

SKELETAL MALFORMATION OF FETUSES FROM PREGNANT *Sprague Dawley* RATS FED *Jatropha curcas* CRUDE OIL (JCO)

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ABSTRACT

*Studies on the teratogenicity effects of *Jatropha curcas* has been reported in pregnant rats. This study was conducted to observe whether feeding *Jatropha curcas* seeds crude oil (JCO) to pregnant rats during early and late gestation will cause fetal skeletal malformations. A hundred sexually mature female rats were divided equally into 2 groups: early and late gestation. Each group was then subdivided equally into 5 groups: positive control (retinylpalmitate), vehicle control (corn oil), low dose (0.175ml/kg), medium dose (0.35ml/kg) and high dose (0.7ml/kg) of JCO. Rats were mated overnight and positive pregnant rats were treated on days 1-7 (early group) and days 8-14 (late group). Rats were sacrificed on day 21 of pregnancy. Fetuses were processed accordingly and any skeletal malformations were recorded. Data show fetuses in high treatment group were significantly smaller as compared to other groups. Skeletal abnormalities in fetuses from all JCO treated groups were observed suggesting fetotoxicity.*

KEYWORDS: *Jatropha curcas* crude oil, teratogenicity, skeletal malformation, fetus, pregnant Sprague dawley rats

1.1. INTRODUCTION

Jatropha curcas, also known as purge nut and pokok jarak pagar in Peninsular Malaysia is naturally distributed in the northeastern part of South America but is now abundantly found in many tropical and subtropical countries throughout Africa, India and Asia (Heller, 1996). *Jatropha curcas* is a deciduous large shrub belonging to the genus *Euphorbiaceae* (Heller, 1996).

Emil et. al., (2009) reported that this plant is a non-edible oil crop with good physiochemical properties, is widely used as bio-diesel, feedstock and industrial applications. The whole plant is also being utilized for water conservation, erosion control and soap production (Gubitzet al., 1997). Parts of this plant has also been used for abortifacient purposes (Gunasekara et. al., 1995), for contraception, as a purgative, anti-inflammatory, antitumor, antihelminthic, diuretic agent, to treat gout, paralysis, skin diseases, rheumatic conditions, fever and jaundice (reviewed by Thomas et. al., 2008; Devappa et. al., 2010).

The seeds of physic nuts are a good diesel substitute source (King et. al., 2009). However, the seeds contain high concentrations of phorbol esters namely curcin which is toxic to human and animals therefore accidental consumption cause several symptoms such as giddiness, vomiting, diarrhea and even death (Makkar et al., 1998).

Retinoids in retinyl palmitate used as the positive control in this study represents one of the better studied classes of teratogens (Hayes et. al.1981). Retinoids are converted effectively into retinoic acid (RA) responsible for the regulation of gene expression (Venkatesh et. al., 2013). Excess vitamin A and its metabolites, particularly retinoic acid can be teratogenic (Sommer, 2008). In addition, retinoids also inhibit limb outgrowth and chondrogenesis, resulting in limb reduction defects (Ali-Khan and Hales, 2003., 2006). Retinylpalmitate is dissolved in corn oil as it cannot be dissolve in water or Tween 20. Corn oil is normally use at 10 ml/kg as a vehicle for test agent in teratogenic studies and has been found to cause nephrotoxicity in pregnant and lactating dams thus providing a confounding factor (Sato et. al., 2000). Therefore corn oil is given in one group of animals to confirm that effects observed may be due to the corn oil itself.

Skeletal malformation or dysplasias are a heterogeneous and complex group of conditions affecting both bone growth and development resulting in various anomalies in the shape and size of the skeleton (Khundan, 1981). Skeletal malformations are clinically important as they are associated with severe disability and may cause death (Cassart, 2010). Alizarin Red is used for staining of the fetal bones to evaluate all variations of the ribs, sternum, dumbbell-shaped vertebral centrum, split vertebral centrum, extra lumbar vertebra and ossifications (vertebral centrum, metacarpal bones, metatarsal bones, sacral and caudal vertebrae) (Tetsushi et. al., 2009).

