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Original Research Paper

EXPERIMENTAL AFLATOXIN B_1 TOXICOSIS IN YOUNG RABBITS - A CLINICAL AND PATHO-ANATOMICAL STUDY

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Summary: A feeding trial was conducted to assess the clinical, gross and histopathological alterations in various organs of New Zealand White rabbits fed an aflatoxin B_1 (AFB_1) contaminated diet. Aflatoxin extract was included in a toxin-free diet to provide the desired level of 0.5 ppm/kg of feed for *ad libitum* consumption by 16 young rabbits for a period of 50 days. Clinical signs of toxicosis were noticed from the 20th day onwards and were initially characterized by dullness, lethargy, reduced feed and water intake, hyperirritability, dyspnoea, oliguria and dehydration, which was followed by paralysis of the hind limbs, reduced heart rate and jaundice at the terminal stage of toxicosis. A decrease in body weight was observed in the treatment group. The carcasses of the rabbits in the toxin-fed group appeared emaciated and anaemic with subcutaneous oedema and gelatinization of fat. The liver and kidneys were the most affected organs followed by the stomach, intestines, lungs, heart, spleen, gonads and brain. Grossly, congestion and focal haemorrhages were observed in the affected organs in the initial stages. At the terminal stage of toxicosis, the liver was enlarged, icteric with greyish-white necrotic foci on all the lobes; nephrosis, catarrhal enteritis, pneumonia and mild testicular atrophy were also observed. Histopathology revealed vascular congestion, leucocytic infiltration and degenerative change in the affected organs during the initial stage of toxicosis. At its terminal stage, coagulative necrosis, perivascular and periductal fibrocellular reactions along with mononuclear-cellular infiltration and distortion of the hepatic chords were observed in the liver. Gastrointestinal ulcerations, hyalinization of the tubular epithelium and a widening of the glomerular capsules (Bowman's capsules) were also observed in the kidneys. The seminiferous tubules showed degeneration/denudation of the epithelium and a reduction in the number of mature spermatids. The study highlighted the toxic effects of a subacute dietary exposure of rabbits to AFB_1.

Key words: experimental toxicosis; aflatoxin B_1; pathology; rabbits

Introduction

Aflatoxin B_1 (AFB_1) is the most abundant and toxic metabolite produced by the *Aspergillus flavus* and *Aspergillus parasiticus* moulds, which are widespread contaminants of foods and feed in different parts of the world (1). In India, where the ambient temperature and humidity is high and long-term storage is often inadequate, high levels of AFB_1 in feed samples have been recorded (2). Animals, as well as human beings, are usually exposed to mycotoxins through their diet (3) and, depending on different factors such as age, sex, route of administration and species involved, this can result in acute, sub-acute or chronic mycotoxicosis (4). In recent years large-scale rabbit farming has been taken up in India, both for meat production as well as for biomedical research purposes. Diseases are the major impediment to profitable rabbit farming. Rabbits are the most sensitive animals to aflatoxicosis, the LD_{50} being only 0.3 mg/kg body weight, which is the lowest among all animal species (5). Because many of the clinical signs and clinico-pathologic changes of experimental aflatoxicosis in rabbits are similar to those reported in other species of animals, rabbits constitute an appropriate model for studying the mechanisms of AFB_1 toxic actions in food-producing animals (6). AFB_1 has been associated with

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outbreaks of aflatoxicosis in Indian rabbit farms (7, 8). Experimental AFB_1 toxicosis is known to cause alterations in enzyme levels along with patho-anatomical changes in vital organs (9, 10, 11, 12). Dietary AFB_1 exposure causes immunosuppression in animals resulting in an increased susceptibility to infection (13, 14, 15). The metabolism of AFB_1 in the liver produces highly-reactive chemical intermediaries, which bind to DNA resulting in the disruption of transcription and abnormal cellular proliferation, leading to mutagenesis and carcinogenesis (16, 17). AFB_1 is a threat to an *in utero* developing foetus producing teratogenicity when administered to pregnant animals (18).

