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Effects of Aflatoxin B1 on the skeletal system of rabbit (Oryctolagus cuniculus) fetuses

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The present study was carried out to determine the effects of aflatoxin B1 (AFB1) on the skeletal system of the fetuses of balady rabbits. The female animals were divided into three groups, one control and two treated, the control group contained four dams, the intoxicated group with 0.1 mg/kg contained six dams while the intoxicated with 0.05 mg/kg contained three dams. A dose of 0.1 and 0.05 mg/kg/day AFB1 was administered by gastric intubation to pregnant rabbits on the 6th-21st day of pregnancy. The fetuses were obtained through the uterine incision at different stages of gestation according to the group. The lengths and weights of the fetuses as well as absolute organs weights were measured, revealing the statistically significant differences between the control and intoxicated with 0.05 mg/kg group at 29th day of gestation (p<0.001) while there were non-significant decrease of control and intoxicated with 0.1 mg/kg at 22nd day of gestation. The observed gross anomalies included wrinkled skin, enlarged eye socket and microphthalmic eyes in both groups. The heart of treated group showed reduction in size with wide ventricular lumen and shallow inter ventricular groove in intoxicated group with a dose of 0.05 mg/kg AFB1. Regarding the skeletal anomalies, there were incomplete ossification in some of the skull bones, the laminae of the vertebral arches throughout the vertebral column remain cartilaginous. The sternum was incompletely ossified. Most of the appendicular skeleton bones were grossly shorter and remained in cartilaginous state.

Key words: Aflatoxin B1 (AFB1), rabbits, teratogenicity, ossification.

INTRODUCTION

Aflatoxins, metabolites of the fungi Aspergillus flavus and Aspergillus parasiticus, are frequent contaminants of a number of staple foods, particularly maize and ground nuts, in subsistence farming communities in tropical and sub-tropical climates in sub-Saharan Africa, Eastern Asia and parts of South America. Increased expression or deregulation of the c-myc and c-Ha-ras genes may play an important role in the development of hepatomas induced by AFB1 (Tashiro et al., 1986). 7,8-Benzoflavone stimulates the metabolic activation of aflatoxin B1 to mutagens by human liver (Buening et al., 1978). A mouse hepatoma cell line, Hepa-1, is highly sensitive to the toxic effects of Aflatoxin B1 (AFB1) (Kärenlampi, 1987). Consumption of a CMRN-containing diet provides substantial protection against the initiation of AFB1 hepatocarcinogenesis in the rat (Kelly et al., 2000).

The metabolism of AFM1 and AFB1 has been studied in vitro using human liver microsomes. Formation of primary

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metabolites associated with metabolic activation to the respect-tive epoxides reflected the differences between the carcinogenic potentials of the two toxins and, similar to AFB1, the conjugation of AFM1 epoxide with reduced GSH was catalyzed by mouse, but not human liver cytosol (Decad et al., 1977). The nuclei and microsomes are capable of metabolizing aflatoxin B1 into aflatoxin M1, aflatoxin Q1, and two unidentified fluorescent compounds in the presence of fortified NADPH generating system (Yoshizawa et al., 1981). The levels of expression of many of the forms of cytochrome P-450 involved in AFB1 metabolism are known to be highly sensitive to environmental factors. This indicates that such factors will be an important determinant in individual susceptibility to the tumorigenic action of AFB1 (Forrester et al., 1990).

Among commonly occurring mycotoxins, aflatoxin B1 (AFB1) gained immense importance due to its biological effects and widespread toxicity (Trail et al., 1995). AFB1 is one of stable mycotoxins commonly produced by toxigenic strains of Aspergillus: Aspergillus flavus, Aspergillus parasiticus and Aspergillus fumigatus, that are ubiquitous in hot/humid conditions and natural contaminants of food and feed stuff (Hussein and Basel, 2001; Diekman and Green, 1992). In 1993, WHO-international agency for research stated that rabbits are exclusively recommended by regulatory bodies for developmental toxicity studies also classified AFB1 as class 1 carcinogen and regulate its level to a very low concentration in traded commodities (20 ppb in grains, 05 ppb in milk and 4 ppb in feeds).

Congenital malformations are structural anomalies which take place during embryogenesis. Several medicines and certain chemicals may cause malformations or permanent defects which may lead to death passing through fetal circulation, which is called (teratogenesis) (Brendel et al., 1989).

Development of embryo is affected by teratogens mostly during the process of organogenesis, which is recognized as the time period from the occurrence of the neural plaque to closure of the plate (Stanley and Bower, 1986; Vickers and Brackley, 2002). It begins usually on the 6th–18th day in rabbit (Petrere et al., 1993; Wangikar et al. 2005) during this period, teratogenic agents can lead to significant congenital anomalies.

Rabbits and ducks are the most sensitive species to aflatoxin B1 (AFB1) with the oral LD50 0.3 and 0.36 mg/kg bodyweight, horses and sheep are moderately susceptible to oral LD50 1.0 and 2.0 mg/kg body weight respectively, while chickens are relatively resistant to oral LD50 6.5 mg/kg body weight (Marquardt and Fronhlich, 1992; Pier, 1992). Also, Clark et al. (1980) determined the acute oral LD50 of AFB1 for rabbits as 0.3 mg/kg body weight, while Wangikar et al. (2005) mentioned that 0.1 mg/kg body weight AFB1 is the minimum teratogenic dose in rabbits which interfere with intrauterine development during gestation days 6-18.

