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A study on experimentally induced aflatoxicosis on the carryover of aflatoxin B_1 into eggs and liver tissue of white leghorn hens

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Abstract
A feeding trial was conducted with 90 commercial White Leghorn layer hens to study the effect of experimentally induced aflatoxicosis on the carryover of aflatoxin B_1 into the eggs and liver tissues. The experiment consisted of five treatments with 0.00, 0.75, 1.50, 2.25 and 3.00 mg/kg (ppm) of dietary aflatoxin. These levels were obtained by including Aspergillus infested rice in the control diet. The experiment was conducted for three 28-day periods from the onset of 50% egg production. Traces of aflatoxin or their metabolites were detected in eggs and livers from all the treatments except control groups.

Keywords: poultry feed, aflatoxin, laying hens, carryover, eggs, liver tissue

Introduction
Presence of fungi or their toxic metabolites, known as mycotoxins are common in poultry rations in the tropical regions of the world. Mycotoxins are naturally occurring compounds that can contaminate crops before or after harvest, during processing, transport or storage (Liau *et al*., 2007; Yaling *et al*., 2008) [27, 47]. Aflatoxins are mycotoxins that are produced by the fungi Aspergillus flavus and Aspergillus parasiticus. Not much importance was given to mycotoxins until 100,000 turkey birds died in England due to the consumption of contaminated peanut (groundnut) meal containing aflatoxins imported from Africa (Blount, 1961) [6]. The magnitude of aflatoxins dwarfed all the previously known cases of mycotoxins like ergotism, mushroom poisoning, brooder pneumonia and others.
Aflatoxins are also called flavo coumarins. as they contain coumarin nucleus Aflatoxins are designated as B_1, B_2, M_1, M_2, G_1 and G_2. Aflatoxin M_1, was found in the liver, kidney and urine of sheep and in the liver of rats when aflatoxin was given to them (Butler and Clifford, 1965; Allcroft et., 1966) [8]. Aflatoxin B1 (AFB1) is the most potent naturally occurring carcinogen known (Moss, 1991; Coulombe, 1993; Binder *et al*., 2007) [34, 11, 4]. AFB_1 has been tested extensively as it induces DNA damage, gene mutation, chromosomal anomalies and cell transformation in mammalian cells *in vitro*. In case of AFB1, it is generally felt that there is no threshold dose below which no tumour formation would occur. Only a zero level of exposure will result in no risk (FAO/WHO, 2004). Acute and Chronic toxicity of aflatoxins have been demonstrated in several animal species including birds, fish, domestic animals and primates. (Eaton and Gallagher, 1994) [13]. Cases of human acute aflatoxicosis have been reported in Kenya where an aflatoxicosis outbreak resulted in 125 deaths (Aziz *et al*., 2005) [2]. Krishnamachari *et al*., (1975) [24] reported 106 deaths in India. Deaths were also reported in Malaysia (Lye *et al*., 1995) [30]. Linsel and Peers (1997) [28] revealed an association between high incidence of liver cancer in Africa and dietary intake of aflatoxin. In 1993, the International Agency for Research on Cancer (IARC) classified AF B_1 and mixtures of aflatoxins as Group I carcinogens (substances that can cause cancers) in humans. There is also mounting evidence that aflatoxin is an important factor in infant under nutrition (Katerere et al., 2008) [23] and impaired growth in children under 5 years of age (Gong *et al*., 2002) [17]. This has been attributed to aflatoxins supressing the immune system and interfering with the absorption of micronutrients (Williams *et al*., 2006) [45]. Aflatoxins are considered to be the cause of acute hepatitis, immune suppression, play a role in kwashiorkor, increase in neonatal susceptibility to jaundice and a relevance to hepatitis B and AIDS (Hsieh and Atkinson, 1999; Bintvihok *et al*., 2002; Kuper-Goodman, 2003; Kovacs, 2004, Masoero *et al*., 2007) [19, 5, 25, 32] reported a 2% conversion and carryover of AF B_1 in milk as AF M_1,. AF M_1, is relatively

stable in raw milk and milk products and is not destroyed by heat (Galvano *et al*., 1996) [16]. Infants and children are most susceptible due to the high amounts of milk consumed and absence of fully developed detoxification process in them. Costamagna *et al*.,(2019) [10] found that cheese retained a portion of AF M_1, from the milk it was made, Shephard (2018) [40] reported a carryover of 98 ug/kg (ppb) aflatoxin into peanut oil when it was extracted through artisanal (traditional expeller) process when contaminated peanuts were used for making peanut oil.

