Effects of Vitamin A Supplementation on Reducing Toxicity of Aflatoxin B1 on the Ovary of Young Female Rats

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Abstract: Aflatoxins are highly toxic, mutagenic, teratogenic and carcinogenic compounds produced by some species of Aspergillus, especially A. flavus and A. parasiticus. This study was designed to investigate the possible therapeutic dose of Vit. A on ovary of young female rats treated with aflatoxin B1 (AFB1). Animals were divided into 5 equal groups each group contains 6 rats. Group I animals of this group had been kept as normal without any treatment and considered as controls. Group 2: Animals of this group were orally administered vehicle 50% DMSO (dimethylsulfoxide) alone. Group 3: Animals of this group were orally administered vehicle 0.05 μg AFB1 per kg dissolved in 50% DMSO (dimethylsulfoxide) . Group 4: Animals of this group were orally administered 0.05 μg AFB1 per kg with Vitamin A (132 IU double the human therapeutic dose). Group 5: Animals of this group were orally administered 0.05 μg AFB1 per kg with Vitamin A (132 IU double the human therapeutic dose). The experiment lasted for 14 weeks, animals were dissected 24 hours after last dose. Ovarian sections of treated female rats showed pathological changes represented by reduction number and deformed follicles, with absence of mature follicles. In addition, semithin ovarian sections exhibited follicles without oocytes, residual in zona granulosa cells with reduction in theca layer. Also serum follicle stimulating hormone (FSH), luteinizing hormone (LH) levels were decreased and estradiol level was increased. Vitamin A showed a partial improvement of histopathological as regards ovary sections observed with numerous follicles in various stages of development (primary, secondary and Graafian follicle, corpora lutea), with presence of some deformed follicles. Also serum follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol levels were improved with supplementation of vitamin A. In conclusion, this study provides evidence that AFB1 adversely indirectly damages ovarian tissue through increasing estradiol, while vitamin A treatment effectively attenuates the toxic effect of AFB1 in the ovary

Keywords: Aflatoxin B1- Vitamin A- Ovary - young female rats

1. Introduction

Aflatoxins are produced by fungi of the genus Aspergillus which grow on corn grain, soybeans, dry beans, cottonseed, wheat and peanuts. The most common AF are AFB1, AFB2, AFG1 AFG2, AFM1 and AFM2. Aflatoxin B1 (AFB1) is the most toxic and is usually predominant (FAO and WHO, 1997). Aflatoxins are not only contaminate our food stuffs, but also are found in edible tissues, milk and eggs after consumption of contaminants for feed by farm animals (Fink-Gremmels, 1999; Bennett and Klich, 2003, Aycicek et al., 2005 and Giray et al., 2007). The toxico-pathological spectrum of AFB1 (in a broad spectrum of vertebrates) is very wide encompassing acute toxicological effects, carcinogenicity, teratogenicity, genotoxicity, immunotoxicity and sometimes death (Wild and Turner, 2002).

The fungal metabolites namely mycotoxins represent the most significant contaminants of food and feed (Aly, 1993). Various members of mycotoxins were detected in animal sera, feed and food and produced severe dangerous changes in active organs (Hassan et al., 2004, 2007 and 2008). AFB1 was reported to exert deleterious effects on the reproductive capacity of lab and domestic female animals (Ibeh et al., 2000; and Abdelhamid et al., 2004). Histopathological examinations of the ovaries in aflatoxin-treated mature domestic fowls showed follicular atresia, accompanied by cessation of egg production during the whole feeding period (Hafez et al., 1982). Aflatoxin is known to be a human carcinogens based on sufficient evidence of carcinogenicity in humans (Yaling et al., 2008).

The mycotoxins in feed consumed by animal and their serum cause disturbances in the hormonal profile related to fertility including follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone (TES.), and can cause abnormal fetal development in farm animals which affect the normal function of reproductive organs and elsewhere the productivity of animals (Tiemann and Vanselow, 2003).