The aim of this study was to assess the sub-acute effects of AFB_1 through the clinical, gross and histopathological alterations in various organs of rabbits.

Materials and methods

An *Aspergillus parasiticus* (strain NRRL 2999) culture maintained at the Division of Nutrition and Feed Technology, Central Avian Research Institute, Izatnagar, India was used to produce aflatoxin on rice following a standard method (19). The determination of crude aflatoxin was carried out using thin-layer chromatography (20) followed by the quantification of toxin as per the standard spectrophotometric method (21).

Twenty-four 3-month-old New Zealand White rabbits of either sex were procured from the Laboratory Animal Research Division, Indian Veterinary Research Institute (IVRI), Izatnagar, India and were individually housed in stainless steel cages on a 12-h dark/12-h light cycle. These rabbits were maintained on a toxin-free base diet supplied by the Feed Processing Unit, IVRI, along with green fodder (Burseem) and water administered *ad libitum*, until they gained about 1 to 1.5 kg in body weight. The body weight of each animal was recorded and the rabbits were randomly divided into two groups, control and experimental, comprising 8 and 16 animals respectively. Before feeding, the basal diet was tested for any possible residual aflatoxin, using the Howell and Taylor method (22), and no detectable levels were found (detection limit 1 ppb kg^{-1} feed). The AFB_1 was incorporated into the basal ration at the rate of 0.5 ppm/kg of feed. The control group was kept on a base diet only, while the experimental group was fed the aflatoxin-mixed ration for a period of 50 days.

The clinical signs and feed and water intake of the rabbits were regularly monitored. At every 10-day interval, the body weight of all the animals was recorded and two animals from the treatment group and one from the control group were sacrificed. All the rabbits that were sacrificed or that died spontaneously were subjected to detailed post-mortem examinations. Representative tissues from the liver, kidneys, stomach, intestines, lung, heart, spleen, gonads (testes/ovaries), pancreas, adrenal and thyroid glands and brain were collected in 10 % neutral-buffered formal saline for detailed histological studies. The tissues were processed and paraffin sections (4-6 thickness) were stained with Haematoxylin and Eosin following the standard procedure (23).

Results

Clinical Signs

The control rabbits did not manifest any abnormal signs during the experimental period. In the AFB_1-treated group, the signs of toxicity were noticeable from the 20th day onwards and were initially characterized by dullness, lethargy, diarrhoea (in a few rabbits) and reduced feed and water intake. From the 30th day onwards, the rabbits showed hyperirritability, dyspnoea, oliguria (with dark yellowish urine), dehydration and emaciation. Towards the terminal stage of the experiment, bradycardia, jaundice and paralysis of the hind limbs were observed. The body weights of the intoxicated rabbits were lower than those measured in the control group. During the course of the experiment, two rabbits of treatment group died on the 25th and 28th days, respectively. At the end of the 50th day, the animals in the experimental group showed poor body condition compared to the control group.

Gross Pathology

The necropsy observations of the AFB_1-fed rabbits are presented in Table 1. All the visceral organs showed varying degrees of congestion with focal haemorrhages. The liver and kidneys were the most affected organs followed by the stomach, intestines, lungs, heart, spleen, gonads and brain. On the 50th day, the livers of the toxin-fed rabbits were enlarged, pale to icteric with multiple, variously-sized, prominent and greyish-white necrotic

