HISTOLOGICAL TERATOGENIC EFFECTS OF VARIED DOSES OF PHENYTOIN ON THE DEVELOPMENT OF FETAL HEART LEFT VENTRICULAR WALL IN ALBINO RATS (RATTUS NORVEGICUS)

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Abstract: Phenytoin, an antiepileptic medicine has been shown to interfere with the development of the fetal heart when used by mothers during pregnancy. This embryological disruptionsmay serve as contributor to the various diseases of cardiovascular system being witnessed in childhood as well as in adult hood including sinoventricular tachycardia, hypertension, and ischemic heart diseases among others. There is lack of data on histological effects of phenytoin on the heart and this study therefore aimed to establish histological teratogenic effects of phenytoin on the heart when varying used at different gestational periods in albino rats. In carrying out the study a total of 30 pregnant female albino rats weighing between 150-250grams were used as the experimental model whereby they were randomly assigned into two broad study groups of 3 control and 27 experimental group. The 27animals in the experimental group were further subdivided into three study groups as follows [low phenytoin group(31mgs/kg/bw), medium (62mg/kg/bw) and high phenytoin group(124mg/kg/bw)] of nine (9) rats each. Each of the nine (9) rats in each of the low, medium and high phenytoin groups were further subdivided into three study sub- groups of 3 rats each as trimesters one (T_1) trimester two (T_2), and trimester three (T_3) in order to determine the effects of phenytoin when exposed on different gestational periods. The control group received food and water adlibitum while experimental animals received food, water adlibitum and varying doses of phenytoin based on their study category and the exposure period. All the pregnant dams 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 humanely sacrificed and their hearts obtained and processed for light microscopy. The left ventricular walls of the processed slides were observed under a light microscope connected to a computer screen with a camera and photomicrographs were captured and saved. The intergroup and intragroup comparison of the left ventricular wall was done. The left ventricular wall layers showed disorganization among the experimental group compared to the of the control particularly the highphenytoin group when phenytoin was administered during the first trimester.

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

1.1 Introduction

Phenytoin is a drug that is used in treating convulsions that occur in disorders like epilepsy and its other name is Dilantin (Patocka*et al.*, 2020). It 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). It has been shown that phenytoin may increase the risk of congenital defects when used by mothers during pregnancy (Lander *et al.*, 2008). The effects of phenytoin may be attributed to its metabolites that accumulate and cause disruption of the developing vessels due to hypoxia hence leading to necrosis of existing and developing structures of the cardiovascular system through disruption of their histo-morphogenesis and cell cytodifferentiation (Webster *et al.*, 1997). This structural dysmorphogenesis of the fetal heart is associated with congenital heart diseases which are the most common birth defects in human that consequently result in some of the heart problems like coronary artery disease, congestive heart problems and pulmonary hypertension among others (Ksoo*et al.*, 2017). Though these malfomations have been associated to genetic and environmental factors, some of the medications such as phenytoin have been shown to have effects on the heart development hence may increase risk of having children with 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).

Study Objectives

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

2. To establish whether the histological teratogenic effects of phenytoin are dose dependent

3. To establish whether the histological teratogenic effects of phenytoin are dose dependent

Hypothesis (H_0)

Prenatal exposures to phenytoindo not affect the fetal heart histological organization

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.

Materials and Methods

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).

Study Design

A static group laboratory based experimental study design was adopted

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).

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).

Sample 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

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.

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.

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}

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.

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, 5 µm 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)

Ethical Approval

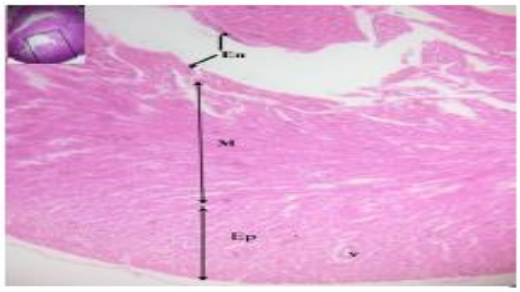
All procedures for animal handling, feeding, humane sacrificing and harvesting of organs were performed as per laid down protocols, with approval from Animal Ethics Committee Jomo Kenyatta University of Science and Technology REF: JKU/2/4/896A**).**

RESULTS

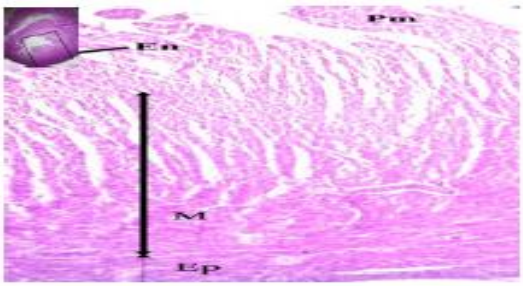
TM_1 comparative histomorphological features of the thickness of the ventricular wall between phenytoin treated groups (LPG, MPG, HPG) against the control.