On the other hand vitamin A is significantly prevents aflatoxin induced alterations in the tissue such as liver, kidney and gizzard of chicks. Considerable interactions exist between vitamin A and aflatoxin. In essence vitamin A is anti-mutagenic, both in vivo and in vitro to prevent aflatoxin induced liver damage. Gradelet et al. (1998) reported that carotenoids exert their protective effect through the deviation of AFB1 metabolism towards detoxication pathways. Carotenoids are also effective in reducing DNA damage but less effective than vitamin A. Various studies have demonstrated toxin binding compounds such as vitamins E and C (Hoehler and Marquardt, 1996). However, no data are currently
available on the ability of these to prevent aflatoxin toxicity in rat ovary. Young animals are more susceptible, with the sex and mode of administration of the toxin affecting the response. Therefore, this study was conducted to evaluate the ability of vitamin A to reduce toxicosis of aflatoxins in ovary of young female rat.

2. Material and Methods
Animals and treatments
The present investigation was carried out on young female albino Wistar rats weighing 80-100 g. Animals were acclimatized for two weeks and the commercial food and tap water were supplemented ad libitum during acclimatization period. The animals were subsequently divided into five groups of 5 rats each.

Group1: Animals of this group had been kept as normal without any treatment and considered as controls.

Group2: Animals of this group were orally administered vehicle 50% DMSO (dimethylsulfoxide) alone.

Group3: Animals of this group were orally administered with Vitamin A (132IU double the human therapeutic dose)

Group4: Animals of this group were orally administered 0.05μg AFB1 per kg dissolved in 50% DMSO (dimethylsulfoxide).

Group 5: Animals of this group were orally administered 0.05μg AFB1 per kg with Vitamin A (132IU double the human therapeutic dose).

The experiment lasted for 14 weeks, animals were dissected 24 hours post treatment then animals were sacrificed by ether overdose.

Chemicals
Aflatoxin B1 used in this study were obtained from Sigma Chemical Company (St. Louis, USA). It was dissolved in sun oil and orally given at dose 0.05 μg/ kg body weight/day. Vitamin A is available in market as capsule contain 50000 IU(132IU double the human therapeutic dose), as described by Sinha and Dharmshila (1992). The therapeutic dose for rat was calculated according to the table given by Paget and Barnes(1964). The dose was given orally and estimated according to the weight of the rat.

Histological preparation
Immediately after sacrificed, ovaries were quickly removed and fixed in Bouin's fluid then dehydrated in an ascending series of alcohol, cleared in two changes of xylene and embedded in paraffin wax. Sections of 5 micrometers thickness were cut using rotary microtome and mounted on clean slides, for histological examination sections were stained with Ehrlich's haematoxylin and eisin.

Semithin sections Studies:

The following steps in preparing sections for semithin were carried out. Fresh small pieces of ovarian tissue up to 1 mm² in size were fixed in 4% glutaraldehyde-formaldehyde for 5 hr. then in (0.2 M) Na cacodylate for 2hrs. at 4°C, then washed in phosphate buffer pH. 7.2 for 30 min. and post fixed in 1% osmic acid (2% OsO₄+ 0.3 M of Na cacodylate) for 2 hrs. at 4°C, then washed in phosphate buffer (pH 7.2) for 30 min. at 4°C. Samples were dehydrated through ascending grades of ethanol and embedded in epoxy resin in an oven at 60°C for 14 hrs. to produce a firm block, then processed for preparation of semithin sections which were stained by 1% toluidine blue (Hunter, 1984). Sections were prepared and examined at the Central Lab., Faculty of Science, Ain Shams University.

Biochemical analyses
For hormone determination, blood samples were withdrawn through a heart puncture and then centrifuged. Serum were stored at -20 ºC until assayed for the biochemical parameters. FSH, Determination of FSH by enzyme-linked immunosorbent assay (ELISA) kits according to Rose (1998). Determination of LH by ELISA kits according to Rebar et al. (1982) and estradiol determination of total E2 by radioimmunoassay (RIA) kits according to Xing et al. (1983).

Statistical analysis
The results were expressed as mean ± SD of different groups. The differences between the mean values were evaluated by Student’s t-test (Fowler et al., 1998).

