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Aflatoxin B1 induced developmental neurotoxicity in RIR egg

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ABSTRACT

Present study has identified developmental neurotoxicity of 5ng and 10ng aflatoxin B1 on day '0' in ovo exposure of RIR eggs. AFB1 exposure to developing chick is associated with significant elevation of total protein and depletion of glucose in brain and serum. The activity of AChE, MAO-A and MAO-B were significantly decreased by in ovo AFB1 exposure. Moreover, AFB1 caused significant depletion of glutamate level where as tryptophan level was increased in AFB1 treatment groups. These results suggest that in ovo exposure to AFB1 may cause alterations in various neurotransmitters which may be resulted into brain deformities in developing embryo.

Key words: Aflatoxin B1, *in ovo*, Brain, Neurotoxicity, Neurotransmitter

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INTRODUCTION

Mycotoxins are toxic metabolites synthesized by some naturally growing fungi which are lethal to living organism. *Aspergillus flavus* and *Aspergillus parasiticus* with aflatoxin B1 (AFB1) as the most potent toxins of teratogenic, mutagenic and carcinogenic effect [1, 2, 3, 4]. Animals are considered the most group exposed to high concentration of aflatoxins through feedstuffs that develop several health problems which lead to huge economical losses. These losses are pronouncing in meat and eggs in terms of quality and quantity as a result of contamination with aflatoxins residues [4, 5, and 6]. Feeds of cereal grain origin demonstrate the most susceptible commodities along with nuts for contamination with AFG1, AFG2 and AFB2 in addition to AFB1 as the main toxin contaminants.

AFB1 is the most acute and chronic toxic member of the aflatoxin and is most frequently contaminated with food [7, 8]. Aflatoxin B1 (AFB1) is a group I carcinogen. It is a potent carcinogen, producing primarily hepatocellular carcinomas in experimental animals [9]. It is also toxic to extra hepatic tissue, such as the nervous system [10, 11]. AFB1-epoxide can bind to cellular macromolecules like DNA, RNA and protein causing chemical lesions which can lead to manifestation of biological effects and acute or chronic diseases in both animals and human [12].

Some studies indicated that aflatoxin B1 altered the level of various biogenic amines and their precursors in rat and mouse brains [13, 14], neuronal ATPases [10], reduces whole brain tryptophan hydroxylase [15] and alters brain AchE activity [16]. Repeated exposure of AFB1 (16 µg/kg daily for 6 weeks) increased central and peripheral nervous system Na^+/K^+-ATPase in male Wistar rats[10]. In some cases AFB1 alters homeostasis of biogenic amines of central nervous system. In rats, AFB1 caused a decrease in brain serotonin and its metabolite 5-hydroxyindoleacetic acid [13]. It is also inhibited brain stem tryptophan metabolism, as evidenced by decreased tryptophan 2, 3 deoxygenase activity [13, 15].

As discussed earlier aflatoxin B1 poses a risk of neurotoxicity, if taken for a long period of time. It is clear that data recorded for aflatoxin B1induced toxicity on central nervous system, especially on neurotransmitter is rare which gives a good indication for planning of the present study.

MATERIALS AND METHODS

Fertile RIR eggs were obtained from the Department of Livestock Production & Management, Anand Agricultural University, Anand. All eggs were wiped with 70% ethanol and numbered. Eggs were grouped in four (control, vehicle control and two groups of treated), 25 in each. Protocol was approved by departmental ethical committee according to CPCSEA. The concentrations of 5ng/5µl 20%alcohol/egg (Low dose, LD) and 10ng/5µl 20% alcohol/egg (High dose, HD) of AFB_1 in treatment group and 5µl 20% alcohol/egg of alcohol were injected in vehicle control group in air sac of eggs with sterile syringe at '0' day of incubation. The eggs were placed into an incubator at 37.5 ^{0}C, 65% relative humidity and turned every 3 hours. Weight mobility of incubated eggs was followed every day, and the mortality was estimated by candling of eggs. After 21 days of incubation, hatchlings were sacrificed after collecting blood through heart puncture. Brain was removed, washed in PBS, blotted on tissue paper and then used to estimate biochemical parameters and neurotransmitters.

Estimation of Total Protein:
The assay is based on the method of Lowry *et al.,* [17]. The assay is dependent on the amount of aromatic amino acids present in protein. The phenolic group of tyrosine and tryptophan residue (amino acid) in a protein will produce a blue purple color complex, with Folin-phenol reagent. Intensity of color is measured at 640 nm.

Estimation of Glucose:
The assay was carried out in serum for glucose estimation that utilizes glucose oxidase peroxidise (GOD-POD) system described by Trinder [18]. Glucose is oxidized by glucose oxidase (GOD) to produce gluconate and hydrogen peroxide. The hydrogen peroxide is then oxidatively coupled with *p*-amino antipyrene (4-AAP) and phenol in the presence of peroxidises (POD) to yield a red quinoeimine dye that is measured at 505 nm.