2.1. MATERIALS AND METHODS

2.1.1. Preparation of *Jatropha curcas* crude oil (JCO)

Jatropha curcas crude oil was purchased commercially from Agolink Sdn. Bhd. in Kluang, Johor, Malaysia. Basically the crude oil was extracted from the seeds by first removing the kernel using a shell remover. After that, a seed separator was used to separate the kernel and the seed. The seeds will then be left to dry for 3-5 days. Subsequently, oil from the seeds was expelled using an oil expeller through a screw -

type method where seeds were pressed until the oil comes out. The oil will be left to settle down after which the top clear yellowish crude oil layer was used.

2.1.2. Determination of Dosage

Dosage testing was performed to determine the LD₅₀ of JCO on *Sprague dawley* rats. Forty-four rats were given a random concentration of JCO for 7 consecutive days. The concentration of JCO tested on the rats (4 rats per dose) were 1.5 ml/kg, 1.2 ml/kg, 1 ml/kg, 0.9 ml/kg, 0.8 ml/kg, 0.7 ml/kg, 0.6 ml/kg, 0.5 ml/kg, 0.4 ml/kg, 0.3 ml/kg, and 0.2 ml/kg. The highest concentration for 50% survivability was 0.7 ml/kg. Hence, concentration higher than 0.7 ml/kg caused 100% mortality of rats before reaching day 7 of administration. Thus, 0.7 ml/kg was considered the highest dose, 0.35 ml/kg the medium dose while low dose was 0.175 ml/kg.

2.1.3. Experimental Animals

One hundred mature fertile female *Sprague dawley* rats weighing 200-250gm was obtained from the Laboratory Animal Unit, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia with ethics approval from the Animal Ethics Committee, UPM (ACUC). The animals were fed on standard pellet diet and water was given ad libitum. The animal were also maintained under standard environmentally controlled room (temperature (25+ 2 °C) and light (12-h light/12-h dark cycle).

Rats were given seven days to adapt to the environment and to undergo two consecutive estrus cycles. Rats were equally divided into 2 groups which were early and late pregnancy (50 rats each). Then, each group were equally sub-divided into 5 groups which consist of positive control, vehicle control, low dose (0.175ml/kg), medium dose (0.35ml/kg) and high dose (0.7ml/kg) JCO group. For vehicle control, the rats were given corn oil while retinyl palmitate was given to positive control rats.

2.1.4. Experimental Procedure

Each female rat was mated with a male rat and kept overnight in their cages with a tray at the bottom of the cage for the purpose of collecting vaginal plugs. In the morning, when a vaginal plug was detected, it will be considered as day 1 of pregnancy and day 1 post-coitum. This was further confirmed with a vaginal smear for the presence of sperm. The mated females were then randomly assigned to different experimental groups which were early and late group where the early group from gestation day (GD) 1-7 and the late group from gestation day (GD) 8-15.

The treatment consisted of ingestion by oral gavage of 30000USP/kg per day of retinyl palmitate (positive control group), corn oil 1 ml/kg (vehicle control group), low dose (0.175ml/kg), medium dose (0.35ml/kg) and high dose (0.7ml/kg) of JCO on GD 1-7 for early group and on GD 8-15 for late group. During the gestation period, the rats were observed closely twice a day for survival, changes in appearance, behavior, signs of vaginal bleeding, feed and water consumption. The dams were also weighed daily to monitor for any weight loss during the experiment. The maternal weight gain was recorded during the entire pregnancy (total weight gain) and during the treatment period.

On day 21 of pregnancy, rats were sacrificed with chloroform overdose and their fetuses were removed by Caesarean section. Dams and the placenta were taken and weighed. The fetuses were further processed and stained to detect skeletal malformations. The number of fetuses was recorded and examined

for obvious external malformations before subsequent processing. For skeletal examination, the number of skeletal elements was counted and any malformations or variations were recorded.

