COMPARATIVE PATHOLOGY OF AFLATOXICOSIS IN THE DUCK AND FISH WITH SPECIAL REFERENCE TO THE IMMUNE SYSTEM

By

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DECLARATION

I hereby declare that this thesis entitled "Comparative pathology of aflatoxicosis in the duck and fish with special reference to the immune system" is a bona fide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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Dedication

I dedicate this work to my beloved wife Anitha,
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Chapter 1

INTRODUCTION

In the Indian Agricultural Economy, duck husbandry and aquaculture have a major role to play. These two agricultural activities are intimately linked to the socio-economic status and well being of the poor and the landless rural population of the coastal areas. India has 23.76 million ducks and this forms 7% of the total poultry population.

India depends heavily on fish production for its export earning and as a source of animal protein. The country produces about 3.6 million tons of fish products. The main source of fish production comes from capture fisheries. The domestic and international demands for fish are increasing very rapidly but this cannot be met from the existing sources. The production in capture sector is almost stagnant. Efforts are already being made to mop up all the available resources. This may ultimately lead to a situation where existing resources may be depleted. This situation can be remedied only by resorting to culture of aquatic animals to augment the production. Recently the Union Government and State Governments have drawn up various special schemes to increase the production and to make aquaculture a popular movement.

The Kerala state has a long stretch of coastal areas and most of the land in these areas are water logged during a significant period of the year. Extensive paddy cultivation is practised by farmers however it is a seasonal occupation. In recent years the paddy cultivation has become more uneconomical due to huge labour cost and there is a tendency to reclaim these lands for raising buildings. This may lead to environmental hazards and social conflicts.

Kerala is ranking fourth in the duck population and has 0.85 million ducks (Anon, 1994). Alleppey, Ernakulam, Kottayam and Pathanamthitta are the main duck rearing districts of Kerala. These areas are also known for their inland fish production. In these localities, it is seen that both duck husbandry and fisheries are practised on traditional basis. The duck farmers lead a nomadic life moving their flocks from one place to another in search of food. The migratory habit of the farming community exposes their stock to variety of diseases which often results in huge mortality of duck population. This threatens the very existence of duck husbandry in Kerala.

There is immense scope of improving duck husbandry through the adoption of scientific management of flocks. This include selection of good breeds, adoption of nutritive feeding practices and better disease control measures. Sustainable agricultural practices are replacing the old concept of intensive monoculture practices through out the world. This has several

advantages and it is more acceptable to the local population. Not only that it never interferes with the ecological balance of the nature but it also regenerates the natural resources. Integrated farming systems are the natural choice when we think of sustainable agricultural practices. It effectively recycles the waste generated in one system to the advantage of the other system. Integrated farming system reduces the input cost per unit of output generated. This is because the animal feed wastes and excreta enrich and fertilize crop fields/fish ponds. The agricultural wastes can in turn be used for animal feeding. Better environmental conditions generated through integrated farming reduces pest and disease causing organisms and thus brings down the cost on medications.

Duck cum fish farming and pig cum fish farming have emerged as major integrated systems in many parts of the world. Chinese had practised fish cum swine husbandry from immemorial times. Countries like Thailand, Indonesia and Philippines have demonstrated that duck husbandry cum aquaculture have immense potential to improve rural income.

Among various integrated farming systems duck cum aquaculture is most suited for water logged areas of Kuttanad. It has several advantages. The feed waste of duck and excreta of duck directly and indirectly enrich the food chain of fishes. Duck feed on aquatic plants and aquatic invertebrates

which are parasitic to fishes or harbour developing stages of parasites. Duck eggs and fish are delicacies for people of Kerala and there is good demand for these in the state.

In the intensive integrated farming system, a major input in duck husbandry and pisciculture is the feed. In order to harvest maximum production both duck and fish are to be fed with nutritive feeds which are less expensive. The major ingredients of the compounded feed of the duck and fish are cakes and grains and these ingredients provide the requirement of protein and energy at low costs. However, due to poor feed processing and storage conditions, the feed gets damaged. India in general and Kerala in particular has long spell of wet season with high relative humidity. During harvest and post harvest operations the grains and oil seeds may not get dried up properly and these contain high undesirable amount of moisture. This makes them good media for the growth of moulds. Many moulds produce potent toxins in the feed which cannot be easily detectable. These mycotoxins, are potent health hazards for livestock.

During 1936 to 1942 and again between 1940 and 1950 epizootics of hepatoma occurred in rainbow trout, a fish extensively cultured in Western countries. Investigation into epizootics of hepatoma brought out one common factor that these epizootics occurred with the change in dietary pattern. In early years trouts were fed on a diet of meat products.

Subsequent years witnessed change of meat based diets to pelleted diets which contained considerable amount of oil cakes. By 1960 trout hepatoma epizootics became a serious problem in USA. Extensive studies were carried out during these periods and in 1965 it was established that aflatoxin, a potent mycotoxin, present in the ground nut oil cake was responsible for hepatoma epizootics in trout farms (Ashley *et al.* 1964; Engebrecht *et al.* 1965).

Subsequent studies in animals and birds indicated that aflatoxin can induce a variety of pathological conditions in addition to carcinogenicity. It is also known that mycotoxins are powerful immunosupressants.

Most of the work in fish on aflatoxicosis pertains to its carcinogenicity. There is considerable lacunae in the knowledge about the pathology of aflatoxicosis in fishes. This is especially true in the Indian context. There is no recorded information about aflatoxin related problems in the commonly cultured Indian species of fishes. In the intensive duck and fish productions, ducks and fishes are fed diets rich in oil cakes and grains which are usually contaminated with mycotoxins. The aflatoxin, which is powerful hepatoxin induces mortality, reduces growth rate and by its immuno suppressive effects leads to outbreak of diseases. Aflatoxin is therefore, considered as a major problem in duck and fish rearing establishments.

With the expansion of aquaculture, nutrition related problems are likely to become acute. Besides this, major portion of fish produced is exported to earn foreign exchange. Since we are using highly contaminated oil cakes and grains to feed the fish there is every possibility of the presence of aflatoxin residues in the fish meat and this has no export market value since aflatoxin standard prescribed in exporting countries are very high.

Taking into consideration the above mentioned factors and the fact that only very meagre informations are available on the pathological effects of aflatoxin particularly in fishes, a study was envisaged to elucidate the pathological effects of aflatoxin in the duck and the Indian major carp (*Labeo rohita*). The main objective of the present investigation is to investigate into the deleterious effects of aflatoxin on the immune system of the duck and fish and make a comparative assessment of the immuno biological effects in these two species.

REVIEW OF LITERATURE

Chapter 2

REVIEW OF LITERATURE

2.1 AFLATOXICOSIS - GENERAL REVIEW

Hepatosis, hepatomas, damaged livers, bile duct proliferation, regeneration and nodular hyperplasia of hepatocytes and adenomatous formation were noticed in ducks which were on a diet of groundnut meal (Yoshida and Kamota, 1952).

Asplin and Carnaghan (1961) could establish that duckling and young ducks were highly susceptible to aflatoxin and hepatic tumours developed in ducks fed aflatoxin. Carnaghan (1964) noticed that the high incidence of hepatomas in ducks were due to contamination of their ration with aflatoxin. In ducks, liver lesions were produced at very low dose of aflatoxin and with short duration of exposure.

Several workers induced aflatoxicosis and studied the lesions in ducks. Lesions in the liver were characterized by extensive bile duct hyperplasia, fatty degeneration of hepatocytes and necrosis of the parenchymal cells (Armbrecht and Fitzhugh, 1964; Butler, 1964). Aflatoxin B_1 was more toxic than aflatoxin B_2. However, both toxins produced bile duct hyperplasia and necrosis of hepatocytes (Newberne *et al.* 1964). Ducklings were found to be the best model for the bioassay of aflatoxicosis

(Carnaghan, 1965). Madhavan and Rao, (1966) demonstrated that 40 micrograms of aflatoxin per day to duckling were lethal within 5 days. According to Yadagiri (1970) liver necrosis, shrunken hepatic cells with pyknotic nuclei and fatty change of hepatocytes were the important lesions of aflatoxicosis in ducks.

Administration of aflatoxin B_1 to rats resulted in the modification of hepatic mitochondrial DNA and long term inhibition of mitochondrial DNA transcription and translation (Bhat *et al.* 1982).

Aflatoxicosis in broiler chicken was manifested by jaundice, coagulopathy, dehydration of combs, shanks, decreased body weight and depression of bursal weight. Haemoglobin content and packed cell volume were affected. Hepatocytes were swollen and had undergone fatty change. Bile duct proliferation was evident (Chattopadhyay *et al.* 1985). Chen *et al.* (1985) fed a diet containing 2057 and 1323 micrograms/kg feed of aflatoxin B_1 and B_2 respectively to broiler chicken for 35 days. Samples were collected on 1, 2, 4, 8 and 16 days after withdrawal of contaminated feed. It was noticed that food conversion was decreased in chicken. Aflatoxins caused depressed growth, enlargement of the liver, kidney, heart and gall bladder. Haemorrhagic spots were present on the liver and muscles. Livers were pale and had fatty degeneration. After 8 days all lesions disappeared.

Crude aflatoxin had adverse affect on the growth and immune response of turkeys and broilers. In turkeys 400 ppb aflatoxin was extremely toxic. Aflatoxin produced liver lesions at 100 ppb and 20 ppb of aflatoxin suppressed cell-mediated immunity. Compared to turkey, chicken were more resistant to the effects of aflatoxin. Liver lesions were evident only at 80 ppb level. Cell-mediated immunity was affected at the 200 ppb level (Giambrone *et al.* 1985b).

In calves aflatoxins caused increase in the serum glutamic oxaloacetic transminase and alkaline phosphatase. Zinc could counteract this effect. However, dietary zinc could not increase the haemoglobin, packed cell volume and total solids in plasma (Wyatt *et al.* 1985).

When pigs were fed aflatoxin B_1 and Ochratoxin A together they exhibited clinical signs of Ochratoxin A intoxication. Mild hepatic degeneration was also present (Tapia and Seawright, 1985).

In rabbits blood clotting factors were affected due to aflatoxicosis. Prothrombin time and activated thromboplastin times of aflatoxin fed rabbits were lengthened. There was significant decrease in fibrinogen, factor IX, factor VIII and factor V. Platelet counts were increased in

subchronically exposed rabbits. Platelet size was decreased in acute aflatoxicosis (Clark *et al.* 1986).

Dalvi (1986) stated that aflatoxicosis was one of the major disease problem in ducklings, goslings, turkey poults and chicken. The toxic effects were mostly localized in the liver where it was characterized by hepatic necrosis, biliary duct proliferation, icterus and haemorrhage. Feed conversion efficiency of birds was lowered. Egg production and weight gain were also affected.

Aflatoxin B_1 administration did not modify hepatic mitochondrial DNA in mice and hamsters. Mitochondria did not metabolise aflatoxin B_1. Solubulized mitoplast containing less than 1% microsome could catalyse the activation of AFB_1 to electrophilic reactive forms *in vitro*. When aflatoxin B_1 was transported into mouse mitochondria through liposome there was 80% inhibition of protein synthesis. Failure of AFB_1 to cross mitochondrial membrane might be the reason for reduced metabolism of AFB_1 in mouse and hamsters (Niranjan *et al.* 1986).

Aflatoxin in broiler birds reduced the weight gain and increased the feed gain ratio. Histological changes were present in the liver (Borisova *et al.* 1987). Dafalla *et al.* (1987) fed seven day old chicks a feed containing aflatoxin at 0.5 ppm for four weeks. Body weight gain and feed conversion

ratio were less compared to the control group. The serum sorbitol dehydrogenase (SDH), Glutamic dehydrogenase (GDH) and total concentration of potassium were elevated in aflatoxin fed birds. Histopathological changes in the liver and kidney were typical of aflatoxicosis.

Erythrocytes from chicken receiving dietary aflatoxin levels had increased sensitivity to staphylococcal beta haemolysin. Chicks which received 10 micro grams of aflatoxin/kg of diet had increased zone of haemolysis compared to the controls (Doerr and Hamilton. 1987).

Low protein diet directly enhanced the effect of aflatoxin in chicken. Aflatoxin also enhanced the production of pancreatic enzymes like trypsin, chymotrypsin, amylase and lipase. Reduction of protein in the diet decreased amylase. Low level dietary fat lowered the toxicity of aflatoxin and reduced intensity of liver lesions (Richardson *et al.* 1987(a); Richardson *et al.* 1987(b); Richardson and Hamilton, 1987).

Coagulation defects were the main symptoms in rabbits intoxicated with aflatoxin B_1. The coagulation defects were due to failure of hepatic synthesis of factors V, VII, VIII and fibrinogen. Serum albumin concentration was also decreased (Baker and Green, 1987).

In dogs aflatoxin caused fatty degeneration of the liver, bile stasis, icterus, anemia, ascites, hydrothroax, hydropericardium, pulmonary oedema and gastro-enterorrhagia. Microscopical lesions were hepatocellular fatty change, necrosis, proliferation of bile ductules, stasis of bile and fibroplasia (Bastianello *et al.* 1987).

In hamsters and rats manganese salts were found to ameliorate the effects of aflatoxin on the liver; bile duct hyperplasia and enlarged hepatocellular nuclei were reduced. Aflatoxin was found to inhibit the excretion of heavy metal (Hastings and Llewellyn, 1987; Katzen and Llewellyn, 1987). Singh *et al.* (1987) noticed decrease in total proteins and 75% reduction in alkaline phosphatase activity.

Increased serum levels of SGOT and lipases and decreased serum amylase were noticed in broiler chicken exposed to aflatoxin (Balachandran and Ramakrishnan, 1988). Pigs received different levels of aflatoxin in feed developed interlobular fibrosis of the liver, periportal lipidiosis, periportal lymphocytic infiltration and bile duct hyperplasia. Lymphocytes were depleted in the thymus. Liver lipid values were increased. Weights of livers did not show much variation. Decrease in body weight gain and increase in serum aspartate transaminase, gamma glutamyl transferase and alkaline

phosphatase were noticed. The total protein, serum albumin, urea nitrogen, phosphorous and cholesterol were also decreased (Harvey *et al.* 1988).

Several mycotoxins together could produce a synergistic toxicity in broiler chicken and swine. Aflatoxin alone produced fatty livers and affected body weight gain. It also produced increase in relative weight of the kidney, heart and decreased mean corpuscular volume and serum potassium levels (Huff *et al.* 1988a & b).

Aflatoxin had a significant effect on the male reproductive system of ducks. 25 micro gram of aflatoxin/duck daily for 3 months reduced the weight of testes and caused significant reduction in feed intake. Testicular tissue was degenerated and there was complete absence of spermatogenesis. Hepatosis and nephrosis were also evident (Jayakumar *et al.* 1988).

Okuda *et al.* (1988) observed that aflatoxin B_1 increased replication of duck hepatitis B virus in hepatocytes and could influence expression of virus in infected ducks.

Coppock *et al.* (1989) reported acute aflatoxicosis in pigs. Out of 600 pigs affected, 400 pigs died. Corn was the source of aflatoxin. Feed contained 2500 to 3500 micro gram of aflatoxin/kg of feed.

Outbreaks of aflatoxicosis in pigs due to feeding of contaminated corn were also reported from Iowa. Corn contained 2020 μg AFB_1 and 1200 μg AFB_2. The pigs lost weight and feed consumption was very low. Mortality and morbidity were high (Cook *et al.* 1989). Sodium calcium aluminosilicate in the diet could ameliorate the effect of aflatoxin in pigs. Body weight gains were restored, feed intake became normal and hepatic changes were reduced. Activity of serum enzymes like alkaline phosphatase, gamma glutamyl transferase and prothrombin time were brought to normal values (Colvin *et al.* 1989 and Harvey *et al.* 1989).

Aflatoxin was implicated as a causative factor in the development of carcinoma of ethmoid region in cattle. Localization of AFB_1 metabolites in the sustentacular cells in the apical portion of the olfactory surface epithelium and Bowman's glands in the olfactory lamina propria mucosae was demonstrated. Higher metabolism of aflatoxin in the nasal olfactory mucosa was due to cytochrome P-450 isoenzyme system. The microsomal electron transport to cytochrome P-450 was facilitated by high level of cytochrome reductase (Larsson *et al.* 1989).

Contaminated corn fed to dairy cattle was identified to be a source of aflatoxin poisoning. Some times aflatoxin exceeded 2365 micro gram/gram of feed. The dairy cattle had staring hair coat, diarrhoea, pale

liver and anasacra. Histopathological changes included cirrhosis of the liver with bile duct hyperplasia and periportal accumulation of mononuclear cells. The feed contained aflatoxin B_1, B_2, G_1 and G_2 (Hall *et al.* 1989; Van-Halderen *et al.* 1989).

Occurrence of large scale mortality among ducks due to aflatoxins was common in North Eastern states of India. Clinical symptoms and hepatic lesions were suggestive of aflatoxin intoxication. Feed analysis confirmed the diagnosis. Histopathological changes included degeneration and necrosis of hepatocytes, with mild proliferation of biliary epithelial cells (Mukit and Kwatra 1989; Roy *et al.* 1989).

It was found that adding hydrated sodium aluminosilicate to feeds prevented many of the toxic effects of aflatoxin in chicken; however, adding activated charcoal to the diet did not have the protective effect against aflatoxins. Hydrated sodium aluminosilicate formed sequestrated aflatoxin in the intestine which resulted in poor bioavailability of AFB_1 (Kubena *et al.* 1990; Phillip *et al.* 1990).

In Japanese Quails aflatoxin produced ataxia, leg weakness, incordination of movements, torticolis and opisthotonos (Rao *et al.* 1990). Aflatoxins caused decrease in the excretion of phosphorous and calcium

concentration in plasma of broiler chicken. This was due to renal tubular damage (Glahn *et al.* 1990).

Duck hepatitis B virus infection did not alter the response of the duck to aflatoxin and AFB_1 was found to be a potent carcinogen in both virus infected and virus non-infected ducks (Cova *et al.* 1990; Cullen *et al.* 1990).

Krishna *et al.* (1991) investigated on the outbreak of diseases in several rabbit farms and they found that the feed used in these farms contained 90 to 540 micrograms of AFB_1/kg of feed. The livers of these rabbits were hard and icteric. Hepatic cells showed degenerative changes and the hepatic sinusoids were engorged. There was periportal fibrosis and biliary hyperplasia.

Kubena *et al.* (1991) and Harvey *et al.* (1991) noticed that feeding of hydrated sodium aluminium silicate reduced the adverse effect of aflatoxins in turkey and lambs.

Aflatoxin was found to be affecting the avian renal functions. It altered vitamin D and parathyroid hormone metabolism and stimulated sodium resorption and decreased glomerular filtration rate. Plasma calcium level was decreased due to increased calcium excretion (Glahn *et al.* 1991).

Tryptophan supplementation in feed reduced lipid accumulation in the liver of hens. However, in aflatoxin fed hens, tryptophan supplementation increased the hepatic accumulation of lipids (Rogers *et al.* 1991).

Among various mycotoxins, aflatoxins exerted their influence on the reproductive system indirectly. The animals reduced their feed intake. Growth was retarded. Impaired liver and kidney functions affected various physiological mechanisms (Diekman and Green, 1992).

Aflatoxin B_1 caused a dose related embryo mortality in chick embryos. There was no apparent difference in the weight gain of hatched chicks. However, the thrombocytes in the blood were reduced. There was increase in the number of lymphocytes and monocytes. Peritoneal lavage yielded less number of cells. Adhesive and phagocytic efficiency of macrophages were reduced (Neldonortiz and Qureshi, 1992).

Pier (1992) stated that aflatoxicosis was a serious and continuing problem in animal production. Toxin producing fungi were universally present in major food crops. Besides, the acute aflatoxicosis in which the signs were apparent, the chronic aflatoxicosis produced signs that were not clinically obvious. In many animal species, chronic toxicosis affected the

immune system. Suppression of the cell-medicated immunity, reduced phagocytosis, depression in complement and interferon production and poor response to vaccination were the main effects. Chronic aflatoxicosis often expressed as increased susceptibility to infectious disease.

Aflatoxin was suspected to be playing an important role in induction of olfactory tumours in cattle. Experimental studies using microsomal preparations of the bovine olfactory mucosa had indicated that bovine olfactory mucosa had a high AFB_1 bioactivity. This led to DNA damage and mutations (Tjalve *et al.* 1992).

Verma and Raval (1992); Raval and Verma (1992) reported the alteration in erythrocyte morphology and biochemical composition of muscles in rabbits during aflatoxicosis.

Leenadevi (1992) experimentally demonstrated the carcinogenic activity of AFB_1 in ducklings.

Seawright *et al.* (1993) compared the aflatoxin metabolism in the duck and rat liver. They studied the metabolism in duck infected with hepatitis B virus and non-infected ducks. Aflatoxin B_1 was more actively metabolized to aflatoxin B_1 -8,9-epoxide by the duck liver than the rat liver. Repair of DNA lesion in the duck and subsequent formation of ring-opened

aflatoxin B_1 FAPy adduct paralleled that in rats. Duck hepatitis virus infection had no effect on any of the biochemical process involving aflatoxin B_1.

However, Cova *et al.* (1993) observed that the hepatocellular carcinoma of the duck in Qudong area of China was always associated with duck hepatitis virus B infection.

Cova *et al.* (1994) analysed a series of 16 duck liver samples collected from the farms of Qudong, China where high incidence of aflatoxicosis and hepatitis B virus infection were prevalent in the human population. Hepatocellular carcinoma was found in eight and cirrhosis in one of these samples. Bile duct proliferation characteristic of aflatoxin B_1 exposure was found in these ducks. Polymerase chain reaction for the demonstration of DNA of duck hepatitis virus B was positive only in one case. However, HPLC immunoassay revealed AFB_1 – DNA adduct formation in one tumour. Aflatoxin exposure was an important factor in duck liver carcinogenesis.

Duflot *et al.* (1994) noted that P 53 mutation at cordon 249 which was common in hepatocellular carcinoma of other animals did not occur in hepatocellular carcinoma induced in ducks by aflatoxin and duck hepatitis virus B.

Kumagai *et al.* (1995) compared activation and inactivation of AFB_1 by microsomes and cytosol prepared from the mammalian and avian livers. They suggested that relative susceptibility of avian and mammalian species to toxic carcinogenicity could be due to combined action of microsomes and cytosol.

Duflot *et al.* (1995) studied the relationship between duck hepatitis virus infection and aflatoxicosis in producing duck hepatocellular carcinoma. Carcinomas were identified in 3.3 year Chinese brown ducks. Hepatocellular carcinomas ranged from well differentiated trabecular to highly anaplastic type. Tumour giant cells, tumour necrosis, tumour thrombi in blood vessels and cirrhosis were also observed. Biliary hyperplasia was present in 86% of cases. Polymerase chain reaction for duck hepatitis B virus DNA, was positive in 68% of cases. There was strong evidence that aflatoxins were involved in the induction of tumour.

2.2 AFLATOXICOSIS IN FISH

As soon as the toxicity of aflatoxin produced by the *Aspergillus flavus* was known it was recognized that the toxin could produce neoplastic transformation in rainbow trout livers. Several workers noticed high incidence of hepatic tumours in rainbow trout farms where groundnut meal formed the part of fish feed (Ashley and Halver, 1961; Nigrelli and

Jakowska, 1961; Ghittino, 1961; Halver *et al.* 1962). Toxicity of aflatoxin for fish was first described by Ashley *et al.* (1964) and Halver (1965). They reported that aflatoxins were extremely carcinogenic to fish. Widespread hepatic carcinoma occurred in many farms due to aflatoxicosis.

When aflatoxin was fed at higher levels to trout it induced an acute toxic syndrome with massive focal hepatic necrosis, branchial oedema and a generalized haemorrhagic syndrome (Halver *et al.* 1966; Halver *et al.* 1969; Ashley, 1970). The effect of aflatoxins varied among fishes, the rainbow trout being the most sensitive to aflatoxins. At 0.01 ppb aflatoxin could produce neoplasia in trout (Halver, 1965; Halver, 1969; Ashley, 1970). Wunder (1976) observed that young rainbow trout developed liver cancers due to feed contamination of aflatoxin. The out break was brought under control after removing the contaminated feed. Feeding of polychlorinated biphenyl/Aroclor could inhibit tumour development in rainbow trout induced by aflatoxin B_1 (Hendricks *et al.* 1977).

High incidence of liver cancer was noticed in cultured rainbow trout during 1957 to 1960. The feed of affected farms contained aflatoxins (Sinnhuber *et al.* 1977). It was found that the presence of fish protein concentrates in feed augmented tumorogenic activity of aflatoxins B_1, in trout (Lee *et al.* 1978).

Several workers studied the effect of aflatoxins on young trouts and embryos of trout. In one experiment Zaleski *et al.* (1979) fed 0.03, 0.045 and 0.096 mg/kg aflatoxin B_1 to trout brood. The stock died (23%) within 50 days of the experiment. Fish which received 0.06 mg/kg aflatoxin did not survive beyond 84 days. There was also 100% mortality in fish which were fed with 0.045 mg/kg aflatoxin B_1 by 90 days. In stocks fed with 0.03 mg/kg aflatoxin B_1 there was 72% mortality. In the control group there was only 40% mortality. Wales (1979) assessed the effect of aflatoxin B_1 bath on embryos. 1 mg/kg aflatoxin B_1bath for 15 minutes to one hour produced hepatoma in 60 to 68% later when embryos were hatched out.

Effect of other hepatotoxin like Dieldrin in combination with AFB_1 on liver pathology was studied. It was found that Dieldrin increased the incidence of AFB_1 induced tumours. Dieldrin alone could not induce tumours (Hendricks *et al.* 1979). Tilapia was found to be highly susceptible to aflatoxin. In Tilapia carcinogenicity was not confined to the liver, but it produced wide range of neoplasms like renal tubular carcinoma, lymphoma and hepatoma (Haller & Roberts 1980). Though Aroclor 1254 was reported to be tumour suppressing Hendricks *et al.* (1980) could not effect any suppression of hepatomas by feeding Aroclor 1254 to aflatoxin exposed trout. Permanent cell lines could be developed from AFB_1 induced

hepatomas (Fryer *et al.* 1981). High incidence of adenomatus stomach and liver carcinoma were traced to high aflatoxin content (Peters *et al.* 1981).

In the rainbow trout, aflatoxin was metabolised in liver and formed adducts with DNA. These adduct formation was proportional to the AFB_1 dose. Unbound AFB_1 metabolites were aflatoxicol, aflatoxin M_1 and polar conjugates (Bailey *et al.* 1982). In Salmon (Cohosalmon) aflatoxin did not produce hepatoma but liver lesions were present which included necrosis of hepatocytes and fatty change (Bruenger and Greuel, 1982). In Tilapia culture aflatoxicosis was a major cause of loses (Roberts and Sommerville, 1982). In carps, aflatoxin neither produced any liver lesions nor any alteration in hematological values. There was no accumulation of aflatoxin in fish muscles (Svobodova and Piskac, 1980; Svobodova *et al.* 1982).

The *invitro* mutagenic potency of several metabolites of aflatoxin qualitatively correlated with *invivo* carcinogenic activity of various aflatoxin in rainbow trout (Coulombe, 1983). Aflatoxin contamination of feed in addition to hepatoma, produced hepatic cirrhosis, cystic liver degeneration, cholangioma and hepatic adenocarcinoma in rainbow trouts (Lopez-Jimenez, 1983).

Naphthoflavone and Aroclor 1254 were found to modify the carcinogenic effect of aflatoxin in the rainbow trout. Naphthoflavone was

shown to metabolise AFB_1 into 7 hydroxy alfatoxicol and aflatoxicol M-1 which were less mutagenic (Loveland *et al.* 1983; Shelton *et al.* 1983).

Parashari and Saxena (1983) studied the toxicity of aflatoxin B_1 in blood parameters of catfish *Claria batrachus* and noticed leukaemogenic effect on blood leucocytes.

Beta naphthoflavone inhibited AFB_1 carcinogenesis in rainbow trout. It decreased DNA binding in hepatocytes, increased rate of detoxification and decreased cyclopropencid fatty acids which were promoters of carcinogenesis. The general excretion of aflatoxin through bile was increased. Aflatoxicol M-1 glucuronide was present in the bile (Bailey *et al.* 1984; Loveland *et al.* 1984).

It was found that trout ambryos were highly sensitive to aflatoxins. Tumours could be induced in embryo by micro injections and bath techniques. Hence trout embryos were considered to be economic model system for carcinogen testing (Hendricks *et al.* 1984; Metcalfe and Sonstegard, 1984).