However, the literatures about the teratogenic study in rabbits are meager and the rabbit is the most sensitive species to mycotoxins and greatly similar to humans in early developmental patterns (Beaudion et al., 2003) and the extra-embryonic membranes of rabbits are closely related to that of human (Foote and Carney, 2000). This study was planned to elucidate the effects of AFB1 on the skeletal system of rabbit fetuses by administering different doses during gestation period of 6-18 day. Literature on this is lacking

**MATERIALS AND METHODS**

**Experimental animals and mating procedures**

Sexually mature (2.5±0.5 kg), virgin apparently healthy balady rabbits of both sexes (2 males &13 females) from 3-4 months old were used. The animals were kept for two weeks for acclimatization with the housing conditions. The female rabbits in estrous were mated with males and considered as day zero of conception. The pregnant animals were divided into three groups and each individual was kept in a separate cage. All animals were kept under a constant day/night cycle (12 h L/12 h D) for 29 days (pregnancy period). All animals received routinely ad libitum standard rabbit diet (18% crude protein containing granule pellet) free of any mycotoxins at Central Laboratory of Residue Analysis of Pesticides and Heavy metals in Food, Agricultural Research Center, Ministry of Agricultural, Egypt. Tap water was supplied ad libitum on a daily basis. The fetuses were taken by uterine incision after slaughter of the dams.

**Method of treatment**

The control group (4 animals): two animals were dosed by gastric intubation with a dose of 0.2 ml corn oil/kg/day from 6th-18th day of the pregnancy, slaughtered at 29th day of gestation. One animal dosed from 6th-13th was slaughtered at 14th day of gestation and the last one dosed from 14th-21th with the same dose, was slaughtered at 22nd day of gestation.

The intoxicated group (3 animals): received AFB1 with a dose of 0.05 mg/kg dissolved in corn oil 0.2 ml/kg/day from 6th-18th day of the pregnancy, was slaughtered at 29th day of gestation. The intoxicated group (6 animals): three received AFB1 with a dose of 0.1 mg/kg dissolved in corn oil 0.2 ml/kg/day from 6th-13th day, died on 14th day of gestation and three received the same dose from 14th-21th day of the pregnancy, died at 22nd day of gestation.

**Fetus preparation**

All the collected fetuses were weighed separately and their crown rump length (CRL) was measured. The fetuses were carefully examined for gross morphology, visceral anomalies (liver, heart, kidney, brain and eye) as well as the skeleton.

**Fetus staining for skeleton**

Ten fetuses from the control group, 10 fetuses from the intoxicated group with a dose of 0.05 mg and 10 from the intoxicated group with a dose of 0.1 mg at 22nd day gestation were randomly selected and fixed in absolute ethyl alcohol and stained with Alizarin Red-S-AlcianBlue to define the mineralized areas and the cartilage, respectively. The amount of mineralization was calculated as the length of stained portion of the bone by Alizarin Red-S.

The fetuses were firstly fixed in 95% ethyl alcohol for 7 days and subsequently put in pure acetone for degreasing for 3 days. Then,
RESULTS

Macromorphometric measurements and gross anatomical findings

During the period of the experiment, there were no mortalities of any control dams as well as dams of intoxicated group with a dose of 0.05 mg/kg AFB1 while the treated group with high dose of 0.1 mg/kg did not complete gestation and all dams died even when they were administered from 14th-21st day of gestation.

The fetuses of control group and intoxicated with a dose of 0.05 mg were alive at 29th day of gestation after slaughtering of the dams. While the dams intoxicated with 0.1 mg/kg AFB1 from 6th day died at 14th day; three fetuses were of olive seed in size and suffered from total hemorrhage and necrosis. Neither the limb buds nor the brain ventricles formed as compared to the control fetuses. The dams intoxicated with the same dose from 14th day of gestation aborted at 22nd day of dead fetuses with severe vaginal bleeding and died (Figures 1 and 2).

The mean fetal weight of intoxicated group with 0.05 mg/kg and control fetuses was 28±1.29 and 54.28±8.26, respectively while the mean crown rump length was 9.84±0.21 and 11.57±0.47 respectively (Table 1 and Figure 3). They were significantly reduced when compared with the control. While the fetuses of the treated group with 0.1 mg/kg at 22nd day of gestation, showed comparable fetal weights of intoxicated and control groups as 16.05±3.28 and 16.34±0.56 respectively and slight decrease in crown rump (CRL), 7.17±0.47 and 7.18±0.37 respectively (Table 2 and Figures 4 and 5).

The gross anomalies of treated fetuses with 0.05 mg/kg at 29th day and 0.1 mg/kg at 22nd day were severe subcutaneous congestion at different regions including abdomen and head mainly in the eye region. In addition, enlarged eye socket with micro-opthalmic eye and wrinkled skin was seen (Figures 3 and 5). The treated fetuses with a dose of 0.05 mg/kg AFB1 showed small sized liver (Figure 8) as well as small sized heart with shallow interventricular grooves and wider ventricular lumen as compared to control group at 29th day of gestation (Figures 6 and 7).

The mean absolute weight of different fetal organs (liver and gall bladder, stomach and intestine, heart and lungs and kidneys) of treated dams with a dose of 0.05 mg/kg was significantly decreased as compared to control fetuses at 29th days as follow (2.52 ± 0.296 and 3.23 ± 0.23, 1.84 ± 0.11 and 2.47 ± 0.11, 1.69±0.11 and 1.89±0.09, 0.247±0.011 and 0.33±0.036, respectively) (Table 1 and Figure 9). While fetuses of treated dams with dose of 0.1 mg/kg AFB1 which were slaughtered at 22nd day of gestation showed non-significant decrease in absolute organs weight (liver and gall bladder, stomach and intestine and kidneys) as compared to control fetuses at the same age as follows (1.31±0.09 and 1.42±0.13, 1.01±0.029 and 1.08±0.13, 0.21±0.028 and 0.23±0.022, respectively), while heart and lungs of treated fetuses were significantly

skin and internal organs were totally removed to achieve better staining results.