Table 1: Necropsy findings of different organs in aflatoxin B_1 fed rabbits

	Congestion					Haemorrhages					Oedema					Degeneration					Necrosis				
Days / Organ	10	20	30	40	50	10	20	30	40	50	10	20	30	40	50	10	20	30	40	50	10	20	30	40	50
Liver	+	++	++	+++	+++	-	-	+	++	++	-	-	-	-	-	-	+	++	++	+++	-	-	+	++	+++
Kidneys	-	+	++	++	+++	-	-	+	+	++	-	-	-	-	+	-	-	+	++	++	-	-	-	+	++
Stomach	+	+	++	++	++	-	-	+	+	++	-	-	+	++	++	-	-	+	++	++	-	-	-	-	+
Intestines	-	+	+	++	++	-	-	-	+	+	-	-	+	++	++	-	-	+	+	++	-	-	-	-	-
Lungs	-	+	+	++	++	-	-	-	+	++	-	-	+	++	++	-	-	-	-	-	-	-	-	-	-
Heart	-	-	+	++	++	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Spleen	-	-	+	++	++	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-

+→+++ = increasing severity of lesion
- = absence of lesion

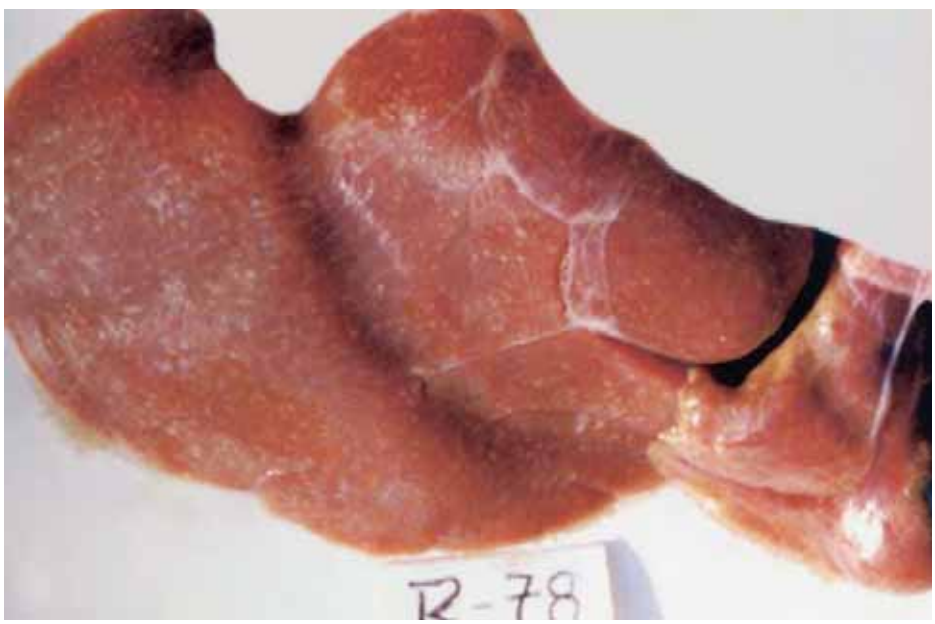

Figure 1: Liver - enlarged with multiple variously-sized greyish-white nodular foci

foci on both surfaces of all the lobes (Fig. 1). The gall bladder was distended, with thick greenish-yellow bile. The kidneys were congested and showed a moderate degree of nephrosis. The urinary bladder was distended with thick yellowish turbid urine. The mucosa of the gastrointestinal tract (GIT) was thickened with focal areas of erosion in the gastric and duodenal regions. There were mild pneumonic changes in the lungs. The heart revealed epicardial congestion. Mild to moderate degrees of congestion were observed in the spleen, meninges, ovaries, pancreas, adrenal and thyroid glands in the later stage of toxicosis. The testes were pale and mildly atrophied. The AFB_1-fed rabbits that died during the experimental period were emaciated and moderate degrees of congestion were found in the liver, kidneys, GIT, lungs, heart and meninges. The sacrificed animals showed emaciation, pallor of the mucus membranes, pronounced subcutaneous oedema and gelatinization of fat depots.