When phenytoin was administered at trimester I (TM_1), it was observed that the myocardial thickness decreased as the doses of phenytoin increase as shown below in figure 4.4 photomicrograph 2, 3 and 4 respectively as compared to that of the control in photomicrograph 1. The endocardium as well as its endothelial (En) lining was poorly outlined shown in photomicrograph 2, 3 and 4. The epicardium (E) showed decreased adipose tissue and poorly outlined mesothelium with increasing dose of phenytoin as shown below in photomicrograph 2,3 and 4 respectively. The papillary muscle was not well outlined among low and medium phenytoin group as shown in photomicrograph 2 and 3 compared to that of the control.

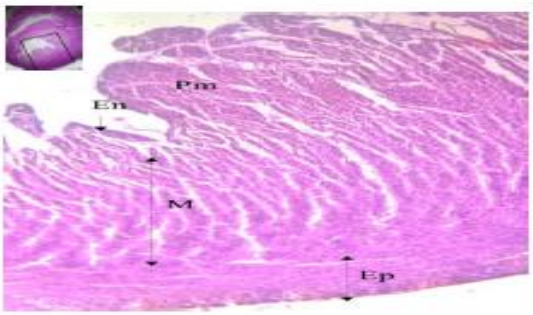
Figure 4.4: Shows the comparative ventricular wall thickness and arrangement of the cellular components of the myocardium (M), epicardium (Ep) and endocardium (En) at TM_1 in: (a) control, (b) the LPG, (c) MPG, (d) HPG Hematoxylin and Eosin (H&E) Mag X10.



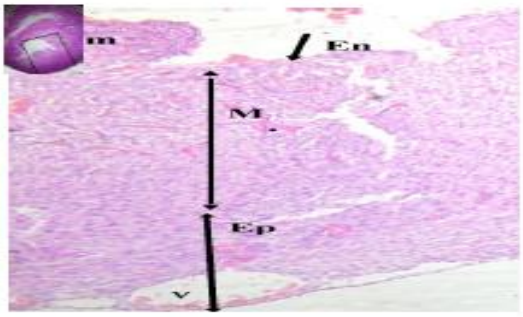
Photomicrograph a: shows well outlined epicardium, myocardium and endothelium as well as well organized myocardial fibers



Photomicrograph b: shows well outlined epicardium, myocardium and endothelium however the myocardial fibers are disaggregated



Photomicrograph c: shows reduced myocardial thicknesss, poorly outlined endothelium and disaggregated muscle fibers



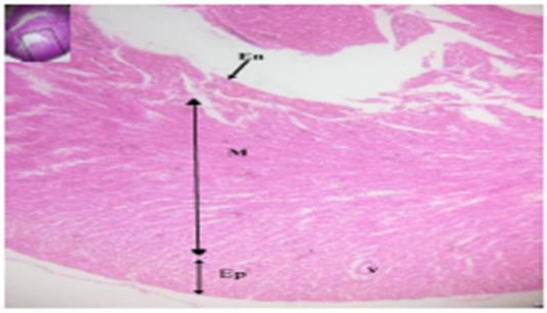
Photomicrograph d: shows reduced myocardial thicknesss, poorly outlined endothelium and disaggregated muscle fibers

TM_2 comparative histomorphological features of the left ventricular wall between phenytoin treated groups (LPG, MPG and HPG) against that of the control.

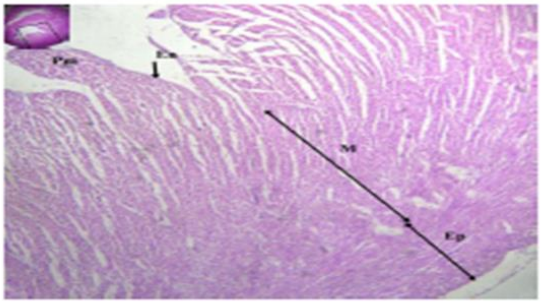
It was observed that when phenytoin was administered in trimester two (TM_2), the myocardium (M) of the left ventricles in high and medium phenytoin dose groups were reduced in thickness and the myocardial muscle fibers were as well loosely arranged and disorganized as shown in figure 4.5 below in photomicrograph 4 and 3 respectively when compared to the control as shown in photomicrograph 1. There was no marked histomorphological change between the low phenytoin groups as shown in photomicrograph 2 when compared with that of the control photomicrograph. The endocardiums as well as its endothelial (En) lining were also poorly outlined as shown in figure 4.5 below and in photomicrograph 2, 3 and 4. The epicardium (E) showed decreased

