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Mycotoxicity and embryonic development: I- Aflatoxin B_1 reduces quality and birth rate during mice embryonic development

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Abstract

Mycotoxins are fungal products often found in food, formed during growth, harvesting, drying or storage of fruits, seeds, or grains, leading to a variety of toxic effects in humans and animals. Aflatoxins are a family of polyketide secondary metabolite produced by the food-contaminating moulds *Aspergillus parasiticus* and *Aspergillus flavus*. Aflatoxin B_1 (AFB_1) is a carcinogenic, teratogenic, mutagenic and growth inhibitory mycotoxin. To explore the negative morphological effects of AFB_1 on the developing mice embryo, 50 adult females albino mice (CD1) were divided into five groups (10 females for each group): Control, positive control and three groups treated with a daily oral dose of 5, 10 or 20 µg/kg bw of AFB_1 extract. Fifteen days after treatments, females were caged with males. Pregnancy, mortality, embryos number rates and body weight of female mice and infants were recorded. At 21 days old, crown rump, head, ear, tail, fore and hind-limb lengths of mice infants were investigated. Current data showed a decrease in pregnancy and embryo number rate among females in the animals treated with 10 & 20 µg AFB_1, while treatment with 5 µg showed an increase in pregnancy and embryo number rate compared to control one, while mortality rate among fetuses increased in the three experimental animals treated with AFB_1. Some cases of abortion were observed in 10 & 20 µg groups, while ulcers were observed in 20 µg groups. Weight and all morphometric parameters showed significant decrease except tail length that showed insignificant increase in the treated groups compared to the control one, while ear length showed significant increase. Different congenital malformations were observed in infants such as tail loss, delayed hair and teeth development and poor ossification in treated groups. The present study demonstrated that AFB_1 induced developmental anomalies and reduced quality and birth rate. Data clarified that minimum concentration of AFB_1 required to induce teratogenicity is far beyond the minimum concentrations estimated in earlier literatures. It is suggested that AFB_1 might change genetic elements and consequently interfere with bone mineralization and induce teratogenicity and anomalies during mice embryonic development. Further studies are required to explore the mechanism of action.

Keywords: Aflatoxicosis, teratogenicity, congenital anomalies, birth rate, abortion.

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Competing interest: The authors have declared that no competing interest exists.

Introduction

Mycotoxins are substantial pollutants in animal feed because they are secondary metabolic compounds of some toxigenic fungus. The molds *Aspergillus flavus* and *Aspergillus parasiticus*, which typically infect poorly preserved foods such as peanuts, pistachios, corn and rice, produce AFB_1 and related aflatoxins (Gross-Steinmeyer and Eaton, 2012).

Mycotoxins cause mycotoxicosis (diseases and death) in humans and animals. Fungal contamination of feedstuffs and food commodities occurs frequently in nature and is often mycotoxins are present along with it. As evidenced by several results, ongoing global warming encourages the occurrence of mycotoxins contamination (Liew and Mohd-redzwan, 2018).

Among the most important types of mycotoxins is aflatoxins (Ellis et al., 1991). Aflatoxin contamination of agricultural commodities is, therefore, not only a serious health hazard but also an economic issue (Jelinek et al., 1989). Aflatoxicosis, an acute poisoning caused by high levels of aflatoxins, can be fatal and is typically characterized by acute liver dysfunction (jaundice, lethargy, nausea and mortality). There is a possibility that eating food with an aflatoxin content of 1 mg/kg or more will result in aflatoxicosis. Aflatoxin immunosuppression could reduce an organism's resistance to infection (e.g., HIV, tuberculosis). Children are much less resistant to acute contact than adults. Aflatoxin exposure can result in genotoxic birth abnormalities in offspring (WHO, 2018).

Aflatoxins are a category of chemically similar molecules with minute variations (Fig. 1). There exist several types of aflatoxins that are produced naturally, like AFB_1, AFB_2, AFG_1 and AFG_2 (Wallace et al., 2015; Wild and Montesano, 2009).

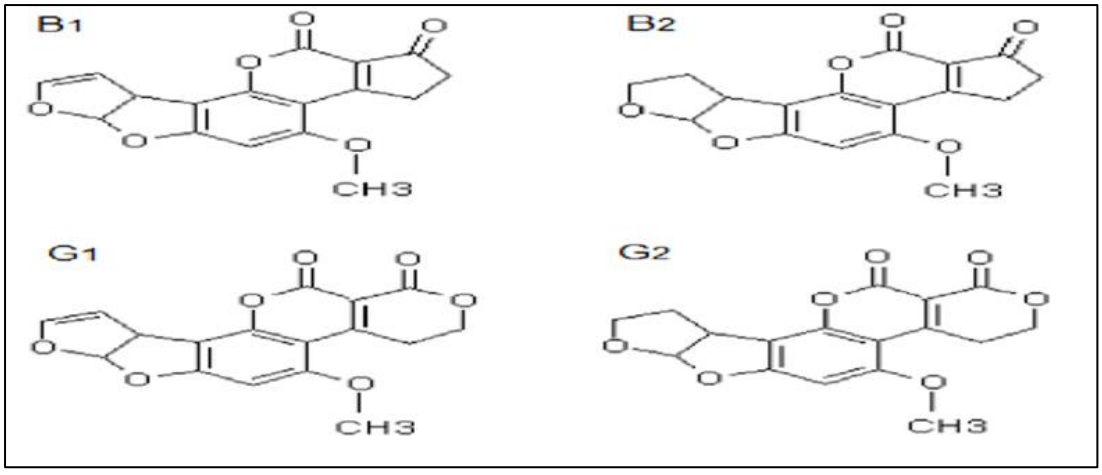

Fig. 1: Chemical structures of aflatoxins (Biehl and Buck, 1987).

AFB_1 is thought to become the most prevalent and powerful one (Yugender Goud et al., 2016). AFB_1-contaminated foods can have more subtle consequences on livestock and poultry; such as immune system suppression, slower development rates, and decreased feed efficiency (Hussein and Brasel, 2001; Zain, 2011). WHO International Agency for Research on Cancer (WHOIARC) classified AFB_1 as Group 1 carcinogen (Hussein and Brasel, 2001). Furthermore, AFB_1 is well known to be genotoxic and carcinogenic and human exposure through the food

chain should therefore be kept as low as possible (Wallace et al., 2015).

The initial phase of AFB_1 breakdown by microsomal cytochrome enzyme (CYP450), which results in AFB_1-exo 8,9-epoxide, has been suggested to be the primary cause of genotoxicity in the instance of AFB_1. However, the oxidative stress brought on by AFB_1 contributes just as much as or more to the mutagenicity of the aflatoxin (Williams et al., 2004). Exposure to AFB_1 has been documented in numerous experimental studies to produce acute and chronic toxicity in people, poultry, livestock and laboratory animals by causing harm to various tissues and systems (Alsayyah et al., 2019).

When given to rats that are pregnant between the sixth and fifteenth days of pregnancy, AFB_1 harms them at a dosage of one mg/kg body weight (Fetaih et al., 2014). When pregnant women or birds are exposed to aflatoxins, the resulting impacts on developing embryos or infertile eggs, respectively, can have a variety of negative health effects and pathological pregnancy/incubation outcomes (Smith et al., 2017). Pregnant woman exposed to increased risk to experience a range of consequences, such as premature birth, fetal loss and restricted fetal growth. There has been evidence of growth limitation in both people and animals, where there is an inverse correlation between birth weight and the concentrations of the right biomarkers in the cord blood (Abdulrazzaq et al., 2002).

This study seeks to explore:

- The negative morphological effects of AFB_1 on the developing mice embryo.
- determine whether a small dose of AFB_1 could induce teratogenicity.
- The effect of AFB_1 on the pregnant females' mice.

Materials And Methods

AFB_1 producing strain

AFB_1 producing strain of *Aspergillus flavus* proved as highly producer in previous study (Zohri et al., 2017), kindly provided from Botany and Microbiology Department, Faculty of Science, Assiut University. This strain was maintained on Czapek's dextrose agar medium aerobically and stored at 4°C until use. Prior to using it for production of AFB_1, it was grown on Czapek's dextrose medium at 28°C for seven days aerobically. Homogenous spore suspension was obtained by scraping fungal hyphae and suspending it in sterilized distilled water containing 0.01% tween 80 and stirring for 30 min then using it as fungal inoculum.