Histopathology

In the treated group, the liver revealed generalized vascular congestion, swelling of the hepatocytes, varying degrees of granular and hydropic degeneration along with mild fatty changes up to 30 days. From the 40th day onwards, marked fatty changes, areas of coagulative necrosis around the central veins, engorged portal areas, hyperplastic bile duct epitheliums as well as perivascular/periductal mononuclear cellular infiltrations were observed. In the terminal stage of toxicosis, there

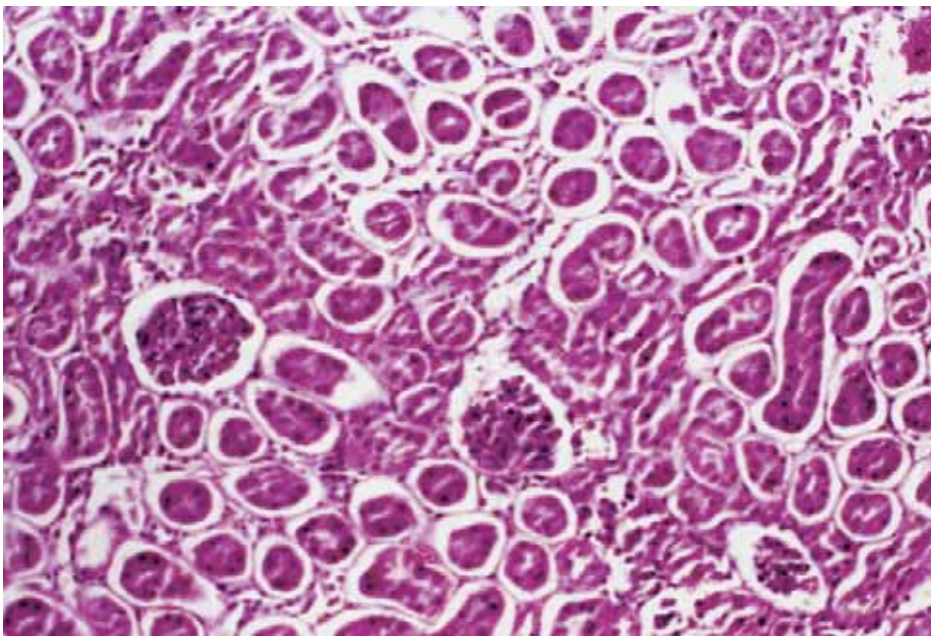

Figure 2: Kidney - degeneration of tubular epithelium. H&E x 190

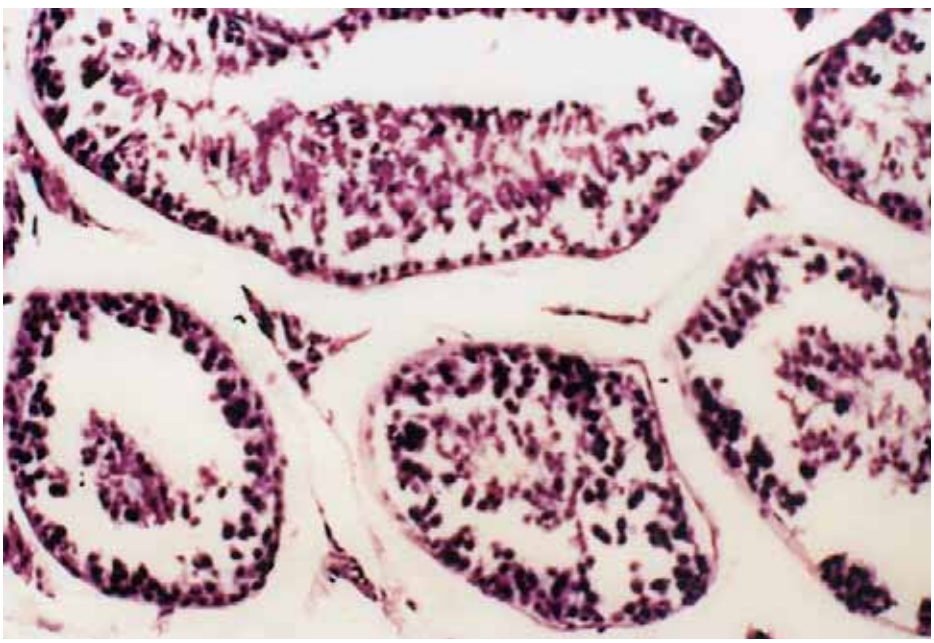

Figure 3: Testes - degeneration and desquamation of germinal layer/spermatogonial cells with a reduced spermatid population in the seminiferous tubules. H&E x 210