3. Results
Histological Observations
Sections of ovary of control rat revealed that it consists of spindle shaped cells, fine collagen fibres and ground substance which together constitute the ovarian stroma. The peripheral zone of the stroma, the cortex, contains numerous follicles in various stages of development (primary, secondary and Graafian follicle). In addition, corpora lutea and atretic follicles are present (Figs.1a,b&c).Ovarian sections of rats treated daily with vehicle 50% DMSO (dimethylsulfoxide) for 14 weeks showed normal structure of germinal epithelium as well as healthy follicles and stromal cells(Fig.1d),Sections in ovaries of rats daily treated with vehicle and Vitamin A (132IU double the human therapeutic dose) for 14 weeks revealed the stroma containing numerous developing follicles (Figs.1e&f). On the other hand section in ovary of rats orally receiving 0.05 μg AFB1 / kg dissolved in 50% DMSO (dimethylsulfoxide), showed decrease in the number of developed follicles where only primary follicles appeared with necrotic cells of their columnar border (Fig.2a). Many
deleterious histological changes were induced on the tertiary follicle such as, destruction and separation of basement membrane of oocyte and the theca follicle from the zona granulosa appeared with vacuoles (Fig.2b). In addition a large number of developing follicles were severely affected including some abnormal Graafian follicles that appeared elongated with eccentric nucleus, and without corona radiata and cumulus oophorus and abnormal feature zona pellucida and zona granulose (Fig.2c). Other follicles appeared containing wide zona pellucida in one and vacuolation another, and dilated congested blood vessel (Fig.2d). Also noted that the histological sections was not show any mature follicles. Ovarian sections of rats treated daily with AFB1 with Vitamin for 14 weeks exhibited marked improvement in the histological state compared with those of animals treated with AFB1 alone. Normal like stages of oogenesis, primary, secondary, tertiary and Graafian follicles were observed. Yet some deformed follicle appeared where the ovarian medulla contained large number of vacuoles and hemorrhagic lesions. On the other hand some sections showed dilated and congested blood vessels, and many deformed follicles including elongated Graafian follicles and antrum follicular. Also oocytes appeared without corona radiata and cumulus oophorus. Zona granulose cell layers and their theca became thinner (Fig.3d). Secondary follicles appeared with degenerated zona granulosa and theca, where zona pellucida was not apparent (Fig.3d).

Semithin sections:
Histopathological examination of the semithin ovarian sections in G1,G2 and G3 showed normal histological pattern including numerous follicles in various stages of development (Fig.4a). Secondary and tertiary follicles, appeared with normal oocytes and zona granulosa and antrum follicular (Figs.4b &c). Sections in ovaries of rats daily treated with AFB1 for 14 weeks G4 revealed many histological changes such as reduced number of developing follicles and hemorrhages (Figs.4d&f). Many follicles appeared without oocytes (Figs.5b,c,d&e). Antrum follicular and zona granulosa were only seen in tertiary follicles (Fig.5c). Some follicles appeared containing residual cells of zona granulosa and thecal layer became thinner (Fig.5c). Examination of ovary sections of rats treated with AFB1 followed by Vit.A showed marked improvement in development follicles including those that contain oocytes, antrum follicular compared with animals treated with AFB1 (Fig.5f).

**Biochemical Results**

**Changes in FSH, LH and estradiol.**

Animals treated with AFB1 (G4) revealed very highly significant ($P < 0.001$) decrease in serum follicle stimulating hormone (FSH) and serum luteinizing hormone (LH) levels reached 5.80±0.43 and (16.24±0.44) compared with control group (8.98±0.45) and (25.56±0.64) as shown in table (1). Animals treated with AFB1 and vitamin A (G5) the results indicate partial improvement in serum follicle stimulating hormone (FSH) and serum luteinizing hormone (LH) levels significant decrease ($P < 0.05$) reach (7.44±1.73d and 22.28±2.37), while animals in (G2) and (G3) showed non-significant changes in FSH and LH levels when compared with control group (Table1). The data shown in table (1) indicate that AFB1 administration(G4) induced very highly significant ($P < 0.001$) increase in estradiol level reached (16.0 ±0.83) compared with control (2.56±1.03) while animals treated with vitamin (G5) showed significant decrease (4.61±1.36) when compared with AFB1 (G4) treated rats showed highly significant increase in estradiol level in serum of animals (G5) compared with control. Treating animals (G2 and G3) showed non-significant difference in levels of FSH, LH and estradiol when compared with control group.