Cultivation of fungal isolates

The prepared inoculum of the fungal strain was transferred to 250 ml flasks, each containing 50 ml potato dextrose broth. The flasks were incubated at 30°C for 10 days as static cultures.

Extraction and purification of AFB_1

After the incubation period, the content of each flask (medium + mycelium) was transferred into a blender jar and homogenized for 2 minutes at low speed and 5 minutes at a high-speed blender (16000 rpm) with 100 ml chloroform. The extraction procedure was repeated again with the same volume of chloroform. Combined, filtered, dried over anhydrous sodium sulfate and then evaporated to near dryness by water bath (Gonzalez et al., 2011). The dry crude extracts were collected and suspended in 50 ml chloroform and applied to a silica gel column (200 mesh, Merck). The column was washed with 50 ml n-hexane and toxins were eluted with 50 ml of

chloroform-acetone (9:1 v/v) solvents system. Elute was collected and evaporated to near dryness (AOAC, 2000).

Detection of AFB_1

Thin-layer chromatography (TLC) analysis

Ten µl of cleaned extract was spotted on TLC plate along with standard AFB_1 purchased from Sigma Aldrich Company. Solvent system used was chloroform: methyl alcohol **(**97: 3 v/v**)**. AFB_1 was detected as fluorescent bright blue under long waves UV light (Ivan, 2005).

High performance liquid chromatography (HPLC) analysis

The concentration of AFB_1 was estimated by HPLC (AOAC, 2000) in Faculty of Veterinary Medicine, Assiut University. The estimated amount of AFB_1 was 6515.9 ng per vial as shown in (Fig. 2).

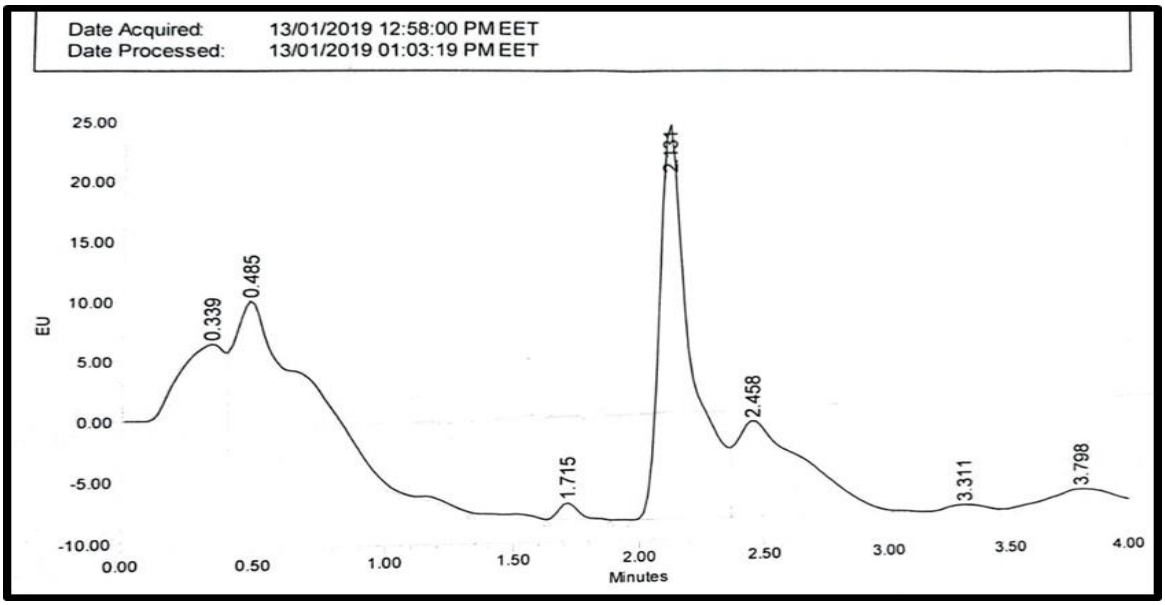

Fig. 2: Estimation of AFB_1 concentration using HPLC.

Experimental design

The AFB_1 residue was dissolved in absolute ethanol (99.9%) and the ethanol concentration was reduced to 10% by adding dist. water to prepare the different doses of AFB_1.

50 Adult females and 15 males albino mice (CD1) of an average body weight 22-25 gm were obtained from the animal house of the Egyptian Organization for Biological Products and Vaccines (VACSERA), Helwan, Cairo, Egypt. Suitable temperature of 23 ± 2 °C and lighting cycle of 12 hours light /dark were also taken into consideration. Adult mice were kept under observation for 10 days before experimentation to exclude any intercurrent infection and to acclimatize the animals to the new conditions. The chosen animals were identified, kept in plastic cages, fed a conventional rat pellet diet and given some vegetables as a source of vitamins. Drinking tap water was provided at libitum.

Five groups of female mice were randomly distributed as Control, positive control treated with 10% ethanol (AFB_1 solvent) and three experimental groups treated with daily oral doses of 5 µg/kg bw, 10 µg/kg bw and 20 µg/kg bw of AFB_1 extract. After treatment for fifteen

days with these doses, females mice were caged in the afternoon with mature males in a ratio of three female/ one male/cage.

Body weight of pregnant mice of both control and treated groups were recorded every day, also body weight of female mice before pregnancy and after delivery (lactation period). Pregnancy rate was calculated by dividing the number of pregnancies for all females in the group throughout the experimental period/the number of females in the group (10 females), also mortality rate of infants and embryo number per mother were calculated.

Morphological investigations

Infants were daily weighed till the age of 21 days old for monitoring the body weight changes and then were examined to investigate morphological changes and abnormalities.

Skeletal investigations

Infants at age 21 days old preserved in 95% ethyl alcohol for Alizarin Red S and Alcian blue stain to investigate the skeletal elements according to the body method of (Salaramoli et al., 2015). Analysis of the optical density (OD) was carried out using the software Image J (the JAVA SE Runtime Environment, version 6).

Morphometric measurements

Crown-rump length, head length, head circumference, head height, tail length, ear length, fore limb and hind limb lengths for 21days old infants mice were measured.

Statistical analysis

The data were expressed as mean ± SE. The results were calculated and statistically analyzed using column statistics and one-way analysis of variance with the Newman-Keuls multiple comparison test as a post test. These analyses were carried out using prism software for windows, version 5.0 & excel (Microsoft office 10). Differences between and among the groups were considered significant if $P < 0.05$, 0.01 or 0.001.

Ethical approval

The research protocol of the present study was performed in accordance with the Ethical Research Committee of the Faculty of science, South Valley University, Qena, Egypt (Approval No. 023/11/22).

Results

Pregnancy, Embryos number and mortality rates

Current data showed a decrease in pregnancy rate among females from 2.6 times per female in the control animals to 2.2 & 1.3 times in the experimental animals treated with 10 & 20 µg/kg bw of AFB_1 respectively, while treatment with 5 µg/kg bw of AFB_1 resulted in an average of 2.9 times per female (Fig. 3). Embryo number decreased from 11.1 per mother in the control to 10.3 & 8.5 per mother in the experimental animals treated with 10 & 20 µg/kg bw of AFB_1 respectively, while treatment with 5 µg/kg bw of AFB_1 resulted in an average of 13 infants per mother (Fig. 4). For mortality rate, 111 infants were born in the control group. Out of them, 7 were dead (or quickly died just after birth) representing a percentage of 6.3%. In the positive control group, 7 infants were dead out of total of 103 representing 6.79%. The dead infants in the treated groups were 10, 12 and 22 out of a total of 130, 103 and 85 representing percentages of 7.69%, 11.65% and 25.88% for the groups treated with 5, 10 and 20 µg AFB_1 respectively (Fig. 5).