1. They were stained according to the method described by Inouye (1976) and Young et al. (2000).
2. Then the specimen were transferred to transparency process using ascending series of glycerol and 1% aqueous solution of KOH after which they were preserved in 100% glycerin.
3. The stained preparations were carefully examined using Stereo microscope (OPTIKA) to illustrate the different parts of the bones of both axial and appendicular skeleton.

Statistical analysis

The individual data on relative organ weight, CRL and fetal weight were subjected to descriptive statistics (mean and standard deviation), followed by analysis of variance (ANOVA) and T-test according to Argyrous (2005) using Statistical Package for Social Science (SPSS) software program version 16.0 and Excel Microsoft office program (2003). Statistical significance was accepted at P < 0.05.

Nomenclature used in this study was adapted according to Nomina Anatomica Veterinaria (2005) and Nomina Embryologica Veterinaria (2003) whenever possible.
Table 1. Mean ±SD for fetuses at 29th day of gestation of both control and treated with 0.05 mg/kg AFB1.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Control fetuses</th>
<th>Treated Fetuses</th>
<th>T</th>
<th>P-value</th>
<th>Df</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>54.28±8.26</td>
<td>28.00±1.29</td>
<td>8</td>
<td>0.001</td>
<td>12</td>
</tr>
<tr>
<td>CRL (cm)</td>
<td>11.57±0.47</td>
<td>9.84±0.21</td>
<td>9</td>
<td>0.001</td>
<td>12</td>
</tr>
<tr>
<td>Liver and gallbladder weight (g)</td>
<td>3.23±0.23</td>
<td>2.52±0.296</td>
<td>5</td>
<td>0.001</td>
<td>12</td>
</tr>
<tr>
<td>Stomach and intestine weight (g)</td>
<td>2.47±0.11</td>
<td>1.84±0.11</td>
<td>10</td>
<td>0.001</td>
<td>12</td>
</tr>
<tr>
<td>Heart and lungs weight (g)</td>
<td>1.89±0.092</td>
<td>1.69±0.11</td>
<td>4</td>
<td>0.002</td>
<td>12</td>
</tr>
<tr>
<td>Kidneys weight (g)</td>
<td>0.33±0.0365</td>
<td>0.247±0.011</td>
<td>6</td>
<td>0.001</td>
<td>12</td>
</tr>
</tbody>
</table>

P-value = 0.0001. Within the same raw means with different superscripts considered as highly significant (p ≤ 0.01).

Figure 3. A photograph showing significant decrease of CRL of control (C) and fetuses of treated dams (T) with a dose of 0.05 mg/kg AFB1 at 29th day of gestation with micro-ophthalmic eye (arrow) and a dose of 0.05 mg/kg at 29th day of gestation.

Table 2. Mean ±SD for fetuses at 22nd day of gestation of both control and treated with 0.1mg/kg AFB1.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Control fetuses</th>
<th>Treated Fetuses</th>
<th>T</th>
<th>Df</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>16.34±0.372</td>
<td>16.005±3.28</td>
<td>0.268</td>
<td>12</td>
<td>0.794</td>
</tr>
<tr>
<td>CRL (cm)</td>
<td>7.18±0.0372</td>
<td>7.17±0.47</td>
<td>-0.063</td>
<td>12</td>
<td>0.951</td>
</tr>
<tr>
<td>Liver and gallbladder weight (g)</td>
<td>1.40±0.13</td>
<td>1.31±0.09</td>
<td>1.677</td>
<td>12</td>
<td>0.119</td>
</tr>
<tr>
<td>Stomach and intestine weight (g)</td>
<td>1.08±0.13</td>
<td>1.01±0.029</td>
<td>1.58</td>
<td>12</td>
<td>0.139</td>
</tr>
<tr>
<td>Heart and lungs weight (g)</td>
<td>0.797±0.035</td>
<td>0.746±0.051</td>
<td>2.20</td>
<td>12</td>
<td>0.048</td>
</tr>
<tr>
<td>Kidneys weight (g)</td>
<td>0.23±0.022</td>
<td>0.21±0.028</td>
<td>1.378</td>
<td>12</td>
<td>0.193</td>
</tr>
</tbody>
</table>

Within the same raw, means with different superscripts are considered significant (p<0.05).

decreased as compared to the control (0.746±0.051 and 0.797±0.035, respectively) (Table 2 and Figure 10).

**Skeletal anomalies**

The bony and cartilage elements of the fetal skeletons were examined using double staining technique. The bony components were stained red with alizarin Red-S while the cartilage component was stained with alcian Blue. The length and rate of ossification of the long bone can be calculated by the amount of mineralization that stained red by alizarin red-S stain.
Figure 4. A photograph showing control fetus at 22\textsuperscript{nd} day of gestation with intact and normal skin.

Figure 5. A photograph showing fetuses of treated dams with 0.1 mg/kg AFB1 at 22\textsuperscript{nd} day of gestation with subcutaneous congestion at different regions including abdomen and head mainly in the eye region (S), sever wrinkled skin (WR) and microphthalmic eye (m).

Figure 6. A photograph showing heart of fetuses of treated dams (T) with 0.05 mg/kg AFB1 at 29\textsuperscript{th} day of gestation with small sized heart and shallow inter-ventricular groove as compared to the control fetus (C). Inter ventricular groove (A).