Ruiz-Perez (1984) and Ruiz-Perez *et al.* (1984) reported that 60% of rainbow trouts of 8 years age had trabecular hepatomas and livers of these fishes contained 2 ppb aflatoxin. Majeed *et al.* (1984) also observed

high incidence of hepatoma in adult females of rainbow trout which were on a special diet and they pointed out that circumstantial evidence indicated the involvement of aflatoxin.

When aflatoxins were fed at higher levels it produced generalized haemorrhagic syndrome in fish (Poppe *et al.* 1985).

Aflatoxin produced chromosomal aberrations in the kidney cells of cyprinids within 48 hours after injection. Micronuclei formation in the erythrocytes of cyprinids increased in response to aflatoxin injection (Al-Sabti, 1985, 1986).

Severe anemia, low haematocrit values and mortality in channel catfish were attributed to aflatoxin contamination of feed (Plumb *et al.* 1986). An outbreak of hepatocarcinoma involving 50,000 salt water reared trouts maintained at Danish trout farms were traced to aflatoxin contamination of feed (Rasmussen *et al.* 1986).

Goeger *et al.* (1986) found that indole-3-carbinol could reduce hepatic DNA binding and RBC DNA binding of AFB_1 in trouts. Polychlorinated biphenyls were found to inhibit carcinogenic effect of AFB_1 in trouts. The blood, liver and bile contained aflatoxin in fish fed with PCBS but pretreatment with PCB shifted metabolite profile of AFB_1

towards polar metabolite aflatoxin M-1 and glucuronide conjugate (Shelton *et al.* 1986).

Compounds like indole-3-carbinol, beta naphthoflavone, polychlorinated biphenyl complex Aroclor 1254 could reduce the incidence of hepatocellular carcinoma in trouts when they were fed these compounds prior or after the exposure to aflatoxin B_1 (Bailey *et al.* 1987).

Tereza *et al.* (1987) reported lipoid liver degenerations with presence of large quantities of ceroid and vacuolar degeneration of mucosal epithelium of the intestine in fishes received from state farms of Portugal. The feeds were found to be contaminated with moulds and yeasts.

Beta-naphthoflavone was reported to be suppressing DNA adduct formation with aflatoxin and tumour production in trouts whereas butylated hydroxy anisole did not have any effect (Goeger *et al.* 1988). This view was contradicted by Nunez *et al.* (1988). They found that Beta-naphtho flavone did not reduce DNA adduct formation with aflatoxin in trouts.

Seventeen beta estradiol, indole-3-carbinol, betanaphthoflavone and DDT enhanced hepatocarcinogenesis due to aflatoxins in trout at embryonic stage. When yolk sac fry of rainbow trout were injected with

aflatoxin they developed tumours in the liver after one year (Metcalfe *et al.* 1988).

There was no relationship between hepatocarcinogenicity and liver glutathione concentration and glutathione transferase activity. There was also no relation between aflatoxin B_1 epoxide glutathione detoxification and hepatocarcinogenicity in the rainbow trout and cohosalmon. The mechanism of hepatocarcinogenicity in the fish might be entirely different from that of rodents (Valsta *et al.* 1988).

In elasmobranch liver, the aflatoxin was reported to be metabolised only to aflatoxicol. This was demonstrated in post mitochondrial preparation. The post mitochondrial preparation of calf liver, clear nose skate and nurse shark were allowed to metabolise aflatoxin. Calf liver produced aflatoxin M, and aflatoxin Q1 as major metabolites. The elasobranch livers produced only aflatoxicol (Bodine *et al.* 1989).

Nunez *et al.* (1989) studied the enhancing effect by 17 beta estradiol on aflatoxin B_1 induction of tumours in rainbow trout. Four groups of 21 day old rainbow trout embryos were exposed to static solutions of water containing 0, 0.005, 0.025 and 0.125 ppm aflatoxin B_1 for 30 minutes. Six weeks after AFB_1 exposure, two groups at each dose level were subjected to dietary 17 beta estradiol-2-treatment while two remaining

groups received control diet. Estradiol-2-promoted hepatic tumour incidence in fish exposed to the 0.025 ppm AFB_1 from 0% to 9% and in fish exposed to the 0.125 ppm dose AFB_1 from 5% to 60%.

In channel catfish subchronic toxicity by aflatoxin B_1 affected the growth rate, reduced hemoglobin concentration, and produced fall in erythrocyte count. 10,000 microgram AFB_1 per kilogram of feed was highly effective in altering haemocrit values. However, the leucocyte counts were high in AFB_1 affected fishes. Necrotic foci were present in the liver. Such foci contained basophilic hepatocytes also. Spaces resulting from necrosis were present in basophilic foci of liver. Sinusoids of head kidney were dilated. Haematopoietic areas contained large number of immature erythrocytes. Intestinal mucosal epithelium accumulated excessive iron pigments. Gastric glands in the stomach were necrotic and contained infilitrate of macrophages (Jantrarotoi and Lovell, 1990).

Acute toxicity in channel catfish caused regurgitation of stomach contents. LD50 values for 19 gram fish was 11.5 mg/kg. Gills, livers, kidneys, spleens and stomach of fish treated with 12 mg AFB_1/kg body were extremely pale. Haemocrits, haemoglobin concentration and erythrocyte counts were about 10% of those values in the control fish. Histological lesions in the moriobund fish were sloughing of the intestinal mucosa, necrosis of the haematopoietic tissues, hepatocytes, pancreatic acinar cells

and gastric glands. The spleens showed reduction in volume of the red pulp and reduction in the number of leukocytes in splenic corpuscles. Renal tubular lumens were dilated (Jantrarotoi *et al.* 1990).

The relationship between aflatoxin B_1 metabolism, cytotoxicity and hepatocarcinogenicity was studied in rainbow trout fry. Fry were exposed to aqueous solutions of 0.05, 0.1, 0.25 and 0.5 mg/l ^3H Aflatoxin B_1 for 30 minutes. Another group of fry was fed 500 mg/l^{-1} beta naphthoflavone for one week before exposure to 0.5 mg/l^{-1} ^3H AFB_1 for 30 minutes. Samples were taken 24 hours and 2 weeks later for DNA binding and histopathological analysis. Histopathological lesions and DNA binding showed a linear dose response. The results indicated that cytotoxicity and carcinogenicity depended on aflatoxin conversion to electrophilic 8,9 epoxide. Cytotoxic phase was characterised by architectural disruption due to severe swelling of hepatocytes and necrosis. The viable remaining cells showed pleomorphic atypical nuclei and foamy cytoplasm. Small basophilic cells emerged from degenerative hepatocytes. These cells showed high mitotic index (Nunez *et al.* 1990).

Mutation of two C-ras genes was detected in liver tumours induced by AFB_1. These were point mutation in exons of C-K_1-ras genes (Chang *et al.* 1991). Studies using ^{14}C labelled AFB_1 in channel cat fish indicated binding of 95% AFB_1 to plasma proteins. AFB_1 level was high after 4 hours

of oral administration in tissues. The absorption and elimination half lives were 1.5 and 3.7 hours respectively. Peak plasma concentration occurred 4 hours after dosing. AFB_1 residues from tissues rapidly declined. More than 5% of administered dose was excreted through the bile and kidney. It was felt that AFB_1 was not completely absorbed (Plakas *et al.* 1991). Lovell (1991) was of the view that sensitivity for mycotoxins varied among different species of fishes.

Nunez *et al.* (1991) studied the ultra structure of hepatocellular neoplasms induced by AFB_1 in trouts. In hepatocellular carcinoma and hepatocellular adenomas, nuclei were large and uniform and contained large nucleoli. Rough endoplasmic reticuli were dilated and glycogen in cells were depleted. In hepatocellular carcinomas, microvilli were poorly developed in the space of Disse and in the bile canaliculi. Size and number of inter cellular spaces increased in carcinomas. Preductule cells were few. All these findings indicated loss of inter relationship between hepatocytes.

Fate of ^3H labelled aflatoxin administered both orally and intravenously in rainbow trout was studied for a period of eight days. Autoradiography and scintillation counting methods were used. Highest amount of radioactivity was observed in the bile, liver, kidney, pyloric caeca, uveal tract of eye and the olfactory rosette for longer duration. The

radioactivity could not be extracted with polar and non-polar solvent indicating covalently bound metabolites (Ngethe *et al.* 1992).

Incidence of aflatoxin induced tumours was influenced by environmental temperature. Two gram size rainbow trouts were acclimatized to 10 and 18 degree celsius for one month. They were immersed in 0.1 ppm ^{3}H labelled aflatoxin B_1 for 30 minutes. The radioactivity in liver was higher in fishes acclimatized for 18^0C than in fishes acclimatized for 10^0C. Hepatic DNA adduction was higher in 10^0C acclimatized fish than in 18^0C acclimatized fish. However, tumour incidence was found to increase with high temperature acclimatization (Zhang-Quan *et al.* 1992).

Enzyme profile of hepatic neoplasm induced by AFB_1 in rainbow trout was studied. Though activities of ethoxy resorufin-O-diethylase (EROD), microsomal and cytosolic epoxide hydrolase (m EH and C EH), aldehyde dehydrogenease (ALOH), DT diapholase, gamma-glutamyl transferase (gamma GT) Glutathione transferase (GST) Uridine diphospho-glucuronyl trnasferase (UDGPT) and P450 IAI were measured only benzaldehyde dehydrogenase and gamma-glutamyl transferase showed increase. Induction of aldehyde dehydrogenase, uridine diphosphoglucuronyl transferase and depression of cytochrome P450 IAI

were also noticed (Parker *et al.* 1993). Hepatic accumulation of aflatoxin B_1 diferred in the rainbow trout and Tilapia (Ngethe *et al.* 1993).

In embryos of rainbow trout aflatoxicol was a more potent carcinogen. Aflatoxicol produced better sequestration and DNA binding which was three fold greater than AFB_1. However, AFB_1 was taken up more by the embryo. The potency of the carcinogen was directly related to DNA adduct formation. Both chemicals produced same type of neoplasm (Bailey *et al.* 1994).

Among cell lines derived from salmonid tissues RTL W_1 derived from rainbow trout liver and RTG_2 derived from rainbow trout gonad were highly sensitive to aflatoxin B_1 treatment. In these cell lines there was inhibition of DNA synthesis and neoplastic transformation whereas cell line derived from chinook salmon embryo CHSE-214 was unresponsive (Bechtel and Lee, 1994). Chavez *et al.* (1994) fed Tilapia with 7 different levels of aflatoxin viz. 0, 0.94, 1.88, 0.375, 0.752, 1.5 and 3.0 mg/kg of feed for 25 days. Fishes were subsequently fed a normal ration and maintained for 50 days. Fish samples from each treatment were taken on days 15, 26, 54 and 75 and preserved for histopathological studies. There was reduction in growth rate and feed consumption directly proportional to aflatoxin levels. Severe changes were observed in the liver. Fatty infiltration of hepatocytes, nuclear and cellular hypertrophy, nuclear atrophy, increase in the number

of nucleoli, cellular infiltration, cellular basophilia and necrosis were some of the histological findings. Kidneys showed congestion, shrinking of glomeruli and melanosis.

Horsberg *et al.* (1994) described tissue distribution of 3 H labelled aflatoxin B_1 after oral and intravascular administration in rainbow trout and Nile tilapia. They detected radioactivity by liquid scintillation and whole body autoradiography. In the case of rainbow trout highest quantity of radioactivity was observed in the bile, liver, kidney, pyloric caeca, uveal tract of the eye and olfactory rosette. A fraction of radioactivity was not extractable with either polar or non polar solvents. The high concentration seen in the anterior and trunk kidney suggested possible effect on the excretory and immune functions. In tilapia blood concentration of radioactivity was comparable to that of the rainbow trout but the liver showed only about 1/5 to 1/10 of radioactivity of that in rainbow trout.

Alpha foeto protein was detected in the serum of rainbow trout with hepatocellular carcinoma induced by micro injection of aflatoxin B_1 at the embryonic stage. The alphafoeto protein cross reacted immunologically with human alphafoeto proteins. These were absent in normal rainbow trout (Sarcione and Black, 1994).

2.3 EFFECT OF AFLATOXIN ON THE IMMUNE SYSTEM

2.3.1 Duck

Brown and Abrams, (1965) observed that the aflatoxin was immunosuppressive and they demonstrated that in the ducklings aflatoxin produced hypoproteinemia and low globulin levels. They observed that ducks became highly susceptable to Salmonella infection. Okuda *et al.* (1988) and Uchida *et al.* (1988) observed that aflatoxin B_1 increased replication of duck hepatitis B virus and enhanced the expression of virus in infected ducks.

Balakrishnan (1992) reported that ducks fed aflatoxin B_1 had T-cell lymphopenia and reduced skin sensitivity reaction to 2,4, dinitrochlorobenzene and PHA-M. The ducks showed reduced phagocytic index. Antibody response to Ranikhet disease F vaccine was reduced compared to the control ducks. There was also necrosis and degeneration of the thymus, spleen, and the bursa of Fabricius.

2.3.2 Fish

Arkoosh and Kaattari (1987) noticed that the antibody production in rainbow trout was reduced when they were fed with aflatoxin B_1.

2.3.3 Other Animals

2.3.3.1 Humoral Immunity

In rats aflatoxin bound to RNA polymerase and affected immunoglobulin synthesis (Clifford and Rees, 1967). When mice were given aflatoxin before or during vaccination there was marked depression in the formation of antibody. When aflatoxin was fed after vaccination there was only moderate depression of antibody secretion (Galikeev *et al.* 1968). It was found that aflatoxin inhibited RNA polymerase. This resulted in the inhibition of immunoglobulin and hence immunosuppression (Lafarge and Frayssinet, 1970).

Consumption of aflatoxin at the level of 0.25 to 0.5 ppm during or after immunization against *Pasteurella multocida* resulted in impaired resistance in 20 to 67% of birds (Pier and Heddleston, 1970).

Thaxton and Hamilton (1971) observed impairment of reticuloendothelial system in aflatoxicosis of chicken which resulted in decreased ability of chicken to form haemagglutinins.

When turkeys were given 0.5 ppm aflatoxin containing feed before, during and after vaccination with New castle disease virus vaccine there was no difference in antibody titre but a lag in interferon production was noticed during the first 24 hours in aflatoxin fed birds (Pier *et al.* 1971).

Pier *et al.* (1972) noticed that turkey vaccinated against Fowl cholera had not gained sufficient resistance to *Pasteurella multocida* infection when they were on diet containing aflatoxins. Density gradient estimation of serum fractions did not reveal any change in the immunoglobulin fractions. They could overcome the defect by giving either normal or immune serum to the bird prior to challenge.

The degree of immunosuppression was found to be directly proportional to the dietary concentration of aflatoxin in the chicken. Feeding of graded levels of aflatoxin from hatching resulted in depression of haemagglutinin titre at 3, 6, and 9 days after injection of sheep RBC (Edds *et al.* 1973).

Tung *et al.* (1975) reported decreased serum IgG levels in chickens fed 2.5 microgram aflatoxin B_1/g of diet.

Paul *et al.* (1977) stated that *in vitro* response of bovine B lymphocytes to pokweed mitogen was inhibited when aflatoxin was fed.

When chicks were fed aflatoxin at the rate of 2.5 microgram/gram feed from hatching to four weeks of age there was significant reduction in the immunoglobulin fractions of the serum. The reduction was confined to

IgG and IgA fractions mainly and not to IgM fractions. Since IgA and IgG were produced late in an immune response and IgM bearing cells were already present; the aflatoxin feeding did not affect the early production of IgM bearing cells. The toxin affected the generation of late antibody producing cells (Giambrone *et al.* 1978).

In pigs, aflatoxin increased the susceptibility to Salmonella infection and antibody response was lowered (Miller *et al.* 1978).

Depressed antibody titre against Ranikhet disease vaccination was noticed in chickens fed with 0.2 ppm aflatoxin over a period of 21 days (Mohiuddin *et al.* 1981).

Aflatoxin in broiler chicken caused severe hypoproteinemia, lymphocytopenia, decreased relative weight of the bursa of Fabricius and depression of complement activity. All these indicated immunosuppression (Campbell *et al.* 1983). A dose and time related response of immunological functions were observed in mice by Reddy *et al.* (1983). These mice were fed 0, 30, 145 and 700 microgram of aflatoxin per kg body weight. A significant decrease in the humoral immunity was observed.

Stewart *et al.* (1985) noticed decreased complement activity in chicks given dietary aflatoxins. Marshaly *et al.* (1986) observed that protein

synthesis was decreased due to poor RNA synthesis in chicks given dietary aflatoxin.

Aflatoxins affected acquired resistence and immunity in different species of animals. Both cell-mediated and humoral immunity were adversely affected. Antibody response some times showed depression and this was dose dependent and only IgA and IgG showed diminishing trends whereas IgM concentration remained stable. Reduction in the size of the bursa of *Fabricius* was also observed (Pier, 1986).

Gopalakrishnan Nair (1986) demonstrated reduction in humoral antibody response in pigs during experimental aflatoxicosis. Panangala *et al.* (1986) reported significant reduction in complement titres in pigs when they were fed aflatoxin at the rate of 560 micro gram/kg of body weight. Balaraman and Arora (1987) reported that aflatoxin at 50 to 250 ppb levels in the colostrum significantly affected passive immunity status of neonatal calves. Continuous presence of aflatoxin B_1 in the diet had an immuno suppressive effect in chicken. Its withdrawal did not restore the immune system activity to the full extent (Kadian *et al.* 1988).

When chicks were fed a diet containing 0.5 ppm or more of aflatoxin the serum protein levels were proportionately decreased. The immune response against Ranikhet disease virus was decreased (Rao *et al.*

1988). Sharma (1988) noticed that the protection level against fowl pox virus vaccinated chicks was lowered when they were fed 0.25 microgram aflatoxin from the 2nd day of hatching onwards.

Padmanabhan (1989) expressed the view that the immunosuppressive effect of aflatoxin was antigen specific. The aflatoxin produced inhibition of RNA polymerase, increased the lysosomal activity, inhibited both Reticulo-endothelial system and the specific immune system.

Virdi *et al.* (1989) stated that antibody response to sheep RBC was decreased in chicks fed with aflatoxin B_1.

Rao *et al.* (1990). Reported that in Japanese Quail (*Coturnix coturnix Japanica*) aflatoxin caused depression of lymphocytes in the bursa of Fabricius.

Pier (1992) stated that in many animal species aflatoxins affected the immune system and pointed out that chronic aflatoxicosis often caused increased susceptibility to infection and poor response to vaccination.

Potchinsky and Bloom (1993) observed that aflatoxin B_1 induced genotoxicity and cytotoxicity. It induced six to eight fold increase in sister chromatid exchanges in B cells of the chick embryo. AFB_1 reduced

progression of B cells and also the mitotic index of B lymphocytes. Aflatoxin had selective genotoxcity towards B lymphocytes.

Raisuddin *et al.* (1994) and Cusumano *et al.* (1995) reported that aflatoxin caused severe immunotoxicity in rats which resulted in poor immune response.

2.3.3.2 Cell-Mediated Immune Response

Pier *et al.* (1972) observed thymic involution and depression of delayed hyper sensitivity in turkeys raised on a diet containing 0.5 ppm AFB_1. Michael *et al.* (1973) induced aflatoxicosis in chicken. They found that the birds had decreased ability to process antigens which was attributed to impaired reticulo endothelial system. Giambrone *et al.* (1978) fed chicken with a diet containing 2.5 microgram of aflatoxin/g diet from hatching upto 84 weeks. These chicks were deficient in cell-mediated immunity as evidenced by poor graft versus host reaction.

Chang and Hamilton (1979) reported that in chicken fed with a diet containing 2.5 microgram AFB_1 resulted in reduced chemotaxis and poor phagocytic activity by hetrophils and monocytes.

When mice were exposed to 30, 145 and 700 microgram/kg body weight of AFB_1 there was dose dependent suppression of lymphopoiesis

(Reddy *et al.* 1983). In steers there was depression of delayed hypersensitivity reaction when they consumed aflatoxin contaminated corn. The steers consumed about 800 microgram of aflatoxin/g of feed (Richard *et al.* 1983).

Bovine lymphocytes were treated with aflatoxin in culture. There was inhibition of blastogenesis. T-lymphocyte functions were depressed. Killer cells, helper cell activity and other immunological activities were reduced (Bodine *et al.* 1984).

In Guinea pigs which received aflatoxin at the rate of 0.06 mg/kg body weight daily for three weeks there was a reduction in the number of T-lymphocytes (McLoughlin *et al.* 1984).

Vishalakshan *et al.* (1984) studied the effect of aflatoxin on ANAE positive lymphocytes in pigs and reported that total lymphocyte count was depressed. When aflatoxin was fed to kids the cell-mediated immune response was decreased (Sinha and Arora, 1985). They stated that aflatoxin suppressed *invitro* response to phytomitogen by bovine and caprine lymphocytes.

Crude aflatoxin had adverse effect on the immune response of turkeys and broilers. In turkeys a low dose of 20 ppb of aflatoxin

suppressed the cell-mediated immunity whereas in broiler chicken the suppression of cell-mediated immunity was observed at higher dose level of 200 ppb of aflatoxin (Giambrone *et al.* 1985b).

Giambrone *et al.* (1985a) also studied the effect of purified aflatoxin on birds and found that both aflatoxin B_1 and aflatoxin B_2 suppressed the cell-mediated immunity.

Gopalakrishnan Nair (1986) reported that single dose of aflatoxin reduced the complement activity and bacteriostatic activity for 16 hours in cattle. However, multiple dose affected only bacteriostatic activity and this perisisted for two weeks.

Aflatoxins had more pronounced effect on the cell-mediated immunity and complement system than humoral immunity in poultry and cattle (Pier, 1986).

In chicken aflatoxin caused marked depression of the phagocytic activity by heterophils and Kuppfer cells (Mohiuddin *et al.* 1986).

Reddy *et al.* (1987) examined the immune system of CD-1 mice exposed to aflatoxin B_1 and reported that synthesis of DNA was decreased in lymphocytes. There was poor response to T dependent antigens.

Singh *et al.* (1987) observed 40% reduction in phagocytic index of rabbit phagocytes when rabbits were exposed to aflatoxin.

Harvey *et al.* (1988) stated that in pigs which received different levels of aflatoxin, there was depletion of lymphocytes in the thymus and the liver showed periportal accumulation of lymphocytes.

Kadian *et al.* (1988) noted reduced phagocytic activity in chicken due to immunosuppressive effect of aflatoxin.

Rao *et al.* (1988) also observed reduced cell-mediated immune response in chicks in experimental aflatoxicosis as measured by 'T' lymphocyte count, cutaneous reaction to 2, 4 dinitro chlorobenzene and graft versus host reaction.

Ghosh *et al.* (1990) opined that aflatoxin decreased the number of ANAE positive cells in chicken.

In chick embryos aflatoxin increased the number of lymphocytes and monocytes. The peritoneal lavage yielded less number of cells. The macrophages were less efficient in adhering and phagocytosing foreign particulate materials (Neldon Ortiz and Qureshi, 1992).

Pier (1992) was of the view that in many animal species chronic aflatoxicosis affected the immune system. Suppression of the cell-mediated immunity, reduced phogocytosis, depression in complement and interferon production and poor response to vaccination were the main effects.

300 to 600 microgram of aflatoxin/kg body weight suppressed the cell-mediated immunity in rats. Continuous low level of aflatoxin enhanced susceptibility to infection and tumorogenesis. Bovine lymphocytes also responded to aflatoxin in a similar way (Raisuddin *et al.* 1993; Sharma, 1993).

Jakab *et al.* (1994) reported that in rats both inhalation of aflatoxin B_1 aerosol and oral administration of AFB_1 produced suppression of alveolar macrophage activity and peritoneal macrophage activities.

Van-Heugten *et al.* (1994) studied the effect of aflatoxin B_1 at different levels on pigs. It was observed that increasing concentration of aflatoxin proportionately affected skin response to PHA.

Aflatoxin was found to cause severe immunotoxicity in rats. Aflatoxin and its metabolites inhibited phagocytic activity of Kuppfer cells and intracellular killing (Raisuddin *et al.* 1994; Cusumano *et al.* 1995).

MATERIALS & METHODS

Chapter 3

MATERIALS AND METHODS

3.1 STUDIES ON THE DUCK

Sixty ducklings straight run four weeks old were purchased from a duck farmer of Niranam of Alapuzha district. These ducklings were weighed and randomly grouped into group I and group II. Group I consisted of forty eight ducklings and Group II consisted of twenty four ducks. The group I formed treatment group and the group II formed the control group. The birds in Group I were further randomly sub-grouped into group IA, Group IB, Group IC and Group ID. Each subgroup had twelve ducks each.

All the ducks were fed with ration consisting of rice and wheat grains together with dry fish and vitamins. The feed was tested and found negative for aflatoxin and ochratoxin

Aflatoxin B_1 was obtained from M/s.SIGMA Chemicals, USA. Aflatoxin B_1 (500 μg) was dissolved in 20 ml of Dimethyl sulphoxide (DMSO). 10 ml of the solution contained 250 micrograms of aflatoxin B_1 and 1 ml contained 25 micrograms of aflatoxin. Each bird in the experimental group was weighed and received aflatoxin B_1 at the rate of 75 microgram/kg body weight on alternate days till their sacrifice (Table 3.1).

Table 3.1: Feeding schedule of aflatoxin B_1 in DMSO (1 ml DMSO contained 25 μg) to ducks

Weight of the ducks (g)	DMSO (ml)	AFB_1 (micrograms)
208	0.6	15
210	0.63	17.75
220	0.66	16.50
230	0.69	17.25
240	0.72	18.00
250	0.75	18.75
260	0.78	19.50
270	0.81	20.25
280	0.84	21.00
290	0.87	21.75
300	0.90	22.50
310	0.93	23.25
320	0.96	24.00
330	0.99	24.75
340	1.02	25.50
350	1.05	26.25
360	1.08	27.00
370	1.11	27.75
380	1.14	28.50
390	1.17	29.25
400	1.20	30.00

Required amount of aflatoxin solution was diluted in DMSO and taken in 5 ml syringe. A 1mm gauge rubber tubing was attached to the nozzle of the syringe. The duck was held and the beak was opened. The tube was introduced into the oesophagus and the solution was ejected into the oesophagus. Birds in the control group (Group II) received the solvent without toxin till the end of the experiment.

3.1.1 Measurement of Weight

Birds from both the groups were weighed each fortnight and weights were recorded.

3.1.2 Sacrifice and Collection of Samples

The birds of Group IA were sacrificed by decapitation at the end of the first fortnight. Samples of liver, kidney, bursa of Fabricius, spleen, thymus, heart and brain were collected for histopathology and fixed in 10% buffered formal saline. The birds of Group IB, Group IC and Group ID were scarified at the end of the 2^{nd}, 3^{rd} and 4^{th} fortnight respectively.

Haematological studies in ducks were conducted as per the method of Valsala (1968). Pieces of liver, thymus, bursa of Fabricius and spleen were collected in 3% buffered glutaraldehyde for electron microscopy. Blood and serum were collected from each bird for estimation of the haematological

parameters, total serum protein, albumin, globulin, albumin globulin ratio and electrophoretic separation of serum proteins.

3.1.3 Demonstration of ANAE Activity

Alpha Naphthyl Acetate Estrase staining as per the methods of Giorno and Baverly (1980) and Valsala *et al.* (1981) were carried out in the peripheral blood smears of ducks.

3.2 STUDIES IN FISH

Sixty fresh water fishes (*Labeo rohita*) weighing 200-300 g were collected at random from the ponds of the Fisheries College, Panangadu, Kerala agricultural University. They were maintained in fibre glass tanks. Water was changed on alternate days. They were fed on a pelleted diet of following composition

Soyabean oil cake	450 g
Dry fish meal	250 g
Gingely oil cake	100 g
Coconut oil cake	100 g
Wheat flour	100 g
Cobadex forte (Glaxo)	10 capsules
Vitamin C	100 mg
Mineral mixture	50 mg

The oil cakes, fish meal and wheat flour were finely ground and mixed. They were steamed, cooked and cooled to 50°C. To this mixture vitamin B complex (Cobadex Glaxo), vitamin C and mineral mixture were added along with 50 ml of 0.5 per cent gelatin solution and pellets were made in a hand press and dried at 45°C. The feed was tested and found free of aflatoxin and ochratoxin.

3.2.1 Experimental Feed

The experimental feed contained all the ingredients of normal maintenance feed and aflatoxin B_1 was added to the feed at the rate of 0.4 mg/kg of feed. Aflatoxin B_1 was dissolved in 10 ml of Dimethyl Sulphoxide (DMSO); added to the feed mixture and was uniformly mixed before pellatization.

