG respectively) which was significantly lower from that of the control (189.07mm^3) p = 0.001. Similarly, the mean total heart septal volume was decreased in HPG (47.57±1.95) followed by the MPG (51.84±0.84) while that of the LPG was 53.65±0.67 when compared with the heart wall and septal volume of the control (64.28±0.78 mm^3).

Similarly, there was reduction in the fetal heart wall volume in HPG and MPG (169.91, 174.96) respectively when phenytoin was administered at TM_3 and this was significantly lower when compared to the control (189.07 mm3) p= 0.001. However, the LPG fetal heart wall and septal volume (188.68.) was not significantly different from that of the control group. The mean total volume density of the fetal heart septum reduced in HPG (50.97±0.62) , MPG (52.49±0.67) and LPG (57.21±1.96) as compared to the control group (64.28±0.78)

Table 2: The TM_1, TM_2 and TM_3 comparative means of the total fetal heart wall and septal volume densities (mm^3) in LPG, MPG and HPG against the control.

Parameter	Control	LPG (31mg/kg)	MPG (62mg/kg)	HPG (124mg/kg)	F-Statistic	P-value
Fetal Heart Wall Volume -TM1	189.07±2.29 ^{a}	176.94±1.87 ^{ab}	169.49±2.18 ^{bc}	155.70±5.20 ^{d}	19.25	<0.001*
Fetal Heart Septum Volume –TM1	64.28±0.78 ^{a}	60.16±0.64 ^{ab}	57.63.87±0.74 ^{bc}	52.94±1.77 ^{d}	15.25	<0.001*
Fetal Heart Wall Volume-TM2	189.07±1.60 ^{a}	178.84±2.24 ^{ab}	172.79±2.80 ^{bc}	158.55±6.50 ^{c}	10.76	<0.001*

Fetal Heart Septum Volume-TM2	64.28±0.78 [a]	53.65±0.67 [ab]	51.84±0.84 [bc]	47.57±1.95 [c]	10.76	<0.001 *
Fetal Heart Wall Volume-TM3	189.07±1.60 [a]	188.68±6.55 [ab]	174.96±2.24 [bc]	169.91±2.07 [c]	7.39	<0.001 *
Fetal Heart Septum Volume-TM3	64.28±0.78 [a]	57.21±1.96 [ab]	52.49±0.67 [bc]	50.97±0.62 [c]	7.39	<0.001 *

Phenytoin, when administered in TM1of gestational periods reduced the fetal heart wall and septal volume densities significantly compared to when administered at TM2 and TM3 as shown in table 2 below

Notes: The means, followed by the same letter in a row are not statistically different at ($p < .05$) using one-way ANOVA. with Tukey test on post-hoc t-tests.

4.0 DISCUSSION

Phenytoins have quantitative effects on fetal heart when administered prenatally at different phenytoin dosages during different gestational periods. Results showed that fetuses of the experimental group had reduced total heart volume, wall and septal volume densities among phenytoin treatment groups, particularly when administered in the first and second trimester (TM1 and TM2) at high doses. In this study, the reduction in fetal heart volumes (total heart volume, wall and septal volume densities) observed in phenytoin treatment groups can be attributed to the fetal heart alterations during its development (Andermann, 1992). This study is in agreement with a previous study by Gelder et al., (2010) on effects of antiepileptic drugs; lamotrigine, carbamazepine, phenobarbitone, valproic acid among others on fetal growth and development when exposed in-utero and especially during organogenesis.This study findings showed decreased among the experimental groups when compared to the control group and this could be attributed to various septal defects seen postnatally as reported by Godbole, 2010 who observed large ventricular septal defect with left to right shunt in a 12year old girl with prenatal history of phenytoin use by her mother. Another study that was done by Avana, 2017 reported biventricular dysfunction with mild tricuspid regurgitation echocardiogram findings at 33 weeks gestation in a mother who was on phenytoin throughout gestation.

This study found reduction in total heart volume that could be attributed to disruption of its development like; alteration of embryonic blood flow and blood pressure (Danielsson,2001), fetal hypoxia, vascular disruption and necrosis of existing and developing structures that may be attributed to generation of reactive oxygen species within the embryo during reoxygenation possibly resulting in free radicle damage (Webster et al., 1997). Study done by Bittigau et al., (2002) demonstrated effects of antiepileptic drugs including phenytoin through apoptotic neurodegeneration on the fetal structures. Patocka et al., (2020) findings showed cerebella atrophy following phenytoin use. These findings help explain the reduction in the total heart volume and vascular tunic volume as well the volume densities of the fetal heart wall and the septum. On the other hand , postnatal study done by Ksoo et al., (2017) on various antiepileptic drugs contrast the findings of this study by demonstrating increase in blood vessel wall thickness resulting in arthrosclerosis that manifests with impairment of the cardiovascular system functions.

CONCLUSION

In conclusion of the study has established phenytoin use during pregnancy have histostereological effects on the fetal heart wall and the septum particularly when administered during the first and second trimester using high and medium doses.

RECOMMENDATION

The study recommends that;
1. The use phenytoin during pregnancy should be avoided as it has been shown to affect the pregnancy outcome particularly in trimester one (TM_1) and trimester two (TM_2) by seeking appropriate alternatives that are safer to the fetus.

2. Should expectant mothers be on prolong use of phenytoin and the drug cannot be withdrawn because of associated withdrawal side effects to the mother, the doses should be adjusted to the minimal effective dosages that would confer the maximum maternal benefits and reduce the teratogenic risks to the pregnancy.

3. Due to time and dose dependent teratogenic effects of phenytoin, health care workers including clinicians, nurses, midwives and others, need to be educated on how they will need to be educating women of reproductive age and are on chronic usage of phenytoin of its teratogenicity during pregnancy, on the need for early planning of their pregnancies for effective introduction of alternative medicines, to enable them avoid use phenytoin during pregnancy.

4. Further studies be carried out in non-human primates that have close phylogenetic relations to humans, to ascertain its effects to the pregnancy outcome in relation to doses.

Acknowledgements

Author is grateful to Dr. Joseph.KKweri, Chairman of Department in Human Anatomy, Jomo Kenyatta University of Agriculture & Technology for his dedication and support to success of this study.

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