The avian immune system mainly consists of lymphoid tissue and lymphatic vessels. The thymus, bursa of Fabricius and bone marrow are primary avian lymphoid organs, whereas the spleen, mucosal associated lymphoid tissues (MALT), germinal centers and diffuse lymphoid tissues are secondary lymphoid organs. The lymphoid organs of chicken are very sensitive and vulnerable to injury/damage leading to depression of immune response and increased susceptibility to infectious agents.

Immunosuppression in chicken is caused by several factors such as nutrition, management, diseases, stress etc. Among nutritional causes, consumption of mycotoxins suppresses the immune function and decrease the resistance against infectious diseases. Among various mycotoxins, aflatoxin (AF) has the most potent biological effects, and affects all the lymphoid organs resulting in hypoplasia by causing lymphocytolysis in thymus, spleen and bursa of Fabricius thereby interfering with both cell-mediated and humoral immunity. AF not only affects the immune system of the layers but also the progeny due to the aflatoxin carry over via the egg to the embryo, which compromises the immune system and phagocytic function in progeny leading to increased susceptibility to various pathogens (2).

Because AF in its various forms presents a strong immune-toxicologic risk to hosts exposed to the mycotoxins, many studies have sought out natural/derived agents that could be employed to mitigate the potential toxicities. Various adsorbent materials (aluminosilicates, bentonite, silicas, zeolite, mycosorb, etc) have been studied for their ability to mitigate the effects of AF on the immune system. One such study evaluated the efficacy of diatomaceous earth (DAE) in reducing the detrimental effects of aflatoxin (AF) in broiler diet.

Study on efficacy of diatomaceous earth to ameliorate aflatoxin-induced pathomorphological changes in lymphoid organs of broiler chicken

Lakkawar A.W.1*, Narayanaswamy H. D.2, M.L. Satyanarayana 2

ABSTRACT

The efficacy of Diatomaceous earth (DAE) in reducing the detrimental effects of aflatoxin (AF) in broiler diet was evaluated. DAE was supplemented 2000 mg/kg of feed along with 0.5 and 1 ppm of AF in feed. A total of 240 healthy day old broiler chicks were divided into 6 groups comprising of control and treatment groups. Feeding of AF resulted in reduction in size of the thymus, spleen and bursa of Fabricius. In addition, petechial haemorrhages were observed on the surface of the thymus. Histopathology revealed varying degree of lymphocytolysis and depletion of lymphoid cells in thymus, spleen and bursa of Fabricius. In addition, the ceacal tonsils also revealed a mild to moderate degree of lymphoid depletion.

The supplementation of DAE to aflatoxin-mixed feed revealed significant improvement characterised by decreased severity of lesions in lymphoid organs. The macroscopic and microscopic changes in the birds fed DAE in combination with AF included those that were observed in AF-alone fed birds, but of reduced magnitude and severity. The study concluded that 2% DAE in feed can be effectively used to reduce the histotoxic effects of aflatoxin on lymphoid organs in broiler chicken.

Key Words: Aflatoxin, 2% diatomaceous earth, amelioration, lymphoid organs, broiler chicken

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Introduction

The avian immune system mainly consists of lymphoid tissue and lymphatic vessels. The thymus, bursa of Fabricius and bone marrow are primary avian lymphoid organs, whereas the spleen, mucosal associated lymphoid tissues (MALT), germinal centers and diffuse lymphoid tissues are secondary lymphoid organs. The lymphoid organs of chicken are very sensitive and vulnerable to injury/damage leading to depression of immune response and increased susceptibility to infectious agents.
evaluated for their ability to remove or diminish the adverse effect of mycotoxins in poultry feed. These compounds must not be absorbed from the gastrointestinal tract and should have the ability to physically bind with chemical substances, precluding their absorption (12). A variety of adsorbents such as bentonite (21), zeolite (12), hydrated sodium calcium aluminosilicate (24), Saccharomyces cerevisiae (3) and activated charcoal (8) have been used successfully in detoxifying AF in contaminated feeds.