adipose tissue and poorly outlined mesothelium with increasing dose of phenytoin as shown in photomicrograph 2, 3 and 4 respectively. The papillary muscle (Pm) was poorly outlined among low and medium phenytoin group as shown in photomicrograph 2 and 3 compared to that of the control.

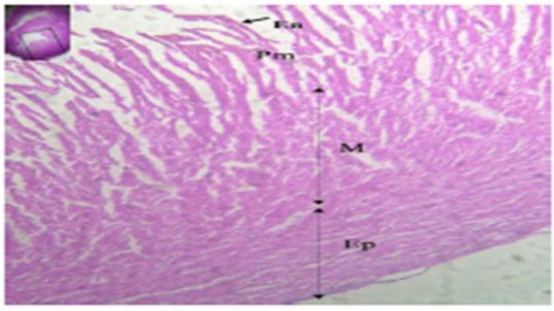
Figure 4.5: Shows the comparative ventricular wall thickness and arrangement of the cellular components of the myocardium (M), epicardium (Ep) and endocardium (En) at TM_2 of: (a) control, (b) the LPG, (c) MPG, (d) HPG Hematoxylin and Eosin (H&E) Mag X10.



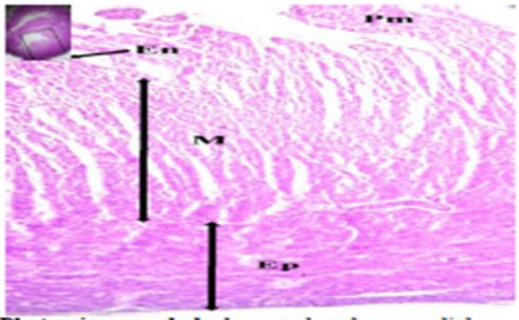
Photomicrograph a: shows well outlined epicardium, myocardium and endothelium as well as well organized myocardial fibers



Photomicrograph b: shows well outlined epicardium, myocardium and endothelium however the myocardial fibers are disaggregated



Photomicrograph c: shows reduced myocardial thicknesss, poorly outlined endothelium and disaggregated muscle fibers

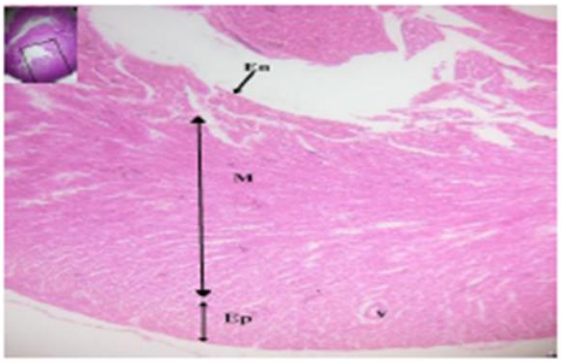


Photomicrograph d: shows reduced myocardial thicknesss, poorly outlined endothelium and disaggregated muscle fibers

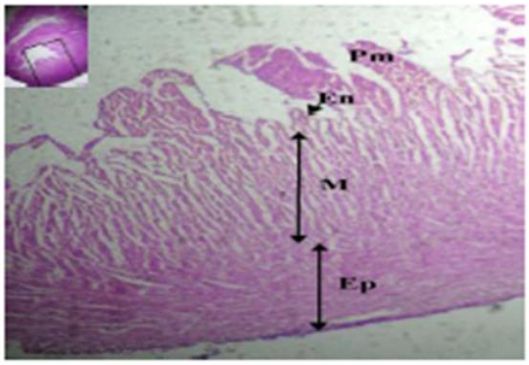
TM_3 comparative histomorphological features of the left ventricular wall between phenytoin treated groups (LPG, MPG and HPG) against that of the control.