2.1.5. Evaluation of skeletal malformations

2.1.5.1. Fetal staining

Fetuses were washed with tap water after being kept overnight in 10% formalin solution. Fetuses were then partially incised at the abdominal region and all organs were taken out. Later, fetuses were placed in clean individual bottles containing 1:4 diethyl ether: methanol v/v and left for a week. Subsequently, fetuses were washed with tap water and placed in clean individual bottles containing 0.3% Alizarin Red and 10% KOH (0.3% of Alizarin is mixed with 1L of KOH by 100-120 drops). Fetuses were left for a week. The red colored solution should turn colorless as Alizarin Red was absorbed by the fetuses. After one week, fetuses were transferred to clean individual bottles consisting of 1:1 glycerine:75% ethanol v/v for 24 to 48 hours. Finally, all fetuses were stored in clean individual bottles containing pure glycerin.

2.1.5.2. Fetal bone observation

Vertebral arches and centers, ribs and sternal centers were examined for size and shape. The malformations observed were dumbbell shape of vertebral center and sternum, ossification center, not well ossified skeleton, hypoplastic vertebrae and sternum, wavy ribs, addition or deletion of bones.

2.1.6. Statistical Analysis

All data were expressed as means \pm S.E.M. (standard error of means). Data on maternal organ weights, fetal weights and placenta weights were subjected to Two-way analysis of variance (ANOVA), followed by Duncan test to compare mean differences between experimental groups while regression analysis was applied for data on body weight. A p value of less than 0.05 was selected as the level of statistical significance.

4.1. RESULTS AND DISCUSSIONS

Rats in high JCO dose group for early and late gestation produced the lowest number of fetuses when compared to other groups suggesting resorption or abortifacient activities of *Jatropha curcas*. In addition, only a few rats with high JCO dose for both early and late groups survived with intact fetuses whereas other rats do not have fetuses. This may be due to fetal resorption which occur in early gestation period group and abortion which may occur in late gestation group. The decrease in number of fetuses of high JCO group suggests the lethal and possible teratogenic effects of *Jatropha curcas*

JCO causes alteration in fetus weight in medium and high JCO dose groups for both gestation periods. The weights of fetuses were significantly decreased ($p < 0.05$) in medium and high JCO dose groups as compared to low JCO dose and control groups for early and late gestation periods. Decreased maternal weight during gestation was considered to be a good indication of toxicity.

In addition, the size of head, body and tail of fetuses in high JCO group were smaller in both gestation periods. The body weight, head size, body size and tail size were decreased in the late gestation group as compared to the early gestation group. This suggests that the toxicity effects of JCO towards the fetuses were much higher during the late gestation period.

The placental weight also showed a significant decrease ($p < 0.05$) in high JCO dose group during the early and late gestation periods. In addition, the weight of placenta was markedly reduced in the early gestation group as compared to the late gestation group suggesting that *Jatropha curcas* was more toxic towards the placenta during early gestation. Evseenko, et.al., (2006) states that even though the placenta was regarded to be a selective barrier, some toxins do transcend the placenta and could cause teratogenic effects.

For the skeletal anomalies, the vertebrae, ribs and sternum showed abnormalities such as dumbbell-shaped vertebrae, split vertebrae and decrease ossification center. The ribs were also wavy. The abnormalities of the sternum include ossification of sternum and xiphisternum, split sternum, hypoplastic sternum and absence of sternum. The dumbbell-shaped vertebrae were observed in fetuses from all groups. Dumbbell-shaped vertebral anomalies are a common abnormality which could be observed even in normal groups of fetuses (Reviewed by Tyl et. al., 2007)..

There were many fetuses with skeletal malformations observed in the treatment and positive control groups while none of the fetuses from vehicle control group have any skeletal malformations. In dumbbell-shaped vertebrae malformation, the high dose treatment had a significant increase ($p < 0.05$) percentage in early and late gestation groups. In addition, there was a significant increase ($p < 0.05$) in percentage of wavy ribs in high dose treatment group during late gestation as compared to other treatment groups during the same period.

All other skeletal malformations evaluated showed no significant difference between treatment groups and gestation periods. This suggests that other skeletal malformations were not dose-dependent. Therefore feeding JCO to pregnant dams during early and late gestation periods did not cause skeletal malformations in fetuses however, the types of malformations that were significantly high were the dumbbell shaped vertebrae and wavy ribs.