Diatomite or Diatomaceous earth (DAE) is a mineral that consists of high levels of silicon dioxide. It is fine-grained, biogenic siliceous sediment, and is available in large quantities at relatively low cost (9). DAE consists essentially of amorphous silica derived from opalescent frustules of diatoms resulting in an inert, lightweight, highly porous, super-absorbent material, and has a fine porous structure with low density (28). DAE is a powerful natural adsorbent, which might effectively absorb the toxins through its polar ends of the toxin (7). DAE has a small mass (0.5-0.8 g/cm³), high porosity and high content of silicon responsible for the high adsorption capacity (30). These properties and ability of DAE might plausibly be responsible for the ameliorating action against this mycotoxin in broiler birds.

Earlier study indicated that diatomite was not effective in reducing the detrimental effects of AF in broiler (6). However, Modirsanei et al (13) reported DAE significantly increased body weight gain, feed intake and improved feed conversion ratio as well as productive efficiency index, increased serum albumin and the activity of serum lactate dehydrogenase in the birds fed AF-mixed diet. The variations in results with DAE are likely due to variations in sources of DAE and processing used in the earlier studies. Diatomites from different sources vary in their mineralogical composition, morphological characteristics and milled particle sizes. Diatoms vary significantly in size and shape as there are several thousand species of diatoms. The diatomite sample used in this study contains beneficial frustules of diatoms resulting in an inert, lightweight, highly porous, super-absorbent material, and has a fine porous structure with low density (28). DAE is a powerful natural adsorbent, which might effectively absorb the toxins through its polar ends of the toxin (7). DAE has a small mass (0.5-0.8 g/cm³), high porosity and high content of silicon responsible for the high adsorption capacity (30). These properties and ability of DAE might plausibly be responsible for the ameliorating action against this mycotoxin in broiler birds.

The present study was conducted to evaluate the gross and microscopic changes in aflatoxicosis induced by 0.5 and 1 ppm AF exposure in feed of broiler chicken, and to determine the possible preventive role of DAE on the AF-induced pathological changes in lymphoid organs.

Material and Methods

Two hundred and forty unsexed day-old healthy broiler chicks were procured from a reputed commercial hatchery and reared in battery cage system in experimental sheds with average temperature ranging from 27 to 31°C and relative humidity of 59% to 62% with 16:8:1 h (Light : Dark) cycle of intensity of 10 to 20 lux. All chicks were vaccinated on days 7 and 11 of age with the LaSota strain of Newcastle disease virus and Infectious bursal disease (intermediate strain), respectively.

Optimum conditions of management was provided to the birds throughout the period of experiment. Toxin-free and DAE-free Starter and Finisher broiler feed was procured from Department of Poultry Science, Veterinary College, Bangalore, India as recommended by the National Research Council. Required quantities of cultured aflatoxin material were added to make the final concentration of aflatoxin in feed to be 0.5 ppm and 1ppm.

The approval of the Institute Animal Ethics Committee (IAEC) was obtained prior to the conduct of the experiment. The birds were randomly divided into 6 groups, each comprising of 40 chicks (Table 1).

Table 1: Experimental groups

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>TREATMENT</th>
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<tbody>
<tr>
<td>I</td>
<td>CONTROL (Toxin free &amp; DAE free feed)</td>
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<tr>
<td>II</td>
<td>AGRIPOWER DAE 2000 mg/kg of feed</td>
</tr>
<tr>
<td>III</td>
<td>AFLATOXIN (1 ppm)</td>
</tr>
<tr>
<td>IV</td>
<td>AFLATOXIN (0.5 ppm)</td>
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<tr>
<td>V</td>
<td>AFLATOXIN (1 ppm) + DAE 2000 mg/kg of feed</td>
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<tr>
<td>VI</td>
<td>AFLATOXIN (0.5 ppm)+ DAE 2000 mg/kg of feed</td>
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All the birds were checked daily for the health status and husbandry conditions. All the sanitary and hygienic precautions were strictly followed throughout the experiment. The birds were observed daily for clinical signs and mortality (if any). Six birds selected randomly from each group were weighed individually and euthanized by cervical dislocation on day 7, 14, 21, 28 and 35 of the experiment.