When varied doses of phenytoin (low, medium and high) was administered in trimester 3 (Gestation Day 14-20), it was observed that the myocardium (M) in high, medium and low phenytoin dose group was reduced in thickness and the myocardial muscle fibers are loosely arranged as shown below in photomicrograph 4, 3 and 2 respectively when compared to that of the control as shown in photomicrograph 1. The endocardium as well as its endothelial (En) lining was poorly outlined shown in photomicrograph 2, 3 and 4 below. The epicardium (E) showed decreased adipose tissue and poorly outlined mesothelium with increasing dose of phenytoin as shown in photomicrograph 2, 3 and 4 respectively. The papillary muscle (Pm) was not well outlined among low and medium phenytoin group as shown below in photomicrograph 2 and 3 compared to that of the control.

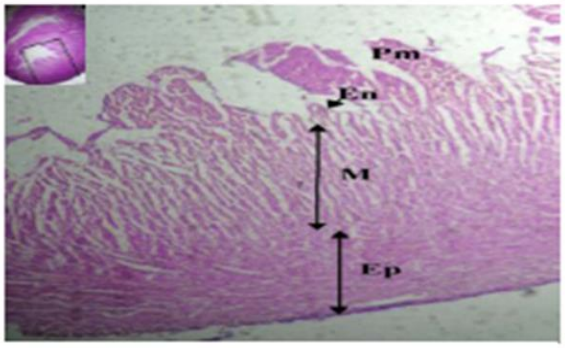
Figure 4.6: Shows the comparative ventricular wall thickness and arrangement of the cellular components of the myocardium (M), epicardium (Ep) and endocardium (En) at TM3 in: (a) control, (b) the LPG, (c) MPG, (d) HPG Hematoxylin and Eosin (H&E) Mag X10.



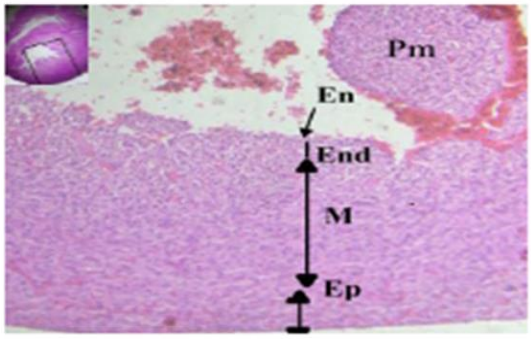
Photomicrograph a: shows well outlined epicardium, myocardium and endothelium as well as well organized myocardial fibers



Photomicrograph b: shows well outlined epicardium, myocardium and endothelium however the myocardial fibers are disaggregated



Photomicrograph c: shows reduced myocardial thicknesss, poorly outlined endothelium and disaggregated muscle fibers



Photomicrograph d: shows reduced myocardial thicknesss, poorly outlined endothelium and disaggregated muscle fibers

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4.0 DISCUSSION

This study examined the histological effects of prenatal exposure to different phenytoin dosages on fetal heart left ventricular wall while administered at different gestational periods. Results showed poor outline of the endothelium, disaggregation of the myocardial fibres, reduced myocardial thickness among others in the experimental groups compared with the control group particularly when phenytoin was administered in the first and second trimester (TM1 and TM2 using high phenytoin dose. These features observed in phenytoin treatment groups may be attributed to the fetal heart alterations during its development (Azarbayjani, 2006). 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 findings may also be attributed to disruption of the heart development like due to 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 and these findings may as well help explain the reduction in the left ventricular myocardium. On the other hand, postnatal study has 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.

The histomorphological findings of this study was poor organization of the ventricular wall layers particularly the myocardium of the left ventricle. In addition, the study findings showed vascular disruption when phenytoin was administered in high doses as shown in fig 4.4 photomicrograph d.This study findings concurred with another study done by Webster *et al.*, (1997) that found phenytoin to cause fetal hypoxia, vascular disruption and necrosis of existing and developing structures of the fetus.

The findings of this study also showed reduced thickness of the left and right ventricular wall with increasing dose of phenytoin as shown in figure 4.7 photomicrograph b, c and d when administered at trimester I of gestation. This findings could be attributed to another study findings that was done by (Katsiki *etal.*, 2014) that showed vascular disruption and necrosis of existing and developing embryonic structures due to generation of reactive oxygen species within the embryo during reoxygenation possibly resulting in free radicle damage. (Appleton & Gill, 2003) also conducted a study of phenytoin and found that embryonic bradycardia which leads to severe hypoxia and alteration in embryonic blood flow and blood pressure induce cardiovascular defects which would possibly present in various histomorphological changes of the heart as described in this study.

CONCLUSION

In conclusion of the study has established phenytoin use during pregnancy have histological effects on the fetal heart wall 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.

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