Analysis of neurotransmitters:
Acetylcholine esterase activity (AChE):
The assay of Acetylcholinesterase activity was performed by the method of Ellman *et al.,* [19]. The acetylthiocholine iodide (ATCHI) was used as a substrate which splits into acetate and thiocholine iodide. The liberated thio group of thiocholine reacts with DTNB which is a chromogen to form a yellow color. Optical density was measured at 412 nm.

Glutamate and Tryptophan:
Glutamate and tryptophan are detected by the Thin Layer Chromatography (TLC).

For Glutamate 5µl and for tryptophan 10µl of brain homogenate was taken with 5µl of 70% Ethanol in it. Ethyl acetate: Methanol: Ammonia (3:4:1) solution was used as mobile phase solvent. According to the reference samples bands, unknown samples band were detected. Then those parts of silica were collected and dissolved into 70% ethanol. After evaporation of alcohol, concentration of amino acid in sample was estimated by the method of Lowry *et al.,* [17].

Monoamine Oxidase A and B:-
The activity of monoamine oxidase A (MAO-A) and of monoamine oxidase B (MAO-B) is estimated by the method of Charles and McEwen [20].

Preparation of sample:-
To acquire mitochondrial fraction, cerebellum of chick brain was dissected out. It was then cut into small pieces and rinsed in 0.25M sucrose, 0.1M Tris, 0.02M EDTA (pH 7.41) to remove blood. The pieces were homogenized in homogenizer with 4 ml of above medium. The homogenate was centrifuged at 800 rpm for 10 min and the pellets were discarded. Then supernatant was centrifuged at 12,000 rpm for 20 min. The precipitate was washed twice with 100ml of sucrose Tris- EDTA and resuspended in 50ml of the same medium.

Estimation of MAO-A:
In the 250µl of homogenate, 250µl of serotonin and 250µl of buffer were added. The reaction tube was placed at 37°C for 20 minute and reaction was arrested by the addition of 200µl of 1M HCL. The reaction product was extracted with 5ml of butyl acetate. The organic phase was separated and measured at 280nm using a spectrophotometer. To prepare blank 1M HCl (200µl) was added prior to the reaction.

Estimation of MAO-B:
In the 250µl of homogenate, 250µl of serotonin and 250µl of buffer were added. The reaction tube was placed at 37°C for 20 minute and reaction was arrested by the addition of 200µl of 1M HCl. The reaction product was

extracted in 2.5ml of cyclohexane. The organic phase was separated and measure at 242 nm using a spectrophotometer. In the blank 1M HCl (200µl) was added before the reaction was carried out.

Statistical Analysis:-
The statistical analysis for all the parameters was carried out using One Way ANOVA followed by multiple comparison using Bonferroni post hoc test by Graph Pad Prism 6 software. Results were analyzed for statistical significance ($p<0.05$).

RESULTS

In ovo toxicated hatchling showed AFB1 induced neurotoxic damage. Intensity to alter neurotransmitters level was observed high in 10ng AFB1 treated group. Value of protein concentration in brain is shown in table 1. Protein concentration in brain and serum was found increased in aflatoxin intoxicated eggs of both the doses to that of control chicks and the values were found significant ($p\leq0.01$). There is no marked difference between vehicle control and control groups of chicks in the concentration of protein of brain and serum.

Table 1 shows the concentration of glucose in brain and serum. Concentration of glucose was observed significantly lower in high dosage treatment of AFB1 than of control group ($p\leq0.05$). Glucose concentration was observed significantly depleted in brain tissue but level was not significantly suppressed in serum of low dose group as compared to control hatchling.

Table 1 Protein and Glucose concentration of brain and serum in AFB1 intoxicated chicks compared to that of the control

Groups	CON	VC	LD	HD
Protein				
Brain(mg/gm tissue)	14.73±0.31	15.01±0.33	16.23±0.43**	17.90±0.45**
Serum (mg/dl)	36.14±0.73	37.36±0.86	40.11±0.88**	41.96±1.46**
Glucose				
Brain(mg/gm tissue)	199.2±11.1	196.02±12.6	163.3±18.4*	162.1±19.4*
Serum(mg/dl)	233.2±11.2	234.3±11.2	222.7±12.4	207.9±13.2*

*Data expressed as Mean ±SE (n=6) * = p ≤ 0.05, ** = p ≤0.001*

The activity of AChE in cerebellum region of brain was found significantly decreased in HD ($p\leq0.001$) and LD ($p\leq0.01$) groups compared to that of control. There was no observed significant difference with VC group when compared to control (Table 2). Activity of MAO-A and MAO-B in brain is shown in table 2. Activity of the MAO-A was observed significantly higher in LD and HD chicks compared to that of control ($p\leq0.001$). There was no significant difference observed in VC when compared to that of control. Significantly increased activity of MAO-B was observed in LD ($p\leq0.05$) and HD ($p\leq0.01$) treatment groups as compared to that of control.