**Table (1):** Showing the effect of administration of aflatoxin B1 and vitamin A on serum follicle stimulating hormone(FSH) and serum luteinizing hormone (LH) concentration and estradiol level in female rats for 14 week.

<table>
<thead>
<tr>
<th>Groups</th>
<th>(FSH) mIU/ ml (M±SD)</th>
<th>(LH) mIU/ ml (M±SD)</th>
<th>(E2) ng/ ml (M±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.98±0.45</td>
<td>25.56±0.64</td>
<td>2.56±1.03</td>
</tr>
<tr>
<td>Control + Vehicle</td>
<td>8.50±0.67</td>
<td>26.42±2.59</td>
<td>2.68±1.04</td>
</tr>
<tr>
<td>Control+ Vit. A</td>
<td>8.84±1.51</td>
<td>26.08±2.21</td>
<td>3.04±0.83</td>
</tr>
<tr>
<td>Aflatoxin</td>
<td>5.80±0.43***</td>
<td>16.24±0.44***</td>
<td>16.0 ±0.83***</td>
</tr>
<tr>
<td>Aflatoxin + Vit A</td>
<td>7.44±1.73*</td>
<td>22.28±2.37*</td>
<td>4.61±1.36**</td>
</tr>
</tbody>
</table>

$P < 0.05$ *Significant $P < 0.01$ **Highly significant $P < 0.001$ ***Very highly significant
Fig. 1: Photomicrograph of ovarin sections of rat (a, b & c) control showing primary follicle (PF), secondary follicle (SF), tertiary follicle (TF), Graaffian follicle (GF) corpus luteum (CL) (d): control with vehicle and (e & f) control with vitamin A showing all types of follicles. (H&E a, b & dX100 - cX40 - cX400).

Fig. 2: Photomicrographs of ovarian sections of rat treated with aflatoxin showing large number of severely affected, developing follicles, (a) showing only primary follicle with nicrosis in columnar cells (N), (b) showing tertiary follicle with loss of nuclear membrane of oocyte (arrow head), vacuoles in theca extrema (arrow) and normal follicular antrum, (c) showing deformed follicle (DF), accentric nucleus (arrow), (d) showing dilated, congested blood vessel, and deformed follicle (arrow) Note that the histological sections do not show any mature follicle (H&E aX40 - bX400 and c, dX100).
Fig. 3: Photomicrographs of ovarians sections of rat treatment with aflatoxin and vit. A., (a&b), showing improvement of developing follicles secondary follicle (SF), tertiary follicle(TF), Graffian follicle(GF), with deformed follicle (DF) hemorrhage(H) and vacuolation(arrow). (c), showing deformed of secondary follicle and, graffian follicle(DF), and dilated congested blood vessel(D.C.B.V.). (d) showing severely affected secondary follicle(arrow). (H&E aX40 - bX40 – c X100, and dX400).

Fig. (4) Semithin ovarian sections of control:
(a) showing large number of developing follicles (arrows), (b&c) magnified of (a) showing normal oocytes (o) zona granulose (zg) and antrum follicular (arrow);
(d,e,f&g) ovarian sections of treated with aflatoxin:
(d) showing malformations in large number of developing follicles (arrows), hemorrhage (H) in (e&f) showing absence of oocytes (short arrow) hemorrhage (H)
(g) showing residual cells of zona granulosa and thin theca layer (arrows);
(h) ovarian sections of treated with afl. + Vit. A. showing normal follicle, oocytes (0), zona granulose (zg) and antrum follicular (Af). Toluidine blue (a&d) x 40, b, c, e,f,g &h x 400.
4. Discussion:

Aflatoxins (AFs) are highly toxic secondary metabolites produced by the species of Aspergillus, especially *A. flavus* and *A. parasiticus*, in most livestock as well as humans by their continuing intermittent occurrence in both feeds and foods (Abdelhamid *et al.*, 1990; Robens and Richard, 1992; Abdelhamid, 2008). Several factors may enhance the occurrence of mycotoxin in the human diet in developing countries. These include eating habits, existing marketing problems which encourage long storage periods; the pre- and post-harvest practices that encourage accumulation of moisture and thus mold growth, ignorance, and poverty. This is aggravated by the fact that there are no strict regulations that impose limits on the concentration of mycotoxins in crops that are marketed in these countries as well as lack of relevant technology required in monitoring fungi and mycotoxins in grains (Wilkister and Nyaora, 2008). In the present study, many histopathological changes were seen in the ovary of albino rats after treatment with aflatoxin including decrease in number, and deformities in of developing follicles present with degenerated zona granulosa cells and absence of mature follicles, hemorrhage and dilated congested blood vessels. Semithin sections displayed the deformed follicles such as absence of oocytes, residual cells in zona granulosa, and reduction in theca layers. This may be attributed to the direct effects of AFB1 on reproductive cycle. Data showed disturbances in estrus cycle, significant reductions in the number of oocytes and large follicles, as well as inhibition and reduction in conception rates (Ibeh and Saxena, 1997).

Decrease of zona granulosa may caused immature follicles. Some studies explained the granulosa cells of the follicles to exhibited a great number of mitochondria, that might afford a greater developmental potential if the oocytes of such follicles were allowed to mature in vitro (Mobarak, 2009). Accordingly failure of in vitro-matured oocytes may be a partly attributed to a reduced number of mitochondria, resulting in insufficient production of adenosine triphosphate required for developmental events (Pizzo and Pozzan, 2007). These results are similar to that obtained by some investigators. Kourousekos and Lymberopoulos (2007) reported deleterious effects of aflatoxin on the reproduction system, i.e., sexual maturation, growth and maturation of the follicles, levels of hormones, gestation, and growth of fetus. Abdelhamid (2005) found that aflatoxin lowered the fertility to 13% and increased the mortality of embryo. Mycotoxins (including aflatoxins) adversely affect the reproductive systems of various animal species (Abdelhamid, 2008). The ovaries showed follicular atresia which has a detrimental effect on egg production (Hafez *et al.*, 1982; Del Bianchi *et al.*, 2005; Pandey and Chauhan, 2007).

These findings may be referred in part due to the adverse effects of aflatoxin. Abd El–Wahhab (1996), noted from microscopic examination of ovaries of female rabbits treated with 0.15 mgAFB1/kg BW that there were some pathological alterations in the form of (1) coagulative necrosis which appeared mainly in the growing and mature follicles and (2) decrease in number and size of Graffian and growing follicles with increased number of atretic follicles and small areas of degenerative changes. Moreover, chronic exposure to aflatoxin decreased reproductive efficiency of ruminants (Diekman and Green, 1992). Fertility of pregnant rats decreased after aflatoxin and embryonic resorptions, malformations, and developmental retardations occurred (Cilievici, *et al.*, 1980).

Effect of aflatoxin-contaminated diet on performance of laying hens showed a significant decrease in egg production and egg weights (Rizzi *et al.*, 2003; Zaghloul *et al.*, 2005; Pandey and Chauhan, 2007). On another way, the results were disagree with Oliveira *et al.* (2000) and Oliveira *et al.* (2003) who found that in laying hens fed on AFB1, production and egg weight were not significantly affected.

So present data indicate that the carry over of aflatoxin B1 residues is relatively most probable to occur in laying hens when the birds are continuously exposed for long periods to low level of aflatoxin in the diet. This fact may be related to the lower capacity of laying hen in detoxifying aflatoxin B1 (Hassan, 1995; Del Bianchi *et al.*, 2005). Aflatoxins incorporated into the feed of laying hens may cause relevant lesions in liver and in kidneys, heart and ovaries. Results also, indicated that prolonged administration of aflatoxins, may cause economic losses to egg producers, besides aflatoxins in egg even in small amounts may cause public health problems due to its cumulative effects for egg consumers as concluded by Chowdhury and Smith, (2004) and Ogido, *et al.* (2004).