The fishes were randomly grouped into GA and GB. GA consisted of forty eight fishes and GB consisted of 12 fishes. GA was designated as the experimental group and received aflatoxin incorporated feed at the rate of 2 per cent of their body weight. GB received the maintenance feed and formed the control group of the experiment. Initially all fishes in both the groups were weighed. The fishes in group GA were randomly subdivided into GA-I, GA-II, GA-III and GA-IV. They were exposed to the toxin containing feed two weeks, four weeks, six weeks and eight weeks respectively. After the

termination of the experiment the fishes were bled to collect blood and serum. Liver, kidney, spleen, thymus, heart and brain samples were collected for histopathology. Liver, kidney, thymus and spleen were collected for electronmicroscopy. The samples were fixed in respective fixatives. The fishes of Group GB were bled only at the end of the 8th week and samples of liver, spleen, thymus, heart and brain were collected from them for histopathology and ultrastructural studies.

Fish in each tank was weighed and body weight was recorded. Total weight of fishes in each tank was calculated and 2% of that weight was estimated. Each day morning feed was weighed equal to the 2% body weight of fishes in tank and tied into small bundles in nylon mesh and suspended into the water using a string. Next day morning the bundle was examined for remains of feed (Table 3.2).

3.2.2 Blood Collection in Fish

The fish was narcotized with intraperitioneal injection of 0.5 to 1.5 ml of 2% xylocane. The narcotized fish was placed on a table with wet cloth covering the fish. The needle of the graduated syringe (which was heparinized) was introduced into the heart from the mid-central portion just posterior to the operculum. Gradually blood was drawn into the syringe and blood was transferred to vials for appropriate tests.

Table 3.2

Feeding schedule of 400 ppb aflatoxin B_1 containing feed to experimental group of fishes

Weight of fish (g)	Feed given (g)
100	2
110	2.2
120	2.4
130	2.6
140	2.8
150	3.0
160	3.2
170	3.4
180	3.6
190	3.8
200	4.0
210	4.0
220	4.4
230	4.6
240	4.8
250	5.0
260	5.2
270	5.4
280	5.6
290	5.8
300	6.0
310	6.2
320	6.4
330	6.6
340	6.8

350	7.0
360	7.2
370	7.4
380	7.6
390	7.8
400	8.0
410	8.2
420	8.4
430	8.6
440	8.8
450	9.0
460	9.2
470	9.4
480	9.6
490	9.8
500	10.0
510	10.2
520	10.4
530	10.6
540	10.8
550	11.0
560	11.2
570	11.4
580	11.6
590	11.8
600	12.0

3.2.3 Anticoagulants

25000 I.U of Heparin sodium (Himedia) was dissolved in 1 ml of distilled water. One to two drops of the heparin solution was added to 10 ml test tubes and spread over the sides by rotation. The tubes were then dried in a desicator. These tubes were used for collection of the blood.

Microhaematocrit method was used to estimate packed cell volume. Hayem's fluid was used to count erythrocytes in a Neubauer counting chamber (Schaperclaus, 1986).

3.2.4 Differential cell count

Panoptic method of Pappenheim employing combined May-Grunwald-Giemesa staining was used for blood smears of fish (Schapercllaus 1986).

3.3 SERUM PROTEIN PATTERN

3.3.1 Estimation of Serum Proteins

Total proteins in duck and fish sera were estimated by the Biuret method (Gornall *et al* 1949).

3.3.2 Estimation of Albumin

Albumin was estimated by the Bromocresol green method. Albumin reacts with bromocresol green solution at pH 4.1 to form a green colour derivative. This colour was measured colorimetrically at 620 nm.

3.3.3 Serum Electrophoresis

Polyacrylamide gel electrophoresis was carried out as per the method of Davis (1964).

3.4 LEUCOCYTE MIGRATION INHIBITION TEST

Leucocytes isolated from the peripheral blood were subjected to leucocyte migration inhibition test as per the method of Indrani and Agarwal (1980).

3.4.1 Separation of Leucocytes from the Peripheral Blood

Leucocytes were separated from whole blood by gradient centrifugation technique of Blaxhall (1985).

The leucocytes isolated from the blood were suspended in RPMI 1640 containing Heparin sodium and antibiotics as described earlier. They were packed into several capillary tubes and one end of the capillary was sealed with clay. The cells were spun down at 400 g for 5 minutes in a refrigerated centrifuge. The capillary tubes were then cut at the top of the leucocyte

layer and short cut portion of the tubes packed with cells were placed each in a migration chamber (M/s.Laxbro Ltd.). The migration chambers were filled with fresh RPMI 1640 solution and chambers were covered with sterile cover glasses the sides of which were sealed with vaseline. Two wells were used for each assay. One of the chamber contained RPMI 1640 without antigen. This was the control while the other chamber contained RPMI 1640 with a few micrograms of antigen which was used to immunize the bird or fish.

The plates were incubated at room temperature overnight and the migration of leucocytes out of the capillary tubes was observed:

3.5 PHAGOCYTOSIS

3.5.1 Assay of Phagocytosis in Ducks

The method described by Glick *et al.* (1964) was adopted.

3.5.2 Assay of Phagocytosis in Fish

3.5.2.1 Isolation of Peritoneal Macrophages

The method described by Secombes (1990) was followed.

The final cell pellet was resuspended in fresh chilled RPMI 1640 with 2% foetal calf serum ml^{-1} 100 I.U. ml^{-1}, pencillin, 100 microgram ml^{-1} streptomycin and 100 I.U. ml^{-1} Heparin. The cell viability was assessed

using 1% trypan blue dye exclusion test. The cell concentration was adjusted to $2x10^6$ ml^{-1}.

The cell suspensions prepared from the blood/peritoneal exudate were made to adhere on sterile cover slips. 500 microlitre cell suspension containing $2x10^6$ cells was put on cover slips and incubated in a chamber containing 5% carbon dioxide for one hour. The cover slips were washed with fresh RPMI 1640 containing antibiotics and Heparin to remove non adherent cells. Suspension of the killed yeast cells at a ratio of 1:10 was added to the cover slips and incubated at room temperature for one hour in a 5% carbon dioxide chamber. Following incubation the cover slips were washed with HBSS and rapidly air dried, fixed with methanol and stained with May Grunwald Giemsa stain and cover slips were mounted on slides with DPX and examined under oil immersion objective of the microscope. Inserted yeast particles in two hundred cells were counted. Phagocytic index (PI) was calculated and counted.

$$PI = \frac{\text{Number of yeast particles phagocyted}}{\text{Number of cells counted}} \times 100$$

3.5.2.2 Preparation of Yeast Cell Suspension

One gram of commercial yeast (*Saccharomyces cereviceae*) was ground in a mortar and pestle to a fine powder. This was autoclaved and

suspended to 25 ml HBSS under sterile precautions. Suspension was adjusted to $10x10^6$ cell microlitre.

3.6 SKIN SENSITIVITY TO PHYTOHAEMAGGLUTININ

The test as described by Rajan *et al.* 1986 was adopted. Skin thickness was measured at 24, 48 and 72 hours. In the case of fishes skin thickness at the caudal peduncle was measured followed by intradermal injection of 0.1 ml PHA-M solution. Skin thickness was measured after 24, 48 and 72 hours respectively.

3.7 IMMUNISATION OF DUCKS AND FISH

Preparation of sheep erythrocytes (SRBC)

Alsever's solution (This was freshly prepared each time)

NaCl 4.2 g

Citric acid 0.55 g

Glucose 20.50 g

Distilled water 1000 ml.

Each ingredient in the above order was added to 500 ml distilled water in a graduated cylinder and dissolved. The volume of the solution was finally made upto one litre with distilled water. The solution was sterilized with steam for 10 minutes and cooled to 4^0C.

The sheep blood (200 ml) was collected using sterile needle into the bottle containing 1 litre of Alsever's solution. The mixture was centrifuged at 300 g for 10 minutes and the cell pellet was suspended in phosphate buffer saline (pH 7.5). This was again centrifuged and washing in phosphate buffer saline was repeated three times. Finally the cell concentration in phosphate buffered saline was adjusted to 10^6 cells/ml of solution. This suspension was used to immunise the birds and fishes.

One ml of SRBC suspension was homogenised with 1 ml of Freund's complete adjuvant. The homogenate was injected intramuscularly in the thigh at two sites of each duck. This was followed by 1 ml of SRBC injections subcutaneously on the third, sixth, ninth and twelfth day. Blood (10 ml) was collected from each bird on the 15^{th} day and allowed to clot. The serum was separated, inactivated at 55^0C for 30 minutes, cooled and stored under refrigeration in sterile vials.

The procedure followed for fish was the same as that of the duck except that the injections were made intraperitoneally.

3.7.1 Assay of Haemagglutination

Materials and Equipments

Serum collected from immunized birds or fishes

2% v/v SRBC in PBS

Microhaemagglutination trays with wells (Laxbro)

Micropipettes of 50 microlitres

Disposable tips for micropipettes

PBS (50 microlitre) was added in each well of 96 well microtitre plate. 50 microlitre of the test serum (50 micro litre) was added to the first well and mixed well. From this well 50 microlitre of diluted serum was added to the next well and continued to the third well and so on. The dilution of the serum in the first well was 1:2 in the second well 1:4 and so on. In the last well no serum was added. This served as the control well.

SRBC (50 microlitre of 2% suspension) was added to each of the 96 wells in microtitre plate and the plate was gently shaken. The plates were incubated for 2 hours at ambient temperature and left at 4^0C for 12 hours. The reactions were read. The highest dilution of the serum which produced haemagglutionation was recorded.

3.8 ULTRASTRUCTURAL STUDIES

3.8.1 Primary Fixation

One mm sized pieces of liver, thymus, spleen, bursa of Fabricius were collected from anaesthetized ducks and immediately transferred to chilled buffered 3% glutaraldehyde solution (in phosphate buffer pH 7.4). In the case of fishes liver, thymus, anterior kidney and spleen were collected in the same way as in the case of ducks and transferred to chilled 3% buffered glutaraldehyde solution. The vials containing tissue pieces in the fixative were kept at 4^0C till they were trimmed. Each piece of tissue was kept on a wooden plank with a drop of 3% Glutaraldehyde. The tissue was cut into pin head sized pieces and again transferred to chilled 3% buffered Glutaraldehyde and kept at 4^0C for 24 hours.

3.8.2 Washing

Following fixation in primary fixative the tissues were washed in 0.1 M phosphate buffer three times (15 minutes each) and post fixed in 1% Osmium Textroxide for one hour at 4^0C.

3.8.3 Dehydration

Dehydration of tissues were carried out in ascending grades of Analar acetone at 4^0C and embedded in Spurr's resin as per the method described by Spurr (1969).

3.8.4 Ultra thin sections

The entire sectioning process was done in an LKB Ultra microtome. The ultra thin sections prepared were lifted on the matted surface of copper grid, having 100 to 300 mesh size. These grids with the sections were stained with uranyl acetate and lead nitrate stains (Spurr, 1969).

3.9 HISTOPATHOLOGICAL OBSERVATIONS

Tissue sections were prepared (4 to 6 micron) from buffered formalin fixed tissues using paraffin embedding technique. The tissue sections obtained were stained by Harris Haematoxylin and Eosin stain as described by Humason (1967).

RESULTS

Chapter 4

RESULTS: EXPERIMENTAL STUDIES IN THE DUCK

4.1 WEIGHT OF DUCKS

Before the commencement of the experiments, the ducks were weighed, which varied between 200 and 400 g.

Twenty five birds were between 300 and 400 g and 47 were having body weight between 200 g and 250 g. The birds were randomly divided into experimental group (Group I) with 48 birds and control group with 24 birds (Group II). In Group I 27 birds were having body weight between 300 and 400 g and 21 birds weighed between 200 and 250 g. In Group II, three birds were between 300 and 350 g and 21 were between 200 and 250 g. At the beginning of the experiment the control group had a mean weight of 257.143± 35.34 g and the experimental group had a mean body weight of 271.43±835 g.

At the beginning of the second fortnight Group I mean body weight was 494.29±138.65 g and mean body weight of the control group was 464.29±186.236 g.

In the third fortnight experimental group of birds had a mean body weight of 429.166±149.28 g while the mean body weight of the control groups reached 950±327.108 g.

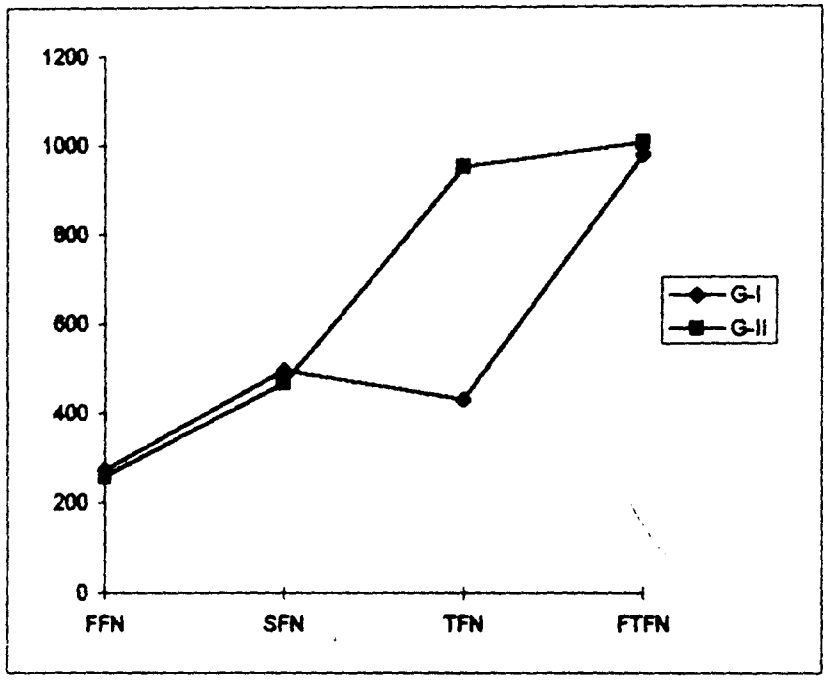
At the end of the fourth fortnight the average body weight of Group I birds reached 978.18±239.730 g. The group II (control) mean body weight was 1005 g±138.4 g. (Table 4.1, Graph 4.1). There was a decrease gain in the body weight of the experimental group of birds.

Table 4.1: Body weights recorded in ducks of Group I and Group II at different intervals

Time interval	1st fortnight g	2nd fortnight g	3rd fortnight g	4th fortnight g
G-I	257.143±35.34	494.29±138	429.16±149.28	978.18±239.73
G-II	271.43±83.5	464.29±186	950.0±327.1	1005±138.4

4.2 CLINICAL SIGNS DUE TO AFLATOXICOSIS

All the ducklings within four days after the administration of aflatoxin, became dull and the feed intake was less. Some of these



FFN	First fortnight
SFN	Second fortnight
TFN	Third fortnight
FTFN	Fourth fortnight

Graph 4.1. Body weight of the experimental group (G-1) and control group (G-II) of ducks.

ducklings developed signs of sinusitis. Mucopurulent nasal discharge was observed in these birds. They were not able to stand and were crawling on their hocks. There was purulent discharge from eyes and corneal opacity developed in these ducks. Skin of these ducks had echymotic haemorrhages. Three of such ducklings died (Fig.1).

4.3 AUTOPSY FINDINGS

Autopsy conducted on dead ducklings and sacrificed ducks revealed significant changes in the liver. Liver was friable, yellowish, mottled and granular. In majority of the cases the liver was enlarged in size. Three ducks revealed haemorrhages in the thymus, and heart. The bursa was reduced in size in most of the ducks (Fig.2 and 3).

4.4 LYMPHOID ORGANS

The thymus and the bursa of Fabricius revealed changes in experimental groups. Bursa revealed reduction in size in four birds. Thymus had petechael haemorrhages in three ducks. The mean weight of the thymus in the experimental group was 1.264±1.18 g and that of the bursa of Fabricius was 0.45±0.22 g. The spleen did not reveal any change. The mean weight of the spleen was 0.64±0.9320 g. There was a reduction in the weight of the bursa of *Fabricius* in the experimental groups of birds. The mean weight of the thymus in the control group was 1.005±0.071 g.

Average weight of the bursa of Fabricius was 0.484±0.2899 g while that of the spleen was 0.475±0.9320 g.

The weight of the Lymphoid organs of the experimental and the control groups are given in Table 4.2.

Table 4.2: Weight of the thymus, bursa and spleen

Group	Thymus g	Bursa of Fabricius g	Spleen g
I	1.264±1.18	0.45±0.22	0.64±0.9320
II	1.005±0.077	0.485±0.2899	0.475±0.219

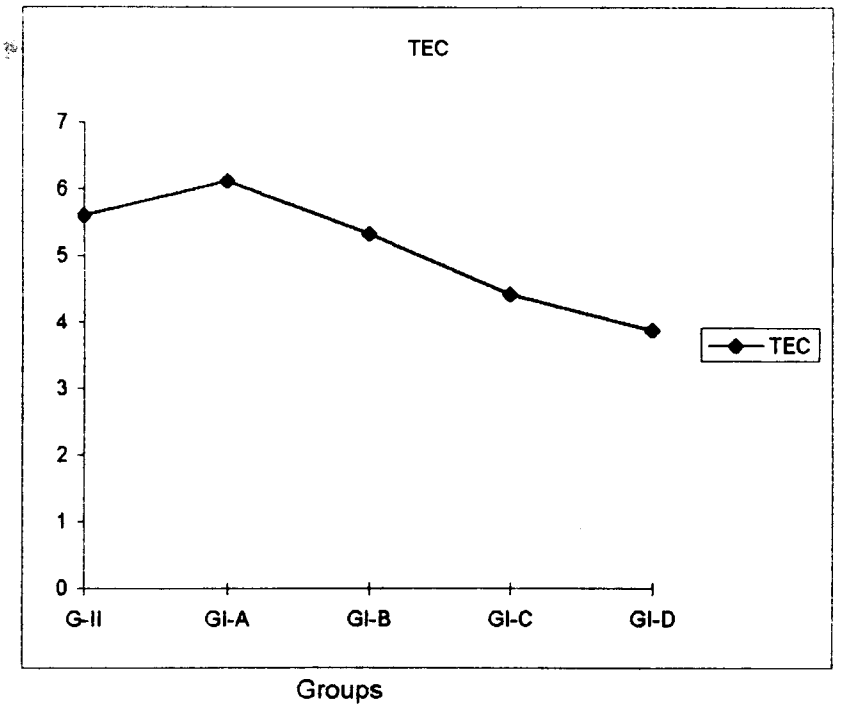
4.5 HAEMATOLOGICAL STUDIES

Mean haematological values for different groups are presented in Table 4.3. Erythrocyte sedimentation rate (ESR) values in Group I varied between 1 mm/hour and 3 mm/hour with overall mean 1.8 mm/h. In the sub-group of GI the mean values of ESR were 2 mm/hour in GI-A, 2 mm/hour in GI-B, 2 mm/hour in GI-C, and 2 mm/hour in GI-D. Packed cell volume (PCV) in Group I varied between 22% and 41%. The mean PCV was 36%. The sub-group means were 32.29, 36.4, 34.33 and 32.5% respectively for GI-A, GI-B, GI-C and GI-D. The mean ESR values did not show variation between aflatoxin treated birds and the birds kept on aflatoxin

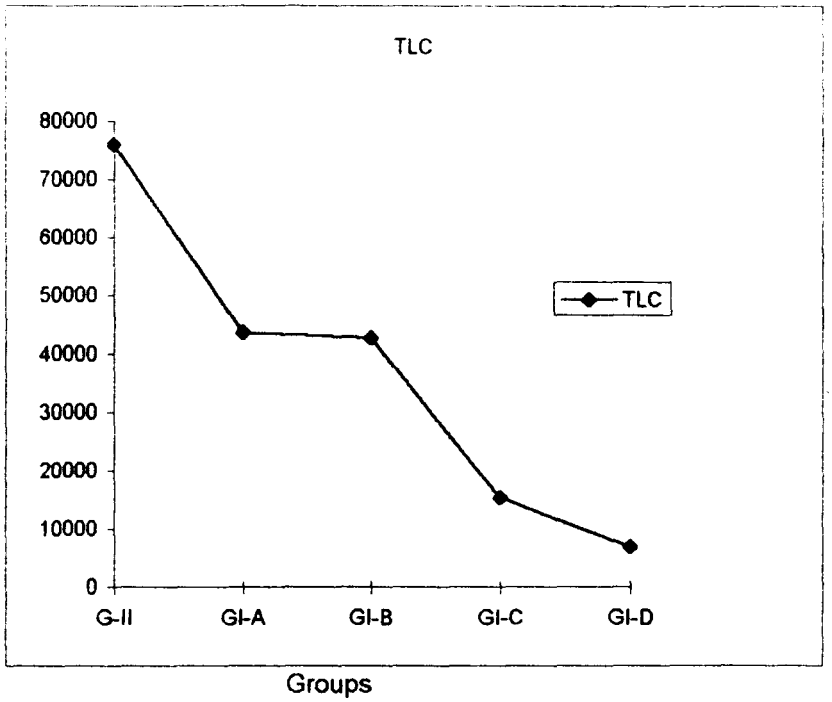
Table 4.3: Mean haematological values in the control and experimental group of ducks

Group	TEC millions/ Cu.mm of blood	TLC Cu-mm/blood	ESR (h)	H_b (g)	PCV (%)	L (%)	H (%)	E (%)	M (%)	B (%)
GII	5.59±1.52	75900±2700	2±0.6	10.50±0.95	40.00±18.13	63.00±18.13	21±8.83	6.50±4.24	6.00±4.24	2.00±1.41
GI-A	6.11±0.71	43644±40390	2±0.8	12.00±0	36.00±	48.75±6.18	39±4.57	2.75±2.2	6.00±2.94	0.50± 0.58
GI-B	5.32±1.60	42619±29588	2±0.4	8.25±	37.00±2.7	53.00±9	33±10.35	2.17±1.94	7.80±3.97	1.17±1.17
GI-C	4.42±0.36	15288±18851	2±0.6	8.00±0.67	35.25±0.95	65.00±16.9	23±12.4	5.33±1.69	2.66±0.47	1±0
GI-D	3.87±0.53	6816±3223.16	2±0.6	7.40±0.54	31.50±5.3	51.33±3.39	39±4.5	1.66±0.47	7±2.16	--±

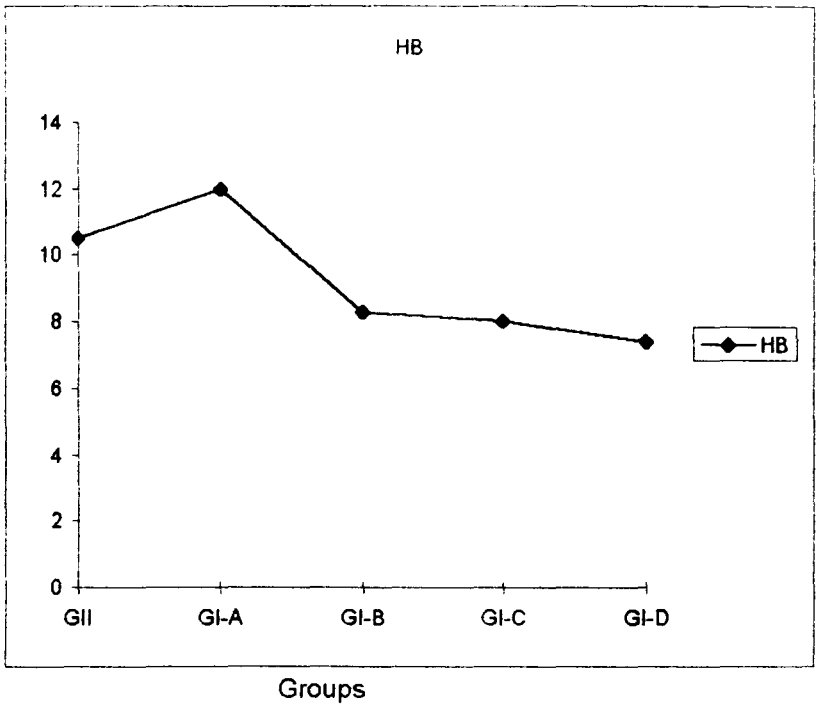
TEC	Total erythrocyte count in millions.cc of blood	L	Lymphocyte count in percentage
TLC	Total leucocyte count/cc of blood	H	Heterophils in percentage
ESR	Erythrocyte sedimentation rate/hour	E	Eosinophils in percentage
H6	Haemoglobin g%	M	Monocytes in percentage
PCV	Packed cell volume %	B	Basophils in percentage



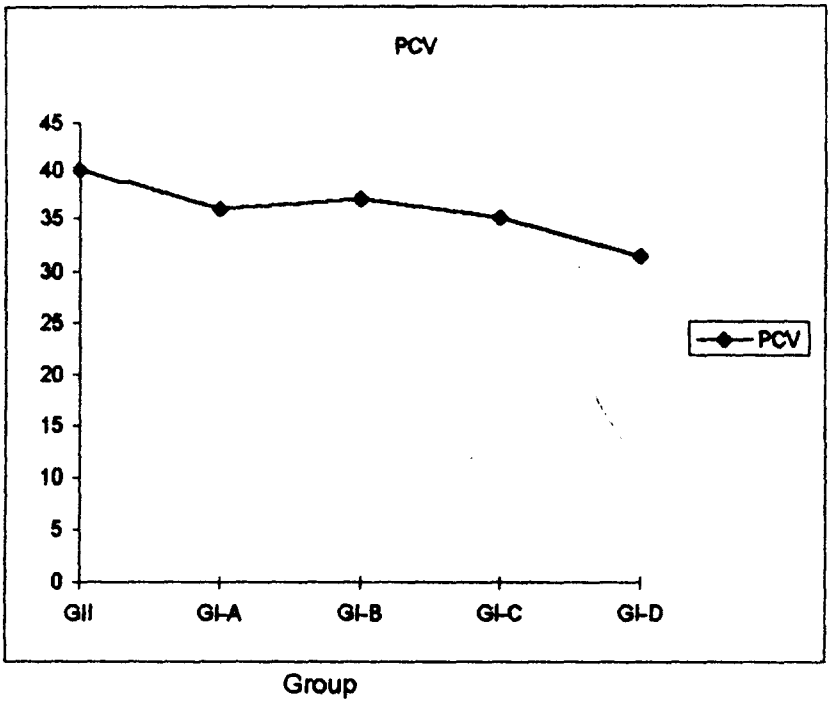
Graph 4.2 Mean total erythrocyte count in the control group (GII) and different experimental groups (G1-A to G1-D) of ducks.



Graph 4.3: Mean total leucocyte counts in the control (GII) and experimental groups (GI-A to GI-D) of ducks.



Graph 4.4 Mean haemoglobin values in the control group and experimental group of ducks



Graph 4.5: Mean packed cell volume of blood in the experimental and control group of ducks

free diet. However, the packed cell volume indicated a decreasing trend with the duration of aflatoxin treatment.

The total erythrocyte count of Group I varied between 6.82 millions/cu.mm and 3.18 millions/cu.mm. the mean values of total erythrocyte count for each sub-group were 6.11±0.71, 5.32±1.60, 4.42±0.36, 3.87±0.53 million/cu.mm, for GI-A, GI-B, GI-C and GI-D respectively. The total erythrocyte count decreased in proportion to the duration of aflatoxin treatment in the experimental group.

The leucocyte count (WBC) for the experimental group had an average of 31291.89±3.2049/cu.mm of blood. The sub-group means were 43664.33±40390; 42619.42±29522.175; 15288.33±18854; 6816.67±3223/ cu.mm of blood for GI-A, GI-B, GI-C and GI-D, respectively. The leucocyte count significantly decreased with the duration of aflatoxin treatment in the experimental group of birds.

The mean differential leucocyte count for Group I were Heterophils 28.375% Lymphocytes 50.875% Eosinophils 2.625%, Basophils 1%, Monocytes 6.8%. The differential leucocyte count did not reveal any significant alteration between the aflatoxin treated ducks and the non-treated ducks.

ESR values in the control group ranged from 1 to 3 mm/h and the mean PCV values ranged from 30% to 48%. Total erythrocyte count varied between 4.83 millions to 7.10 millions with mean 5.59±1.52 million/cu.mm. Total WBC counts ranged from 73200 to 78600 with mean of 75900±2.5/cu.mm. The mean values of differential blood cell count was Hetrophils 27.5% Lymphocytes 49%, Eosinophils 8%, Basophils 3%, and Monocytes 9.5%. The mean ESR value and the mean differential leucocyte count did not reveal any significant difference between the control and experimental groups. The packed cell volume, the total erythrocyte count, and the leucocyte counts were higher in the control group of ducks compared to the experimental group of ducks.