All the six birds euthanized on weekly intervals were subjected to detailed post-mortem examination and gross lesions (if any) were recorded. Representative tissue samples from thymus, spleen, bursa of Fabricius and intestines were collected and fixed in 10% buffered formalin for histological analyses. Tissue samples were dehydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin. 3-5 μm thick sections were stained with haematoxylin/eosin (H&E) (11). Light microscopy was used to evaluate congestion, degeneration, necrosis, fatty changes and leucocytic infiltration.

Results

In the present study, gross pathological changes were recorded in the thymus, spleen, bursa of Fabricius, intestinal Peyer’s patches and caecal tonsils on days 7, 14, 21, 28 and 35 of the experimental study. The birds of group I (Control) and group II (DAE treated) revealed normal morphological appearance of all the lymphoid organs throughout the experimental study. The aflatoxin fed birds without DAE (Group III and IV) showed weakness, emaciation and dehydration throughout the period of 35 days.
Macroscopically, pronounced reduction in size of the thymus, spleen and bursa of Fabricius was observed in group III and IV birds. In addition, petechial haemorrhages were also observed on the surface of the thymus. The size of the lymphoid organs of birds in group V to VI (AF + DAE) showed improvement in size as compared to toxin-alone fed birds (Fig. 1, 2). In addition, the spleen showed enlargement and mild congestion. Intestines in Groups III and IV showed mild to moderate degree of serosal and mucosal congestion. The intestinal contents were catarrhal in nature and showed areas of congestion and haemorrhages in the mucosa of small and large intestines. The condition of intestines improved on supplementation with DAE in feed. In the present investigation, the birds of Groups I and II did not reveal gross changes in any of the lymphoid organs throughout the experimental study.

**Group III and IV (Aflatoxin-fed birds)**

**Thymus**
The microscopic examination of thymus of aflatoxin-fed birds revealed mild to moderate degree of congestion and haemorrhages in the medulla from the second week onwards. A mild degree of lymphocytolysis, lymphoid depletion and necrosis in the medullary area was observed during the second and third week in birds of Group III fed 1 ppm AF. At the end of the fourth and fifth week, severe lymphoid depletion with homogenous cellular debris in the medulla and prominent reticulo-endothelial proliferation were recorded. In addition, thinning of cortical areas, widening of medulla, degeneration of Hassall’s corpuscles was visualized as eosinophilic mass and increase in the number of histiocytes containing cellular debris in cortex were also observed at the end of the experiment. Mild degree of lymphoid necrosis was observed during the third and fourth week in Group IV (AF, 0.5 ppm), and at the end of day 35, moderate degree of lymphoid necrosis with an increase in number of Hassall’s corpuscles comprising of degenerated cells (Fig 3)

**Figure 1-** Comparison of Thymus from bird of control, AF 1 ppm and AF 1ppm + 2% DAE groups on day 35 of the experiment.

**Figure 2-** Comparison between Bursa of Fabricius from bird of control, AF 1 ppm and AF 1ppm + 2% DAE groups on day 35 of the experiment.

**Figure 3-** Thymus from bird fed 1 ppm of AF showing severe haemorrhages, depletion of lymphoid cell and thinning of cortex on day 35 of the experiment. H&E x 200

**Spleen**
Spleen of aflatoxin-fed birds showed mild to moderate degree of congestion with cellular sparsity of the periartriolemar lymphoid component along with mild histiocytosis from day 14 onwards and continued till the end of the experiment. Lymphoid cell necrosis around the periartriolemar sheath with eosinophilic debris and depletion in the germinal centres and reticular cell hyperplasia were consistently observed in spleen of the toxin-fed birds. The lesions were diffuse in birds fed 1 ppm AF (Group III), while focal distribution was observed at 0.5 ppm of AF (Group IV) from the fourth week onwards. In addition, degenerating cells with pyknotic and fragmented nuclei with macrophage accumulation were observed at the end of the experiment.