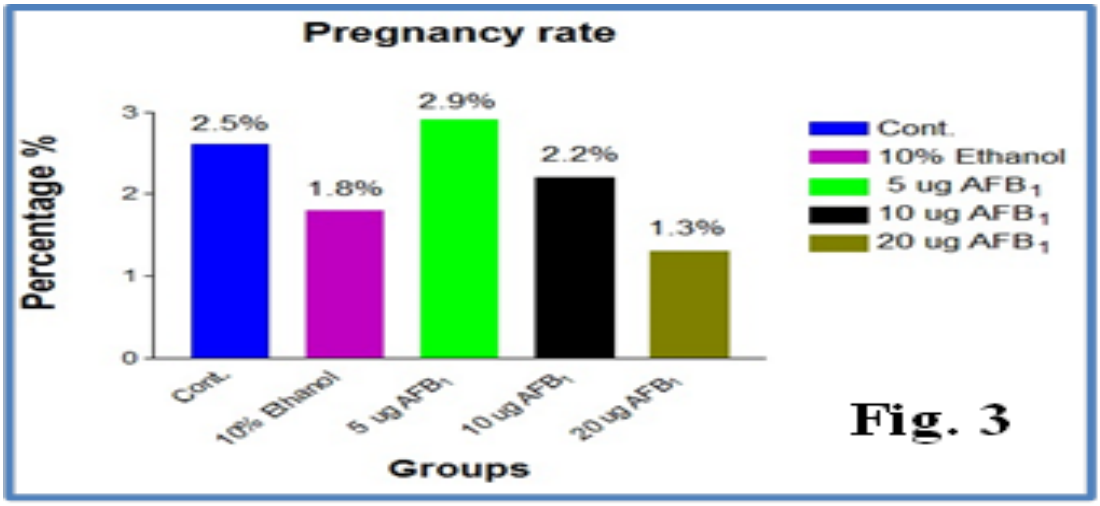

Fig. 3: Pregnancy rate (%). Comparison between different treated groups.

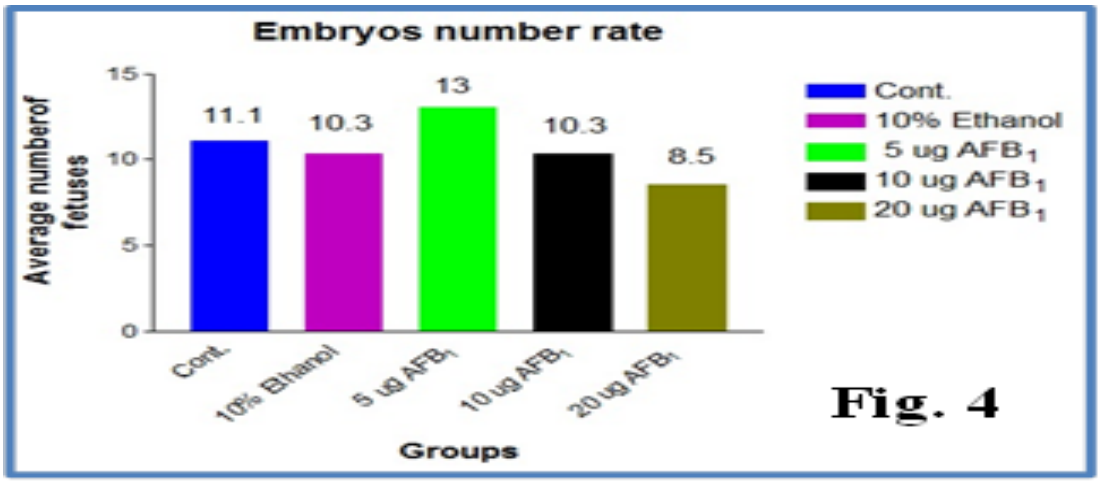

Fig. 4: Embryos number rate. Comparison between different treated groups.

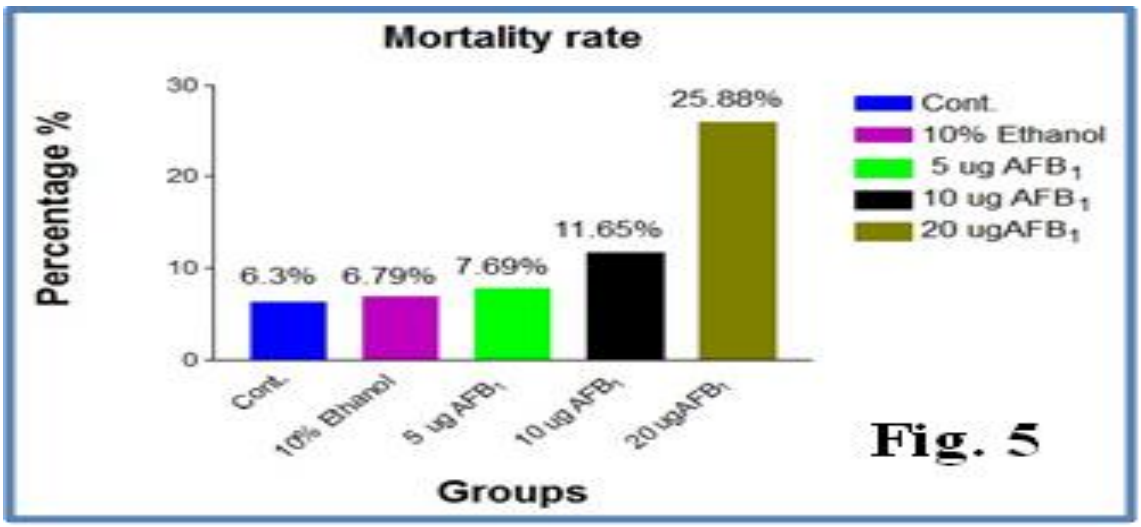

Fig. 5: Mortality rate (%). Comparison between different treated groups.

Measurements of body weight

Statistical analysis of the body weight of females mice before pregnancy indicated that all treated groups showed significant decrease compared to control mothers (Fig. 6). During lactation (after delivery), the two highest doses (10 & 20 µg AFB_1) showed significant decrease compared to control while the lowest dose was insignificantly decreased (Fig. 7). While during pregnancy period, the comparison between the progress of weight of pregnant mothers in different groups showed that the two highest doses caused the lowest progress among all groups (Fig. 8).

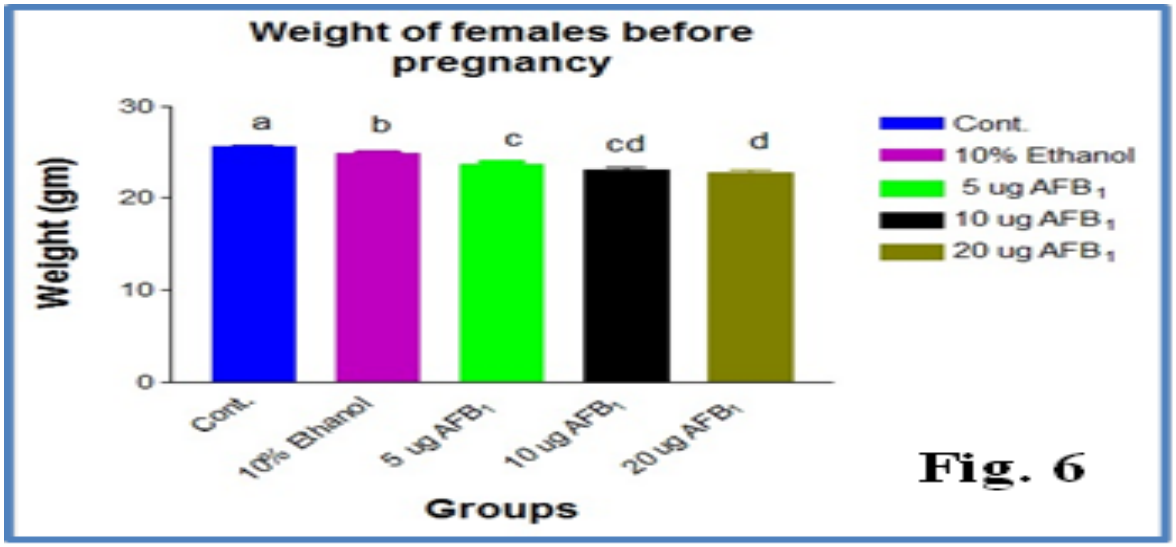

Fig. 6: Weight of females before pregnancy. Comparison between different treated groups. For all figures a, b, c & d significant difference between groups. Means ± SE is presented by columns.