The mean erythrocyte values were found decreasing as the exposure time to aflatoxin increased. It was found that there was significant variation between the treatment groups and the control (Table 4.4). The changes in the total erythrocyte count, total leucocyte count, packed cell volume and haemoglobin values are presented in Graphs 4,2, 4,3, 4,4 and 4,5.

Table 4.4: Analysis of variance for total erythrocyte count in the experimental and control group of ducks

Source	Degree of freedom	Sum of squares (SS)	Mean sum of squares (MSS)	F
Treatment	5-1	38.34	9.59	6.03
Error	60-5	87.42	1.59	
Total	60-1	125.76		

The leucocyte counts were also found to decrease significantly as the exposure time to aflatoxin increased (Table 4.5). The lowest mean values were obtained in GI-C and GI-D groups of the experiment. The means were 15288.33±1885 mm^2 of blood and 6816±3223 mm^2 of blood respectively.

Table 4.5: Analysis of variance for total leucocyte count in the experimental and control group of ducks

Source	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MSS)	F
Treatment	5-1	8151.99	2038	10.43
Error	60-5	4884.17	195.37	
Total	60-1	13036.16		

4.6 SERUM PROTEIN PATTERN OF DUCKS

The serum proteins of the control and experimental groups were estimated. Initially the total protein of different groups were estimated followed by albumin, total globulins and A/G ratio. Finally the duck sera were subjected to electrophoresis to elucidate the pattern of different fractions.

Mean total protein of different groups as well as different fractions are represented graphically in graphs 4.6 to 4.10 and in Table 4.6. In the control group, the total protein value ranged from 4.06 g% to 7.21 g% 100 ml. The mean value was 6.03 ±1.31 g%. In the experimental group the first group (GI-A) had a mean value of 6.96±1.23 g%. The value ranged between 5.33 and 9.48 g%. GI-B had a mean total protein value of 7.13±1.15 g%. The values ranged from 5.74 to 7.4 g%. In GI-C mean protein value was 6.55±1.03 g%. The range was between 5.33 to 8.5 g%. In GI-D which was exposed to aflatoxin for 60 days the mean protein value was 6.75±0.91 to 8.55 g%. The total protein values revealed less variations between groups.

Table 4.6: Serum protein values in the experimental and control group of ducks

Group	Total protein g%	Albumin g%	Globulin g%	A/G Ratio
G-II	6.03±1.31	2.56±0.45	2.47±0.054	1.100±0.2
G-IA	6.96±1.23	2.60±0.56	4.29±1.53	0.730±0.38
GI-B	7.13±1.15	2.42±0.24	4.76±1.33	0.550±0.16
GI-C	6.50±1.03	2.20±0.21	3.90±1.26	0.650±0.36
GI-D	6.75±1.31	2.20±0.21	4.56±0.96	0.504±0.14

Table 4.7: Analysis of variance for total serum protein values of the experimental and control group of ducks

Source	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MSS)	F
Treatment	5-1	8.83	2.2	
Error	60-5	70.55	1.28	1.712
Total	60-1	79.38		

The serum albumin values of different experimental groups and control group revealed considerable variation. This is apparent from the graph 4.6.

The serum albumin values of the control group ranged between 1.95 g% and 3.2 g%. The mean value was 2.56±0.45 g%.

In the experimental groups GI-A and GI-B the albumin values were similar to the control group. In GI-A the albumin values varied from 1.97 to 3.74 g%. The mean value was 2.68±0.56 g%. In GI-B the values ranged between 2.2 and 2.84 g% with mean 2.42±0.24 g%.

In GI-C and GI-D there was reduction in albumin value. Both groups had same mean value 2.2±0.92 (2.21 g% for GI-C and 2.2±0.32 g% for GI-D). In GI-C the values ranged between 2 and 2.6 g%. In GI-D the values varied from 2.1 to 2.5 g%. There was significant reduction of albumin values in the experimental group of birds (Table 4.8).

Table 4.8: Analysis of variance for serum albumin values of experimental and control group of ducks

Source	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MSS)	F
Treatment	5-1	2.19	0.547	3.8194
Error	60-5	7.91	0.144	
Total	60-1	10.1		

The serum globulins revealed high variations among the experimental and control group. The mean serum globulin values of the

control and the experimental groups are represented in the table 4.6 and graph 4.9. The mean serum globulin value of the control group of ducks was 2.74±0.54 g%. The values of this group ranged from 1.5 to 3.2 g%.

In the experimental group GI-A the serum globulin values ranged from 2.5 g% to 5.9 g% with mean of 4.29 ± 1.53 g%. In the second experimental group GI-B the serum globulin value varied between 3.12 g% and 6.23 g% with mean 4.76 ± 1.33 g%. GI-C had a mean globulin value of 3.9±1.269 g% and the range of values were between 1.66 and 6.1 g%. GI-D group had mean serum globulin value of 4.56±0.96 g%. The values ranged from 3.5 to 6.1 g%. It was found that there was a relative increase in globulin levels of experimental ducks as the albumin values decreased. There was significant variation in the level of globulin of the experimental group (Table 4.9).

Table 4.9: Analysis of variance for serum globulin values of the experimental and control group of ducks

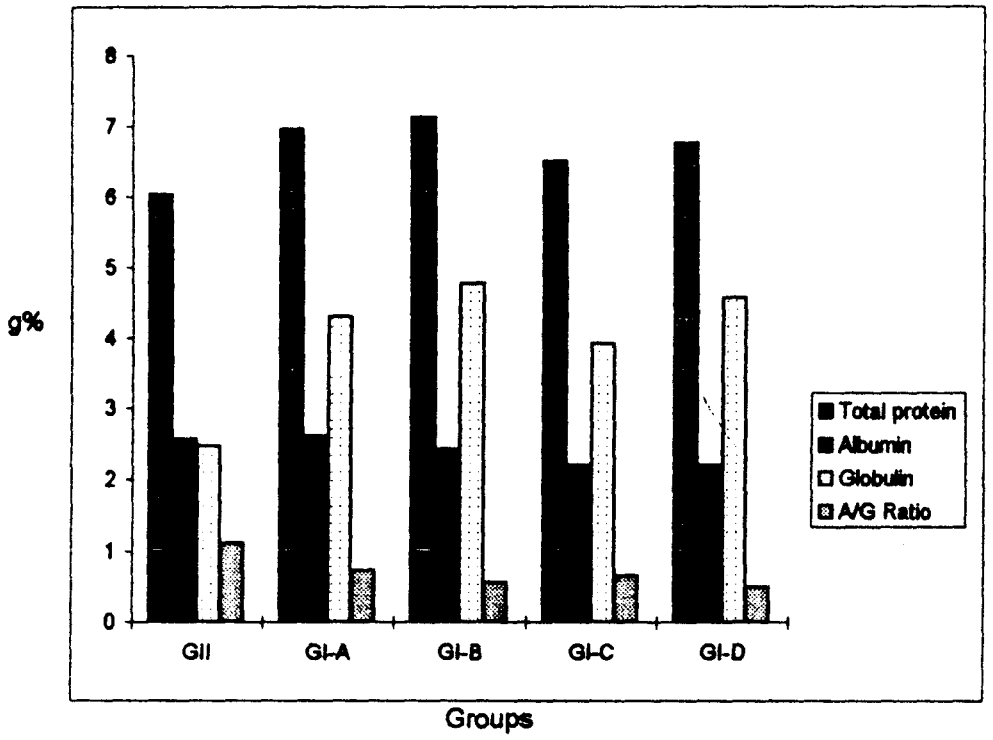
Source	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MSS)	F
Treatment	5-1	39.77	9.99	7.24
Error	60-5	76.07	1.38	
Total	60-1	116.04		

4.6.1 Albumin : Globulin Ratio (A/G Ratio)

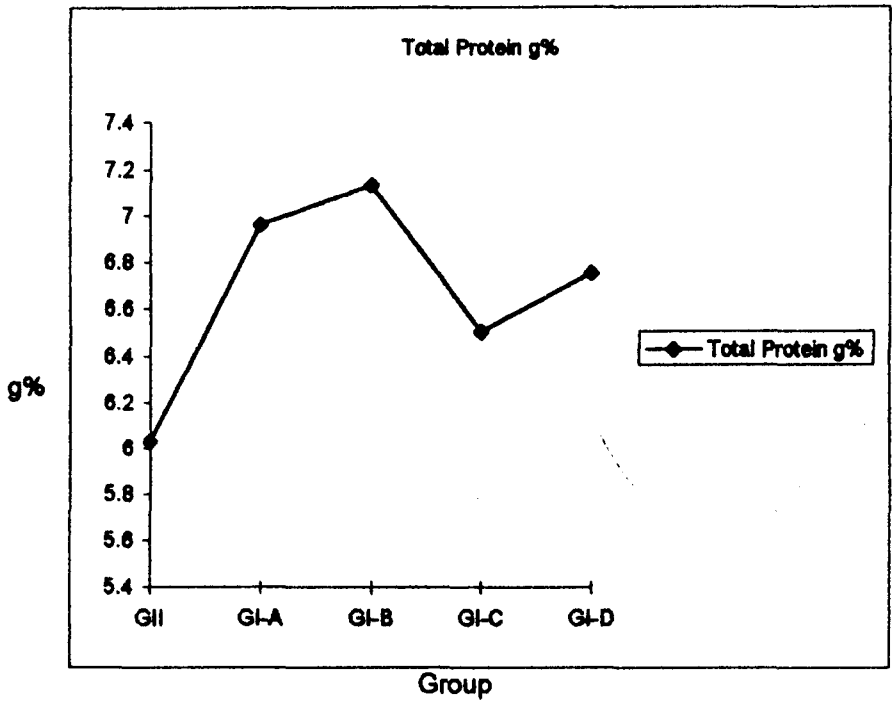
The A:G ratio showed considerable difference between the groups. This is depicted in the graph 4.10. In the control group A/G ratio mean was 1.1±0.2. In majority of the cases the ratio was one. In the group GI-A the mean was 0.73±0.38. The values ranged between 0.03 and 1.5. In the GI-B the mean was 0.55±0.16 and the value varied from 0.33 to 0.83. The GI-C had a mean value of 0.65 and the values ranged between 0.4 and 0.8. The group GI-D the values ranged from 0.252 to 0.7 with mean 0.5±0.139. Significant variation of the A:G ratio occurred in the experimental group.

The overall of picture of serum protein values of ducks are given in Table 4.6 and in Graphs 4.6 to 4.10.

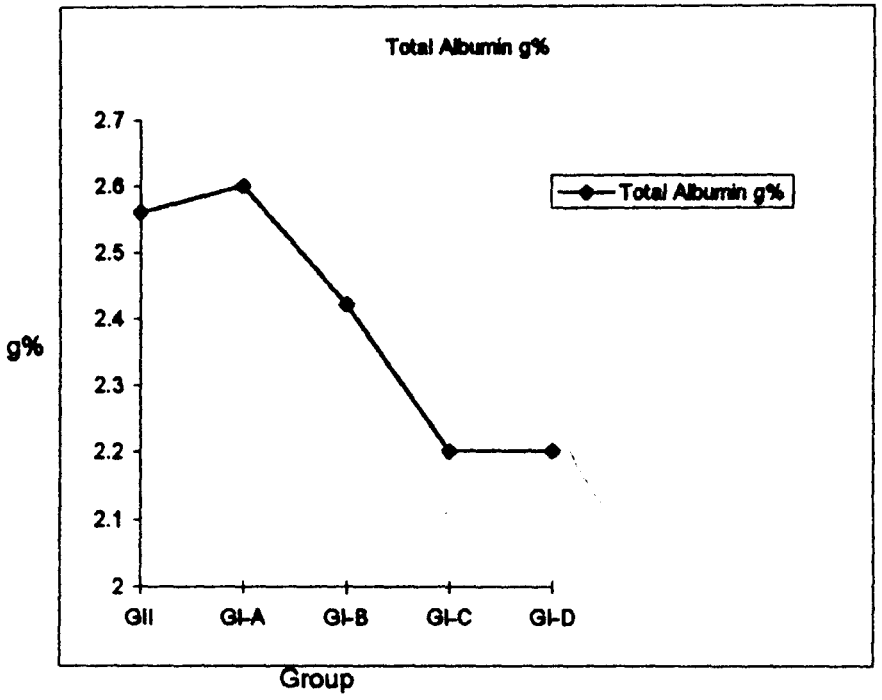
Electrophoretic studies of the serum revealed fall in the albumin fractions and relative rise in the fractions of globulin in GI-C and GI-D groups. There was general fall in the albumin fractions in the experimental group. The GI-A and the GI-B, sera did not show much variation whereas GI-C and GI-D exhibited severe changes. Electrophoretic pattern of serum protein exhibited changes in proportion to the duration of aflatoxin exposure and this was characterized by fall in albumin fraction and relative increase in globulin fractions.



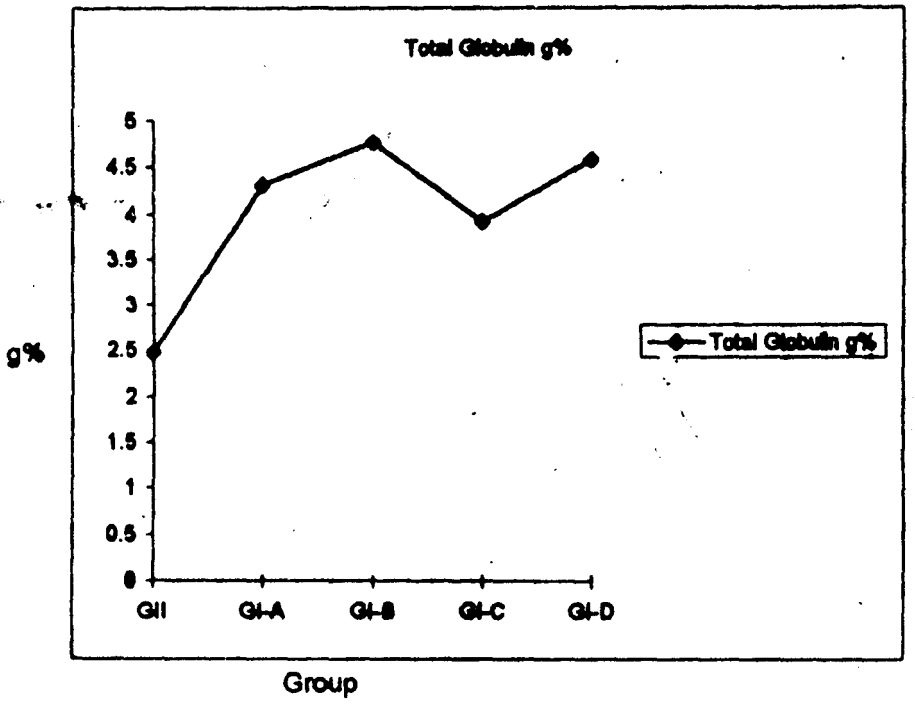
Graph 4.6 Serum protein pattern in the control and experimental group of ducks



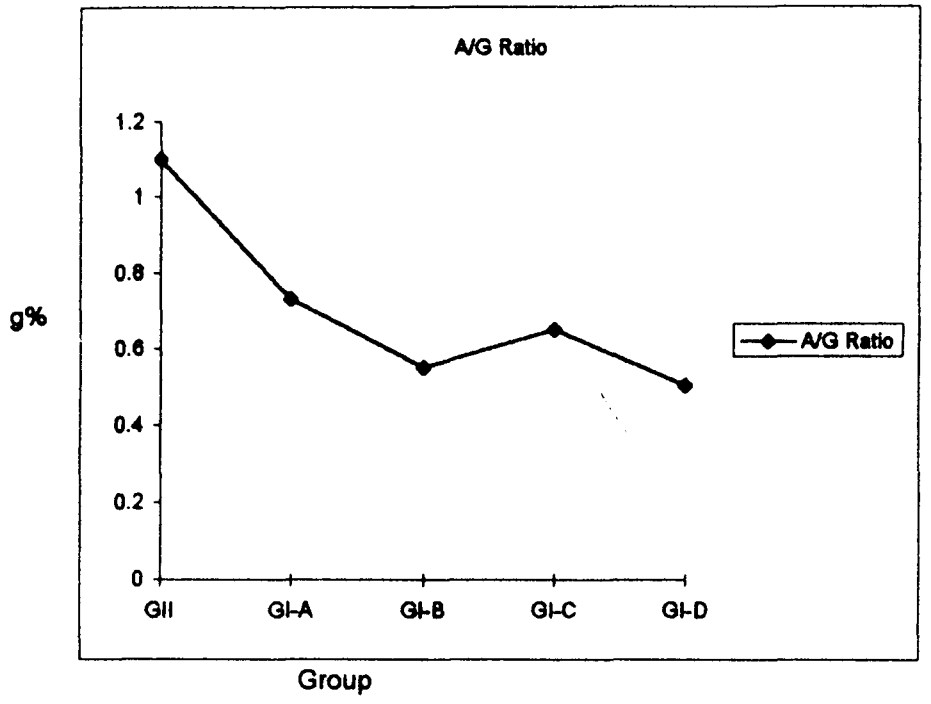
Graph 4.7 Mean total serum protein values in the control (GII) and experimental (GI-A to GI-D) groups of ducks



Graph 4.8: Mean serum albumin values in the control and experimental group of ducks



Graph 4.9: Mean serum globulin values in the control and experimental group of ducks



Graph 4.10: Mean albumin/globulin ratio in the control and experimental groups of ducks

4.7 SENSITIVITY TO PHA-M (PHYTOHAEMAGGLUTININ-M)

Skin thickness of ducks were measured on the 50th day of the experiment. The mean skin thickness of ducks initially was 0.1 cm when PHA-M was injected intradermally. The skin thickness of each duck was measured after 24 hours. The skin thickness of the control group ranged from 0.19 to 0.2 cm with mean 0.186±0.21 whereas the thickness of the experimental group varied from 0.9 cm to 0.19 cm with mean to 0.12±0.1.

After 48 hours the mean thickness of the control group was 0.23±0.068 cm while in the experimental group the mean skin thickness was 0.2±0.052 cm indicating a reduced response to PHA-M.

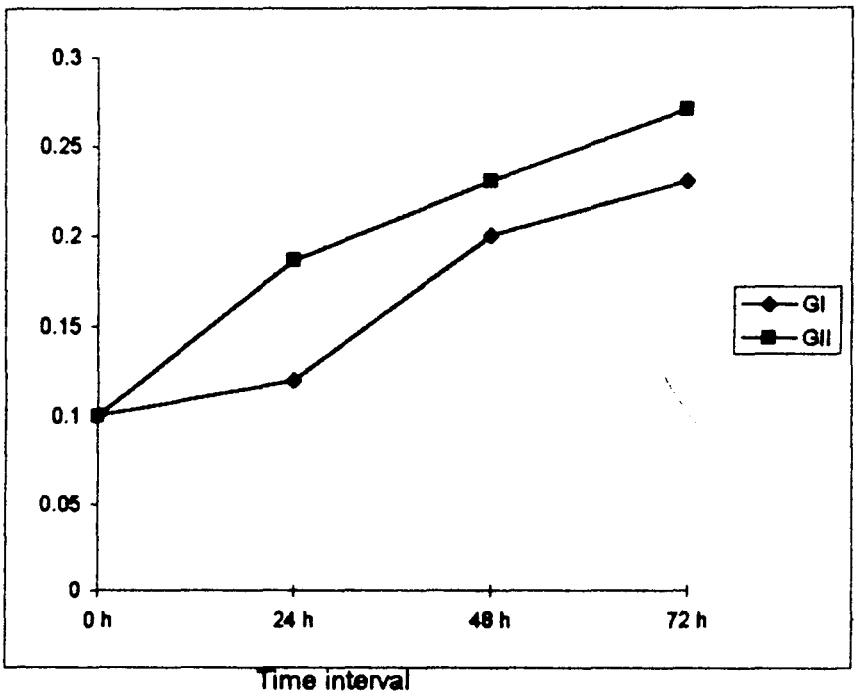
The skin thickness was measured after 72 hours and the mean thickness of the control group was 0.27 cm and the thickness within the group ranged between 0.2 cm to 0.3 cm. In the experimental group the skin thickness varied between 0.165 cm and 0.3 cm with mean of 0.23±0.072 cm. The mean skin thickness of the control group and the experimental group at various time interval are represented in a graph 4.11 & 4.12 and table 4.10. The skin reaction at 0 to 72 hours is depicted in Table 4.10 and Graph 4.11. There was significant reduction in the skin thickness in the experimental group indicative of immuno suppression.

Table 4.10: The mean skin thickness of the control and experimental groups of ducks before and after intra dermal PHA-M injection

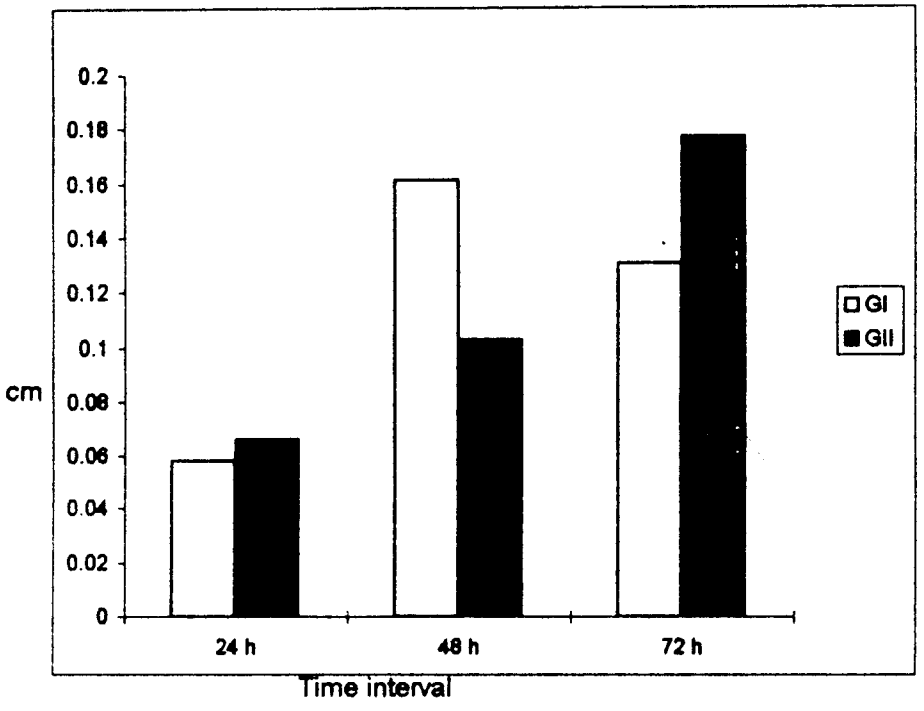
Groups	0 h cm	24 h cm	48 h cm	72 h cm
GI (Treatment group)	0.10±0.14	0.12±0.1	0.2±0.052	0.23±0.72
GII Control group	0.10±0.18	0.186±0.21	0.23±0.068	0.27±

The increase in skin thickness over the base line skin thickness at 0 hour, 24, 48 and 72 hours was calculated individually. The mean increase in each group are plotted in the Graph 4.12.

In the control group the mean increase in thickness over the baseline thickness at 24, 48 and 72 hours was 0.066±0.043 cm, 0.1026±0.727 and 0.177±0.061 respectively. Whereas in the experimental group the mean value increase in thickness at 24, 48 and 72 hours was 0.058±0.043 cm, 0.161±0.26 cm and 0.1306±0.06 cm respectively (Table 4.11 and Graph 4.12). There was significantly reduced response to intra dermal injection of PHA-M in the ducks treated with aflatoxin and this poor response was more pronounced after 72 h of the injection.



Graph 4.11: Mean skin thickness of the control and experimental group of ducks before and after intradermal injection of PHA-M



Graph 4.12 Mean increase in skin thickness of the control and experimental group of ducks after intra dermal injection of PHA-M

Table 4.11: Mean increase in skin thickness over the base line thickness in the control and experimental group of ducks after intra dermal injection of PHA-M

Groups	24 h	48 h	72 h
GI (Experimental)	0.058±0.043	0.161±0.26	0.1306±00
GII (Control)	0.066±0.043	0.1026±0.0727	0.177±0.061

4.8 PHAGOCYTIC INDEX

Phagocytic ability of the phagocytes in the experimental and control group of ducks was assessed by the carbon clearance test of Glick (1964). The optical density of citrated plasma was measured by spectrophotometer using 675 nm filter at zero minute, five minutes and fifteen minutes after intravenous injection of colloidal carbon. Blood for separation of plasma was collected from the vein on the opposite side. In the control group of ducks the OD at 5 minutes showed increase and then at 15 minutes fell below the 5 minute value. In the experimental group of ducks, the fall in optical density at fifteen minute was very low compared to the control group. The optical density values of the control and experimental group of ducks are given in Table 4.12. There was considerable reduction in the

phagocytic activity in the experimental group of ducks indicating suppression of CMI response in these birds.

Table 4.12: Optical density of plasma measured at 0 minute and 5 minutes and 15 minutes after 1/v injection of colloidal carbon in ducks

Groups	Minute		
	0	5	15
Control	0.0	0.1	0.05
	0.01	0.2	0.15
	0.2	0.8	0.5
	0.00	1.5	0.02
	0.02	0.8	0.3
	0.00	0.4	0.3
	0.00	1.5	0.2
	0.00	0.1	0.05
Experimental	0.00	0.2	1.5
	0.00	0.716	1.00
	0.01	0.1	0.1
	0.02	0.224	0.248
	0.01	0.084	0.192
	0.02	0.249	0.230
	0.01	0.261	0.247
	0.00	0.159	0.192

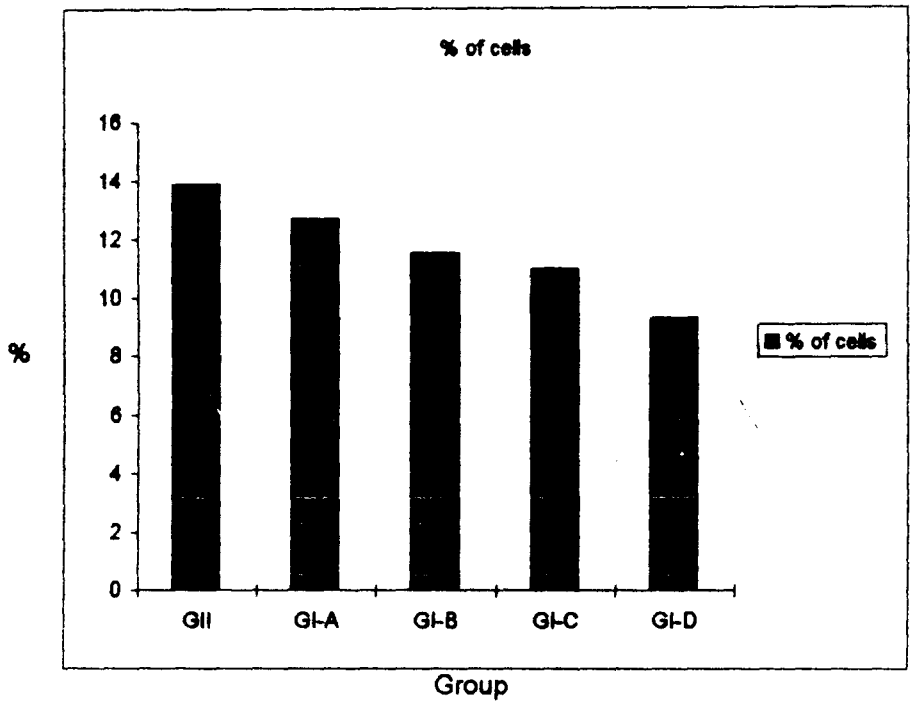
4.9 LEUCOCYTE MIGRATION INHIBITION TEST

The leucocyte migration inhibition test revealed LMIT index between 0.57 and 0.7 with mean index of 0.63±0.065 for the control group. In the case of the experimental group index varied between 0.52 and 0.7 with the mean 0.593±0.078. These two values did not show significant variation.

4.10 ALPHA NAPHTHYL ESTERASE ACTIVITY IN LEUCOCYTES

Blood smears from the control and experimental group of ducks were fixed and stained for alpha naphthyl estrease activity (ANAE). Two hundred lymphocytes were counted at random in each smear. Percentage of cells having positive reaction were calculated. In the control group the reacting cells ranged between 11% and 15% with mean of 13.83±0.8. In the experimental group GI-A which was fed aflatoxin for two week, the range was between 10% and 14% with mean of 12.67±1.18.