Figure 7. A photograph showing cross section of ventricular mass of fetuses of treated dams (T) with 0.05 mg/kg having wider ventricular lumen than that of control fetus (C) at 29\textsuperscript{th} day of gestation.

Figure 8. A photograph showing small sized liver of fetuses of treated dams (T) with 0.05 mg/kg at 29\textsuperscript{th} day of gestation as compared to control one (C).

**Skeleton axiale (axial skeleton)**

Many of the skull bones developed through the process of intramembranous ossification, which does not involve a cartilaginous template. While bones at the base of the skull including the occipital and basisphenoid bones developed through endochondral ossification.
Figure 9. A histogram showing gross parameters for both control and fetuses of treated dams with a dose of 0.05 mg/kg AFB1 at 29th day of gestation.

Figure 10. A histogram showing gross parameters for both control and fetuses of treated dams with a dose of 0.1 mg/kg AFB1 at 22nd day of gestation.

Control fetuses and fetuses of treated dams with a dose of 0.05 mg/kg AFB1 at 29th day of gestation

The skull of control fetuses showed incomplete ossification between parietal and frontal bones where the rostral fontanelle was small and still open and the closure of the sutures (sagittal suture between the two parietal bones, the coronal suture between the frontal and parietal bones,
the interfrontal suture between the two frontal bones as well as the occipito-parietal suture between the parietal and occipital bones) were established but still not closed, while the interfrontal, frontonasal and internasal sutures were well established and closed (Figures 11 and 12). There was nearly complete ossification between basioccipital and basisphenoid bones as well as between the presphenoid and vomer bones while the basisphenoid and presphenoid bones were still connected by a plate of cartilage (Figure 13A and B). Meanwhile, the treated fetuses with dose of 0.05 mg/kg AFB1 showed wide membranous rostral fontanelle. All the developing sutures (sagittal suture between the two parietal bones, the coronal suture between the frontal and parietal bones, the interfrontal suture between the two frontal bones, the occipito-parietal suture between the parietal and occipital bones, the frontonasal sutures between the frontal and nasal bones as well as the internasal suture between the two nasal bones) had smooth adjacent margins and separated from each other by membranes (Figure 14). The ventral surface of the skull of this group of fetuses showed cartilaginous plates between basioccipital and basisphenoid bones, between the pre sphenoid and vomer bones as well as between the basisphenoid and pre sphenoid bones (Figure 15).

Regarding the vertebral column (columnavertebralis), the bodies and arches of all vertebrae of the control fetuses were markedly stained red due to their ossification (Figure 16) while in the one treated with a dose of 0.05 mg/kg AFB1, the lamina of the vertebrae is the only part stained blue because they remain cartilaginous and other parts of the vertebrae (bodies and pedicles) stained red (Figure 17). The transverse processes of the lumbar vertebrae were reduced in the treated fetuses as compared to the control (Figures 18 and 19).
Figure 13B. A photograph of ventral view of skeleton craniale of control fetus at 29th day of gestation showing nearly complete ossification (A) between basioccipital (bo) and basisphenoid (bs) bones as well as between the presphenoid (ps) and vomer (V) bones (C) while the basisphenoid and presphenoid bones were still connected by a plate of cartilage (B).

Figure 14. A photograph of dorsal view of skeleton craniale of fetus of treated dams with a dose of 0.05 mg/kg AFB1 at 29th day of gestation showing wide rostral fontanelle (rf), well established smooth edged sutures (sagittal suture (SS), coronal suture (Cs), occipito-parietal suture (Ops), internasal suture (In), frontonasal suture (Fn) and interfrontal suture (If).

The sternebrae of the control fetuses formed clearly six bony segments completely ossified and stained red while that of treated fetuses with a dose of 0.05 mg/kg AFB1 had five segments with faint red coloration due to delay in their ossification (Figures 20 and 21). The ribs of the control fetuses were markedly stained.
Figure 15. A photograph of ventral view of skeleton cranial of fetus of treated dams with a dose of 0.05 mg/kg AFB1 showing cartilaginous plates (A) between pars basilarisoccipitale (bo) and pars basisphenoidale bones (bs), (C) between the pre sphenoidale (ps) and vomer (V), as well as (B) between the osbasisphenoidale and ospre sphenoidale bones.

Figure 16. A photograph showing ossified cervical vertebrae of control fetus at 29th day of gestation (arrow).

red due to their complete ossification (Figure 22C) while the treated fetus with a dose of 0.05 mg/kg AFB1 show dark violet due to incomplete ossification of ribs (Figure 22T).

Control fetuses and fetuses of treated dams with a dose of 0.1 mg/kg AFB1 at 22nd day of gestation

The skull of control fetuses showed wide rostral fontanelle and all the sutures (sagittal suture between the two parietal bones, the coronal suture between the frontal and parietal bones, the interfrontal suture between the two frontal bones, the occipito-parietal suture between the parietal and occipital bones, frontonasal suture between the frontal and nasal bones and inter nasal suture between the two nasal bones) were well established but still not closely connected by membranes (Figures 23 and 24). While, in the treated group, the rostral part of the parietal bones and the caudal part of the frontal bones was still membranous (unossified), and there was very wide rostral fontanelle. Also, all the developing sutures (sagittal suture between the two parietal bones, the coronal suture between the frontal and parietal bones, the interfrontal suture between the two frontal bones, the occipito-parietal suture between the parietal and occipital bones, frontonasal suture between the frontal and nasal bones and inter nasal suture between the two nasal bones) could be defined by membranes between their smooth adjacent margins and the caudal fontanelle represented by a wide membrane (Figure 25).