Materials and Methods

Production of Aflatoxin on rice

Aflatoxin was produced on rice according to Shotwell *et al*., (1966) [41]. The procedure was carried out in a 300ml Erlenmeyer flasks containing 50 gm of polished rice. To these flasks 25 ml of tap water was added and allowed to stand for about 2 hours with frequent shaking. The flasks were plugged with cotton wool over which a paper cap was fixed. The flasks were then autoclaved at 15 psi for 15 minutes and cooled. They were inoculated with the fungus Aspergillus parasiticus (NRRL 2999) grown artificially on potato agar medium. The flasks were left at room temperature and shaken regularly at short intervals. Care was taken not to allow rice to remain as a compact mass. At 48 hours after incubation, the rice showed small white areas at the sites where the mould had begun to grow. After some period, rice assumed a bright yellow colour which darkened to a dull light brown. Then as the gonidia started forming, the rice turned greenish and after 7-8 days all the rice grains assumed olive green colour. This was assumed as the end point of the fungal growth, after which the flasks were briefly steamed to destroy the fungus. This material was then taken out and dried in a hot air oven. When completely dried, the infested rice was ground to a fine powder in a grinder and representative samples were drawn for drawn for determining the content of aflatoxin in the samples.

Extraction of aflatoxin

Extraction of the toxin in a weighed quantity of the feed was done according to the procedure recommended by Tropical Products Institute, London (TPI Report, 1972). A 10gm representative sample was taken in a 250 ml wide mouthed bottle and 120 ml of acetone-water (70:30 v/v) mixture was added. The bottle was stoppered by with a rubber bund line with aluminium foil. The sample was shaken for 30 minutes on a wrist action shaker to extract the toxin. The supernatant was filtered through a Whatman No.1 filter paper. A measured volume of the filtrate (60 ml) was extracted with chloroform (50 ml) in a 250 ml separatory funnel. The lower chloroform layer was allowed to run off through a bed of anhydrous sodium sulphate (10 gm) placed on a filter paper. The extraction was repeated two more times using 25 ml portions of chloroform, using the same sodium sulphate bed which was then washed with more chloroform and the extracts were pooled. The combined chloroform extracts and washings were warmed on a water bath in order to remove the chloroform. Finally, the solution was concentrated to 25 ml and was used for estimation of aflatoxin.

Estimation of Aflatoxin

Infested mouldy rice and diets were analysed for aflatoxin content using Velasco Fluorotoxin Meter (James Velasco, 1983) [22].

Preparation of microcolumns

One end of the 8" microcolumn of the fluorotoxin meter was plugged with glass wool plug up to 3mm in depth. On this, a layer of sand was added up to 5-7 mm in depth. Then, florisil layer (100-200 mesh) was added up to 5-7 mm. A second layer of sand was added on this. A layer of silica gel about 15 mm in depth was added. To top it all a layer of neutral alumina (80-200 mesh) was added up to 3-7 mm. All the above substances were in powdered form.

Preparation of standard Aflatoxin B_1 microcolumn

1 ml of standard aflatoxin B_1 solution containing 50ng was charged with into the microcolumn using a 1 ml syringe. After this solution was eluted, 1 ml of chloroform + methanol (96:4) was added to drain. This step was reported twice to ensure complete elution of the toxin into the florisil layer.

Operation of the fluorotoxin meter

The unit was plugged into a standard electric outlet. The LAMP START button was pushed and held in position for approximately 20 seconds and then released. A bluish-white glow from the hole on the front of the Velasco Flurotoxin Meter (VFM) indicated that the Ultra Violet (UV) lamp was on. The instrument was allowed to stabilize for one hour.

Determination of Aflatoxin concentration

A blank microcolumn was wetted and inserted into the sample holder and the cap was placed. Using the ZERO control, the instrument was adjusted to ZERO. Then the standard aflatoxin B_1 microcolumn (known to contain 50 ng/ml, provided by Municipal Corporation of Bilthoven, The Netherlands) was inserted and using the CALIBRATION control, the instrument was calibrated to read 20 ppb. The calibration was repeated till a concurrent reading was obtained. The microcolumn containing the unknown sample was inserted into the sample holder and the reading were noted. The toxin content of the unknown sample was calculated based on the calibration factor (20 ppb = 50 ng), dilution and weight of the sample.

Aflatoxin extraction in eggs and liver tissue

Aflatoxin in the eggs and livers was extracted according to the procedure recommended by Tropical Products Institute, London ((TPI Report, 1972).

Effect of experimentally induced aflatoxicosis on the carryover of Aflatoxin B_1 into eggs and liver tissue

An experiment was conducted with laying hens fed varying levels of dietary toxin for three 28-day periods. The detail s of the experiment are given below.