In GI-B the range was between 10% and 13% with mean of 11.5±2.97. In GI-C and GI-D group which were fed aflatoxin, for six and eight weeks, the mean values were 10.92±1.1 and 9.25±1.0, respectively. The range for GI-C was between 9% and 13% whereas in GI-D it ranged from 8% to 12%. The mean was 9.25±1. On statistical analysis the values were not found to be significant. There was decreasing trend as the exposure time to aflatoxin increased. This is depicted in Table 4.13 and Graph 4.13.



Graph 4.13: Mean percentage of alpha naphthyl estrase positive lymphocytes in the peripheral blood of the experimental and control group of ducks

Table 4.13: Mean percentage of Alpha Naphthyl Esterase positive lymphocytes in the peripheral blood of experimental and the control groups of ducks

Groups	Percentage of cells	Range (%)
Experimental groups		
GI-A	12.67±1.18	10-14
GI-B	11.5±2.97	9-13
GI-C	10.92±1.1	9-13
GI-D	9.25±1.0	8-12
Control Group		
GII	13.83±0.8	11-15

4.11 IMMUNE RESPONSE OF THE DUCK TO FOREIGN ANTIGEN

There was considerable variation in the immune response of experimental and control groups. Ducks were immunised with sheep RBC and haemagglutination of sheep RBC was estimated using serial dilusions of sera in a microtitre plate. In the control group haemagglutination was observed above 1:64 dilution and increased up to 1:256 whereas in the experimental group haemagglutination was observed only upto 1:64. The

pattern of humoral immune response is given in Table 4.14. The immune response was significantly low in the experimental group.

Table 4.14: Humoral immune response of duck to sheep RBC

Titre value	Group I No.of animals responded	Group II No. of animals responded
256	--	4
128	--	6
64	4	2
32	4	--
16	4	--
8	--	--

4.12 HISTOPATHOLOGICAL STUDIES

4.12.1 Liver

Histopathological study of the liver from the control group did not reveal any abnormality.

In the GI-A group which were on toxin diet for two weeks the liver revealed fatty change. Focal areas of hepatocyte necrosis were noticed. There was mild biliary hyperplasia accompanied by fibroblastic proliferation in the periportal area which was found to extend to the interior

parts of the lobule. Though there was necrosis and destruction of the parenchyma, the lobular architecture was still discernible.

In GI-B further advancement of the changes seen in GI-A were observed. They were characterized by extensive fatty change, necrosis of the hepatocytes and severe biliary epithelial hyperplasia. Periportal fibrosis was extensive. Architecture of the lobules was disrupted in many areas with fibrosis and regeneration of hepatocytes.

In GI-C multiple areas of necrosis were evident in many sections. This was accompanied by extensive fibrosis and biliary epithelial proliferation. Focal areas of hepatic cell proliferation were also evident. The architecture of the liver was highly disrupted.

Liver revealed severe advanced pathological changes in GI-D which received the toxin for eight weeks. The architecture of the liver lobules was lost in many sections. Hepatic degeneration characterised by extensive fatty change was evident in some cases while in others severe necrosis and replacement of parenchymatous tissue with proliferating biliary epithelium and fibrous connective tissue were observed. The regenerating hepatic cell cords were seen to replace the lost tissue leading to nodular islands of parenchymatous tissue in many sections (Fig.4 and 5).

4.12.2 Thymus

In the control group the thymus had differentiated into lymphoid follicles containing cortical and medullary regions. Cortex had large number of lymphocytes while the medulla contained less number of lymphocytes. The medulla was restricted to small central area. There were isolated polygonal or stellate epithelial cells.

The thymus in GI-A did not reveal any difference when compared to the control group. However, isolated follicles revealed focal areas of necrosis.

In GI-B several follicles of the thymus revealed haemorrhages and central necrosis. There was severe hyperaemia of the inter follicular blood vessels.

In GI-C the thymus revealed interfollicular haemorrhages and necrosis of the central area of lymphoid follicles.

The changes were more acute in the thymus of the GI-D. The interfollicular haemorrhages were extensive and necrosis of the lymphoid follicles was more severe. There were focal areas devoid of lymphoid cells and the cells were loosely arranged (Fig.6 and 7).

4.12.3 Bursa of *Fabricius*

There was shrinkage of the lymphoid follicles of the GI-A and focal necrosis of lymphoid tissues. The changes were mild in nature.

In GI-B there was focal areas of necrosis of the lymphoid follicles, collapse of the follicles and mild to moderate interfollicular oedema. Follicles revealed focal necrosis of the lymphoid tissue and the follicles were seen separated by oedema..

In the GI-C the necrotic changes were more severe and advanced. The architecture of the follicles was disrupted and follicles were seen fused and collapsed. Interfollicular oedema was more pronounced.

In GI-D there was severe depletion of the lymphoid cells. In several follicles necrotic cellular debris was seen along with corpora amylacea. Many follicles were collapsed and coalesced (Fig.8, 9 and 10).

4.12.4 Spleen

The sections in group GI-A did not indicate much histological changes. However, in a few of birds severe congestion of the sinusoids and haemorrhages were noticed.

In the GI-B spleen sections revealed mild depletion of the lymphoid tissue. The reticular connective tissue became more prominent. Sinusoids were congested and scattered foci of haemorrhages were present.

In the GI-C the lymphoid tissue became much depleted. The Malpighian corpuscles became smaller in size with reduced lymphoid tissue. The reticular stromal tissue became more prominent.

The GI-D spleen showed further advanced changes. The reticular tissue became very prominent and the lymphoid cells were much depleted. Malpighian corpuscles and the germinal centres became reduced in size. (Fig.11).

4.12.5 Kidney

GI-A Group

Kidneys revealed nephrotic changes. The cells of the proximal convoluted tubule had lost the brush border. The cells were swollen and had granular cytoplasm. The distal convoluted tubules became more eosinophilic and swollen. The lumen of the tubules contained hyaline casts. The cells lining the Bowman's capsule became more columnar and hypertrophied.

In the GI-B further advancement of changes were evident. In addition to the degenerative changes observed in the GI-A there was

desquamation of tubular epithelial cells and peritubular haemorrhages. Glomeruli were swollen and there was proliferation of mesenchymal cells.

In the GI-C the changes described for GI-B were present in a more pronounced way.

In the GI-D, kidney revealed severe nephrotic changes. It was characterised by the swelling of epithelial cells, desquamation and complete absence of tubular epithelial cells. Intertubular haemorrhages were seen. Periglomerular fibrosis, shrinkage of the glomeruli and mesenchymal proliferation was also observed (Fig.12).

4.12.6 Heart

Cardiac muscle fibres did not show any abnormality in the control group and GI-A. The GI-B, GI-C and GI-D groups revealed loss of striations in the myocardial fibres. Granular appearance of sarcoplasm and focal areas of hyalinisation were also seen.

4.12.7 Brain

In the GI-C and GI-D groups the brain showed significant changes. This included neuronal vacuolation, neuronophagia and satellitosis. Many areas of chromatolysis and focal encephalomalacia were noticed.

4.12.8 Skin

The skin pieces from the control group revealed initially a serous excudation and mild infiltration of leucocytes. AT 48 h extensive exudation of leucocytes which included lymphocytes, monocytes and heterophils were seen. These changes were also seen in the skin collected at 72 h also.

The skin pieces collected from the experimental group revealed mild changes at 48 h which included mild to moderate infiltration of leucocytes and serus exudation. The skin collected at 72 h also revealed moderate infiltration of leucocytes. The changes were mild compared to the control group indicating lowered immunological response.

4.13 ULTRA STRUCTURAL STUDIES

4.13.1 Liver

The structure of the hepatocytes in all the treatment groups showed significant alterations. In the treatment groups GI-A and GI-B there was loss of microvilli from the hepatocytes. Separation of desmosomes and appearance of lipid droplets were observed. The RER's were degranulated. The nucleus of the hepatocytes became irregular and heterochromatin clumps increased. Perichromatin granules appeared in the nucleus. In GI-C and GI-D further damage to the hepatocytes was evident. Both SER and RER revealed fragmentation. The dilation of the nuclear envelope, RER and SER cisternae led to the formation of several vesicles. The nucleoli in

many of the hepatocyte nucleus disappeared. The nucleus had vacant areas and numerous chromatin granules. Mitochondria lost their cristae and appeared swollen. Some of the mitochondria had electron dense material inside their matrix (Fig.13).

4.13.2 Thymus

In GI-A and GI-B groups majority of the lymphoblasts maintained structure similar to the control group. In some there was dilation of the cisternae of ER and nuclear envelope. The chromatin in the nucleus had become more dense hence there was increase in heterochromatin clumps.

In GI-C and GI-D groups absence of the lymphoblasts from the follicles was evident. Nuclei with large amount of heterochromatin were seen. The nucleus of many of the lymphoblasts had invagination of the nuclear membrane leading to formation of vesicles. Perichromatin granules were seen in many of the nuclei. Dilatation of the nuclear envelope and ER was also visible.

4.13.3 Spleen

In the experimental group the cells lost their organellar morphology. The cells had numerous vesicles in their cytoplasm and nucleus of some of the cells had lost the chromatin and contained a few clumps of heterochromatin. In other cells there was increase of heterocrhomatin and

some of the nucleus appeared to contain only condensed chromatin (Fig.14 and 15).

4.13.4 Bursa of *Fabricius*

In the treatment groups the nucleus of the lymphocytes contained numerous heterochromatin clumps and many of them developed vesicles in their cytoplasm. Nuclear envelope in the epithelial cells and lymphoblast revealed fusion and dilation. The nuclear membrane became irregular and RER dilatations were seen. Autophagic multivesicular bodies were seen in the epithelial cells (Fig.16 and 17).

Fig.1: Photograph of a duckling died after feeding aflatoxin B_1. Note the haemorrhages on the skin.

Fig.2: Liver of the duckling exposed to aflatoxin B_1 for eight weeks. Note the pale colour and swollen appearance of the liver.

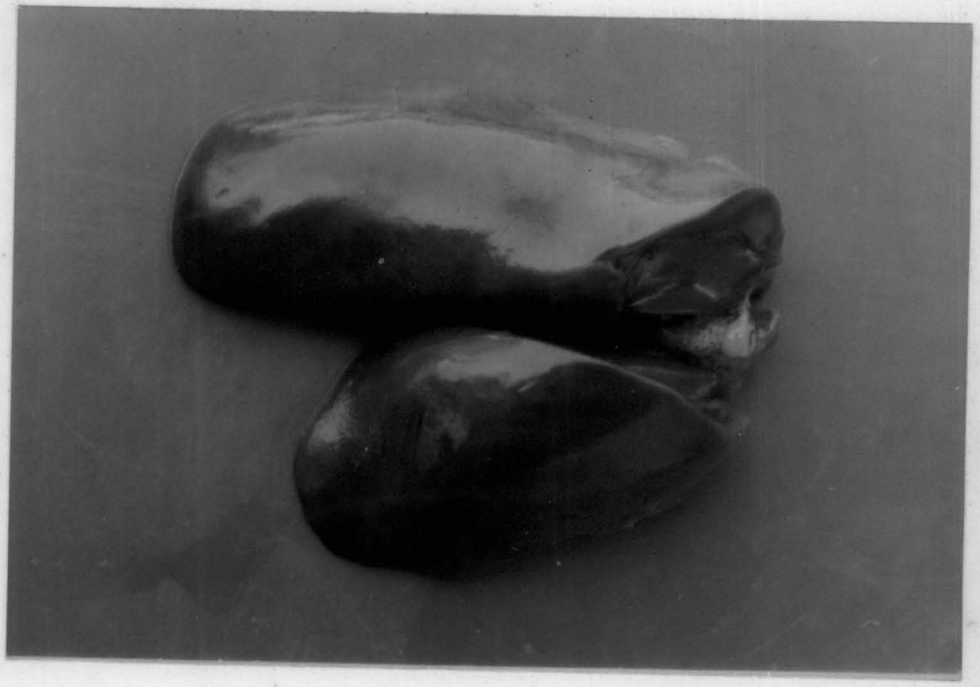
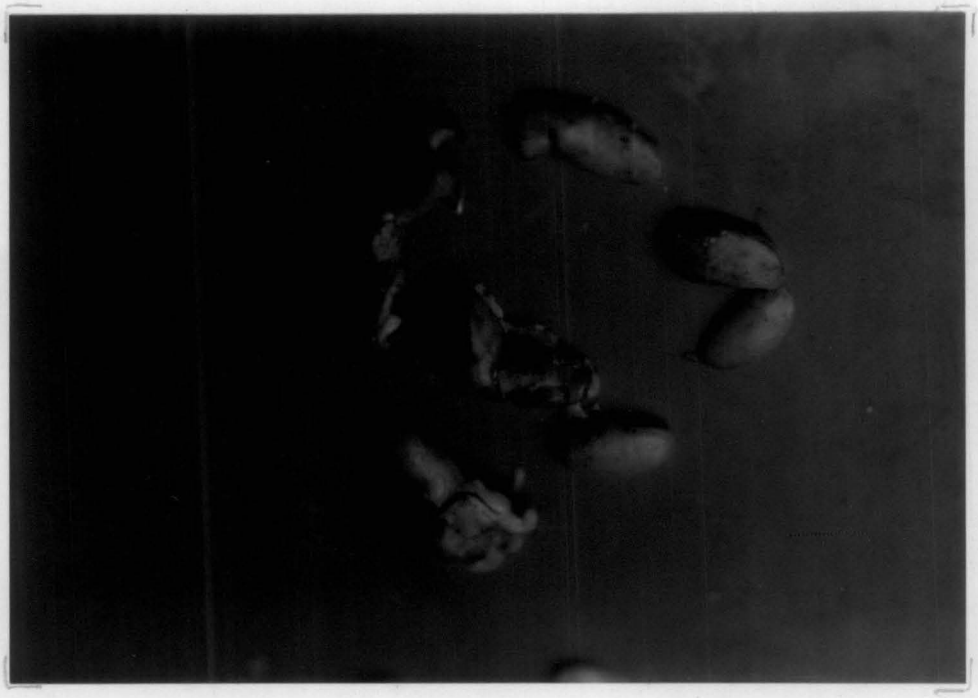


Fig.3: Thymus glands of the duckling exposed to aflatoxin B_1 for four weeks showing haemorhages

Fig.4: Section of the liver from the aflatoxin exposed duckling showing extensive vacuolation of hepatocytes and occasional hepatocytes undergoing apoptosis (H&E 20X).

(2)



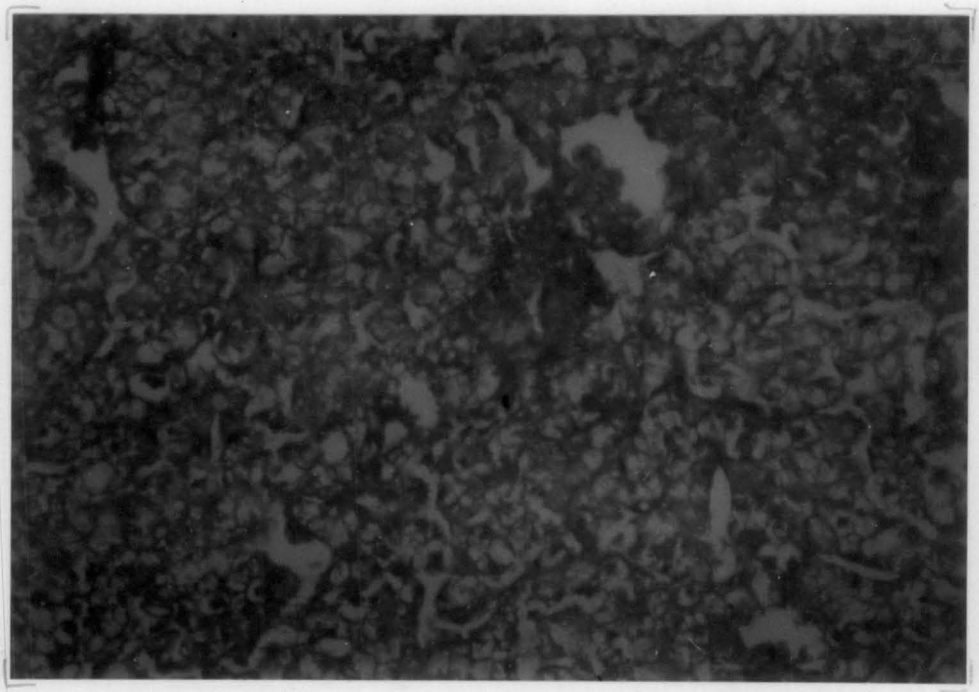
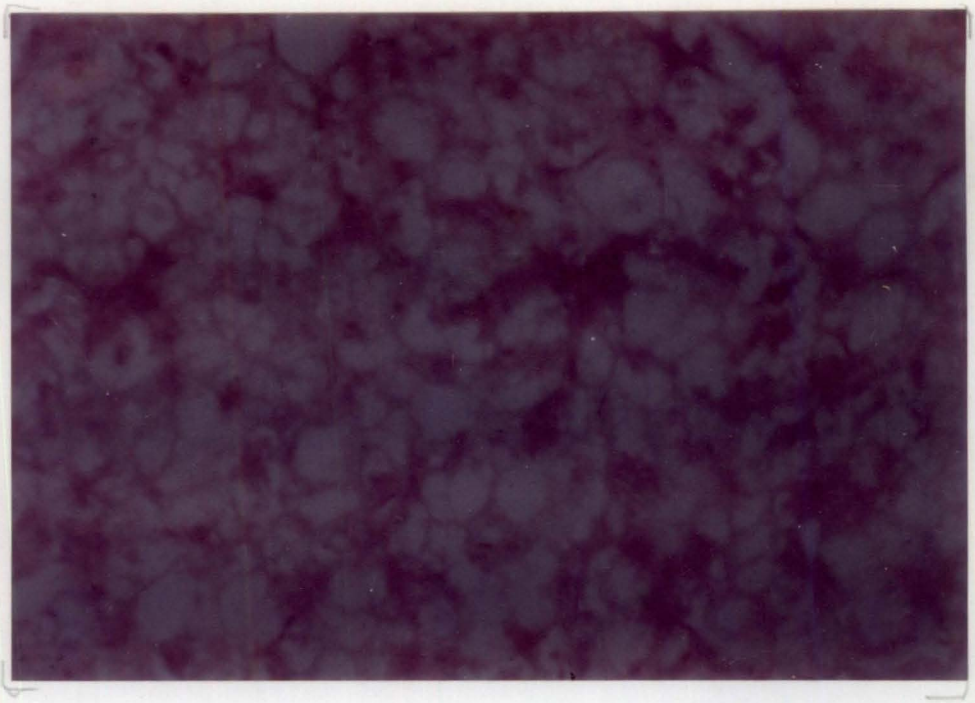


Fig.5: Section of the liver from the aflatoxin exposed duckling showing extensive vacuolation of hepatocytes indicating fatty degeneration of hepatocytes (H&E 40x).

Fig.6: Section of the thymus from the aflatoxin B_1 treated duckling. Note the depletion of lymphoid cells in the follicle (H&E 20x).



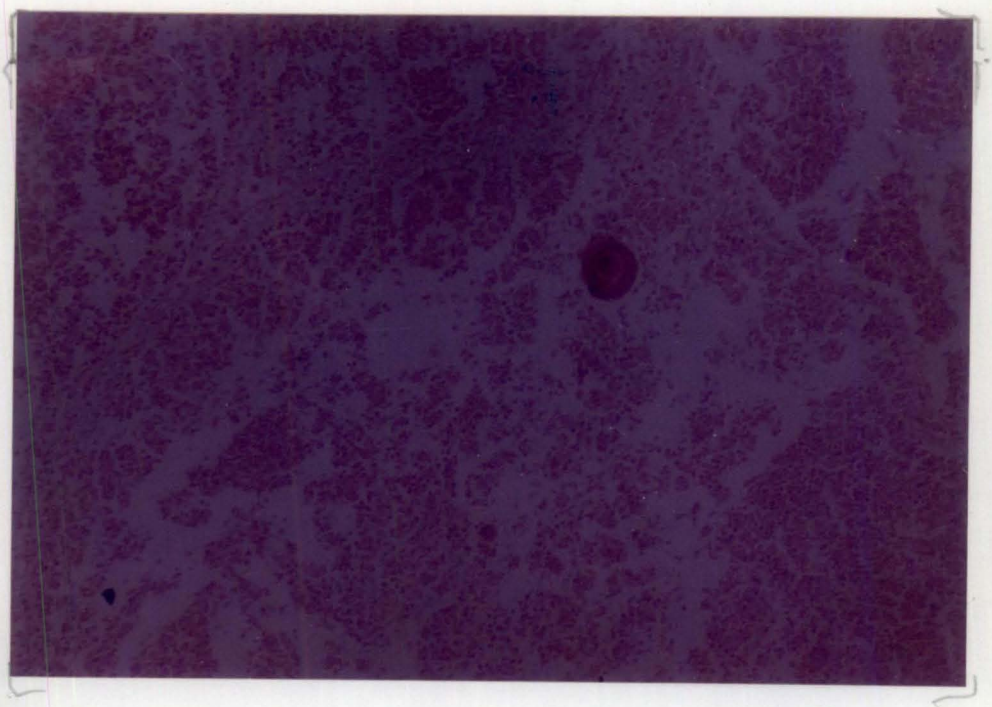
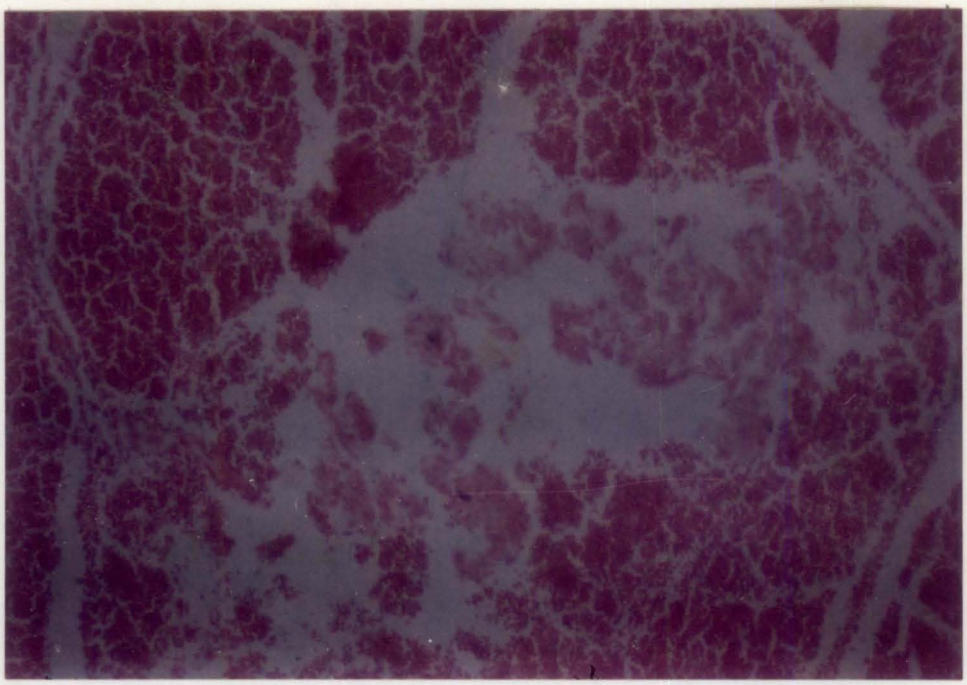


Fig.7: Section of the thymus from the aflatoxin treated duckling depicting the destruction of the lymphoid tissue (H&E 20x).

Fig.8: Section of the bursa of Fabricius from the aflatoxin treated duckling depicting atrophy of the follicle and loss of the lymphoid cells (H&E 20x).



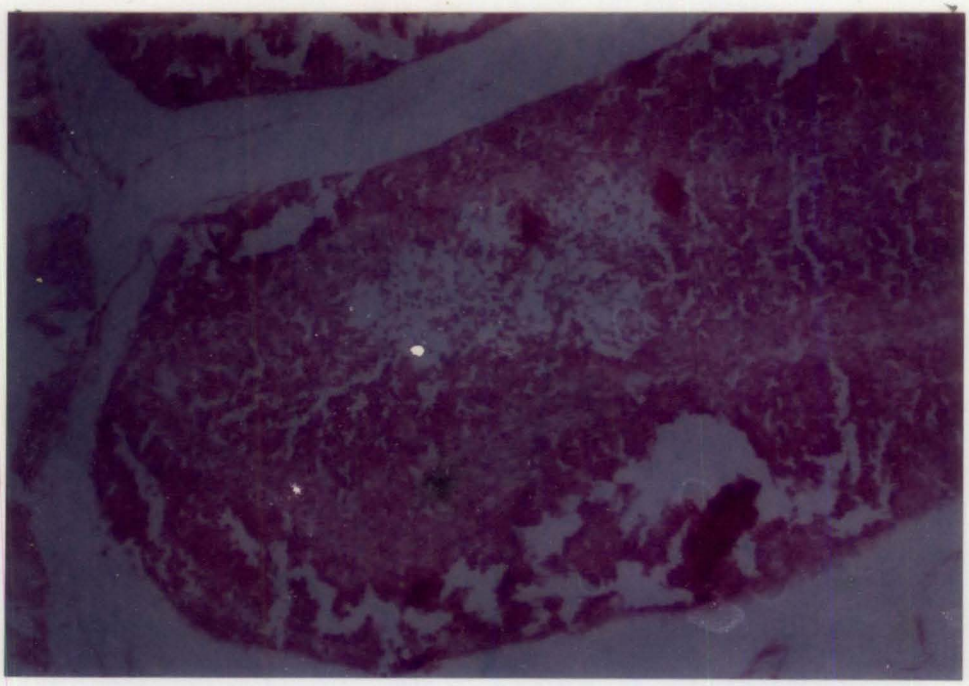


Fig.9: Section of the bursa of Fabricius from the aflatoxin treated duckling showing atrophy of the lymphoid follicle and loss of the lymphoid cells (H&E 20x).

Fig.10: Section of the bursa of Fabricius from the aflatoxin treated duckling showing extensive depletion of the lymphoid cells (H&E 20x).