The ventral surface of skull control fetuses showed incomplete ossification between pars basilarisoccipitale and osbasisphenoidale and between osbasisphenoidale and ospre sphenoidale (Figure 26) while the treated fetuses with a dose of 0.1 mg /kg AFB1, showed complete separation between the developing pars basilarisoccipitale and osbasisphenoidale, between osbasisphenoidale and ospre sphenoidale as well as between ospre sphenoidale and the vomer. There were
no cartilaginous templates between the previous mentioned bones in this group of fetuses. The vomer seemed to be a thin delicate plate of bone (Figure 27).

Regarding the vertebral column in the control group, there were incomplete ossification of the vertebral arches of the cervical vertebrae, thoracic vertebrae and lumbar vertebrae and ossification of their bodies (Figure 28A and B) while the caudal vertebrae showed ossification in their bodies (Figure 30). The treated fetuses with a dose of 0.1 mg/kg AFB1 showed all the vertebrae throughout the vertebral column were smaller in size than that of the control ones. Also, there was delay in the development of the vertebral arches, there were wide separation between the pedicles in the cervical vertebrae and this space became narrow in the thoracic region containing cartilaginous plate. Also, this separation between the pedicles became wide again in the lumbar and sacral regions contained a faint blue cartilaginous plates (Figure 29A and B) while the caudal vertebrae showed the commencement of ossification in the bodies of the 1st two caudal vertebrae and the rest of the vertebrae remained cartilaginous (Figure 31).

The sternum of the control fetuses showed ossified five
sternebrae and one sternebra still cartilaginous (Figure 32). While, the treated fetuses sternum had three ossified sternebrae and the rest sternebrae were still cartilaginous (Figure 33).

**Appendicular skeleton**

**Control fetuses and fetuses of treated dams with a dose of 0.05 mg/kg AFB1 at 29th day of gestation**

The bones of the fore limb (scapula, humerus and radius and ulna) of the control fetuses were grossly longer than those of the treated fetuses with a dose of 0.05 mg/kg AFB1 (Figures 34, 35 and 36). In the control group, the proximal and distal extremities of the radius and ulna and metacarpi as well as the carpus were still cartilaginous while, the treated group showed blue coloration for both extremities of radius and ulna and their shafts showed violet color due to incomplete ossification of the diaphysis. The carpus, the proximal and distal extremities of the metacarpi and the second phalanges of all digits had no cartilaginous templates in this treated group (Figure 36).
Figure 24. A photograph of dorsal view of control fetus skull at 22nd day of gestation showing well established interfrontal suture (A), frontonasal suture (B) and internasal sutures (C). Frontal bone (Fb) and nasal bone (Nb).

Figure 25. A photograph of dorsal view of the skull of fetus of treated dam with a dose of 0.1 mg/kg AFB1 showing wide caudal fontanelle (cf) and very wide rostral fontanelle (rf) in addition to membranous connection between the developing sutures: sagittal suture (Ss), coronal suture (Cs), interfrontal suture (Ifs), frontonasal suture (Fns) and internasal sutures (Ins).

The bones of the hind limb (oscoaxe, femur and tibia and fibula) of the control fetuses were grossly longer than those of the treated fetuses with a dose of 0.05 mg/kg AFB1 (Figures 37 and 38). The oscoaxe of the treated fetuses had a very small ossified pubis than that of the control (Figure 37). The distal extremity of tibia as well as
the distal raw of tarsus in the control group had cartilaginous templates and the talus and calcaneus showed incomplete ossification while the treated group showed no cartilaginous templates for the distal extremity of tibia, central and distal raws of tarsaus and showed commencement of ossification of talus and calcaneus (Figures 39, 40 and 41).

Control fetuses and fetuses of treated dams with a dose of 0.1 mg/kg AFB1 at 22nd day of gestation

The bones of the fore limb (humerus, radius and ulna) of the control fetuses were grossly longer in length than that of the treated fetuses with a dose of 0.1 mg/kg AFB1 (Figures 43 and 44). The scapulae of both groups were

Figure 26. A photograph of ventral view skull of control fetus at 22nd day of gestation showing incomplete ossification (A) between pars basilarisosoccipitale (bo) and osbasisphenoidale (bs), (B) between osbasisphenoidale (bs) and ospresphenoidale (ps) and (C) between ospresphenoidale (ps) and vomer bone (V).

Figure 27. A photograph of ventral surface of skull of fetus of treated dam with 0.1 mg/kg AFB1, showing separation between pars basilarisosoccipitale (bo), osbasisphenoidale(bs), ospresphenoidale (ps) and vomer (V) (arrows) at 22nd day of gestation.
somewhat similar in length (Figure 42). The control group showed incomplete ossification of distal extremities of radius and ulna. There was beginning of ossification in the carpus proceeding from its medial toward the lateral aspect where the lateral bones (ulnar carpal and 4th carpal bones) had cartilaginous templates. The shafts of the metacarpi were ossified while their extremities still cartilaginous and the phalanges of all digits were ossified.

Figure 28. A: A photograph showing incomplete ossification of the vertebral arches of the cervical vertebrae. B: Incomplete ossification of the vertebral arches of thoracic and lumbar vertebra of the control fetuses at 22nd day of gestation (arrows).

Figure 29A. A photograph showing reduced vertebral size as well as a wide separation of the thoracic vertebral arches. 29B: wide separation of pedicles of sacral vertebra with faint cartilaginous templates of fetus of treated dam with 0.1 mg at 22nd of gestation (arrows).

Figure 30. A photograph showing complete ossification of the bodies of the caudal vertebrae of control fetuses at 22nd day of gestation (arrows).