In the present study, Aflatoxins significantly decreased the levels of both LH and FSH. Aflatoxin treatment significantly increased serum estradiol level compared with control group. This decrease and increase may be due to enhanced synthesis or impaired metabolism. Similar to the obtained results of decrease of both LH and FSH, it was reported that mycotoxins produced a variety of adverse health effects reduced progesterone synthesis by inhibition of the follicle stimulating hormone secretion (FSH) (Tiemann and Vanselow, 2003). The main effect of these toxins is the inhibition of protein synthesis throughout binding with DNA and RNA perhaps as a result of interference with nitrogen metabolism produced immunosuppression and reduced antibody formation (Zaghboul and Shehata, 1991; Hassan *et al.*, 1997 and 2004). Comparison between groups administrated
Aflatoxin B1 resulted in detection of a significant difference in levels of LH and FSH of female rats compared to controls. The aflatoxin has a hypophysotoxic effect, especially on adenohypophysis (Clarke et al., 1987). Thus, decreases in levels of the LH could be related to the effect of the toxin on hypophysis. Some organophosphates inhibit G-protein activities and could lead to inactivation of LH receptors (Zou et al., 2006); hence, it may reduce progesterone level.

Serum FSH levels tend to be elevated when the testes are damaged and circulating inhibin-B is reduced (Jensen et al., 2004). It is obvious that this degenerative effect of the toxin on germinal epithelium of the seminiferous tubules would breakout into sertoli cells, bringing about a decrease in inhibit B1 level and, consequently, due to reduction of the inhibitory effect of the inhibit B1 on the production and secretion of FSH, the level of this hormone increases. According to the results of a study in female rats, increase in levels of estradiol and inhibitin B causes a decrease in the level of the FSH in the follicular phase (Erickson and Shimasaki, 2001; Padhy et al., 2009). Aflatoxin contamination can reduce the birds’ ability to withstand stress by inhibiting the immune system. This malfunction can reduce egg size and possibly lower egg production. In addition, one must pay special attention to the use of contaminated corn in layer rations because eggs are promptly used as human food and aflatoxin metabolites have been found in egg yolks (Bray and Ryan, 2006).

The presence of aflatoxins in egg is a potential threat to the health of the consumer. Growing children are more sensitive than adults, as egg is one of their main sources of nutrients that indicated the above-mentioned histopathological changes in ovary of young female rats.

Vitamin A (consisting of retinol and its active metabolites) is vital for vision; controlling the differentiation program of epithelial cells in the digestive tract and respiratory system, skin, bone, nervous system, and immune system; and for hematopoiesis (Gursu et al., 2002).

The administration of vitamin A in the present investigation showed partial improvement of induced histopathological lesions as regards ovary sections observed with numerous follicles in various stages of development (primary, secondary and Graffian follicle, corpora lutea) with presence of some deformed follicles. Also follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol levels were improved with supplementation of vitamin A.

In poultry, immune responses and disease susceptibility have been linked to vitamin A deficiencies. In fact, there is a current interest in the relationship between vitamin A status or availability and overall health of poultry (Aye et al., 2000a, b; Dalloul et al., 2002).

Some studies have shown that a vitamin A deficiency in the diets of coccidiosis-challenged broilers resulted in compromised immune defenses as reflected in lymphocyte profiles, oocyst shedding, and interferon- levels (Dalloul et al., 2002). Alpsoy et al. (2009) showed that AFB1 significantly decreased the level of GSH and the activities of superoxide dismutase and GPx and increased level of malondialdehyde. Simultaneous supplementation with vitamin A, C, and E restored these parameters to that of normal range. Webster et al. (1996) reported that vitamin A thus may control carcinogenesis by manipulating molecular events at the initiation stage. As a result, more studies are needed to understand the mechanism of vitamin A antioxidant activity in mycotoxicosis.

5. Conclusion

Present results reported the significant influence of mycotoxins on some endocrine function of reproductive organs which were reflected on the low productivity. The present study concluded that both physiological and histopathological the main source of these changes is attributed to the environmental pollution of food and feeds by fungi and their toxins. Therefore, every hygienic care must be undertaken during all steps of feed and food production and other factors related to the environment of animal to prevent such pollution. Hence the productivity of animal and human health become under control. These results demonstrate that vitamin A plays a complex role in the process of chemical aflatoxicosis and when added at double therapeutic dose in the diet can provide protection against the harmful effects of AFB1 for experimental period.

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