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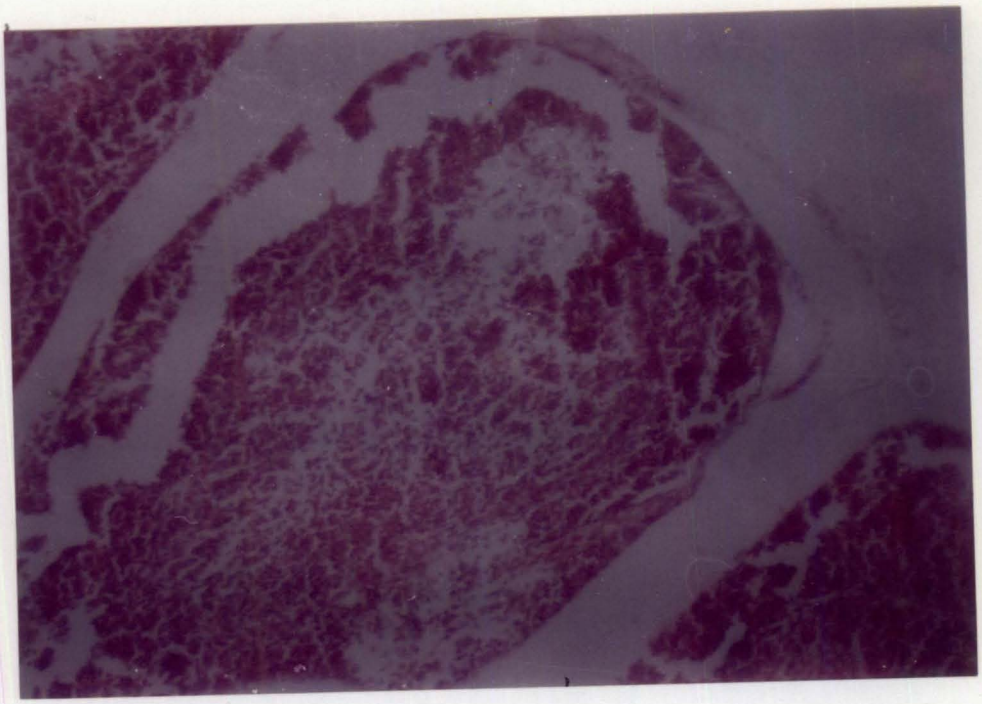

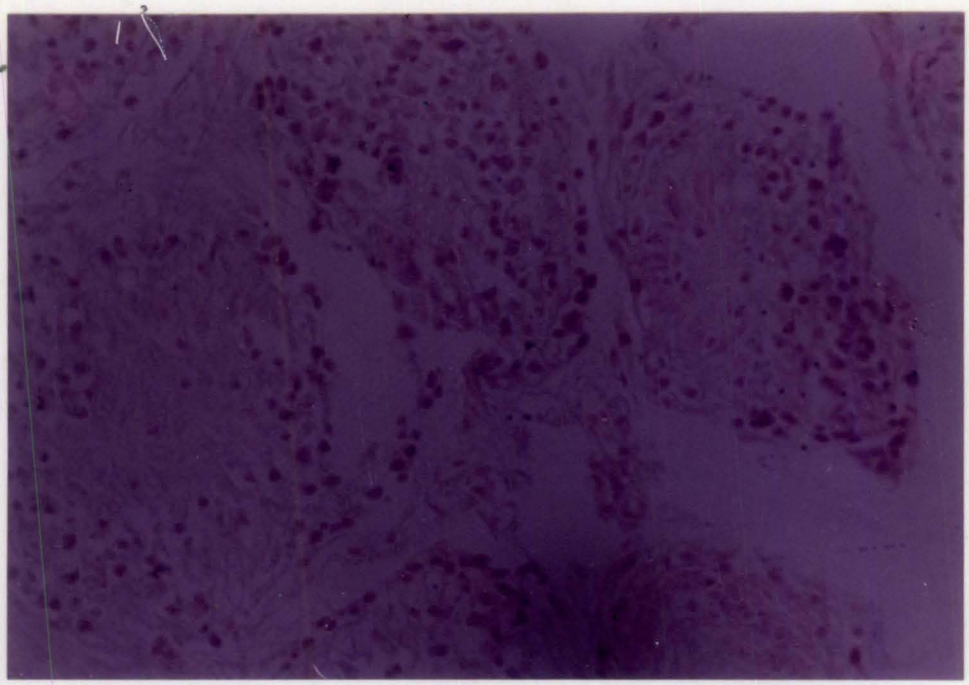

Fig.11: Section of the spleen from the duckling exposed to aflatoxin. Note the depletion of the lymphoid cells in the parenchyma (H&E 20x).

Fig.12: Section of the kidney from the aflatoxin treated duckling. Note the tubular nephrosis and periglomerular fibrosis (H&E 20x).

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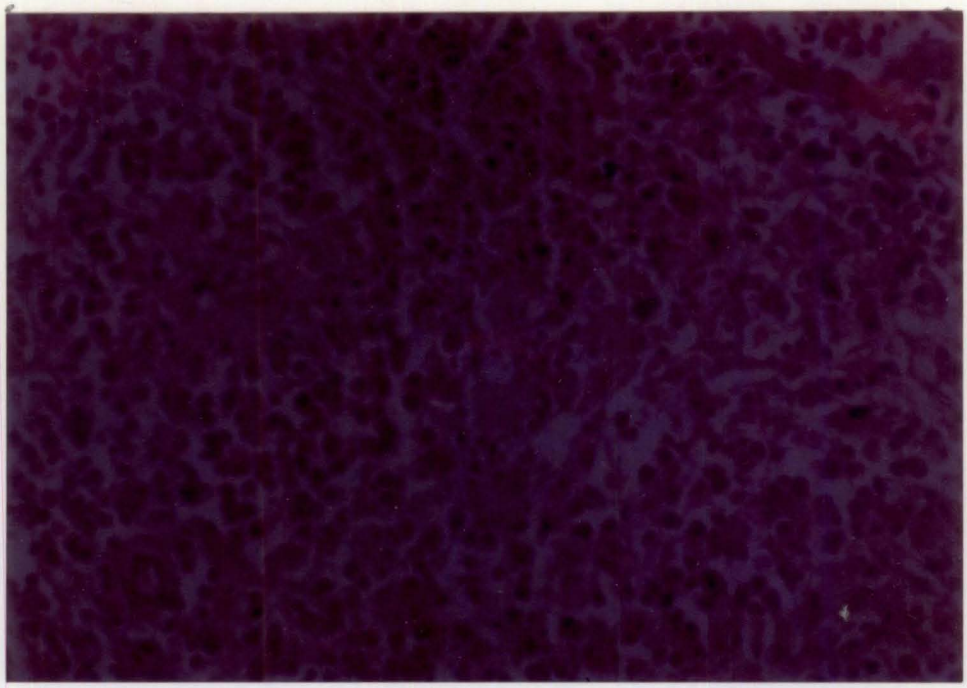


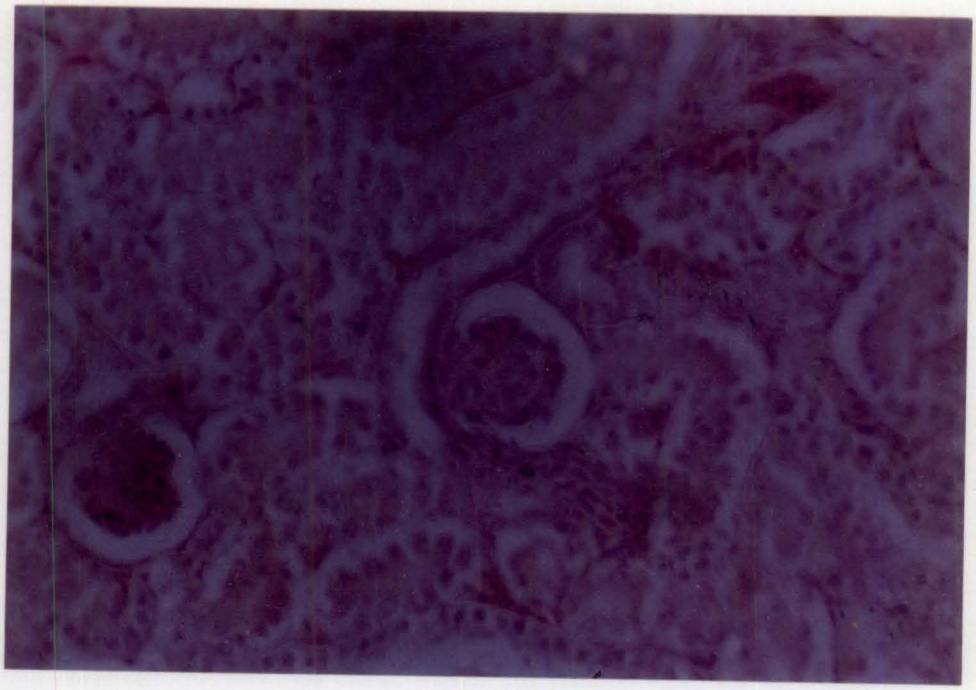


Fig.13: Electron micrograph of the liver from the duckling treated with aflatoxin B_1 for eight weeks. Note the dilatation of the endoplasmic reticulum, loss of contents from the nuclei presence of perichromatin granules in nuclei and separation of the desmosomes (6000x).

Fig.14: Electron micrograph of the spleen from the aflatoxin treated duckling. Note the heterochromatin clumps in the nuclei of some cells (6000X).

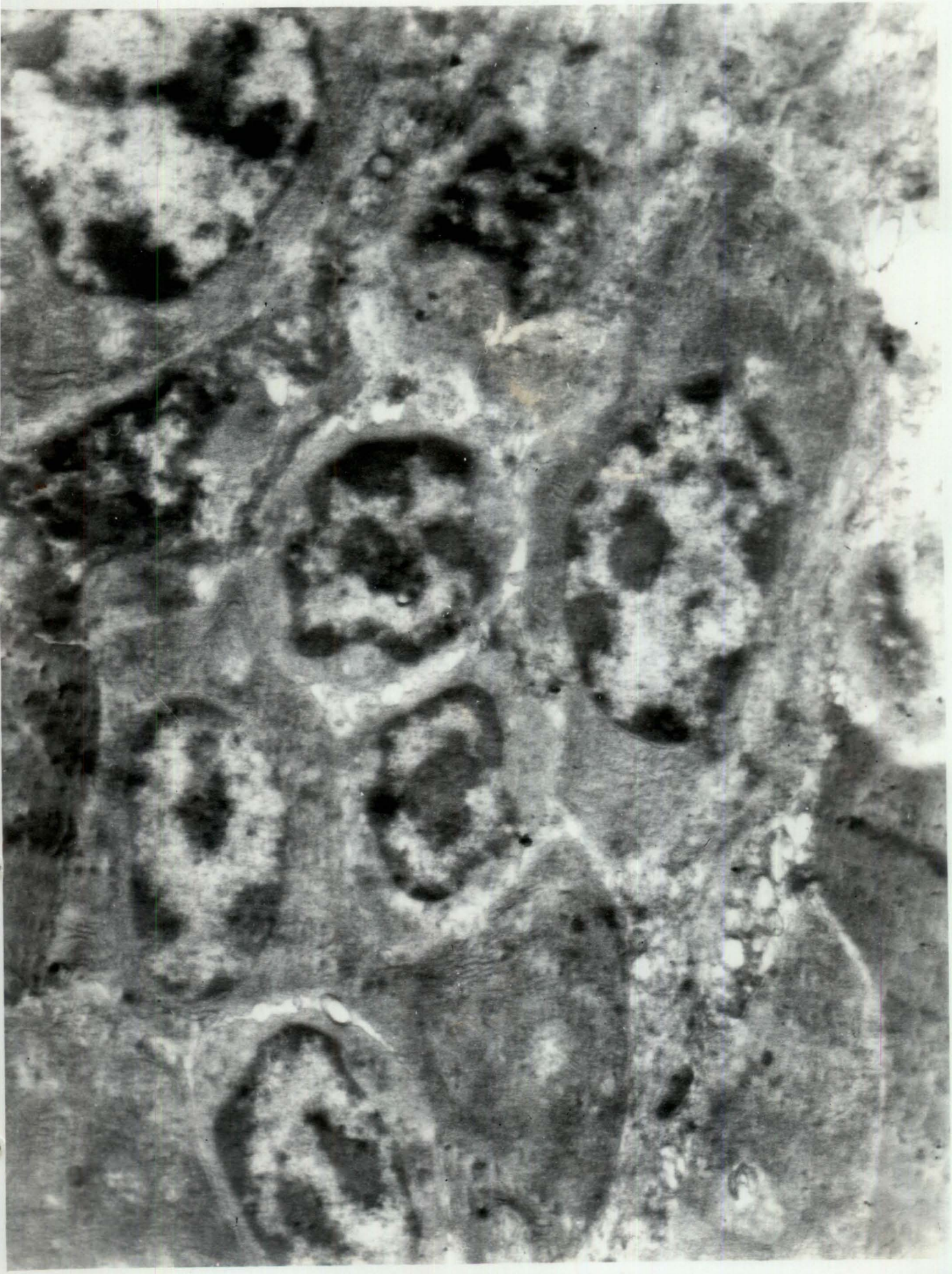


Fig.15: Electron micrograph of the spleen from the aflatoxin treated duckling (6000X).

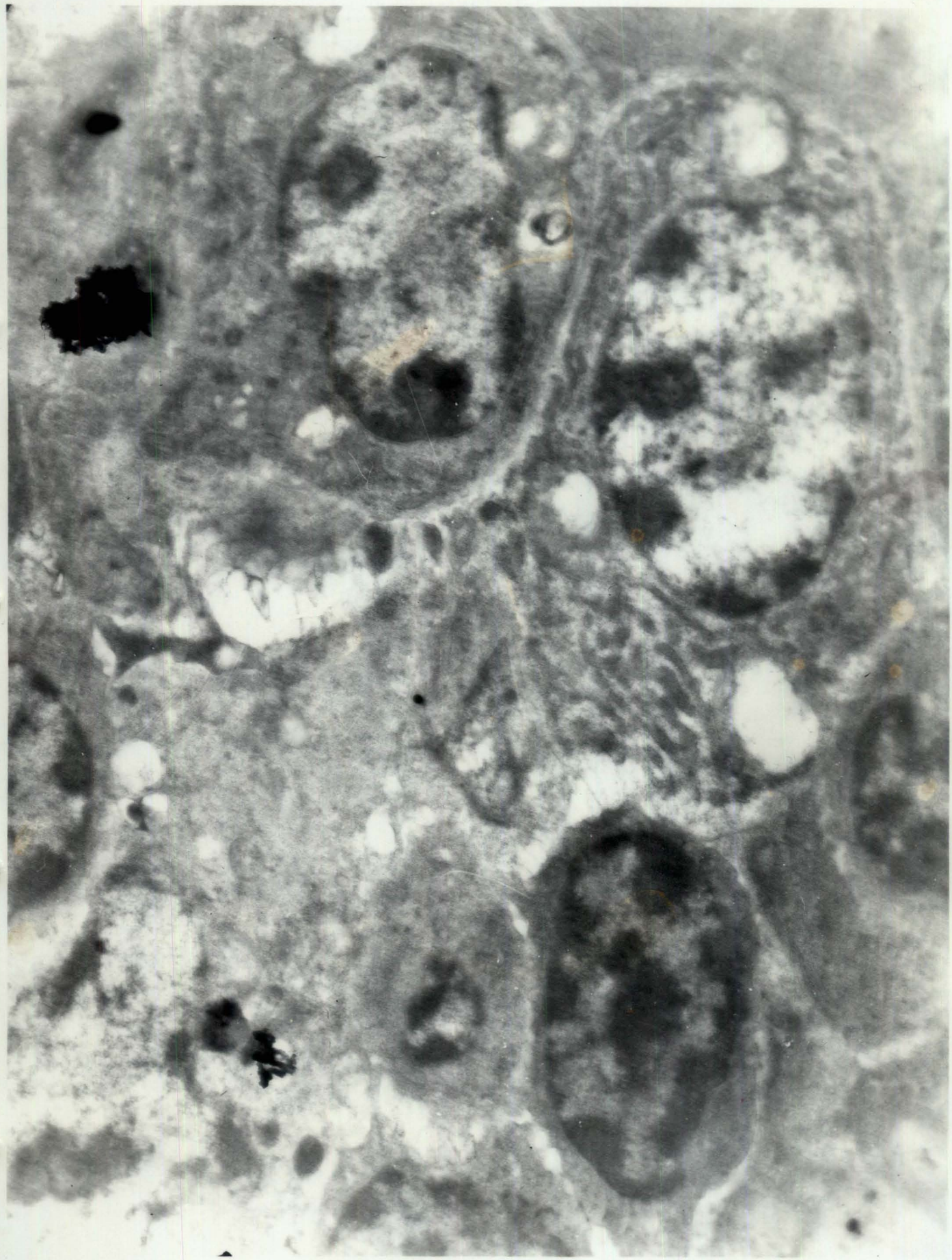


Fig.16: Electron micrograph of bursa of *Fabricius* from the aflatoxin exposed duckling. Note the disorganisation of cytoplasm around many nuclei which has large amount of heterochromatin clumps. (8000X).

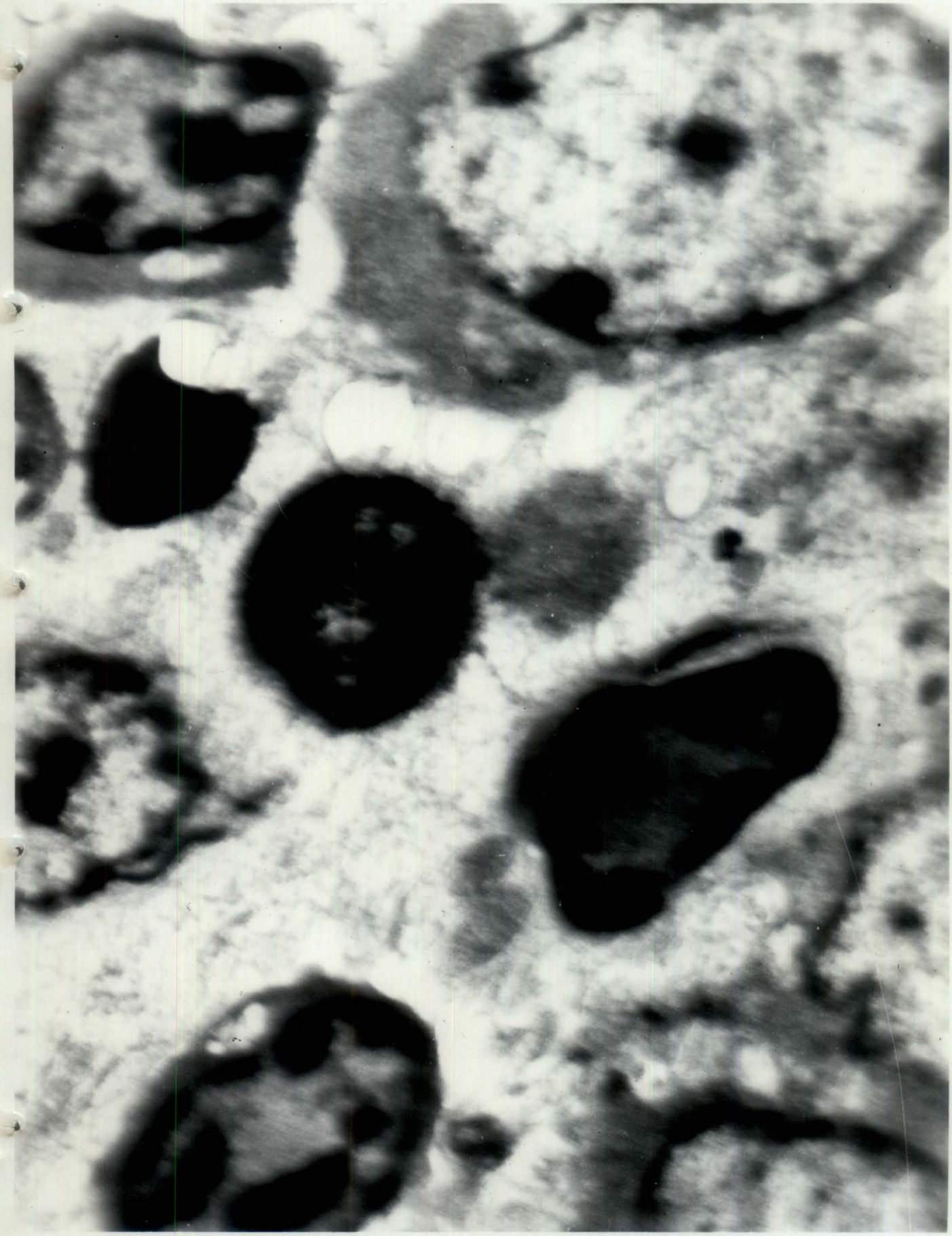
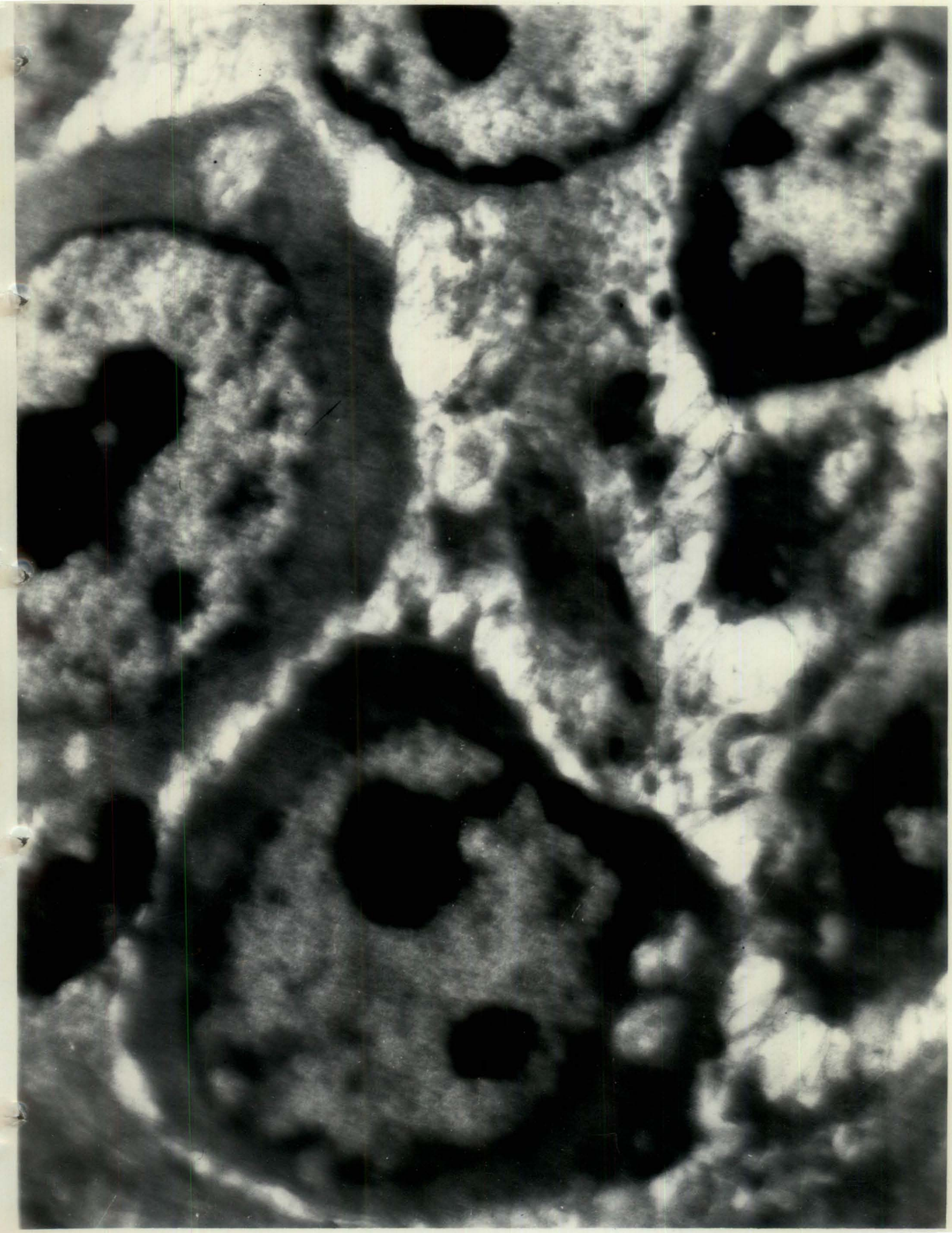


Fig.17: Electron micrograph of the bursa of *Fabricius* from the aflatoxin treated duckling. Note the chromatin clumps in many nuclei and loss of organisation of the cytoplasm in other cells (8000X).



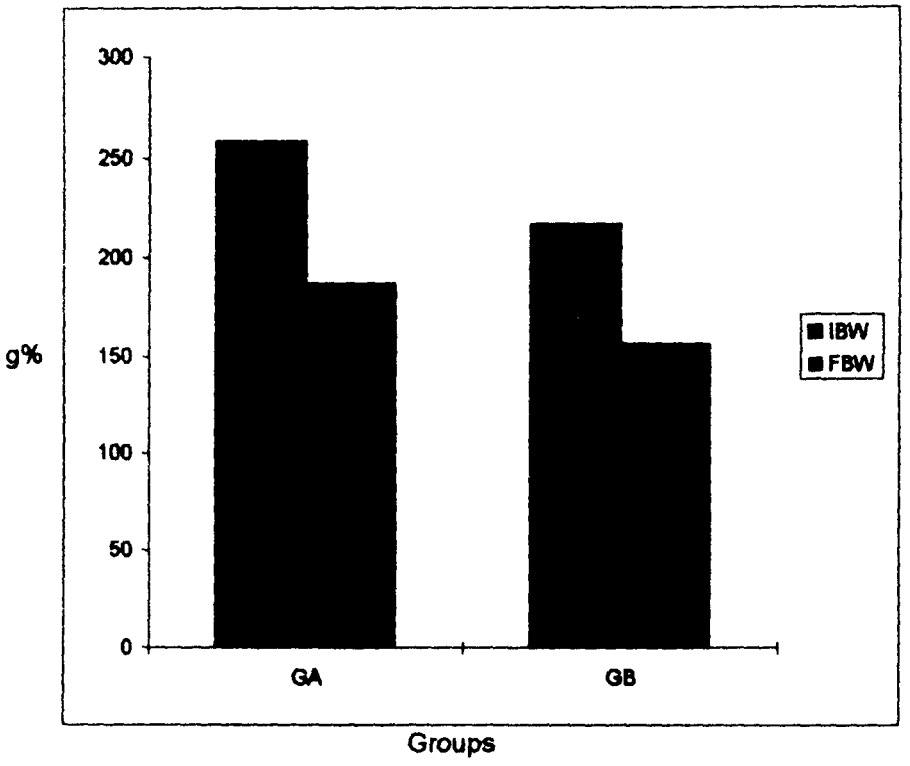
Chapter 5

EXPERIMENTAL STUDIES IN FISHES

Fishes belonging to the species of *Labeo rohita* were acclimatized in fibre glass tanks. They were grouped into experimental group GA and control group GB. GA received pelleted feed containing 0.4 mg of aflatoxin B_1/kg of feed on alternate days. GB group received the aflatoxin free feed.

5.1 WEIGHT OF FISHES

The body weights of fishes ranged from 50 g to 600 g. However, except two fishes (one weighing 50 g and other weighing 600 g) all other fishes were having the weight between 100 to 400 g. The mean weight of fishes at the start of the experiment was 235.94±112.45. The mean body weight of the control group was 186.76±99.71. The mean body weight of the experimental group was 258.1±112.87. Considerable body weight loss was observed in the experimental group. The mean body weight of the control group at the time of sacrifice/death was 156.39±101.93 while the mean body weight of experimental group was 211.5±103.83. (Table 5.1). The mean fall in body weight of the experimental fishes was 46.6±98 g while that of the control group was 30.37±1.04.



Graph 5.1 Mean body weight of the control and experimental group of fishes at the beginning and at the end of the experimental trial

Table 5.1: Mean body weights of fishes of Group GA (Treatment) and Group GB (Control) at the beginning and at the time of sacrifice

Group	Initial body weight (g)	Body weight at the time of sacrifice (g)	Fall in body weight (g)
GA	258.1±112.87	216.5±103.83	46.6±98
GB	186.76±99.71	156.39±101.93	30.37±0.04

The feed intake became very poor in the experimental group after one week. Fishes were taking only very small amount of feed in some tanks. Fishes started dying when they were fed aflatoxin containing feed for 45 days. These fishes were having internal haemorrhages indicated by blood clots in the body cavities. The liver appeared pale and highly friable. Gall bladder was enlarged. Failure of the blood to clot was also observed in fishes fed with aflatoxin for two months. However, no apparent lesions were observed in fishes sacrificed earlier. There was no reduction in feed intake and mortality in the control group (Fig.18.19, 20 and 21).

5.2 HEMATOLOGICAL STUDIES IN FISHES

Since the amount of blood obtained for each test was minimal, the blood from different individuals were pooled and values were calculated. The experimental group GA was divided into sub groups GA-1, GA-2, GA-3 and GA-4. GA-1 was given aflatoxin containing feed for 2 weeks, GA-2 for 4 weeks, GA-3 for 6 weeks and GA-4 for 8 weeks respectively. The mean erythrocyte count for GA-1 was 9.1±0.69 millions/cu.mm of blood and that of GA-2 was 9.7±8.1 millions/cu.mm. The mean erythrocyte counts for GA-3 and GA-4 were 8.25±0.21 and 2.85±0.91 respectively. There was considerable reduction in the erythrocyte count of GA-4 which received toxin containing feed for two months. The mean erythrocyte count for the control group (GB) was 10.15±0.71 millions/cu.mm of blood.