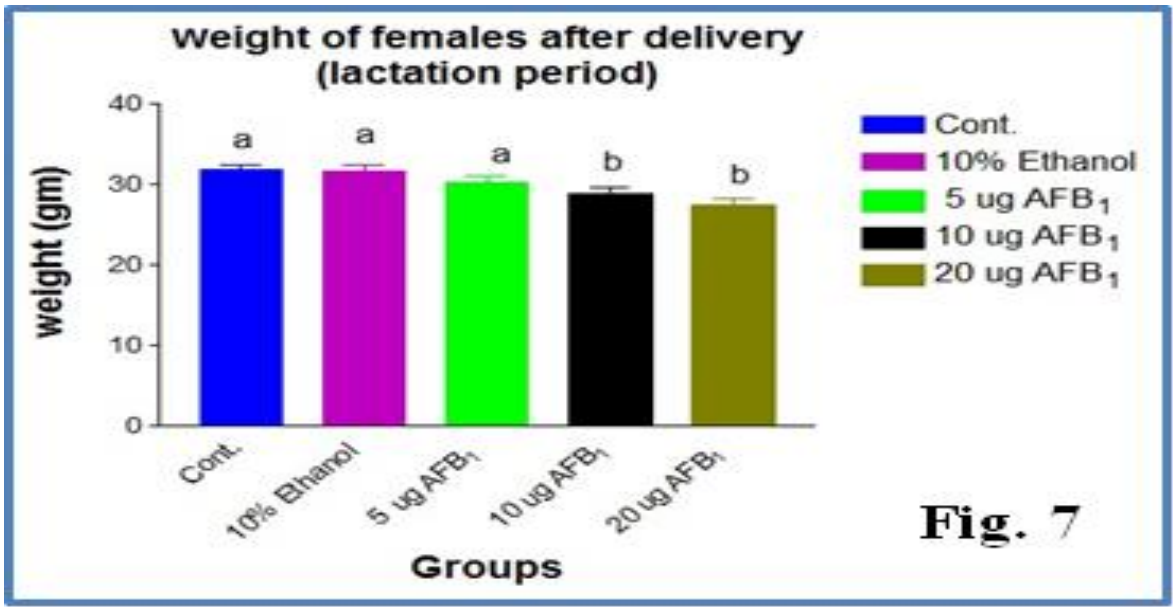

Fig. 7: Weight of females during lactation period. Comparison between different treated groups.

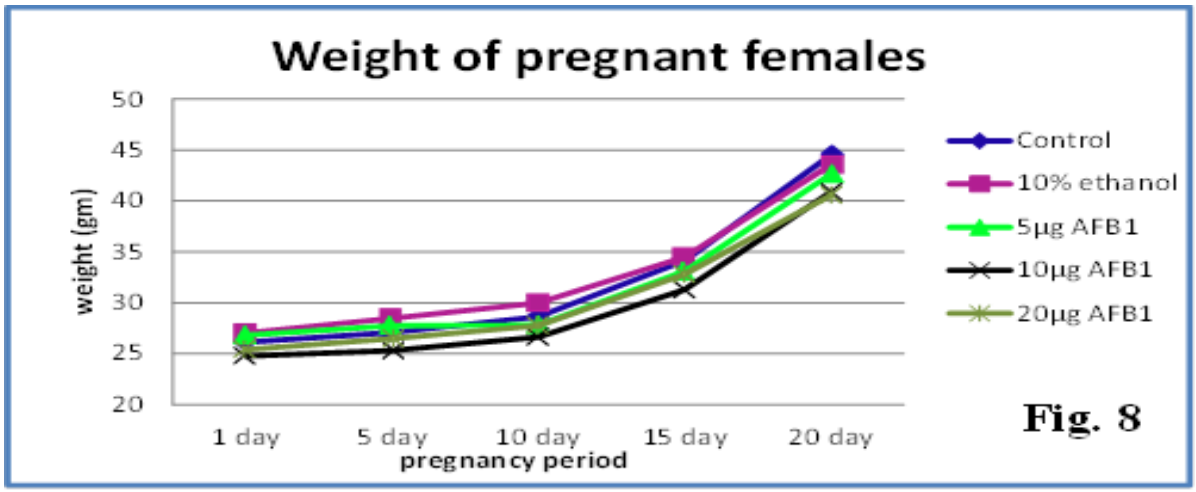

Fig. 8: Weight of females during pregnancy period. Comparison between different treated groups.

Disorders in AFB_1 groups

In 10 µg AFB_1 group, cases of abortion were observed in 4 (18.18%) pregnant females (Fig. 9-a). At 20 µg AFB_1 group cases of abortion were observed in 6 (46.1%) pregnant females (Fig. 9-d), there are many harmful effects of 20 µg AFB_1 dose on female mice such as the appearance of ulcers and abscesses on the body surface (Fig. 9-c & f), tumor in the head region near to the ear (Fig. 9-b). Hair loss in some areas of the body (Fig. 9-e).

In the highest dose treated group, at 7 days old infants hair did not appear (Fig. 10), but hair started to show up on the skin at the 9 days old infants, four days behind the control animals. Congenital anomalies appeared in tail where a constricted area appeared in the middle or at the beginning of the tail at birth and falling of the part of the tail occurs at the constricted area (Fig. 11 & 12).

At 21days old infants mice, the ear length significantly increased in the treated groups (5 µg, 10 µg & 20 µg) AFB_1 and abnormal ear pinnae were observed compared to the control group (Fig. 13). The teeth in 20 µg AFB1group showed a decrease in length and growth in general compared to all other groups (Fig. 14).

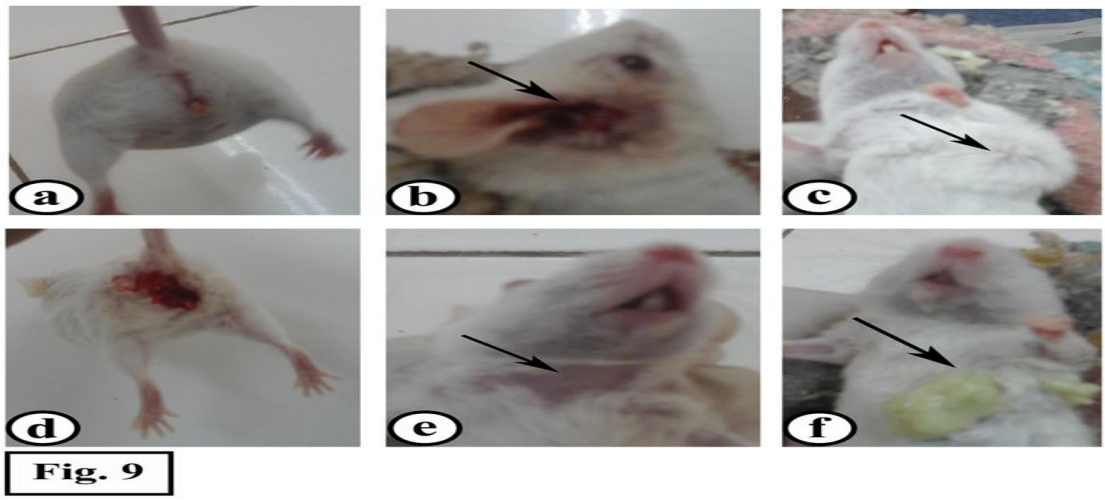

Fig. 9: Photographs of mother mice treated with 10 & 20 µg AFB_1 groups showing, cases of abortion in 10 & 20µg AFB_1 groups (a & d) respectively, tumor (b), ulcers and abscess (c & f) and hair loss (e) in 20 µg AFB_1 group (arrows).

Fig. 10: Photograph of infants mice at 7 days old infants mice treated with 20 µg AFB_1 dose showing, hair delaying and not show up on the skin (left one) compared to control (right one).

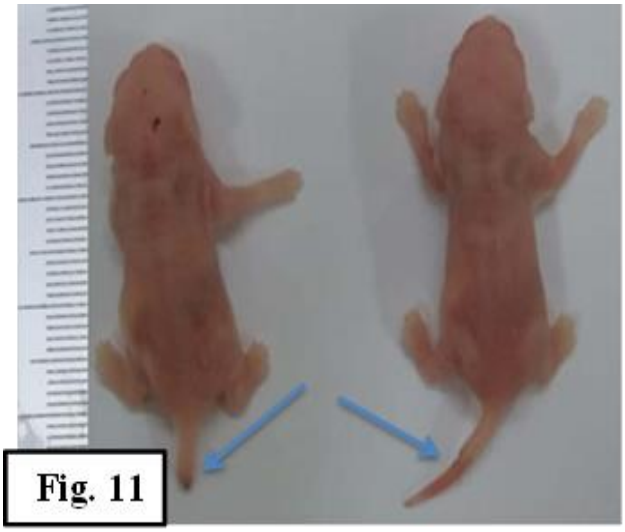

Fig. 11: Photograph of neonatal infants mice treated with 20 µg AFB_1 dose showing, tail loss the middle of tail (right one, arrow), constricted area appeared in the middle of the tail (left one, arrow).