Selection of birds

A suitable number of Single Comb White Leghorn (SCWL) pullets (Babcock strain) of the same parentage and hatch were obtained. These birds were housed in California cages singly. The birds were fed a standard layer ration. By 24th week, 50% egg production was registered. From this pullet stock, 90 birds of comparable body weight and general health were selected. These birds were divided into 5 groups with 18 birds in each group, that represented the five treatments. The 18 birds in each group were subdivided into 3 groups of 6 birds each that represented the treatments within each group.

Experimental diets

The test rations were prepared from the standard layer diet (control diet, Table 1) to which was added a weighed quantity of experimentally infested powdered rice so as to give a calculated aflatoxin content of 0.75, 1.50, 2.25 and 3.00 ppm aflatoxin in the diet. The infested rice was pre analysed for aflatoxin content by Velasco Flurotoxin Meter (VFM) and was found to contain 937.5 ppm. The control and the four experimental diets were analysed for aflatoxin content in the diet. Whilst the control had zero content of aflatoxin, the analysed aflatoxin in the experimental diets 0.60, 1.25, 2.12 and 2.85 ppm respectively. The infested rice was included in various diets by replacing an equivalent quantity or rice polish contained in these, weight by weight. Feeding of the control and aflatoxin diets to the birds were started from the first day of 24th week of age. Birds were housed singly in cages. Feed and were offered ad libitum. The experiment was continued from 24th week of age for three 28-day periods.

Toxin analysis

Representative eggs and livers from each dietary level were analysed for aflatoxin content according to the method recommended by Tropical Products Institute, London (TPI Report, 1972), using the VFM. The colouring matter from liver tissue was removed using column chromatography (Eppeley *et al*., 1968) [14]

Table1. Composition of control diet used in the experiment and Chemical composition of the diet (Calculated)

Ingredient	Percent	Ingredient	Content
Maize	51	ME (Kcal/kg)	2700
Rice Polish	11	Lysine (%)	0 .84
Deoiled rice bran	4	Methionine (%)	0.35
Soybean meal	18	Calcium (%)	3.61
Fish meal	8	Available Phosphorus (%)	0.55
Shell grit	5	Crude Protein (analysed %)	17.7
Dicalcium phosphate	0.5	Aflatoxin (analysed)	Nil
Mineral mixture	2.5		
Total	100		

*Mineral mixture contained : Ca, 32% ; P, 6% ; Fe, 1000 ppm; Cu, 100 ppm ; Co, 60 ppm ;
Mn, 2700 ppm ; I, 100 ppm and Zn. 2600 ppm
**Vitamin premix was added to supply the following vitamins per kg diet:
Vitamin A, 16000 IU; Vitamin D, 2000 ICU; Riboflavin, 8 mg; Thiamine, 16 mg; Pyridoxin, mg;
Vitamin B 12, 16 mcg; Niacin 24, mg; Calcium pantothenate, 16 mg; Vitamin E, 16 mg

Results and Discussion

All eggs collected on the last day of each period from hens fed different levels of AF B_1 were analysed for AFB_1 Or their toxic metabolites. Eggs from all the treatments and periods except the control group were found to contain AFB_1 or their metabolites. (Table 2). The toxin deposition in all the groups increased with the increase in the duration of the experiment, the concentration in the period 3 being the highest. The treatment effect was also found to influence the aflatoxin deposition in eggs. The eggs form birds fed the highest level of 3.00 ppm were found to contain an aflatoxin concentration of 11.1 ppb by the end of period 2. By the end of period 3 there was a slight decrease to 10.5 ppb of toxin deposition in this group. Representative birds from each treatment fed varying levels of dietary aflatoxin have been slaughtered at the end of the experimental period. The aflatoxin content of the livers increased with increasing dietary aflatoxin content up to 2.25 ppm, which was 2245 ppb. There was a marginal decrease at 3.00 ppm dietary aflatoxin level, which was 2186 ppb (Table 3).