The mean total leukocyte count was 32800±20261.5, 21380±17047.63, 17400±7503 and 6200±2774.89/cu.mm respectively for experimental groups GA-1, GA-2, GA-3 and GA-4 showing a reduction of the leucocyte count in GA-4. The lecucocyte count of the control group GB was 24300±3615/cu.mm of blood. The PCV values estimated for GA-1, GA-2, GA-3, GA-4 were 24.5%, 25%, 25%, and 25% respectively while the PCV value estimated for GB was 30.2% (Graph 5.3). Though the packed cell volume of the experimental group of fishes were reduced, the reduction was not in proportion to the duration of exposure to aflatoxin, whereas the reduction in total erythrocyte count and total leucocyte count observed in

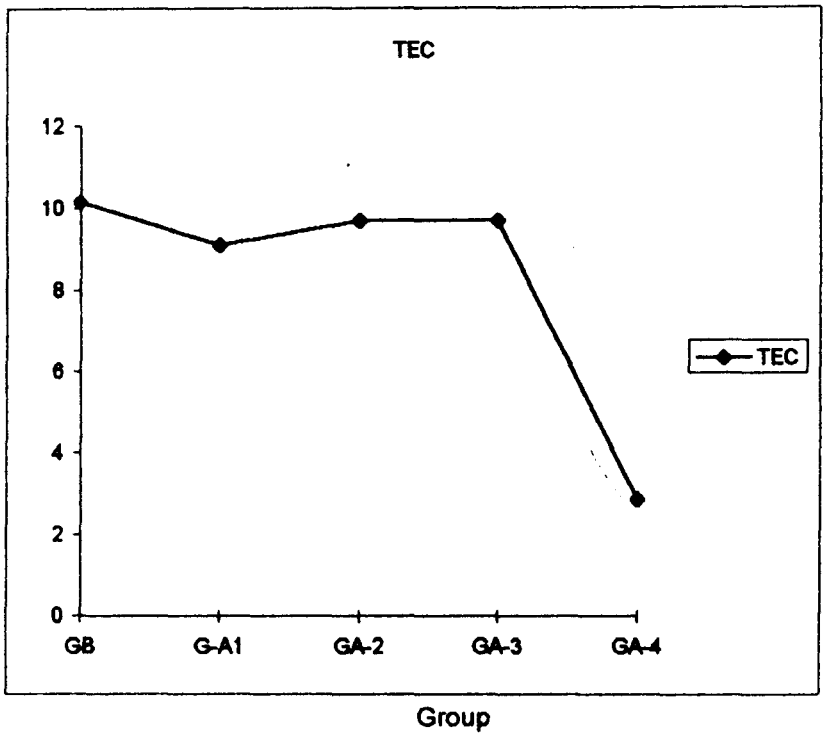
aflatoxin treated fishes were in proportion to the duration of aflatoxin treatment.

The erythrocyte values continued to decrease as the exposure time to toxin increased. Trend in the reduction is represented in the Graph 5.2. The leucocyte count was continued to decrease as exposure time increased. The trend of the reduction in TLC values are given in Graph 5.4. The differential leucocyte count did not reveal much variation in different groups. The packed cell volume values of experimental groups were also lower than the control group values. Thus there was a reduction in most of the haematological values of aflatoxin treated fishes. The haematological values in the control and experimental groups of fishes are given Table 5.2.

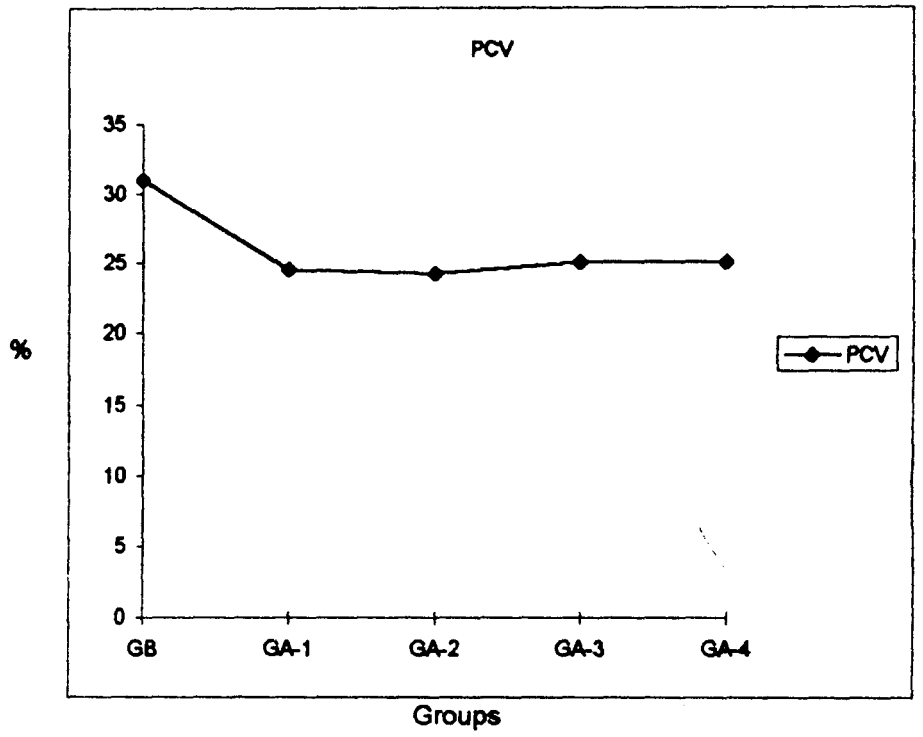
Table 5.2: Mean haematological values in the experimental and control group of fishes

Group	TEC millions/ mm3	TLC	PCV (%)	N (%)	L (%)	E (%)	B (%)	M (%)
GB	10.15±0.71	24300±3615	31	25	65	6	2	2
GA-1	9.10±0.69	32800±22261.5	24.5	30	60	4	1	5
GA-2	9.70±8.10	21380±17047.63	25	28	64	5	2	5
GA-3	8.25±0.21	17400±7503	25	27	64	6	2	5
GA-4	2.85±0.91	6200±2774.87	25	29	67	2	1	1

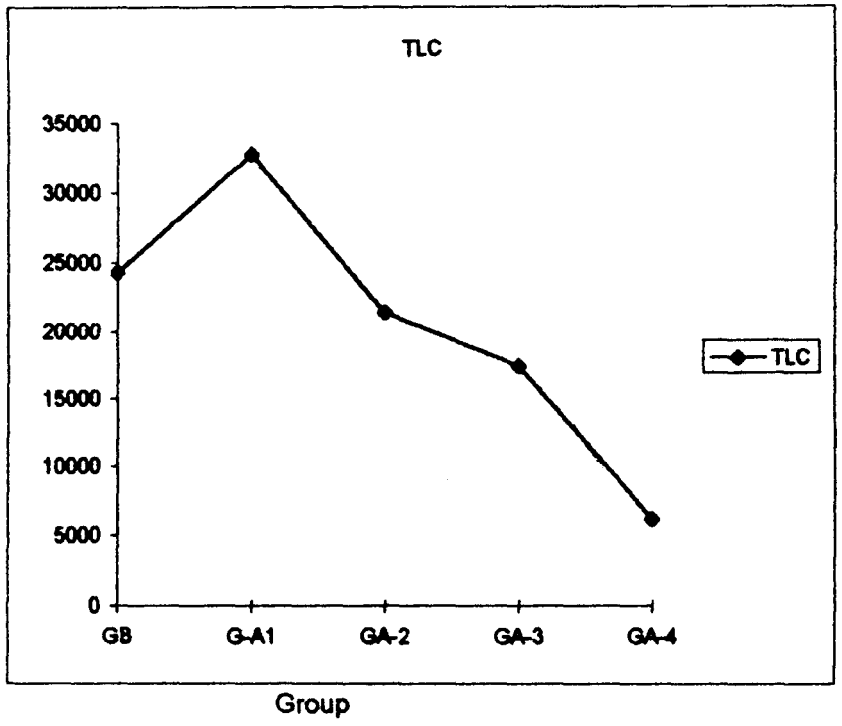
TEC = Total erythrocyte count
PCV = Packed cell volume
L = Lymphocytes
B = Basophils
TLC = Total leucocyte count
N = Neutrophils
E = Eosinophils
M = Monocytes



Graph 5.2: The mean erythrocyte count in the blood of control and experimental group of fishes



Graph 5.3: The mean packed cell volume in the blood of the control and experimental group of fishes.



Graph 5.4: The mean total leucocyte count in the blood of control and experimental group of fishes

5.3 WEIGHT OF THE LYMPHOID ORGANS

The thymus, kidney and the spleen were weighed at the time of the autopsy. The mean weight of the thymus in the experimental group was 187.14±60.9 mg whereas that of the control group was 250±37.8 mg. The mean weight of the kidneys of the control group was 255.83±118.89 mg and that of the experimental group was 147.2±56.7 mg. The mean weight of the spleen of the experimental group was 135±52.73 mg and that of the control group was 146±69.5 mg. The weight of the lymphoid organs did not reveal any significant difference between groups at 5% level by student 't' test.

5.4 ESTIMATION OF THE TOTAL SERUM PROTEINS

The total serum protein level was estimated in the control group and experimental groups of GA-1, GA-2, GA-3 and GA-4. In the control group the protein values ranged between 2.2 g% and 8.5 g%. The mean was 3.73 g%. In the experimental groups there was reduction in the protein values. In GA-1 group the total protein values were between 1.45 g% and 3.3 g%. The mean was 2.81±0.7 g%. In the GA-2 group, the total protein values varied between 1.45 g% and 4.29 g% with mean 2.42±0.68 g%. In GA-3 group the protein values ranged between 2.2 g% and the mean was 2.19±0.81 g%. In GA-4 the protein values ranged between 2.2 g% and 4.4 g% with mean 2.9±0.81 g%. (Table 5.3). The aflatoxin caused reduction in the total protein values in the fish plasma.

Table 5.3: Mean serum protein values in the experimental and control group of fishes

Group	Total protein g (%)	Albumin g (%)	Globulin g (%)	A/G Ratio
GB	3.73±2.37	2.040±0.71	1.62±1.9	2.380±1.04
GA-1	2.81±0.7	1.500±0.88	1.13±0.73	1.940±1.17
GA-2	2.42±0.68	1.372±0.66	1.04±1.18	2.570±2.35
GA-3	2.90±0.81	1.460±0.49	1.54±0.94	1.840±1.93
GA-4	2.90±0.81	1.140±0.51	1.76±0.69	1.028±0.82

Though the protein values of experimental groups were apparently lower than that of the control group the analysis of variance did not reveal any significance (Table 5.4).

Table 5.4: Analysis of variance for total serum proteins of the experimental and control group of fishes

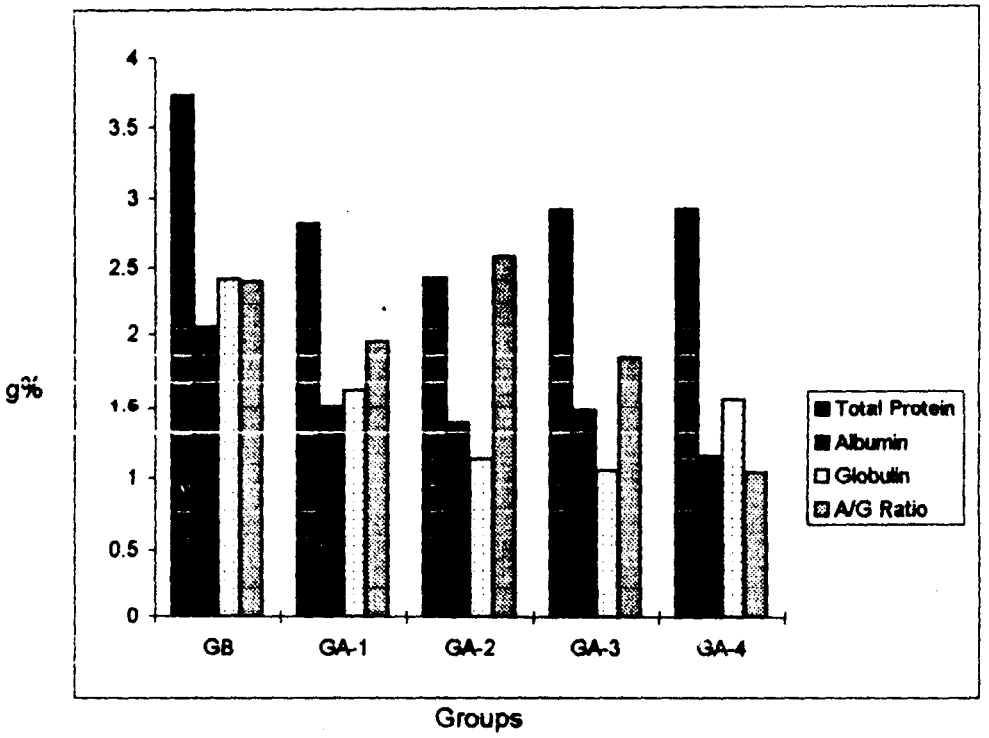
Source	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MSS)	F
Treatment	5-1	5.53	1.38	
Error	30-5	42.76	1.7	0.81
Total	30-1	48.29		

Albumin values showed a progressive reduction within the experimental groups compared to the control group GB. In GB, values varied between 1.33 g% and 3 g% with a mean of 2.04±0.7 g. The first experimental group GA-1 which received the toxin for two weeks the albumin values ranged between 0.48 g% and 2.5 g% with mean 1.68±0.88 g%. In GA-2 the values ranged from 0.7 g% to 2 g% with mean value of 1.37±0.66 g%. In GA-3 the values were between 0.86 g% and 2 g the mean was 1.5±0.49. In GA-4 which received aflatoxin for eight weeks the mean albumin value was 1.14±0.5g%. The range was between 0.7 g% and 2 g%. Thus the aflatoxin affected the albumin level of the blood. The analysis of variance revealed F value 1.137 which was not significant (Table 5.5).

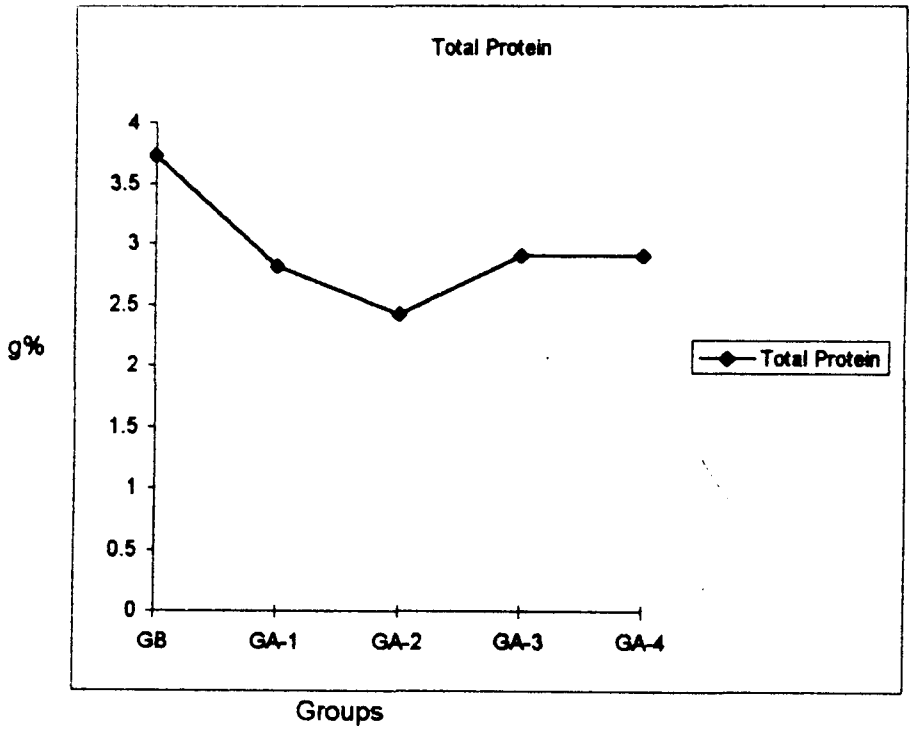
Table 5.5: Analysis of variance for albumin values in the experimental and control groups of fish

Source	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MSS)	F
Treatment	5-1	3.03	0.7575	
Error	30-5	16.64	0.6656	1.137
Total	30-1	19.67		

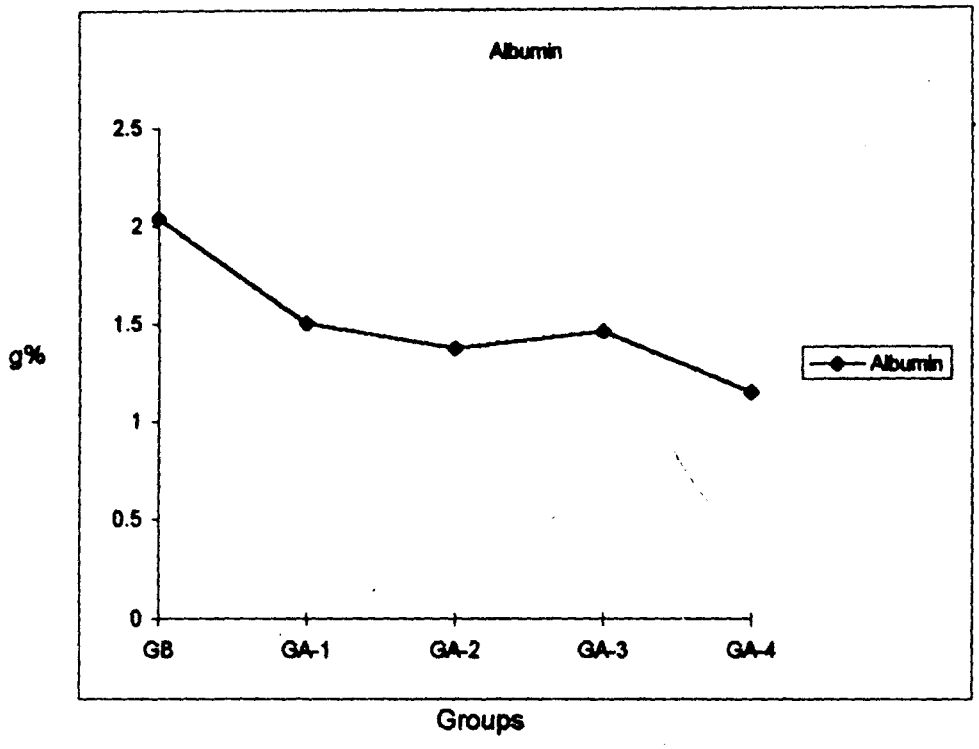
The mean globulin values of the control group was 9.7 g% while GA-1, GA-2, GA-3, GA-4 groups had 6.7 g%, 6.26 g%, 9.26 g%, 10.56 g% as



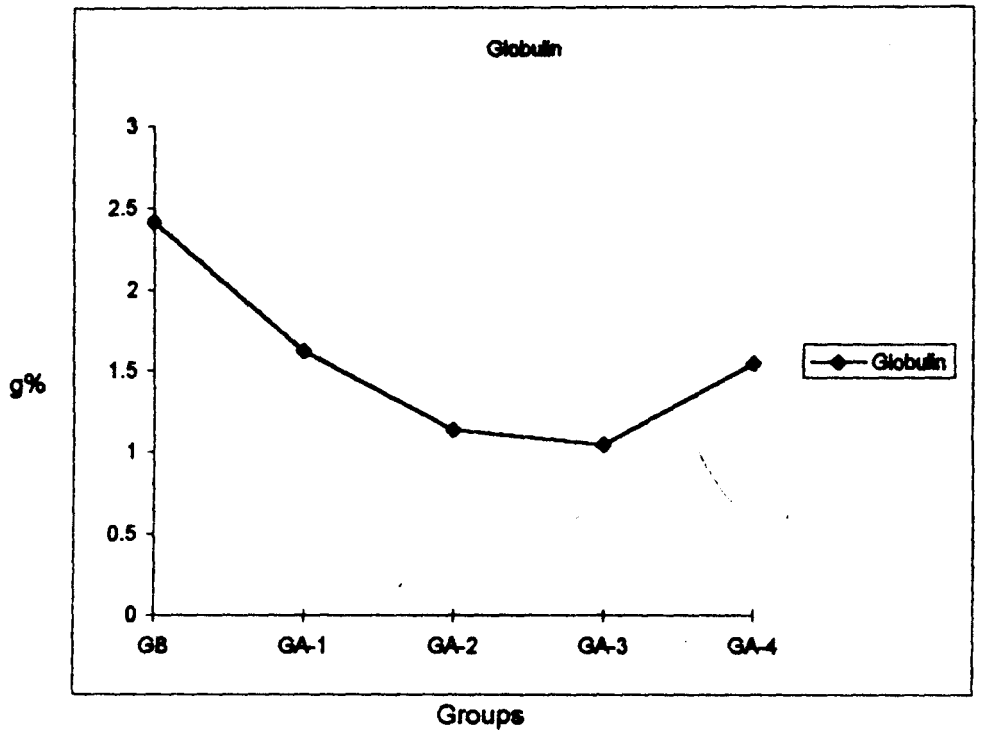
Graph 5.5: Mean serum protein, albumin, globulin and A/G ratio values in the control and experimental group of fishes



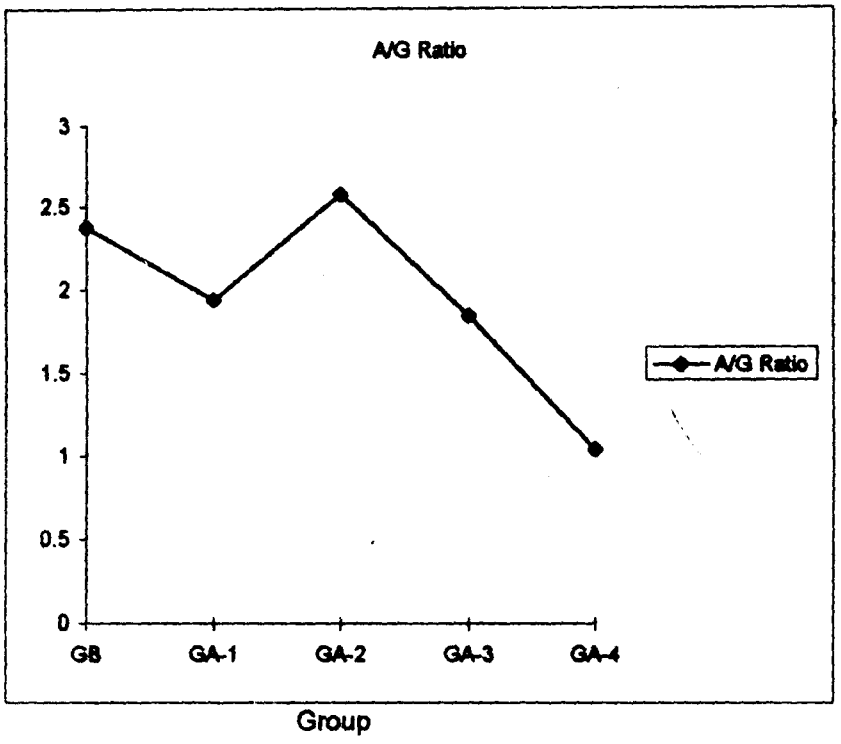
Graph 5.6: Mean total serum protein values in the control and experimental group of fishes



Graph 5.7: Mean serum albumin values in the control and experimental group of fishes



Graph 5.8: Mean serum globulin values in the control and experimental group of fishes



Graph 5.9: Mean albumin/globulin ratio in the control and experimental groups of fishes

mean values. The globulin values in the fish serum did not show any significant difference in the experimental and control group of fishes.

The mean albumin globulin ratio for GB was 2.38±1.04. The mean A/G ratio for GA-1 was 1.94±1.17. The mean A/G ratio for GA-2, GA-3 and GA-4 were 2.57±2.35, 1.840±1.95 and 1.028±0.82 respectively. Though A/G ratio showed difference between the control and experimental groups. The values were not statistically significant.

Electrophoretic picture of the different groups did not reveal much difference. However, reduction in the albumin fractions was noticed in GA-1, GA-2, GA-3 and GA-4. Electrophoretic studies also revealed fall in the serum albumin values in aflatoxin treated fishes (Fig.22). The mean values of the total protein, albumin, globulin, albumin globulin ratio are represented in Table 5.3 and Graphs 5.5, 5.6, 5.7 and 5.9.

Table 5.6: Analysis of variance for globulin values of the experimental and control groups of fish

Source	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MSS)	F
Treatment	5-1	2.39	0.6	
Error	30-5	104.45	4.18	0.14
Total	30-5	106.84		

Table 5.7: Analysis of variance–A:G ratio in the experimental and control groups of fish

Source	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MSS)	F
Treatment	5-1	8.62	2.15	
Error	30-5	70.46	2.82	0.76

5.5 PHAGOCYTIC INDEX

In the control group high level of *in vitro* phagocytosis was noticed. The mean phagocytic index for the control group was 6.22±5.6%. The cells which did not ingest any particle was 6% in this group. 27% cells had 4 to 6 particles per cell. 20% had 7 to 9 particles per cell. 7% cells ingested 10 to 12 particles per cell. 6% cells phagocytosed 13 to 15 particles per cell. Five per cent cells ingested 16 to 18 particles per cell and 2% cells had 19 to 21 particles per cell (Fig.23).

Table 5.8: Phagocytic index of the control and experimental group of fish (percentage of cells ingesting killed yeast particle and the number of yeast particles per cell)

Yeast particles/cells	Group GB (% of cells)	Group GA (% of cells)
No particles/cell	6%	17%
1 to 3 yeast/cell	27%	54%
4 to 6 yeast/cell	27%	20%
7 to 9 yeast/cell	20	8%
10 to 12 yeast/cell	7%	
13 to 15 yeast/cell	6%	
16 to 18 yeast/cell	5%	
19 to 21 yeast/cell	2%	

Compared to the control group the experimental group demonstrated very low phagocytic index. The average phagocytic index for the experimental group was 2.49±1.98. The cells which did not ingest any particles formed 17% and majority of cells had only 1 to 3 particle per cell (54%). Twenty per cent of the cells had 4 to 6 particles per cell. Nine per cent cells had 6 to 8 particles per cell. The experimental group did not have

cells ingesting more than 8 particles. The frequency distribution of cells containing particles is depicted in Table 5.8.

The student 't' test comparing the means of experimental group and control group gave a 't'-value of 1.8203 which was higher than table value of 1.684 at 5% level indicating significant difference between the two groups indicating that aflatoxin adversely affected the phagocytic ability of fish phagocytes.

5.6 INTRADERMAL PHYTOHAEMOAGGLUTINNIN-M SENSITIVITY TEST

Intradermal injection of PHA-M in the caudal peduncle was performed on the 50th day of the experiment. Initial thickness of the skin in the control group varied between 0.344 cm to 0.6 cm. The mean skin thickness was 0.47±0.113. After 24 hours the skin thickness of the control group of fishes ranged between 0.4 cm and 0.7 cm and the mean thickness was 0.54±0.1 cm. After 48 hours the thickness further increased. It ranged between 0.46 cm and 1 cm with mean 0.7314±0.25.

In the experimental groups the skin thickness before the intradermal injection was between 0.3 cm and 0.6 cm. The mean thickness was 0.42±0.11 cm. After 24 hours the skin thickness varied from 0.47 cm to 0.69 cm. The mean thickness was 0.53±0.09 cm. After 48 hours skin thickness

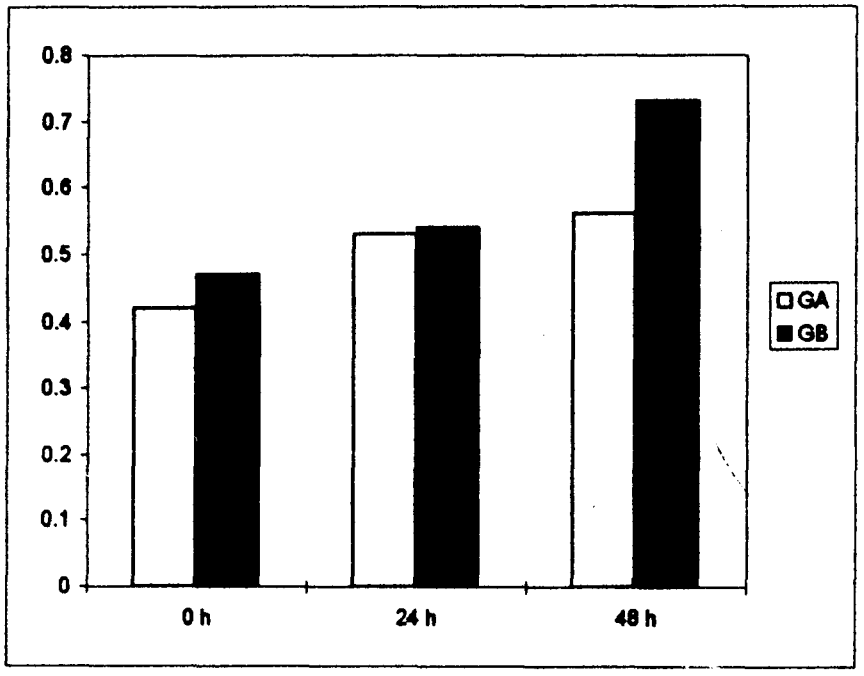
ranged from 0.4 cm to 0.77 cm. The mean thickness was 0.56±0.1416 cm (Table 5.9 and Graph 5.10).