**Bursa of Fabricius**
A mild lymphoid necrosis, sparse cellularity in occasional follicles and depletion in medulla were noticed in bursa of Fabricius in Group III (fed AF 1 ppm) birds during the second week. A moderate degree of lymphoid depletion with formation of multiple varying- sized cysts with well-defined wall within follicle and corrugation of plical epithelium was observed during the third and fourth week. At the end
of experiment, a marked lymphoid depletion, increase in number of histiocytes, single cell necrosis, lymphocytolysis with atrophy of bursal follicles, microcyst formation and interfollicular fibrous tissue proliferation were noticed. The plical epithelium showed hyperplastic changes with corrugations and occasional microcyst formation. Similar changes along with less severe lymphoid tissue depletion were observed in Group IV fed 0.5 ppm of aflatoxin (Fig. 4, 5).

Intestines
Degeneration and necrosis of villus epithelium with infiltration of large number of mononuclear cells in lamina propria was seen at the end of the experiment (day 35). Peyer’s patches/ceacal tonsils revealed mild to moderate degree of lymphocytolysis followed by lymphoid depletion along with histiocytic activity in the fourth and fifth week of the experiment in Group III. Group IV birds fed 0.5 ppm AF toxin showed similar changes of lesser severity throughout the experimental study.

Group V and VI (aflatoxin and DAE)
Thymus
In Group V and VI mild to moderate degree of congestion of medulla along with mild histiocytic filtration was observed from day 14 till the end of the experiment. Mild lymphoid depletion with hyperplastic changes, repopulation of lymphoid cells and widening of cortex with compact arrangement of lymphoid cells were also observed at the end of the experiment (Fig 6).

Spleen
Birds in Groups V and VI revealed mild degree of congestion of the red pulp on day 14 of the experiment. There was widening of lymphoid component of some of the periarteriolar sheath along with formation of occasional secondary lymphoid follicles appreciable from day 14 onwards and then continued till the end of the experiment. The widening of lymphoid component and an increasing number of secondary follicles causing repopulation of lymphoid cells was observed by the end of the fourth week. At the end of the experiment, the follicles showed varying degree of regeneration and repopulation.

Bursa of Fabricius
The bursa of Fabricius in Groups V and VI revealed cellular sparsity in a few follicles with histiocytic infiltration from day 14 of the experiment till the end of the third week. The number and size of the microcyst in the plical epithelium wall also reduced on day 28 of the experiment. At the end of the experiment, there was marked hyperplasia with repopulation of lymphocytes and formation of large follicles consisting of compactly arranged lymphoid cells (Fig. 7) and numerous mitotic cells.

Figure 4 - Bursa of Fabricius from bird fed 1 ppm of AF showing lymphocytolysis and depletion of lymphoid cells on day 35 of the experiment. H&E x 400

Figure 5 - Bursa of Fabricius from bird fed 0.5 ppm AF showing marked depletion of lymphoid cells and microcyst formation in the plical epithelium on day 35 of the experiment. H&E x 100

Figure 6 - Thymus from bird fed 1 ppm of AF and supplemented with 2% of DAE showing mild congestion, regeneration/repopulation of lymphoid cells as well as differentiation of cortex and medulla on day 35 of the experiment. H&E x 200

Figure 7 - Bursa of Fabricius from bird fed 1 ppm of AF and supplemented with 2 % of DAE showing regeneration/repopulation of lymphoid cells in the follicle on day 35 of the experiment. H&E x 400
Intestinal sections showed marked improvement with no significant changes within the villi and appeared almost normal except for mild congestion. At the end of the experiment, the Peyer’s patches/caecal tonsils revealed varying degree of regeneration and repopulation of lymphocytes.