5.1. CONCLUSIONS

The number of female rats that survived with fetuses was decreased in high JCO dose group as compared to medium and low JCO dose groups. This effect was more pronounced in the early gestation group as compared to the late gestation group having the same high JCO dose. This suggests that fetuses in the early gestation group have decreased viability as compared to fetuses in the late gestation group. JCO has toxicity effects towards the weight, size and number of fetuses in high JCO dose groups as compared to medium and low JCO dose groups during the late gestation period. JCO transcends the placenta causing the toxins to reach the fetuses. Toxicity effects were more pronounced in fetuses from the late gestation groups because the fetuses were already 8 days old. Exposure of fetuses to JCO during the early gestation period resulted in fetal resorption which explains the low number of dams with fetuses or low number of fetuses that survive.

Based on the skeletal malformation observed, we conclude that *Jatropha curcas* has teratogenic effects to fetuses when fed to pregnant female *Sprague dawley* rats during early and late gestation. However the skeletal malformations observed were mainly dumbbell-shaped vertebrae and wavy ribs which were highly significant in JCO treated groups. We suggest more samples, more groups and other dosages to be used in the future to elucidate the teratogenic effects of JCO especially on skeletal malformations.

6.1. REFERENCES

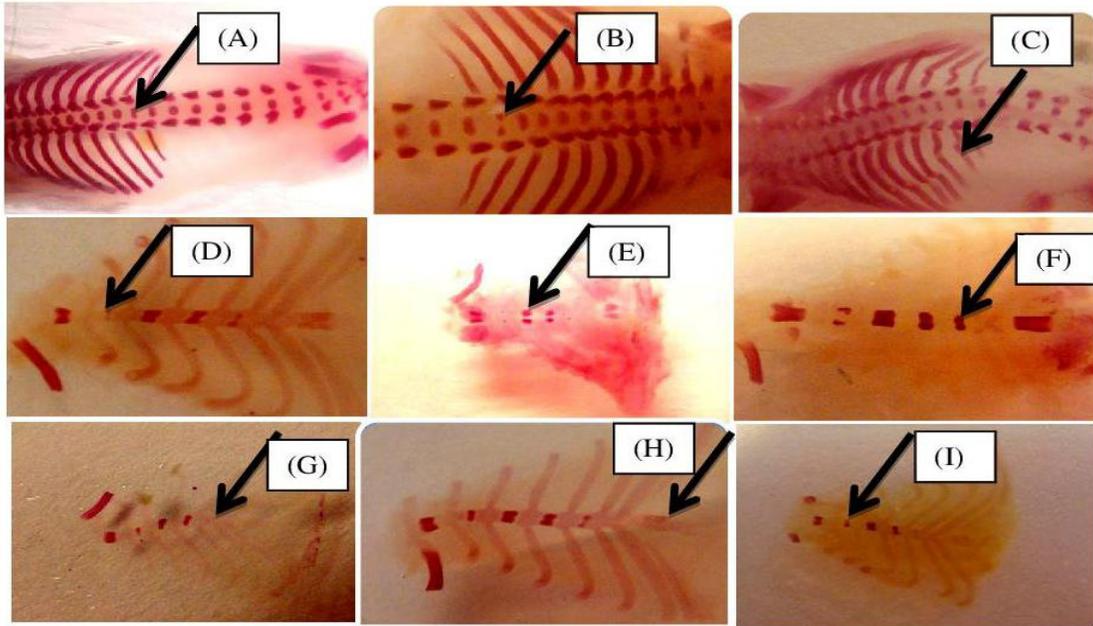
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Figure 1: Body size of fetuses.



(a) Body size of fetus in high dose treatment. (b) Body size of fetus in vehicle control.
 Note: Body size is smaller in high dose treatment compared to vehicle control group.

Figure 2: Skeletal Malformations of fetuses.



(A) Dumbbell-shaped vertebrae (B) Split vertebrae (C) Wavy ribs (D) Not well ossified sternum (E) Split sternum (F) Dumbbell-shaped sternum (G) Absent of sternum (H) Not well ossified xiphisternum (I) Hypoplastic sternum