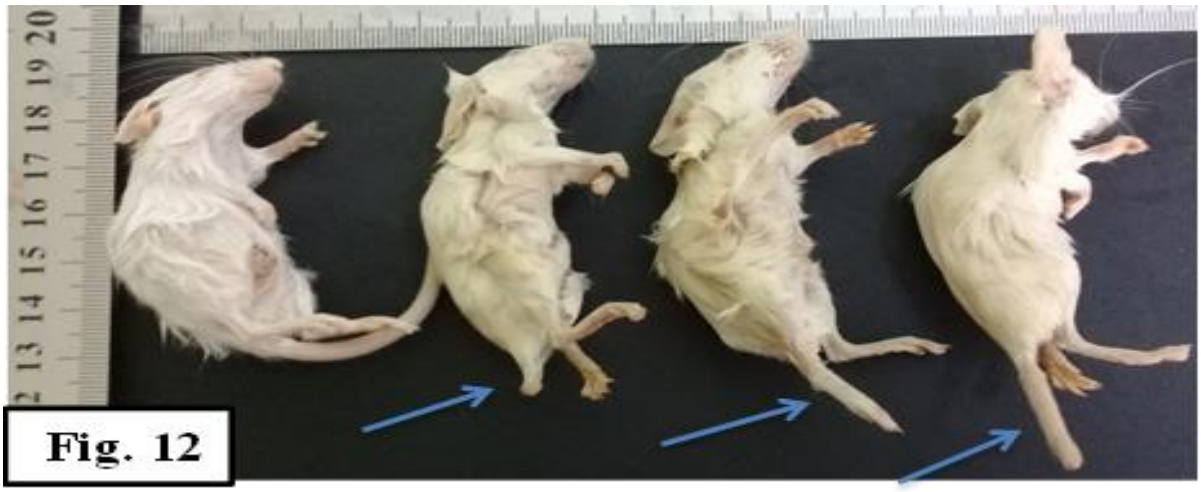

Fig. 12: Photograph of 21 days old infants mice treated with 20 µg AFB_1 dose showing, tail loss and abnormalities (three left mice, arrows) compared to control (right one).

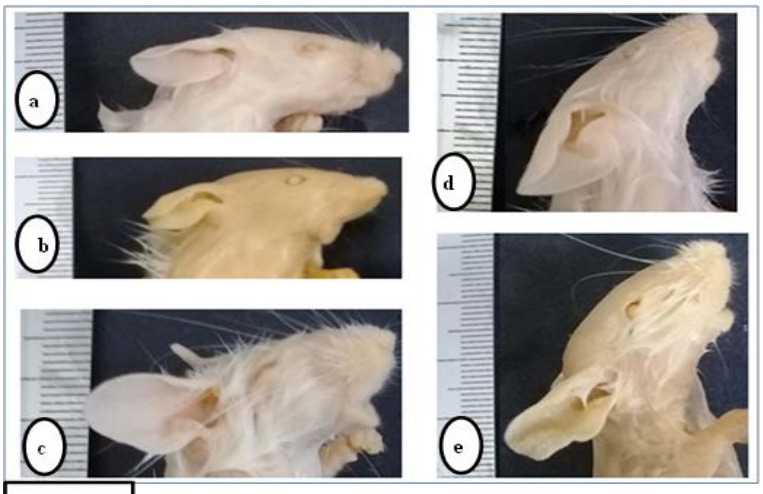

Fig. 13: Photographs of 21 days old infants mice. Comparison between different treatments showing normal ear in groups control (a) and 10% ethanol (b), abnormal ear pinnae and increase in ear length compared to head size in the treated groups (5 µg, 10 µg & 20 µg) AFB_1 (c, d & e) respectively.

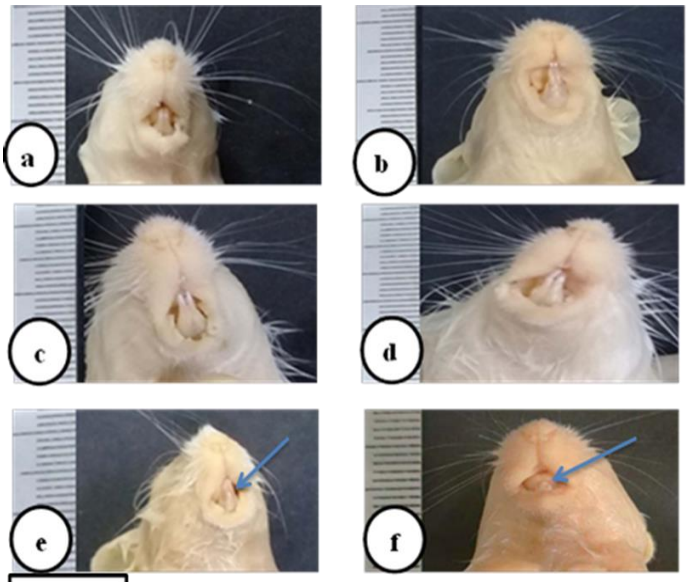

Fig. 14

Fig. 14: Photographs of 21 days old infants mice. Comparison between different treatments showing normal teeth growth in control (a), 10% ethanol (b), 5 µg AFB_1 (c) and 10 µg AFB_1 (d) groups, while in 20µg AFB_1 group (e & f) there was a decrease in length and growth of teeth in general compared to all other groups(arrows).

Skeletal investigation

Skeletal investigation with Alizarin Red - Alcian blue transparencies revealed that the ossification in the treated animals was behind that of the control ones. This was judged by the density of red color of the ossified areas and the greenish blue cartilaginous areas (Figs 16-20). The optical density of red color of the ossified areas significantly decreased in AFB_1treated groups (5, 10 & 20 µg) compared to control and 10% ethanol groups, where $P < 0.001$ (Fig. 15).

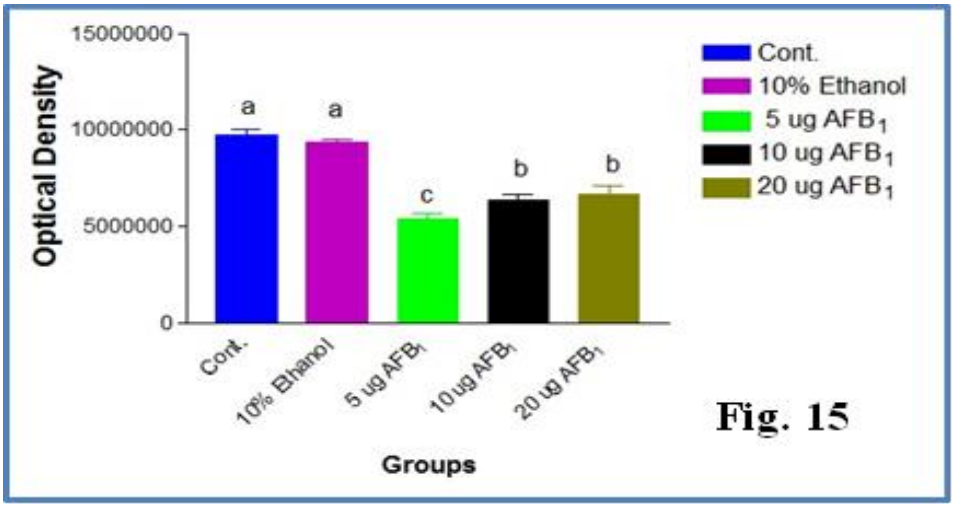

Fig. 15

Fig. 15: Histogram showing the optical density of the ossified areas at the age 21 days old infants mice (Alizarin Red - Alcian blue stains). Comparison between control, 10% ethanol and the three different concentrations of AFB_1 (5, 10 &20 µg).

Fig. 16: Photographs of 21 days old infants mice in control group showing normal ossification of skeletal elements (a & b).