was extensive coagulative necrosis with disruption of the hepatic cords, moderate degrees of portal fibrosis along with focal mononuclear cellular infiltrations and bile duct proliferation. The mucosa of the gall bladder was oedematous.

In the kidneys, initially there was vascular congestion throughout the parenchyma followed by focal areas of haemorrhages and degeneration of the tubular epithelium up to the 30th day (Fig. 2). At the terminal stage of the experiment, the renal tubules showed a marked degeneration and hyalinization of the tubular epithelium along with widened Bowman's spaces of glomeruli.

The mucosa of the stomach and intestines initially showed vascular engorgement, focal areas of haemorrhages, thickening, hyperplastic mucus glands and a heterophilic inflammatory reaction that was followed by epithelial degeneration and desquamation up to the 40th day of toxicosis. On the 50th day, the superficial epithelium of the gastric and duodenal mucosae showed erosions along with mononuclear cellular infiltrations. In the spleen, the initially mild to moderate degrees of vascular congestion were followed by mild distension of the sub-trabecular sinuses, which was noticed after the 30th day of toxicosis. On the 50th

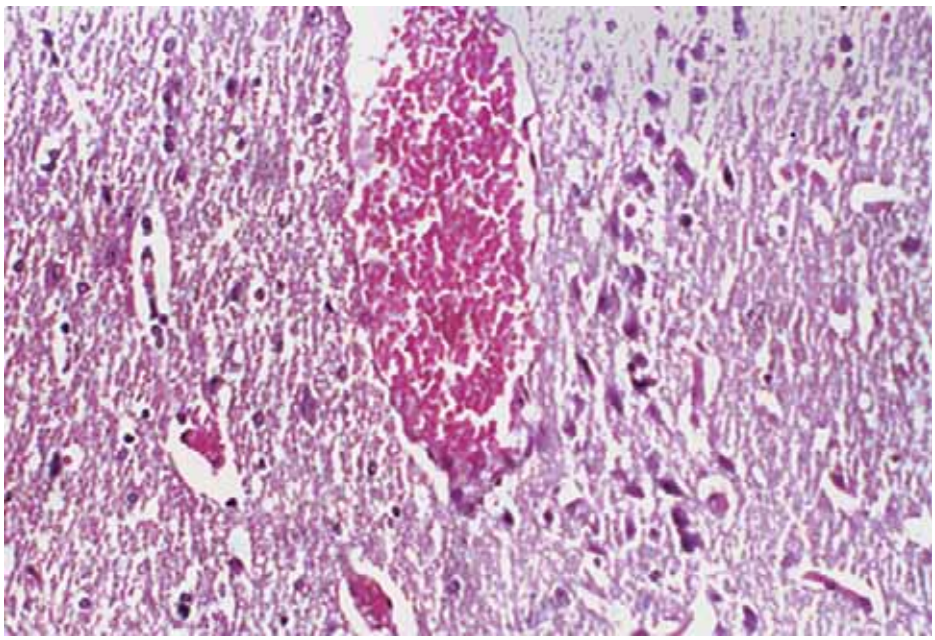

Figure 4: Brain - vascular congestion, widened Virchow-Robin space and mild perivascular oedema. H&E x 300