Table 2 AChE, MAO-A and MAO-B activity of brain in Aflatoxin B1 intoxicated eggs

Groups	CON	VC	LD	HD
AChE	4.2± 0.19	4.19± 0.66	3.09± 0.59**	2.62± 0.41***
MAO-A (nmol/gm protein)	33.1±1.1	34.21±1.9	22.3±1.8***	25.74±1.3***
MAO-B (nmol/gm protein)	1.1±0.09	1.16±0.1	0.87±0.12*	0.77±0.13**

*Data expressed as Mean ±SE (n=6) * = p ≤ 0.05, **=p≤0.01, *** = p ≤0.001.*

Altered change in concentration of glutamate and tryptophan is shown in Table 3. There was significantly depleted level of glutamate in both the treatment group of eggs. Level of glutamate in AFB1 exposed brain was found significantly decreased in LD ($p\leq0.05$) and HD ($p\leq0.001$) groups compared to that of control hatchlings. Concentration of tryptophan in brain in the region of cerebellum was observed significantly elevated in both the dosage groups of AFB1 as compared to control animals ($p\leq0.001$).

Table 3 shows Glutamate and tryptophan concentration in brain tissue of RIR egg hatched chick by *in ovo* intoxication of Aflatoxin B1

Groups	CON	VC	LD	HD
Glutamate (µMole/g tissue	0.93±0.02	0.92±0.03	0.85±0.05*	0.64±0.053***
Tryptophan (µMole/g tissue	0.91±0.05	0.93±0.059	1.55±0.16***	2.3±0.27***

*Data expressed as Mean ±SE (n=6), *=p≤0.05, *** = p ≤0.001.*

DISCUSSION

The present study investigated the effect of aflatoxin (10ng and 5ng ofAFB1) in developing nervous system after *in ovo* injection at day '0' incubation. The eggs of RIR in present study were exposed to apparently sub toxic doses of

AFB1. The doses were chosen to stimulate a possible intermittent exposure to relatively low concentration of this toxin, and to ensure that possible neurochemical alterations would not be a secondary phenomenon. The treated RIR chick exposed to *in ovo* AFB1 showed no noticeable changes in their behaviour.

In the present study, eggs intoxicated by AFB1 show relatively increase in the amount of protein. Ikegwuonu [10] also reported increase total protein concentration in nervous tissue after AFB1 intoxication. Decreased glucose concentration was observed in serum and brain homogenate due to AFB1 intoxication. Decrease in glucose contents of chicks in response to aflatoxicosis accompanied by a decrease in the glycolytic enzymes, suggesting that AFB1 may interfere with the cellular energy supply through its inhibitory action on glycogen synthesis, glucose uptake (a decrease in glucose transport from blood to cells) and glycolysis. Similar result was obtained by Tessier *et al*. [21] who stated low glucose level in aflatoxin treated rats. Also, it was found that AFB1 reduces glucose transport and affects some enzymes engaged in glucose metabolism in the liver [22]. The reduction in glucose uptake may be attributed to the decrease in the number of GLUT 1 and GLUT 4 transporters in response to aflatoxicosis, these proteins are normally stored in cytoplasmic membranes and can be recruited to the plasma membrane as needed [23].

In the present study, significant inhibition of AchE activity in brain of treated chicks was recorded in both the dosage group. Results of present study are in accordance with Cometa *et al*. [24], Sayed and Abeer [25] who reported that AFB1 non-competitively inhibited brain AchE by blocking access of the substrate to the active site or by inducing a defective conformational change in the enzyme through non-covalent binding thus interacting with the AchE peripheral binding site or through both mechanisms. The prolonged release and accumulation of acetylcholine in response to the continued inhibition of AchE may result in neuronal degeneration in chicks.

Further, *in ovo* AFB1 intoxication reduced MAO-A and MAO-B activity in chick brain of low and high dose groups as compared to control hatchling, hence exerted an antidepressant- like action by inhibiting the metabolism of monoamines. MAO regulates the metabolic degradation of catecholamines, serotonin and other endogenous amines in central nervous system. Inhibition of this enzyme causes a reduction in metabolism and subsequent increase in the concentration of biogenic amines [26].

Administration of AFB1, effectively modulate the lowered levels of glutamate and raised level of tryptophan in brain of chicks in response to aflatoxicosis. Our results are in agreement with many investigators, who reported significant alterations in amino acids levels and their metabolites during aflatoxicosis [13, 27] Glutamate and glutamine brain levels showed marked decrease due to aflatoxin exposure to rats [25]. Several studies have shown that central and systemic tryptophan level and its metabolism were altered during aflatoxicosis [13, 14 and 25]. The significant increased in tryptophan level in the course of aflatoxicosis may be occurred as a result of direct alteration in their metabolic pathways, changes in tissue uptake process or as a result of modifications in tissue proteolysis. On the other hand, the disturbance in tryptophan metabolism and its major metabolite, serotonin, eventually disturb the blood-brain barrier which might gave the way for the entrance of aflatoxin B1 or its metabolites into the brain resulting in neuronal deformities [25, 28].

CONCLUSION

Aflatoxin exposure during developing stages alters the level of neurotransmitters that may be resulted into neuronal damage. The research can be further extended to find the relation of neurotransmitters and neurobehavioral study on AFB1 exposed chicks.

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