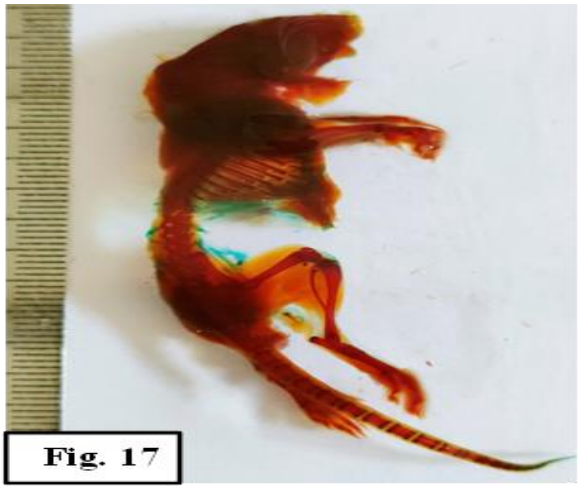

Fig. 17: A Photograph of 21 days old infants mice in 10 % ethanol group showing nearly normal ossification of skeletal elements.

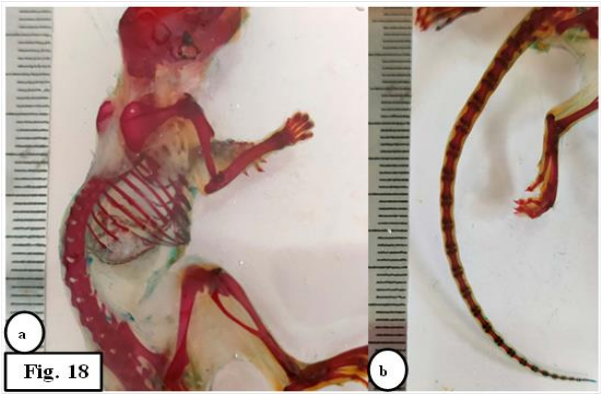

Fig. 18: Photographs of 21 days old infants mice in 5 µg AFB_1 group showing poor ossification of skeletal elements and this was indicated by density of red color of the ossified areas and the remaining greenish blue cartilaginous areas compared to control group (a& b).

Fig. 19: Photographs of 21 days old infants mice in 10 µg AFB_1 group showing poor ossification of skeletal elements and this was indicated by density of red color of the ossified areas compared to control group (a & b).

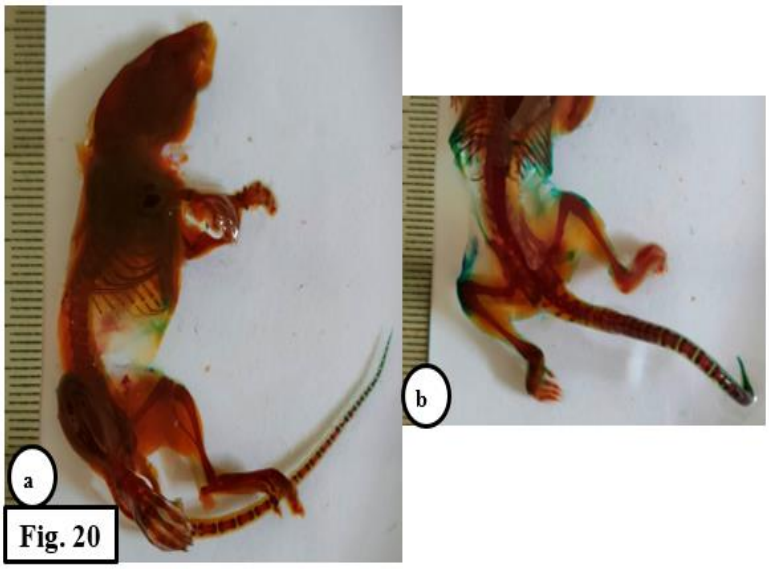

Fig. 20: Photographs of 21 days old infants mice in 20 µg AFB_1 group showing poor ossification of skeletal elements and this was indicated by density of red color of the ossified areas and the blue cartilaginous areas compared to control group (a & b).

Morphometric measurements

Statistical analysis of all the considered morphometric parameters of infants at the age of 21 days such as body weight, crown rump, head length, head height, head circumference, ear pinna, fore limb, hind limb and tail lengths indicated that treatment with different doses of AFB_1 significantly affected such parameters (Figs.21-29).

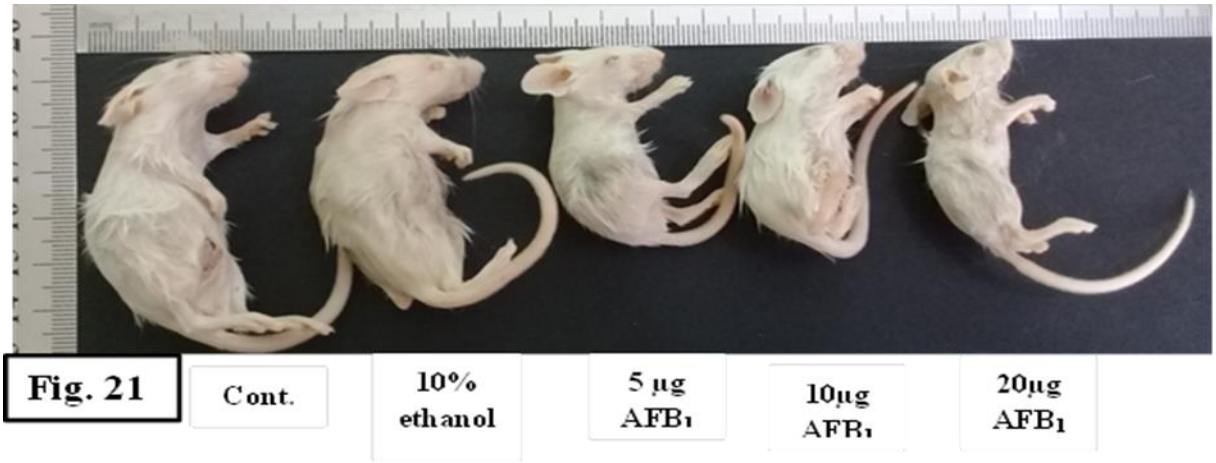

Fig. 21: Photograph of 21 days old infants mice. Comparison between different treatments showing, the size of the body, head size, crown rump length and body weight were decreased. Decrease in hair density and increase in tail length compared to control**.**

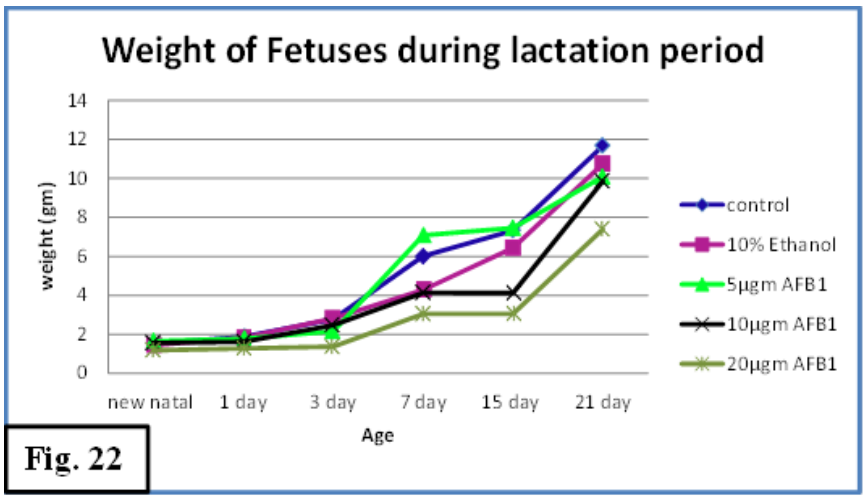

Fig. 22: Weight of infants mice during lactation period. A comparison between different treated groups showed that the two highest doses caused the lowest progress among all group.