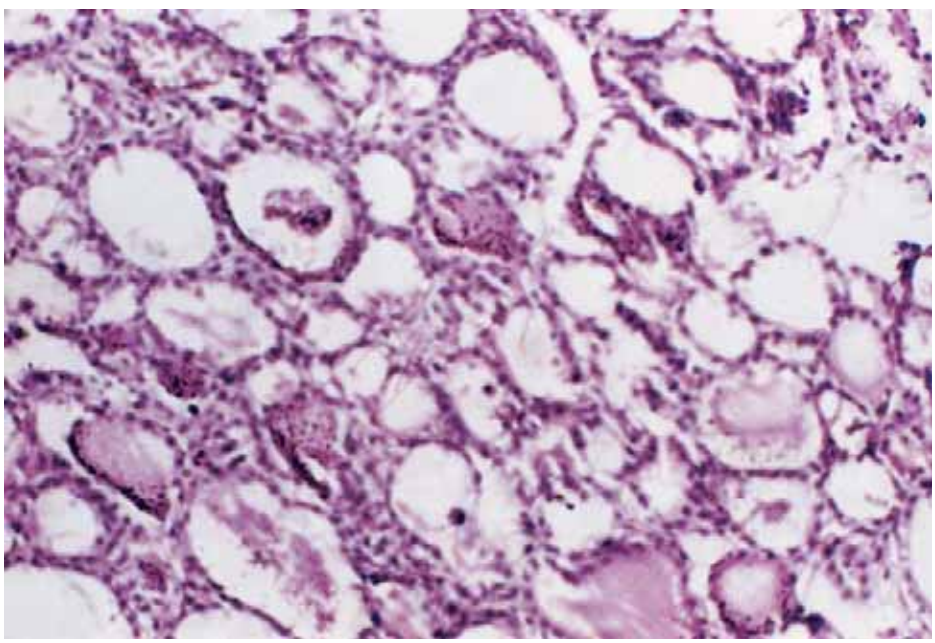

Figure 5: Thyroid gland - follicular degeneration with reduced colloid matter. H&E x 240

day of toxicosis, there was capsular sclerosis, a mild degree of depletion of the lymphoid follicles along with reticuloendothelial cellular hyperplasia.

During the first 30 days following the toxin's administration, the lungs developed alveolar congestion and focal areas of haemorrhages followed by varying degree of oedema and heterophilic and lymphocytic infiltration in the later stages. There was degeneration and desquamation of the bronchial epithelium and mild peribronchial lymphoid hyperplasia. Heart lesions comprised of congestion and mild degrees of haemorrhaging up to the 40th day of toxicosis. During the terminal stage, mild fatty changes and focal mononuclear cell infiltrations along with a degeneration of muscular fibres were noticed.

The testes initially showed engorgement of the intertubular blood vessels followed by degeneration, detachment and denudation of the germinal and spermatogenic epithelium of the seminiferous tubules after the 30th day of toxicosis. The denuded spermatogenic cells accumulated in the lumen. The population of mature spermatids appeared to be reduced by the terminal stage of experiment (Fig. 3).

In the ovaries, up to the 30th day, mild to moderate degrees of vascular congestion were observed

followed by mild degenerative changes in the follicular epithelium by the 50th day of toxicosis.

The brain showed mild to moderate degrees of meningeal and parenchymal vascular congestion (Fig. 4) throughout the experimental period. After the 30th day of toxicosis, there was a focal mononuclear cellular infiltration along with a widening of the Virchow-Robin space followed by a perivascular cuffing, mild perivascular oedema, neuronal degeneration and gliosis by the terminal stage of experiment.

In the early stage of toxicosis, the adrenal glands showed vascular congestion and by the 50th day showed capsular sclerosis and vascular congestion in the cortex and medulla.

Mild to moderate degrees of vascular congestion followed by mild thickening of interfollicular space developed in the thyroid glands during the first 30 days of toxicosis. A reduction of colloids and microfollicular formations were observed in the terminal stage of the experimental toxicosis (Fig. 5). The pancreas revealed vascular congestion and mild degenerative changes in the Islet cells of the acini by the end of 50th day of toxicosis.