Figure 31. A photograph of the caudal vertebrae of fetuses of treated dam with a dose of 0.1 mg/kg AFB1 showing ossification of the bodies of the 1st and 2nd caudal vertebrae (A) and the rest remain cartilaginous at 22nd day of gestation (arrows).
In the treated group, the proximal and distal extremities of radius and ulna, the carpus, the metacarpus of the 1st and 5th digit as well as the 1st and 2nd phalanges of all digits had cartilaginous templates (Figure 44).

The bones of the hind limb (femur and tibia and fibula) of the control fetuses were grossly longer in length than that of the treated fetuses with a dose of 0.1 mg/kg AFB1 (Figures 46 and 47). The oscoaxe had ossified ilium and ischium while the pubis was still small cartilaginous part in both control and treated group and also appeared approximately equal to each other in length (Figure 45). The control fetuses showed complete fusion between the distal extremities of tibia and fibula, the tarsus had a

**Figure 32.** A photograph of control fetus sternum at 22nd day of gestation showing ossified 5 bony sternebrae (arrows) and one sternebra still cartilaginous (A).

**Figure 33.** A photograph of fetuses of treated dam, with 0.1 mg/kg AFB1 at 22nd day of gestation showing ossified three sternebrae (arrows) while the rest remained in cartilaginous template.

In the treated group, the proximal and distal extremities of radius and ulna, the carpus, the metacarpus of the 1st and 5th digit as well as the 1st and 2nd phalanges of all digits had cartilaginous templates (Figure 44).

The bones of the hind limb (femur and tibia and fibula) of the control fetuses were grossly longer in length than that of the treated fetuses with a dose of 0.1 mg/kg AFB1 (Figures 46 and 47). The oscoaxe had ossified ilium and ischium while the pubis was still small cartilaginous part in both control and treated group and also appeared approximately equal to each other in length (Figure 45). The control fetuses showed complete fusion between the distal extremities of tibia and fibula, the tarsus had a

**Figure 34.** A photograph showing scapula of control fetuses (C) was grossly longer than that of the fetuses of treated dams (T) with a dose of 0.05 mg/kg AFB1 at 29th days of gestation.

**Figure 35.** A photograph showing humerus of control fetuses (C) was grossly longer than that of the fetuses of treated dams (T) with 0.05 mg/kg AFB1 at 29th days of gestation.

**Figure 36.** A photograph showing grossly longer radius and ulna of control fetuses (C) than that of the fetuses of treated dams (T) with 0.05 mg/kg AFB1 at 29th days of gestation. also there were no cartilaginous templates of carpus (Ca), proximal and distal extremity of metacarpi (Mt) and 2nd phalanx of the treated fetuses. 1st phalanx (1), 2nd phalanx (2) and 3rd phalanx (3).
Figure 37. A photograph showing os coxa of control fetuses (Cont) was grossly longer than that of the fetuses of treated dams (T) with 0.05 mg/kg AFB1 at 29th days of gestation, also the os pubis of treated fetuses represented a small ossified bone (C).

Figure 38. A photograph showing os femur of control fetuses (C) was grossly longer than that of the fetuses of treated dams (T) with 0.05 mg/kg AFB1 at 29th days of gestation.

Figure 39. A photograph showing completely ossified metatarsus (MT) and digital phalanges (1, 2 and 3) of the control fetuses at 29th days of gestation as well as tarsal bones, the talus (Ta) and calcaneus (Ca) were the only ossified and the remaining bones of the tarsus had a cartilaginous templates.

Figure 41. A photograph of planter aspect of pes region of control fetuses (C) and fetuses of treated dams (T) showing tarsus with beginning of ossification of talus (Ta) and calcaneus (Ca) while the rest had no cartilaginous templates in the treated one.

Figure 42. A photograph showing scapula of control fetuses (C) was grossly similar to that of the treated one (T) with a dose of 0.1 mg/kg AFB1 at 22nd day of gestation.

Figure 43. A photograph showing humerus of control fetuses (C) was grossly longer than that of treated one (T) with a dose of 0.1 mg/kg AFB1 at 22nd day of gestation. Also, the extremities of the treated one were stained faint blue.
Figure 44. A photograph showing radius and ulna of control fetuses (control) was grossly longer than that of the treated one (treated) with a dose of 0.1 mg/kg AFB1 at 22nd day of gestation. Also, the proximal (A) and distal (B) extremity of radius and ulna and carpus (C) of treated fetuses had cartilaginous template. In addition, 1st and 5th metacarpi of the treated fetuses and 1st and 2nd phalanx of all digits had cartilaginous templates. 1st phalanx (1), 2nd phalanx (2) and 3rd phalanx (3).

Figure 45. A photograph showing oscoaxe of control fetuses (C) was grossly similar to the treated one (T) with 0.1 mg/kg AFB1 at 22nd day of gestation. cartilaginous template except the calcaneus bone as well as the phalanges of the digits became ossified. While, in the treated group, the tibia and fibula were still completely separated long bones. The distal extremities of the previous bones as well as tarsus except calcaneus bone were still cartilaginous. Also, the shafts of metatarsi were the only ossified parts as the distal and proximal extremities of these bones in addition to the 1st phalanges of all digits had cartilaginous templates. The 2nd phalanges of the digits had no cartilaginous templates and the 3rd phalanges were ossified (Figure 48).

Figure 46. A photograph showing tibia and fibula of control fetuses (C) was grossly longer than that of treated one (T) with a dose of 0.1 mg/kg AFB1 at 22nd day of gestation, the fibula of the treated fetus was still long bone with incomplete fusion (F) and the proximal ext. of tibia did not form cartilaginous template (P).