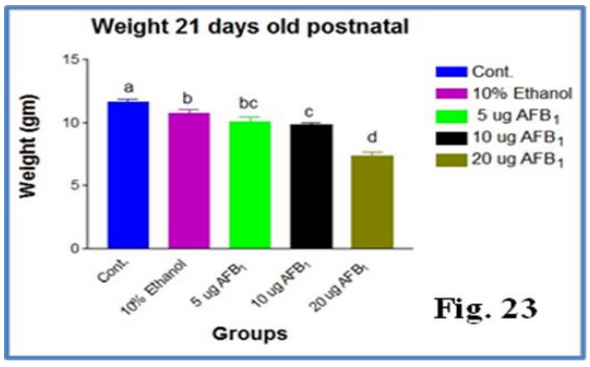

Fig. 23: Comparison of weight (gm) at 21 days old infants mice between different treated groups

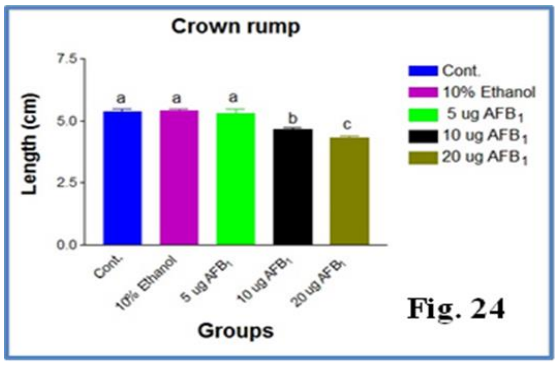

Fig. 24: Comparison of crown-rump length (cm) at 21 days old infants mice between different treated groups.

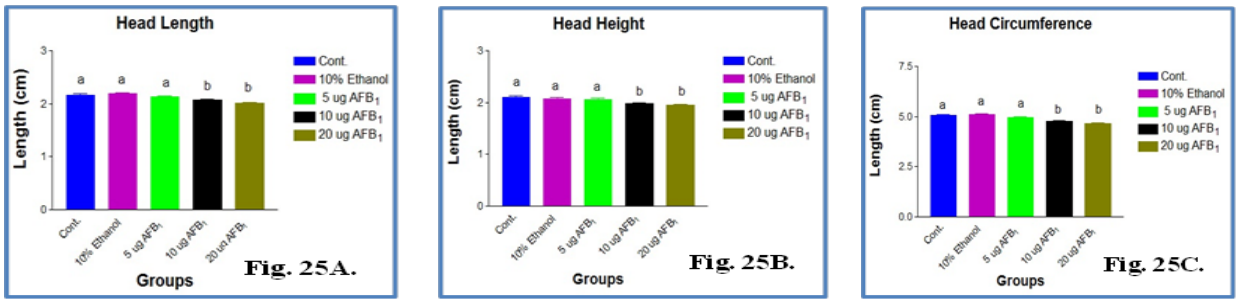

Fig. 25: Comparison of different measurements of head (A. head length, B. height and C. circumference) at 21 days old infants mice between different treated groups.

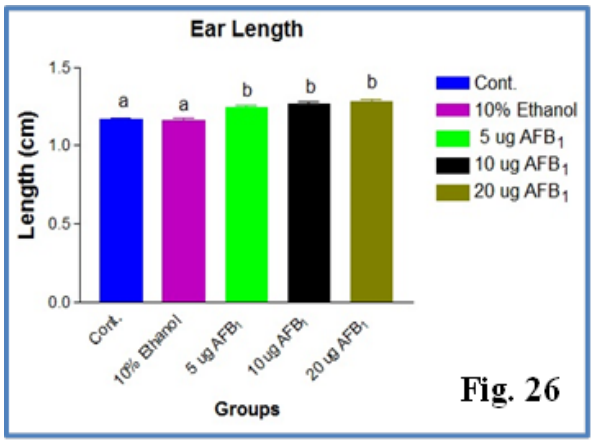

Fig. 26: Comparison of ear length (cm) at 21 days old infants mice between different treated groups.

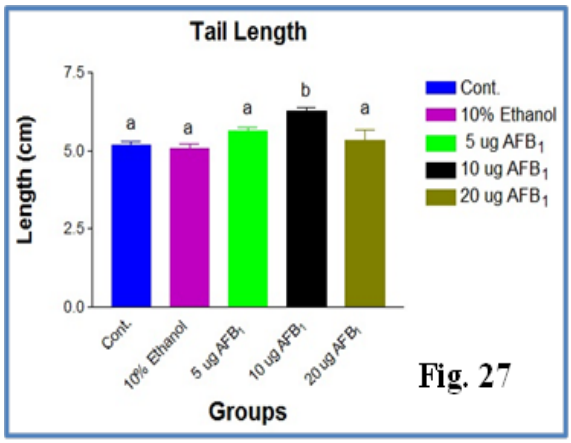

Fig. 27: Comparison of tail length (cm) at 21 days old infants mice between different treated groups

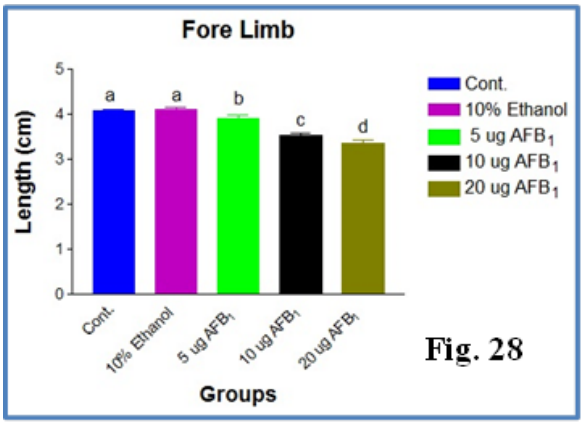

Fig. 28: Comparison of fore-limb length (cm) at 21 days old infants mice between different treated groups.

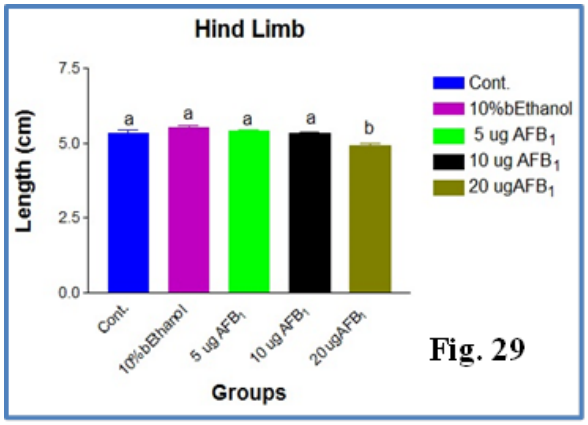

Fig. 29: Comparison of hind-limb length (cm) at 21 days old infants mice between different treated groups.

Discussion

The present study revealed that exposure of pregnant mice to AFB_1 negatively affected of the offspring. AFB_1 increased the mortality rate of the infants while weight of the newborns was decreased. AFB_1 induced ulcers, tumors, hair loss in the pregnant dams and poor ossification of the growing infants in addition to other morphological malformations such as tail loss, abnormal

ear pinnae, delayed hair and teeth development.

Humans can be exposed to AFs at any time during their lives, in utero, through breast milk, through infant formula milk and through infant meals used up to the age of two. Early-life AFs exposure has been associated to a variety of health issues, including poor birth outcomes, stunted growth and development, a weakened immune system and hepatic dysfunction (Ismail et al., 2021). Aflatoxin has several negative effects in animals, it causes liver damage in hens, reduced productivity and reproductive efficiency, lower egg output, poor eggshell quality, poor carcass quality and increased illness susceptibility. Aflatoxin poisoning affects pigs as well, with the chronic effects primarily manifesting as liver impairment. Reduced weight increase, liver, renal damage and decreased milk output are the most common signs in cattle (Fouad et al., 2019).

AFB_1 doses used in the current study were determined deeply lower than the LD_{50} that was previously determined. In rats, LD_{50} of AFB_1 was estimated to be 2.71 mg/kg ranged from 2.0 to 3.7 mg/kg bw (McKean et al., 2006). In rats, oral treatment of AFB_1 at 5 mg/kg for 9 weeks resulted in 100% hepatocellular carcinomas (Newberne and Butler, 1969).

Current study demonstrated that the mortality rate of infants increased while pregnancy rate and average embryos number per labour decreased in the groups treated with AFB_1 compared to control group. Khan et al. (2014) found that in-ovo administration of AFB_1 by embryo resulted in significant mortalities, embryonic malformations and hatchery of chicks with deficient immune system. Such findings are coinciding with the present results.