Discussion

Experimental aflatoxicosis was induced in young rabbits of either sex following their intake of a diet mixed with AFB_1 at a rate of 0.5 ppm/kg feed. Surveys in India have shown that the level of AFB_1 detected in animal-feed ingredients ranges between 0.4 – 8 ppm (2, 24).

In general, the AFB_1-fed animals showed emaciation and a decrease in body weight. The decrease in the body weights of the rabbits in the experimental group can be correlated to a reduction in their feed/water intake, which corresponds with earlier reports (10, 11, 25, 26, 27, 28). It has been reported that a decrease in body weight is one of the earliest indicators of clinical aflatoxicosis in animals (6).

The manifestation of diarrhoea, observed in a few animals in the early stages, may be due to the acute toxic effects of AFB_1. Studies on ileum isolated from guinea pigs have shown that aflatoxin can cause acute gastrointestinal effects by indirectly inducing a contractile effect through the cholinergic system following the stimulation of an acetylcholine release from the postganglionic-parasympathetic nerve endings (29).

The icterus observed in terminal stages may be due to an increase in the levels of bilirubin as a sequela to hepatic necrosis and cholestasis. Similar findings have been recorded in the aflatoxicosis of rabbits dosed with 0.5 and 0.1 ppm/kg b.wt (25). Guerre et al. (25) attributed hyperbilirubinemia to liver damage resulting from decreased cytochrome P450, increased heme oxygenase and biliverdin reductase activities along with an increase in heme catabolism in the liver.

The vascular changes observed in the various organs and tissues in the present study are indicative of an AFB_1-induced endothelial injury. Most of the coagulation factors are synthesized in the liver, which is the target organ for aflatoxicosis. The haemorrhages observed in this study may be attributed to the impairment of coagulation. Blood coagulation defects involving the impairment of prothrombin, factor VII, X and possibly factor IX of the extrinsic pathway have been implicated as contributory factors for vascular changes in various organs following aflatoxicosis (12). Spontaneous haemorrhaging and bruising, in animals fed with aflatoxin, together with alterations in their coagulative profiles have been reported (30, 31).

The liver followed by kidneys were the most affected organs and the lesions observed in this study concurred with earlier findings following aflatoxicosis in rabbits (7, 8, 11, 26, 32, 33). Orally-ingested AFB_1 is most efficiently absorbed from the small intestine at the duodenum (34) and is metabolized in the liver by the cellular cytochrome-P450 enzyme system to form the reactive intermediate, AFB_1-8, 9- epoxide, which in turn reacts with macromolecules such as lipids and DNA, leading to lipid peroxidation and cellular injury (35). Shen et al. (36) suggested the role of reactive oxygen species (ROS), such as superoxide radicals, hydroxy radicals and hydrogen peroxide, in AFB_1-induced cellular injury. The fatty infiltrations/changes observed in the study could be due to impaired lipid transport rather than increased lipid biosynthesis. In addition, the mitochondrial damage following aflatoxicosis can result in a decrease in the oxidation of fats by these organelles, with a concomitant accumulation of lipids (37). The hepatomegaly observed in the AFB_1-fed rabbits appears to be associated with a higher lipid content. The necrotic changes, fibroblastic proliferation, bile duct hyperplasia along with the mononuclear cellular infiltration observed in this study are in accordance with those observed by Sahoo et al. (11).

The renal lesions are secondary to those observed in the liver and are in agreement with the findings of Sahoo et al. (11). Glahn et al. (38) reported that the target site of action of AFB_1 in the kidneys is the glomerular region.

Stetinova et al. (39) suggested that the effects of AFB_1 on the GIT were indirect following alterations in the liver's detoxification mechanism and a possible reduction in nutrient uptake.

The pulmonary inflammation and oedematous changes observed in the animals of the treatment group might also be due to the production of eicosanoids stimulated by the AFB_1 (40). This might have also contributed to the manifestation of dyspnoea in the early stages of toxicosis.