Figure 47. A photograph showing oscoaxe of control fetuses (C) was grossly similar to the treated one (T) with 0.1 mg/kg AFB1 at 22nd day of gestation. cartilaginous template except the calcaneus bone as well as the phalanges of the digits became ossified. While, in the treated group, the tibia and fibula were still completely separated long bones. The distal extremities of the previous bones as well as tarsus except calcaneus bone were still cartilaginous. Also, the shafts of metatarsi were the only ossified parts as the distal and proximal extremities of these bones in addition to the 1st phalanges of all digits had cartilaginous templates. The 2nd phalanges of the digits had no cartilaginous templates and the 3rd phalanges were ossified (Figure 48).

Figure 48. A photograph of pes region showing ossified shaft of metatarsi (Mt) and 3rd phalanx, the proximal and distal ext. of metatarsi as well as the 1st phalanx had cartilaginous templates while the 2nd phalanx had no cartilaginous template in treated fetus (T) with a dose of 0.1 mg AFB1 at 22nd day of gestation and control fetus (C) show ossified shaft of metatarsi (Mt), 1st, 2nd and 3rd phalanges (1, 2 and 3).

DISCUSSION

Animal experiment is the first scientific preference to learn whether a substance does have a teratogenic effect or not. It is the most acceptable way for drug producers to determine potential teratogenic effect of any given drug on human health. In relation to that, this study documented the teratogenic effect of aflatoxin B1 on the skeletal development of the rabbit fetuses which have
similarity to human in the early developmental pattern (Beaudion et al., 2003) and the extra-embryonic membranes were more closely related to that of human (Foote and Carney, 2000). It was worthy to mention that the selection of the rabbit in this study might be attributed to its economic importance and is the most sensitive species to AFB1 as mentioned by Marquardt and Fronhlich (1992) and Pier (1992).

In the present study, the oral route of administration of AFB1 to pregnant rabbits was selected to ensure the administration of exact amount of mycotoxins to the animals and was considered to be the most accurate way to give a fixed dose. This method of administration was confirmed by Arora (1982). Also, aflatoxins are absorbed mainly from GIT by passive diffusion transferred to hepatic portal blood and very little amount appeared to be transferred into the lymphatic system as reported by Hsieh and Wong (1994).

In pregnant rabbits, the teratogenic doses of AFB1 mentioned by Reddy and Rao (2001) were 1-3 ppm during day zero of conception to one weak post partum. While, Wangikar et al. (2005) used different doses of 0.025, 0.05 and 0.1 mg/kg by gastric intubation during the whole length of organogenesis period and inferred that 0.1 mg/kg body weight was the minimum teratogenic dose. In our study, we administrated the doses of 0.05 and 0.1 mg/kg dissolved in 0.2 ml corn oil by gastric intubation. In this respect, the dose of 0.05mg/kg in rabbits under investigation was the minimum teratogenic dose while 0.1 mg /kg was lethal for both dams and fetuses.

In the current work, the dosing administered during period of organogenesis from 6th-18th day of gestation simulate that recorded by Petrere et al. (1993) and Wangikar et al. (2005) in rabbits. The chosen time of dosing was during the process of organogenesis in the present study due to the teratogenic agents which could lead to significant congenital anomalies as documented by Stanely and Bower (1986) and Vickers and Brackley (2002).

**Fetal observations**

Our results revealed that the fetuses of dead dams at 14th day of gestation intoxicated orally with a dose of 0.1 mg/kgAFB1 were necrosed with total hemorrhage as well as no closure of the neural tube, while Le Breton et al. (1964) showed that the dosing of pregnant rats intoxicated AFB1 with a dose of 0.3 mg/ kg intraperitoneal causes fetal deaths and hemorrhage at utero placental junction. The death of fetuses of the treated dams with a dose of 0.1 mg/kg AFB1 at 22nd day of gestation did not correlate with that given by Wangikar et al. (2005) where there was no fetal mortalities for the same dose in pregnant rabbits.

It was also found that the fetal body weights and crown rump lengths in treated fetuses with a dose of 0.1 mg/ kg at 22nd day of gestation were comparable to the control fetuses; the results were not in accordance with that of Wangikar et al. (2005) for treated rabbit fetuses with the same dose at 29th day of gestation and El-Tahan (2013) in rats with the same dose at 21st day of gestation.

In agreement with Wangikar et al. (2005) in rabbits and Wangikar et al. (2004a, b) in rat the present study revealed no fetal mortalities for the dose of 0.05 mg/kg body weight throughout the period of gestation.

In the rabbit fetuses of the dams treated with a dose of 0.05 mg/kg AFB1, there were significant decrease in both fetal body weight and CRL as compared to control fetuses at 29th day of gestation, the results disagreed with that of Wangikar et al. (2005) in fetal weights and agreed with CRL for the same dose and animal. Fetuses gross anomalies found in our work, including wrinkled skin and enlarged eye socket by both doses were also observed by Wangikar et al. (2005) in rabbits with a dose of 0.1 mg/kg and in rat by Sharma and Sahai (1987) by different doses (7, 3.5, 1.4 and 0.7 mg/kg) body weight and El-Tahan (2013) by a dose of 0.1 mg/kg.

The current study revealed other fetal gross anomalies for both treated doses as sever subcutaneous congestion at different regions including abdomen and head mainly in the eye region; such observations have not been recorded before.

The visceral anomalies as microphthalmiceyes and cardiac defects (small sized heart and wide ventricular lumen) in the fetuses in the study of Wangikar, et al. (2005) showed microphthalmiceyes and fusion of auriculoventricular valves.