**Discussion and conclusions**

Avian immune system is capable of responding to numerous factors such as dietary, environmental, physiological, genetic and toxicological factors. Amongst all dietary factors, presence of mycotoxins or their metabolites in feed may be highly reactive and may destroy lymphoid tissues. Aflatoxins are one of the best studied mycotoxins due to their direct tropism for any of the primary or secondary lymphoid organs resulting into either the organ necrosis or atrophy or depletion of specific sub-populations of the lymphoid cellular components (21).

In broiler chickens fed 0.2 ppm of AF, Sakhare et al (23) demonstrated necrotic foci in germinal centres, central artery sclerosis, reduction in lymphocyte counts, hyperplasia of artery sclerosis, reduction in lymphocyte counts, hyperplasia of the bursa of Fabricius, and cells in lymphoid follicles core were less numerous. Similar changes with haemorrhagic foci were also observed in lymphoid follicles of the thymus. In broiler chickens receiving 4 mg/kg of aflatoxin with the feed, the lymphoid organs (thymus, spleen and bursa of Fabricius) exhibited atrophy, reduction of lymphoid cell counts, lymphoid follicles necrosis and haemorrhages (17, 14).

In the present investigation, feeding of different levels of AF showed lymphocytolysis, depletion of lymphocytes and focal hemorrhages in the cortex of thymus, thinning of cortex and hemorrhages in medulla and cortex of bursa of Fabricius as well as atrophy of thymus and spleen of AF-fed birds. A mild to moderate degree of lymphoid depletion, lymphophagocytic areas giving a starry-sky appearance, atrophy of bursal follicles, multifocal cyst formation and hyperplastic changes in plical epithelium with corrugations were recorded consistently in bursa of Fabricius of AF-fed groups. Focal areas of congestion and haemorrhage, lymphoid cell necrosis and depletion around periarteriolar sheath were observed microscopically in the spleen of toxin-fed birds. Caecal tonsils exhibited mild to moderate degree of lymphocytolysis with prominent histiocytic activity. The lesions recorded in lymphoid organs were dependent on dose and duration of exposure to AF. Similar observations were made earlier by several workers in lymphoid organs of birds fed AF-mixed diet (4, 15, 10).

Similarly, Sur and Celik (26) also reported regression of lymphoid organs and depletion of lymphoid cells following experimental aflatoxicosis in broilers. Other researchers observed mild to moderate depletion of lymphocytes, cystic degeneration and fibrous tissue proliferation in the bursa from 2 weeks old Japanese quails orally exposed to aflatoxin 1ppm for a period of 8 weeks (18). Dietary aflatoxin in chicken results in destruction of thymic cortex, decrease of splenic T cells and degeneration of follicles in bursa of Fabricius (29).

In the present study, aflatoxin-(0.5 and 1ppm) fed birds, which received DAE supplementation (2000 mg/kg feed) showed hyperplastic changes in different lymphoid organs with normal population of lymphoid cells giving a histological appearance comparable to control birds. Based on the above findings, it could be construed that the improvement in lymphoid organs could be attributed to strong aflatoxin-binding/adsorptive capacity of DAE resulting in a decrease in the bioavailability of aflatoxin for absorption through gastrointestinal tract and subsequent reduction in the distribution of AF in different organs and systems.

Inclusion of new adsorbent products in the diet such as DAE can significantly ameliorate most of the adverse effects of AF. There are a few conflicting reports on efficacy of DAE in mitigation of toxic effects of aflatoxins in relation to some of the tested parameters. The differences among studies could be explained by different levels of adsorbents or the AF exposure dose tested. In the present study, effective protection against AF was obtained by incorporation of 2000 mg of DAE/kg of broiler feed.