The testicular changes observed in the present study are in agreement with earlier reports (11, 31). Studies of adult male rats fed AFB_1 for prolonged periods, showed that AFB_1 caused regressive changes of different intensity in the germinal epithelium of the seminiferous tubules resulting in a severe dystrophic alteration of the spermatogenic epithelium along with oedematous changes in the interstitial tissue (41). Salem et al. (28) reported a relative decrease in testes weight and an increase in the number of abnormal/dead sperms following a 9-week administration of sublethal doses of AFB_1 to mature male rabbits.

The lesions observed in the brain corresponded with earlier findings following aflatoxicosis in rabbits (31). AFB_1 is known to alter the distribution of acetylcholine esterase (AChE) in the brain affecting cholinergic transmissions at the nerve endings and thus can result in manifestations of nervousness and behavioural deficiency (42). Sahoo et al. (11) reported vascular congestion and focal mononuclear infiltration in the meninges along with perivascular cuffing, mild neuronal degeneration and gliosis following the oral administration of AFB_1 to New Zealand White rabbits at the rate of 0.0625 mg/day/animal for a period of 30 days.

In conclusion, the prolonged feeding of AFB_1 in a diet, beyond 40 days, results in cumulative toxicosis, which is manifested by altered clinical signs as a result of lesions occurring in different vital organs.

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EKSPERIMENTALNO POVZROČENA TOKSIČNOST AFLATOKSINA B_1 PRI MLADIH KUNCIH – KLINIČNA IN PATOANATOMSKA ŠTUDIJA

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Povzetek: V študiji smo kuncem pasme beli novozelandec dajali krmo, okuženo z aflatoksinom B_1 (AFB_1), in nato ugotavljali klinične, patoanatomske in patohistološke spremembe v različnih organih živali. Izvleček aflatoksina smo dodajali krmi, ki je bila brez toksinov, v koncentraciji 0,5 ppm/kg krme. 16 mladih kuncev je imelo to krmo na voljo *ad libitum* 50 dni. Klinični znaki toksikoze so se pojavili po dvajsetih dneh, in sicer otopelost, letargija, zmanjšana poraba krme in vode, razdražljivost, oteženo dihanje, oligurija, dehidracija, kateri je sledila paraliza zadnjih okončin, upočasnjeno bitje srca in v končnem stadiju toksikoze še zlatenica. Živalim iz poskusne skupine se je v primerjavi s kontrolno tudi zmanjšala telesna teža. Trupla kuncev, ki so bili krmljeni s toksinom, so bila videti shujšana, anemična s podkožnimi edemi in želatinastim maščobnim tkivom. Najbolj prizadeti organi so bili jetra in ledvice, nato želodec, črevesje, pljuča, srce, vranica, spolne žleze in možgani. Na splošno so bile v začetnih stadijih bolezni najbolj opazne žariščne krvavitve in kongestija v prizadetih organih. V končnih stadijih toksikoze so bila jetra povečana in ikterična s sivobelimi nekrotičnimi žarišči na vseh režnjih,

opisali pa smo še nefrozo, kataralni enteritis, pljučnico in blago atrofijo mod. S histopatološko preiskavo smo v začetnih stadijih toksikoze ugotovili žilno kongestijo in degenerativne spremembe z infiltracijami levkocitov v prizadetih organih. V končnih stadijih toksikoze smo v jetrih ugotovili koagulativno nekrozo, perivaskularno in periduktalno fibrocelularno reakcijo skupaj z infiltracijo monocitov in spremenjenim položajem jetrnih trakov. Opisali smo tudi gastrointestinalne razjede ter hialinizacijo epitelija cevk in razširitev Bowmanovega prostora v glomerulih ledvic. Semenski kanalčki v modih so imeli degeneriran ali celo ogoljen epitelij, število zrelih spermatid je bilo zelo zmanjšano. Študija jasno poudarja toksične učinke subakutne izpostavljenosti kuncev AFB_1 v krmi.

Ključne besede: toksikoza; aflatoksin B_1; patologija; kunci