The results of the present study where in the highest aflatoxin content was reported in the treatment that received the highest aflatoxin content of 3.00 ppm is in agreement with the findings of Jacobson and Wiseman(1974) [21], who found a positive correlation between toxin content in the eggs and number of days toxin was fed. Salwa and Anwer (2019) [39] while studying the effect of feeding graded doses of 25, 50 and 100 ppm ug/kg (ppb) of AFB_1 to laying hens for 60 days reported a carryover in the range of 0.02 to 0.09 ug/kg. They also observed that AFB_1 was stable even after 20 minutes of boiling. AFB1was specifically carried over to the yolks of eggs (Barly and Vadehara, (1999) [3]; Bray and Ryan (2006) [7]. Wolzak *et al*., (1986) [46] conducted a feeding trial to determine the aflatoxin deposition in the tissues of hens feed a diet containing 330 ppb and 1680 ppb for 4 weeks and detected a highest level of 3 ppb in the gizzard, kidneys and liver. The lowest level of 0.1 were detected in the breast muscle. Eight days after removal of contaminated feed, AFB_1 residues were not detected in the breast, leg muscles, gizzard and ovaries. However, one of the hens had measurable amount of AFB_1 in the liver even after 32 days of removal of the contaminated diet. Jacobson and Wiseman (1974) [21] detected AFB_1 in measurable amounts in eggs from hens fed rations containing varying levels of AFB_1. The toxin was found to be unstable over long periods of time. Trucksess *et al*., (1983) [44] reported that eggs and tissues of hens that were fed a diet containing 8 ppm of AFB_1 for 7 days, contained AFB_1 and Aflatoxicol (Ro). Aflatoxin M_1 was found only in the kidneys. They further reported that 7 days after withdrawal of contaminated feed, AF B_1 (0.08 ppb) was found in one of the nine livers and Ro (0.01 to 0.04 ppb) in eight of the nine muscles analysed, no aflatoxin was found in any other tissues. This suggests that clearance of of aflatoxin in eggs occurs within a short period of time when aflatoxin in the feed is withdrawn. Smith *et al*., (1971) reported that inclusion of a high level of dietary fat in aflatoxin diets reduced mortality in chicks and left their growth unaffected.

Lotzsch *et al*., (1977) [39] while working o hen and quail eggs, reported a carryover of aflatoxin into eggs and egg products although no aflatoxin was detected below 3.00 ppm level. The highest level of residue was obtained at 10 ppm dietary aflatoxin, and at this level egg production ceased after a few days. They also found that rate of transmission was lower in brown eggs comparatively. They further reported that more aflatoxin was carried over into yolks than albumen. Patterson *et al*., (1978) [37] found that 0.04% of dietary aflatoxin was transferred into hens' eggs.

Table 2: Aflatoxin content of eggs of hens fed varying levels of dietary aflatoxin

Dietary Aflatoxin (ppm)	Aflatoxin Period1	Period2	Period3 (ppb)
0.00	Nil	Nil	Nil
0.75	5.0	5.5	5.9
1.50	5.5	5.9	6.3
2.25	8.3	9.7	10.0
3.00	10.0	11.1	10.5

Table 3: Aflatoxin content of liver tissue of hens fed varying levels of dietary aflatoxin

Dietary aflatoxin	Aflatoxin content
(ppm)	(ppb)
0.0	Nil
0.75	569
1.50	1244
2.25	2245
3.00	2186

Lotzsch *et al*., (1977) [39] suggested that the possible health hazards due to the consumption of eggs from hens receiving dietary aflatoxin must be considered minimal. However, given the fact that AF B_1, being a cumulative toxin and eggs being consumed on a daily basis, Chowdary and Smith (2004) [4] and Ogido *et al*., (2004) [35] *et al*., (2004) cautioned that even small amounts of aflatoxins in eggs may cause public health problems. The variation of aflatoxin residue in eggs confirm that only small quantities of AF B_1. Are likely to be deposited in them while the majority are detoxified and / or stored in the liver, ovaries, kidneys, crop, breast muscle, thigh muscle and excreted in the faecal material (Trucksess *et al*., (1983) [44]; Micco *et al*., 1988; Madden and Stahr, 1995; Bintvihok *et al*., 2002; Rizzi *et al*., 2003) [33, 31, 5, 38]. When laying hens are continuously exposed to diets containing low levels of aflatoxin for a long time, the capacity of laying hens to detoxify gets reduced (Del Bianchi *et al*.,2005; Pandey and Chauhan, 2007) [12, 36] resulting in the carryover of AF B_1 into the eggs and tissues of the birds. Hassan *et al*., (2012) [18] however, reported that during long exposure of AF B_1, the elimination of AF B_1 in the chicken increases and carryover decreases with increasing age of chicken, their finding could explain a marginal decrease in the aflatoxin concentration in the livers of chicken in the group fed 3.00 ppm in the present study (Table) in period 3. There was also a marginal decrease in the carryover of aflatoxin into the eggs in period 3 compared to the eggs in period 2, birds in period 3 were older than the birds in period 2 by 28 days. Contrary to the present study, Sims *et al*. (1970) did not find any carryover of aflatoxin or their metabolites into the eggs and livers of aflatoxin fed birds.

The melting point of aflatoxins is in the range of 237 to 289 in their dry form, making it difficult to destroy them by routine cooking procedures. They are also tasteless, thereby evading detection in human foods. Addition of toxin binders, vitamins and minerals to the animal and poultry feeds are the general practices adopted to minimise the transmission of aflatoxins into human foods. Slight overage of crude fat over the minimum requirement in poultry/animal feeds may also bestow some protection to the human foods.

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