Based on the available scientific literature, the chemical complexity of mycotoxins means that the effectiveness of compound in sequestering one mycotoxin does not mean an equal ability to sequester other mycotoxins. Each mycotoxin has different functional groups, thus, the binding capacity of an adsorbent depends on its chemical and physical properties and its relation with the physical structure of the target mycotoxins (20). Thus, the physicochemical differences among the adsorbents used in the studies mentioned above could explain the higher or lower efficacy among them. However, the ability of the toxin-binder to bind mycotoxins depends on other factors such as pH, molecular arrangement and its geographic region of origin (27). Natour and Yousef (16) reported significantly higher in-vitro adsorption ability of DAE to aflatoxin, which is directly proportional to the number of diatom valves. In vitro study showed that DAE has high (94.71 %) ability to absorb AF from the feed at pH 6.5 (24). The normal pH of the chicken intestinal tract contents is 5.7–6.0 in the duodenum/jejunal, 6.3–6.4 in the ileum/rectum and finally up to pH 7.0 or higher in the caecum (5). Considering the correlation between the pH and ability of mycotoxin-binder in in vitro studies, higher binding ability of DAE to the aflatoxins can be expected at the pH of 6–7 in the intestinal tract of chicken to reduce the absorption and systemic availability of this mycotoxin.

In conclusion, the incorporation of DAE in the diet during the period of exposure to AF in the present study could prevent the toxic effects of aflatoxin. This result confirms the protective effects of DAE, which might be due to its capability of specific chemisorption of
AF in gastrointestinal tract, which reduces aflatoxin bioavailability (1). These results clearly demonstrated that slight and moderate histological lesions were observed in chickens fed a diet containing 0.5 and 1 ppm of AF while no macroscopically lesions were seen, and that the simultaneous addition of DAE to the AF-containing diet provided a moderate amelioration in AF toxicity. Furthermore, DAE was found to be inert and non-toxic for broilers. These improvements can plausibly contribute to a solution of the AF problem in broiler chickens, when used with other mycotoxin-management practices.

At the same time it is well documented that clay, zeolite minerals and DAE are structurally and functionally diverse; they vary considerably from source to source and may not have equal affinities and capacities for binding of aflatoxin and other mycotoxins (20). Thus, these adsorbents should be rigorously tested one by one and thoroughly in in vivo conditions, paying particular attention to their effectiveness and safety in sensitive animal/avian models and their potential for harmful interactions. Similarly, generalisations should be avoided for all potential mycotoxins detoxifying agents, as adsorbing compounds can differ in efficacy even within the same category.

Considering the results of the present study and earlier work done on the effect of different levels of DAE on aflatoxin further studies employing the broader perspectives seem to be necessary to determine whether lower levels of DAE in broilers diet will be effective in controlling or preventing the occurrence of aflatoxicosis in chicken.

DISCLOSURE OF INTERESTS
The authors have no conflicts of interest to declare. All authors participated and approved the manuscript for publication.

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References

U istraživanju je ispitivana efikasnost dijatomejske zemlje (DAE) u reduciraju stetnih učinaka aflatoksina (AF) u brojlerskoj ishrani. Dijatomejska zemlja je dodavana u ishranu u koncentraciji od 2000 mg/kg hrane zajedno sa 0.5 i 1 ppm aflatoksina u obroku. Ukupno 240 zdravih jednodnevnih tovnih pilića je podijeljeno u 6 grupa, uključujući kontrolne i tretirane grupe. Unošenje aflatoksina putem hrane je rezultiralo smanjenjem veličine timusa, slezena i bursa Fabricii. Dodatno, petehijalna krvarenja su uočena na površini timusa. Histopatologija je otkrila različite stepene limfocitolize i smanjenja broja limfoidnih ćelija u timusu, slezeni i bursi Fabricii. Također, cekalne tonzile su također pokazale znakove blagog do umjerenog smanjenja premaka limfoidnih ćelija. Dodavanje dijatomejske zemlje u hrani u koju je, također, dodan i aflatoksin, pokazala je značajna poboljšanja u vidu smanjenja težine lezija u limfoidnim organima. Makroskopske i mikroskopske promjene kod ptica hranjenih dijatomejskom zemljom u kombinaciji sa aflatoksinima uključuju iste promjene koje su zapažene kod ptica koje su unosile samo aflatoksin, ali sa smanjenom veličinom i težinom promjena. Istraživanje je zaključilo da dodatak 2% dijatomejske zemlje u obroku efikasno smanjuje histotoksične efekte aflatoksina na limfoidne organe brojlerskih pilića.