The absolute fetal organs: liver, kidneys, stomach and intestine, heart and lung weights was significantly deceased in treated fetuses with 0.05 mg/kg at 29th day as compared to control fetuses, similar observations were found in liver and kidneys in male rabbits intoxicated with AFB1 (Orsi et al., 2007).

**Fetal skeletal anomalies**

As a result of the rarity and unavailability of the literature on the skeletal anomalies induced by AFB1 among the laboratory animals, it was not possible to make a detailed discussion for our results.

Calcium transfer from the mother to the fetus and neonate during pregnancy and lactation plays an extremely important role in the bone health of the mother and infant. Calcium aids in bone health through all ages but is especially crucial during pregnancy and lactation. Development of a novel class of BMP antagonists could lead to new treatments for traumatically and genetically induced heterotopic ossification (Weber et al., 2001). The skeletal elements of the axial and appendicular skeleton are preformed as cartilage templates by a mechanism called endochondral ossification. During this process, a cartilage
template is formed in which chondrocytes proliferate and differentiate into hypertrophic chondrocytes and are gradually replaced by bone (Wuelling and Vortkamp, 2011). Osx regulates chondrocyte differentiation and bone growth in growth plate chondrocytes, suggesting an autonomous function of Osx in chondrocytes during endochondral ossification (Oh et al., 2012). Molecules regulates chondrocyte formation, chondrocyte maturation, and osteoblast differentiation, all key processes of endochondral bone development. These include the roles of the secreted proteins IHH, PTHrP, BMPs, WNTs and FGFs, their receptors and transcription factors such as SOX9, RUNX2 and OSX, in regulating chondrocyte and osteoblast biology (Long and Ornitz, 2013).

The available literature recognized only the teratogenesis of the skeleton from the aspects of incomplete or delay ossification of the bones of axial and appendicular skeleton or reduction or absence of some bones. So, the present thesis focused on tracing the effects of AFB on by two doses (0.1 and 0.05 mg/kg) body weight on craniofacial intramembranous bones which grow normally by ossification at the sutures. Also, the endochondral ossification of the skull base progressed from caudal to rostral direction, from occipital bone, then proceeded to sphenoid, presphenoid and finally to the vomer. Also, the thesis focused on the endochondral ossification of the appendicular skeleton.

The fetuses of treated dams in this work by a dose of 0.1 mg/kg AFB1 showed the commencement of the membranous ossification of the bones of the dorsal surface of skull and no cartilaginous template were formed between the bones of the ventral aspect of the skull while Wangikar et al. (2004b, 2005) mentioned incomplete ossification of the skull bones in the same animal with same dose of AFB1 as well as El-Tahan (2013) in rat fetuses with the same dose.

Regarding the vertebral column, Wangikar et al. (2004a and 2005) observed agenesis of the caudal vertebrae in the rabbit fetuses treated by a dose of 0.1 mg/kg OTA and AFB1 respectively, while the treated group of fetuses with the same dose of AFB1 in the present study showed the commencement of ossification in the bodies of the 1st two caudal vertebrae while the rest of the vertebrae remained cartilaginous.

In this work, all the vertebrae were reduced in size in treated group with a dose of 0.1 mg/kg AFB than the control one, the result which was in a line with that of El-Tahan (2013) in intoxicated rat fetuses with the same dose and toxin.

The sternum of the control rabbit fetuses showed five ossified sternabrae and one sternebra still cartilaginous. While in treated fetuses with a dose of 0.1 mg/kg, sternum had three ossified sternabrae only and the rest still cartilaginous, which was conflicting with El-Tahan (2013) in the rat fetuses with the same dose with failure of sternabrae ossification.

Our results revealed detailed information on each bone in both limbs about length and state of ossification for the treated groups by both doses. In the treated fetuses with a dose of 0.1 mg/kg AFB1, the distal extremities of radius and ulna, the carpus, the metacarpus of the 1st and 5th digit as well as the 1st and 2nd phalanges of all digits had cartilaginous drafts. Also, the distal extremities of the tibia and fibula as well as tarsi except calcaneus bone were still cartilaginous and the shafts of metatarsi were the only ossified parts in addition to the distal and proximal extremities of these bones and the 1st phalanges of all digits had cartilaginous templates while the 2nd phalanges of the digits had no cartilaginous templates. El-Tahan (2013) observed failure or incomplete ossification of long and flat bones of both fore and hind limb in rat fetuses of intoxicated dams with a dose of 0.1 mg/kg AFB1.

3D culture system reveals numerous biological features and widely accepted to be useful for the assessment of toxic compound, but animal experiments are essential considering the effect of microenvironment. For instance, Yoshida et al. (2013) performed the 3D-culture of sebaceous gland cells and was successful in inducing the polarity as epithelial cells and basal membrane formation, but it remains to be known, the interaction between sebaceous glands and hair follicle niche cells. Further, recent cancer researchers emphasized the importance of spheroids rich in cancer stem cells (Yoshida et al., 2013). Invasive behavior of tumor cells in a 3D environment mimick the tumor microenvironment (Brekhan and Neufeld, 2009). Bone morphogenetic proteins (BMPs) regulate cell proliferation, differentiation and motility, and have also been reported to be involved in cancer pathogenesis. BMP4 reduces breast cancer cell proliferation. BMP4 partly recapitulates in 3D culture growth suppressive abilities previously seen in 2D culture (Ampuja et al., 2013).

**Conflict of Interests**

The author(s) have not declared any conflict of interests.

**REFERENCES**