Current study demonstrated that the body weight of mothers was decreased, and pregnant dams suffered from appearance of ulcers, tumors and falling hair. Also, pregnancy loss (abortion) occurred in the groups treated with different doses of AFB_1. Fetaih et al. (2014) demonstrated that AFB_1 caused harm in pregnant rats when given at a level of 1 mg/kg body weight. The body weights of pregnant females showed a considerable reduction and teratogenic changes in the fetuses, represent the impacts and damage. Ivanovics et al. (2021) found that in domesticated animals and humans, AFB_1 contaminated feeds and meals caused a variety of health concerns, including tumors formation and hepatotoxicity. Exposure to AFs may result in negative pregnancy outcomes such as baby growth impairment, preterm delivery and pregnancy loss (Shuaib et al., 2010).

According to Xu et al. (2021), AFB_1 addition reduced body weight of mice. Wangikar et al. (2004) found that the body weight of pregnant Wistar rats significantly decreased at 1.00 mg/kg dosage. Cases of abortion were observed in of 0.50 and 1.00 mg/kg dose groups. One rat each died from treated groups 0.25, 0.50 and 1.00. In the highest (1.00 mg/kg) dose group, the percent implants resorbed was significantly higher whereas the percent live fetuses significantly decreased as compared with those in controls. The only dead fetus observed was in the 1.00-mg/kg dose group.

Also, Smith et al. (2017) demonstrated that Aflatoxin exposure during pregnancy may affect fetal growth, animal research implies that aflatoxin exposure may raise the chance of preterm delivery and pregnancy loss. The fetus may be harmed by maternal aflatoxin exposure

by direct and indirect toxicity, such as maternal systemic inflammation, reduced placental growth, or increased placental cytokines. Aflatoxin's cytotoxic and systemic actions may have a role in maternal anemia, intrauterine growth restriction, fetal death, premature birth and low birth weight.

The current study positively confirms the earlier findings of Wangikar et al. (2004), Shuaib et al. (2010), Fetaih et al. (2014), Smith et al. (2017), Ivanovics et al. (2021) and Xu et al. (2021) in this aspect.

Abdulrazzaq et al. (2002, 2004) demonstrated that the harmful effects of AFs on fetuses are caused by maternal consumption of contaminated food during pregnancy. In rats, AFB_1 at 2 mg/kg was found to be maternolethal, embryolethal and embryotoxic. Pregnant women and their growing fetuses are sensitive to a variety of environmental stresses, including aflatoxin exposure, a mycotoxin that might contaminate up to 25% of the world's food supply (Mayura et al., 1998).

The International Agency for Research on Cancer has classified Aflatoxin B_1 as Group 1 (agents that cause cancer in humans) (IARC, 1993). Acute exposure to high levels of AFB_1 is uncommon, but it can lead to mortality through aflatoxicosis and liver destruction. Chronic exposure to low levels of AFB_1 is fairly frequent, and while it is not immediately fatal, it can cause immunosuppression as well as carcinogenesis in the liver and possibly the lungs (Ubagai et al., 2008; Gursoy-Yuzugullu et al., 2011).

In the present study, the weight and all morphometric parameters of infants showed significant decrease in treated groups with 5 µg, 10 µg and 20 µg of AFB_1 compared to control except for the ear and tail lengths that showed insignificant increase respectively in all experimental groups compared to control. Different congenital malformations were observed in treated infants such as tail loss, abnormal ear pinnae, delayed hair and teeth development in AFB_1treated groups.

In the studies of Wangikar et al. (2004, 2005), the mean weights and crown-rump lengths significantly decreased and the percent gross anomalies significantly increased in fetuses of rabbits and rats fed diet contained 0.05 to 0.1 and 1.00 mg/kg AFB_1 respectively compared to controls.

El-Nahla et al. (2013) and Partanen et al. (2010) have reported that AFB_1 can pass the placental barrier and cause teratogenic and embryotoxic effects in mammals, implying that maternal exposure may pose a major health risk to the developing embryo.

Ali et al. (2019) demonstrated that, AFB_1 causes developmental abnormalities in the chick embryos, such as decreased hatchability, increased mortality, severe congenital deformities, weight and all morphometric parameters were significantly lower in treated groups. mortality rates in the treatment groups increased. Growth retardation, limb deformities such as limb buds or loss of limbs and poor ossification were all found in treated chick embryos.

The current study positively confirms the earlier findings of Wangikar et al. (2004, 2005), Partanen et al. (2010), El-Nahla et al.(2013) and Ali et al. (2019).

Lauer et al. (2019) demonstrated that birth weight is seen as an indicator of the child's short and long-term growth as well as an index of in utero health and nutritional status. Low birth weight is linked to an increased risk of various negative outcomes in later life, including

preterm morbidity and mortality, child stunting, poor immunological function, reduced cognitive development and chronic illnesses. When the foetus is exposed to a considerable amount of AFs, the rate of low birthweight rises.

Skeletal investigation with Alizarin Red - Alcian blue transparencies in the current study revealed that the ossification rate in the treated animals was behind that of the control ones. Wangikar et al. (2004) demonstrated that when pregnant rats were given AFB_1 with 1.00 mg/kg dose, there was incomplete ossification of skull bones, failure of ossification of small bones in some fetuses and gross immaturity. Wangikar et al. (2005) found that skeletal anomalies of fetuses of rabbits fed diet AFB_1 were agenesis of caudal vertebrae, incomplete ossification of skull bones, bent metacarpals and failure of ossification of small bones. Gross immaturity of all bones. El-Nahla et al. (2013) found that when pregnant rabbits were given AFB_1, in terms of skeletal abnormalities, there was partial ossification in some of the skull bones and the laminae of the vertebral arches remained cartilaginous throughout the vertebral column. The sternum was only partially osseous. There were no cartilaginous draughts in the 2nd phalanx, carpus, or metacarpi extremities. The central and distal tarsal rows, as well as the metatarsal extremities, were cartilaginous. Fetaih et al. (2014) found that AFB_1 causes incomplete ossification of skull bones and failure of ossification of small bones. Some of the skeletal defects that involved mostly ribs and soft-tissue anomalies in rat fetuses.

(Gündüz and Oznurlu, 2014; Yarru et al., 2009) showed how AFB_1 can impact bone mineralization, which can lead to skeletal issues because of decreased reabsorption of calcium (Ca) and phosphorus (P) in chicks fed with AFB_1. Skeletal system anomalies were caused by in-ovo treatment of AFB_1, which interfered with the embryo's ability to grow and build its bone tissue. In limbs, effects are more obvious. According to the current study, the inhibiting action of AFB_1 on bone mineralization may be to blame for the poor ossification and consequently low weight and delayed teeth growth that was manifested in the treated groups. Our findings agree with the earlier studies mentioned above.

There are some mechanisms already known on how aflatoxin can cause cell and DNA damage. Wild and Montesano, (2009) and Khlangwiset et al. (2011;) demonstrated that as soon as aflatoxins enter the body, they are absorbed through cell membranes and into the bloodstream. They are transported through the bloodstream to other tissues and the liver, the primary organ for xenobiotic metabolism. Aflatoxin-8,9-epoxide is converted by cytochrome P450 (CYP450) microsomal enzymes in humans and vulnerable animal species to aflatoxin-8,9-epoxide, a reactive form that binds to DNA and albumin in the blood serum, producing adducts and causing DNA damage.

Woo et al. (2012) illustrated that exposure to genotoxic chemicals at an early age increases cancer incidence later in life. AFB_1 is a potent genotoxin that induces hepatocellular carcinoma (HCC) in many animal species and in humans, mice treated with AFB_1 shortly after birth develop a high incidence of HCC in adulthood. G: C to T: A transversions and G: C to A: T transitions were the most common mutations in AFB1-treated mice. Infant male and female mice experience similar amounts of DNA damage and

mutation from AFB_1 that may initiate the neoplastic process.

Conclusion

The present study demonstrated that AFB_1 induced developmental anomalies and reduced quality and birth rate. Data clarified that the minimum concentration of AFB_1 required to induce teratogenicity is far beyond the minimum concentrations estimated in earlier literatures. It is suggested here that the congenital anomalies observed in the present study might be attributed to the interference of $AFB_1$with the genetic material and induction of mutations and DNA damage and due to inhibiting effects on mineralization of skeletal elements.

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