Table 5.9: Mean thickness of caudal peduncle in the experimental and control group of fishes before and after intra dermal injection of PHA-M

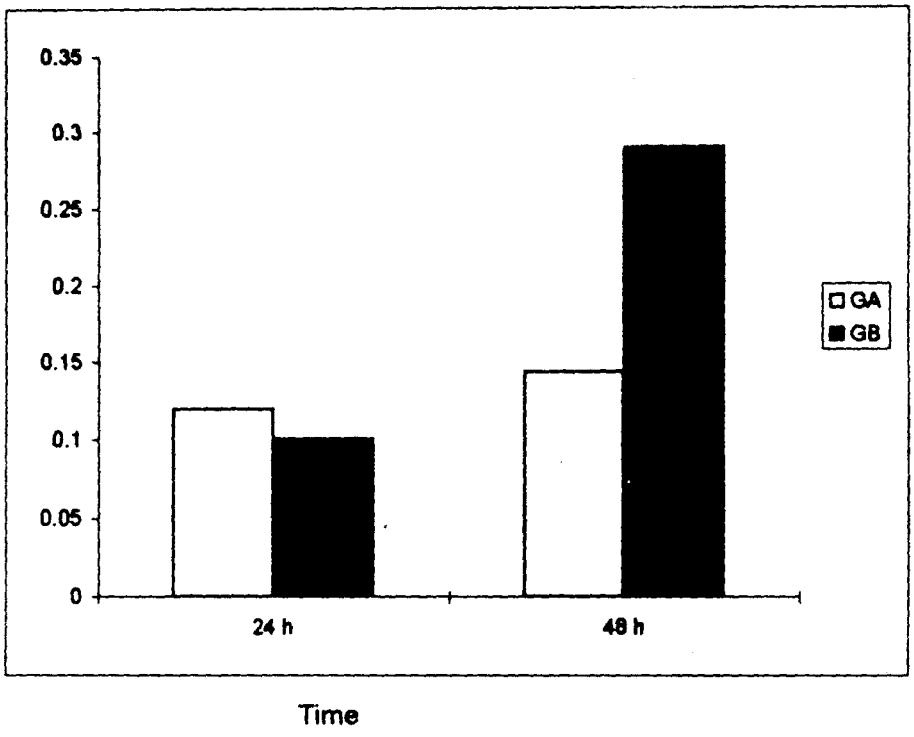
Groups	0 h	24 h	48 h
GA (Experimental group)	0.42±0.11	0.53±0.09	0.56±0.1416
GB (Control group)	0.47±0.11	0.54±0.1	0.7314±0.25

In the control group, increase in thickness in 24 hours was between 0.01 cm and 0.16 cm with mean increase of 0.1±0.456 cm. In the experimental group the increase in thickness ranged from 0.08 cm to 0.17 cm with mean 0.12±0.003 cm.

After 48 hours increase in skin thickness was more pronounced in the control group. The increase was between 0.05 cm and 0.56 cm over the initial skin thickness. The mean was 0.289±0.16. In the experimental group increase in skin thickness after 48 hours ranged from 0.03 to 0.2 cm with mean 0.143±0.07 cm.



Graph 5.10: **Mean thickness of caudal peduncle in the experimental and control group of fishes before and after intra dermal injection of PHA-M**



Graph 5.11: Mean increase in the skin thickness in the control and experimental group of fishes after intra dermal injection of PHA-M

Though the 48 hours skin thickness of the experimental group was lower than the control group the mean between the two group did not show any significant difference by the student 't' test. Increase in skin thickness in 24 and 48 hours of different groups is represented in the Table 5.10 and Graph 5.4. The reduced skin response to PHA-M in aflatoxin treated fishes indicated suppression of CMI response by aflatoxin in these fishes.

Table 5.10: Mean increase in skin thickness in the control and experimental group of fishes after intra dermal injection of PHA-M

Groups	24 h after injection	48 h after injection
GA (Experimental)	0.12± 0.003	0.143±0.07
GB (Control)	0.1±0.0456	0.289±0.16

5.7 LEUCOCYTE MIGRATION INHIBITION TEST

Leukocyte migration inhibition test revealed that leucocyte migration inhibition index ranged between 0.5 to 0.54 in the control group with mean 0.53±0.012. In the experimental groups the leucocyte migration inhibition index ranged between 0.51 and 0.58 with mean 0.53±0.25. There was no significant difference between the two groups.

5.8 IMMUNE RESPONSE IN THE EXPERIMENTAL AND CONTROL GROUPS OF FISH

The fishes of both groups were immunised against sheep RBC and the response was assessed by testing their serum for haemagglutination using serial dilutions of sera from each fish. In the control group fish sera gave haemagglutination from 128 to 512 (1:128 to 1:512) four fishes gave haemagglutination titre of 128 (1:128) another four gave 256 (1:256) and rest gave a titre of 512 (1:512).

In the experimental groups the titre did not cross 32. Four fishes gave titre of 4 (1:4) two had given 8 (1:8), another two had given 16 (1:16) and four had given 32 (1:32). Thus the experimental group of fishes revealed suppression of humoral immune response.

The haemagglutination titres are depicted in Table 5.11.

Table 5.11: Humoral immune response of fishes to sheep RBC

Titre	GB No.of animals gave the titre	GB No. of animals gave the titre
512	4	Nil
256	4	Nil
128	4	Nil
64	--	Nil
32	--	4
16	--	2
8	--	2
4	--	4

5.9 HISTOPATHOLOGICAL STUDIES

5.9.1 Liver

The GA-I group which received aflatoxin containing feed for two weeks exhibited mild changes. Hepatic parenchyma showed focal areas of necrosis accompanied by areas fatty change. In many areas the stromal connective tissue became more prominent.

In GA-2 the changes were more advanced. Focal areas of coagulative necrosis of the hepatocytes and hepatocytes undergoing fatty change were evident. The stromal tissue became more prominent and hypertrophic in many areas.

In GA-3 group which received aflatoxin for six weeks, the liver changes were severe. The necrosis of hepatocytes was advanced and extensive. The liver contained large number of macrophages containing ceroid. Diffuse infiltration of leucocytes was noticed. Parenchyma revealed congestion and haemorrhages. The stroma became very prominent with abundant fibrous tissue.

Liver sections of GA-4 exhibited severe necrosis of the hepatocytes. The apotosis of the hepatocytes leading to formation of hyalinized bodies were apparent. Focal areas of hyperplasia of the hepatocytes or diffuse areas of hyperplasia were noticed in some of the sections. Large number of

macrophages containing ceroid were seen in the liver. Diffuse infiltration of leococytes was also seen in the liver (Fig.24. 25 and 26).

5.9.2 Thymus

In GA-1, GA-2, GA-3 and GA-4, the thymus revealed only sparse lymphoid cells and extensive adipose tissue (Fig.27). There was no histological changes in the control group.

5.9.3 Spleen

In GA-1, the spleen structure did not differ significantly from that of the control group. In GA-2 there was increase in the number and size of the melanomacrophage centres. In GA-3 and GA-4, the spleen revealed reduction in the haemopoietic tissue. The stromal tissue became more prominent, focal necrosis of lymphoid tissue was seen in many areas (Fig.28).

5.9.4 Kidney

The control group kidney consisted of haemopoietic tissue, endocrine tissue and excretory tissue. The anterior lobe exclusively consisted of haemopoietic tissue. The reticular fibre network supported proliferating stem cells giving rise to erythroblast, myeloblasts, monocytes and lymphocytes. This tissue was anastamosed with a network of blood

sinusoids lined incompletely by endothelial cells. There were islands of adrenal cortical epithelial cells and medullary neuroendocrine cells.

The posterior lobe contained the renal tissue, Bowman's capsule containing capillary tufts forming glomeruli and the interstitial haemopoietic tissue. The proximal convoluted tubules with epithelial cells, indistinct cell boundaries, and the distal convoluted tubules with less eosinophilic columnar cells were also seen.. The Henles loop was absent. The interstitial tissue was rich in haemopoietic tissue. The haemopoietic tissue in the anterior and posterior lobe contained pigment bearing macrophage accumulating centres; melanomacrophage centres.

In GA-1 group, kidney exhibited focal necrosis in the haemopoietic tissue and mild cellular degeneration in the proximal convoluted tubules characterised by epithelial swelling and loss of apical striations.

In GA-2 the proximal convoluted tubules exhibited further degenerative changes characterised by eosinophilic droplet formations. Many glomeruli showed increased cellularity and congestion of the glomeruli.

In GA-3 the haemopoietic tissue revealed areas of necrosis. The interstitial tissue was sparse in many sections. Both proximal convoluted

tubules and the distal convoluted tubules revealed degenerative change of epithelial cells characterised by swelling of epithelial cells, granularity of the cytoplasm, vacuoles in the epithelial cells and desquamation of cells. The glomeruli revealed hypercellularity, and in the Bowman's capsule parietal epithellium showed proliferation.

In GA-4 the tubular epithelial necrosis was extensive and the tubules contained hyaline casts. Interstitial cells became rarefied, haemopoietic tissue revealed areas of necrosis. Glomerular capillary thickening was apparent in many glomeruli. Some of the glomeruli were shrunken and sclerotic. Periglomerular fibrosis and epithelial crescent in the Bowman's capsules were seen. (Fig.29 and 30).

5.9.5 Heart

The control group hearts had no significant change. In the GA-1 and GA-2 groups, the heart did not show much significant variations from the control group. In GA-3 and GA-4 groups cardiac muscle fibres had vacuolation and accumulation of mononuclear cells in the epicardium.

5.9.6 Brain

The brain in GA-1 and GA-2 did not show much difference when compared to the control group; whereas in groups GA-3 and GA-4 areas of

gliosis, neuronophagia, satellitosis and areas of encephalomalacia were seen.

5.9.7 Skin

Fishes were killed at 24 and 48 hours after intradermal injection of PHA-M at the caudal peduncle. Pieces of tissues from the area of caudal peduncle were fixed in 10% buffered formol saline and processed for histopathological studies.

The skin tissues from the control group revealed moderate infiltration of lymphocytes and necrotic changes in the Malpghian cells at 24 hours. At 48 hours extensive infiltration of lymphocytes was seen.

In the experimental group skin section at 24 hours revealed fibrinous exudate and mild infiltration of lymphocytes. At 48 hours diffuse inflammatory reaction with fibrinous exudate and moderate infiltration of lymphocyte was noticed. However, the histological reactions were much reduced compared to the control group skin.

5.10 ULTRA STRUCTURAL STUDIES

5.10.1 Liver

In the control group the hepatocytes had rich profile of rough endoplasmic reticulum (RER). Peripheral to the RER, there were numerous

rod and round shaped mitochondria with numerous cristae and granules. The peripheral area of the cytoplasm had stacks of smooth endoplasmic reticulum (SER). Cytoplasm had numerous electronlucent bags and a few number of liposomes. Hepatocytes had well developed stack of Glogi apparatus. The endothelial cells lining the sinusoid had irregular nucleus and were elongated. The nucleus of the hepatocytes appeared spherical with abundant euchromatin and well developed nucleolus. There were free ribosomes in the cytoplasm of the hepatocytes. Hepatocytes had microvilli projecting into the space of Disse.

In the experimental group of GA-1 and GA-2, the hepatocyte nucleus became irregular in shape. In some of the cells heterochromatin appeared in the nucleus. The nucelus contained chromatin granules and perichromatin granules. The mitochondria appeared swollen and lost their structure, cristae had disappeared. Some of the mitochondria contained electron dense material. There was increase in the profile of SER and numerous electron lucent spherical structures with dense core probably peroxisomes appeared. The RER lost many ribosomes and large number of liposomes were visible in the cytoplasm.

In GA-3 and GA-4 hepatocytes organelles lost their structure. Complete degranulation of RER occurred. The ER developed large dilations and vesicles. Duplication of ER and nuclear envelope were seen in many of

the hepatocytes. The vesicles contained electron dense flocculant material (Fig.31, 32, 33 and 34).

5.10.2 Thymus

The stroma of the thymus in the control group contained islands of cells resembling lymphoblasts which had spherical nucleus and cytoplasm had some electron dense granules. The other cells in the thymic stroma had large number of liposomes in the cytoplasm and had dilated endoplasmic reticulum. The nucleus of the lymphoblasts had numerous heterochromatin clumps.

In the treatment groups, the endoplasmic reticulum made invaginations into the nucleus and the nuclear membranes were duplicated. Endoplasmic reticulum developed dilatation leading to vesiculation of cytoplasm. The cisternae of the endoplasmic reticulum contained electron dense material. The nuclear chromatin appeared lost and the nuclear membranes were discontinuous (Fig.35 and 36).

5.10.3 Kidney

In the control group, kidneys had both excretory and haemopoietic tissues. The proximal convoluted tubular epithelial cells had elongated appearance with apical microvilli. Below the microvilli a thick coat of glycocalyx could be seen. Numerous vacoules or vesicles were seen in the

apical portion of the cell. The cell showed the basal infolding and rich profile of RER. Numerous rod shaped mitochondria were also seen in the basal part of the cell.

The distal section of the proximal convoluted tubules showed cells with numerous basal tubular infolding which were continuous with the RER. Numerous mitochondria were seen in between the infoldings. The nucleus of the cell was having prominent nucleolus.

Intertubular area was occupied by cells having different morphology. Many were having in their cytoplasm numerous granules. The nucleus of these cells were having large amount of heterochromatin. Some of the cells contained granules having crystalloid core and other cells had abundant cytoplasm with scanty granules. Some of the cells were having scanty cytoplasm.

In the GA-1 and GA-2, there was no significant deviation from the control group. In some of the tubular epithelial cells there was dilatation of the endoplasmic reticulum. In the interstitial cells, some showed appearance of vesicles and increased heterochromatin clumps in the nucleus. In the GA-3 and GA-4 separation of desmosomes and loss of microvilli were evident in the epithelial cells. The RER lost ribosomes and dilated forming large vesicles. Endoplasmic reticulum had undergone

reduplicaton in some areas. Condensation of the mitochondrial inner compartment with relative expansion of intracristal spaces were seen. Some of the mitochondria were swollen and contained amorphous densities. The nuclear membrane revealed discontinuities and dilatation.

Interstitial cells contained myelin bodies and numerous multivesicular bodies. Mitochondria revealed condensations. Lysosomes contained electron dense amorphous material (Fig.36, 37, 38, 39 and 40).

5.10.4 Spleen

In the control, the GA-1 and GA-2 groups contained more or less the same type of cells. This cells were of different morphology. Many cells had abundant cytoplasmic granules and these cells had nucleus with large amount of heterochromatin. A large number of cells showed lymphoblast morphology described earlier.

In the GA-3 and GA-4 the cells showed numerous multivesicular bodies, the mitochondria revealed condensation. The lyosomes contained electron dense amorphous materials. Many cells developed cytoplasmic vesicles and nucleus contained condensed chromatin.

Fig.18: Haemorrhages on the skin of aflatoxin treated fish (*Labeo rohita*).

Fig.19: Liver of the aflatoxin treated fish. Note the pale patches along with brownish red colour.

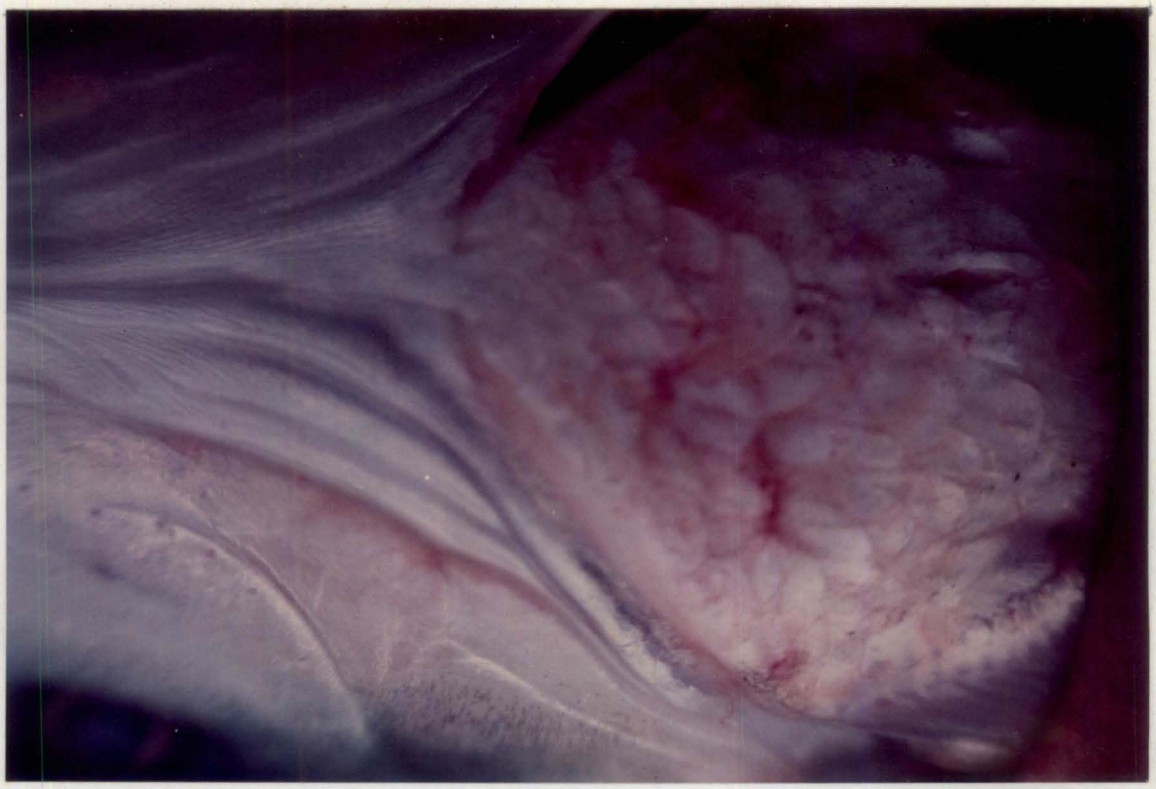
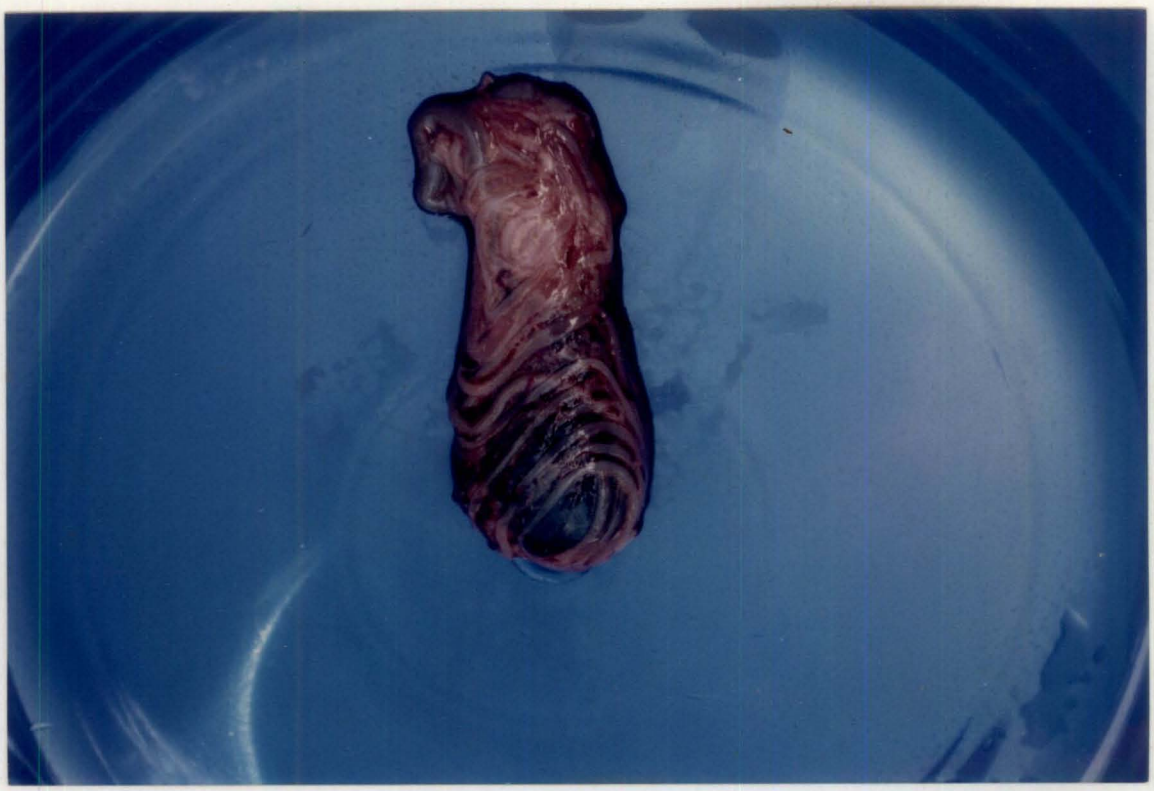


Fig.20: Abdominal viscera from the aflatoxin treated fish. Note the haemorrhages on the peritoneum and enlargement of the gall bladder.

Fig.21: Abdominal viscera from the aflatoxin treated fish showing extensive enlargement of the gall bladder.



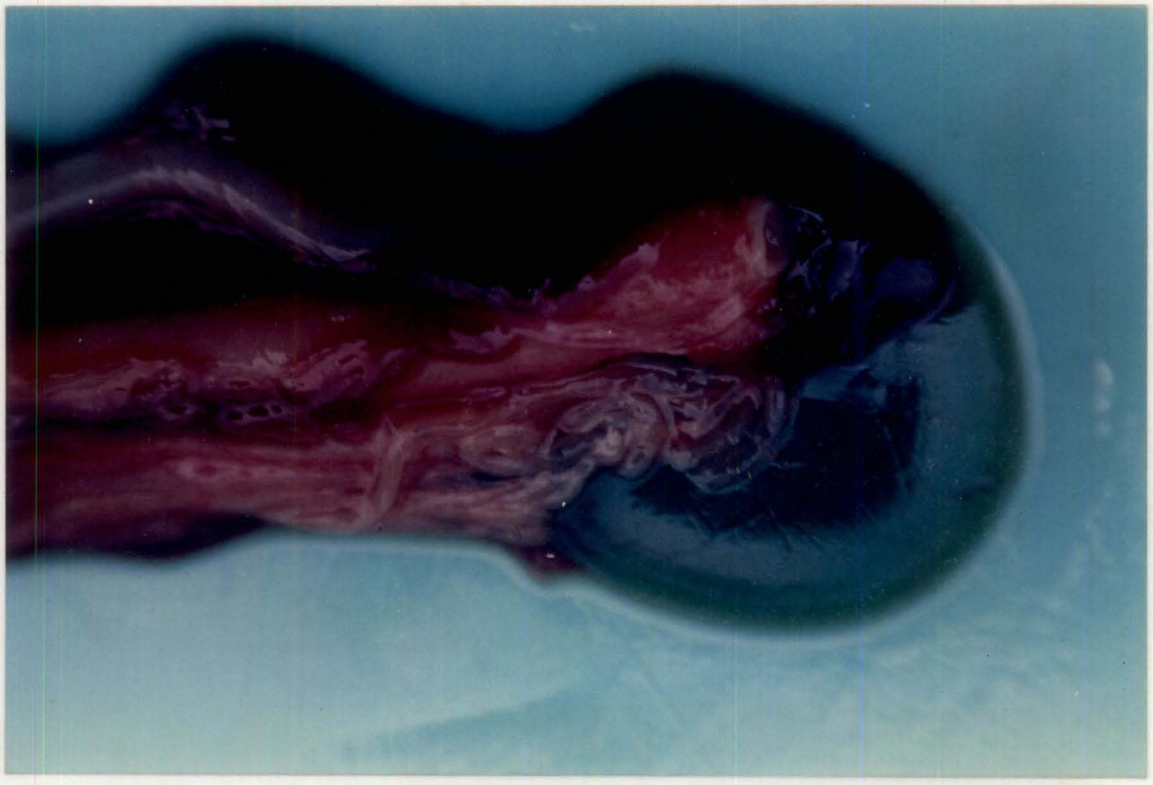
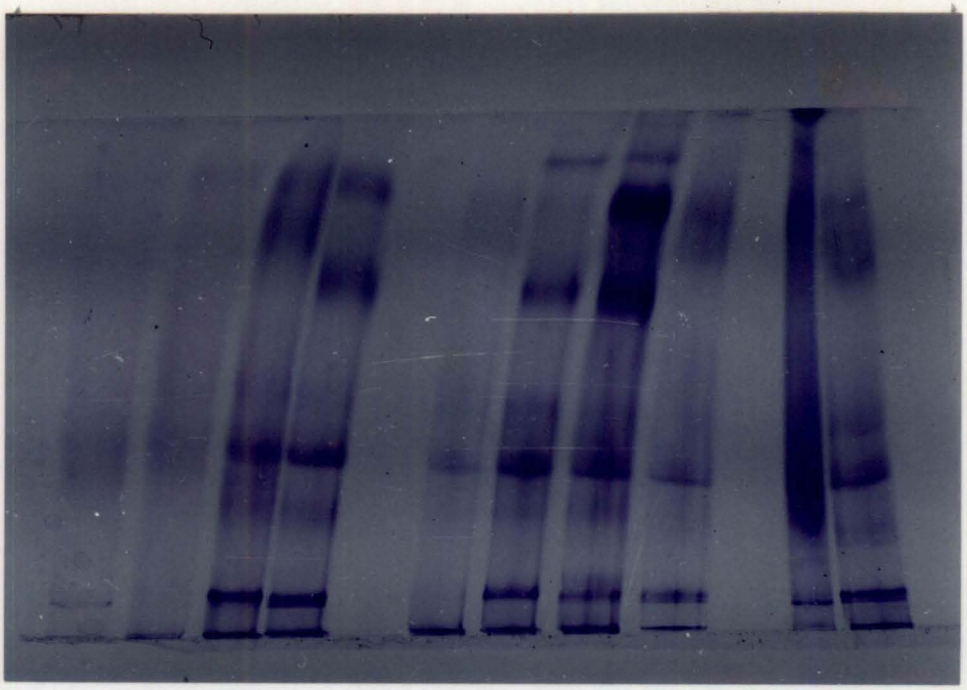


Fig.22: Electrophoretic pattern of the serum proteins in the aflatoxin treated fishes. Note the reduction in the albumin fraction in majority of the cases.

Fig.23: A fish macrophage with the ingested yeast particles. (May Grunwald Giemsa 100x).



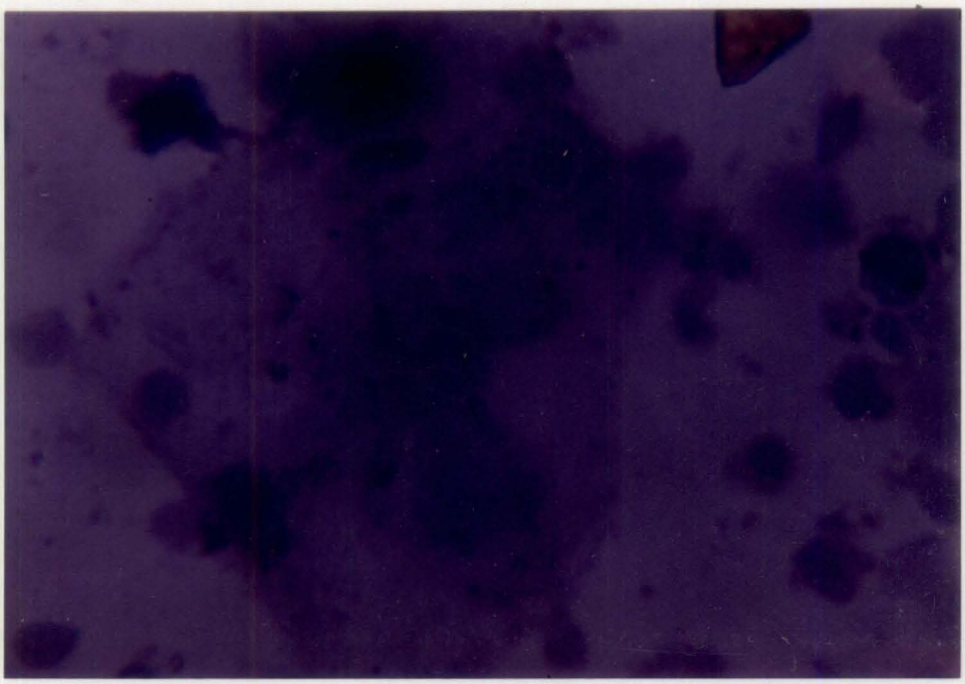
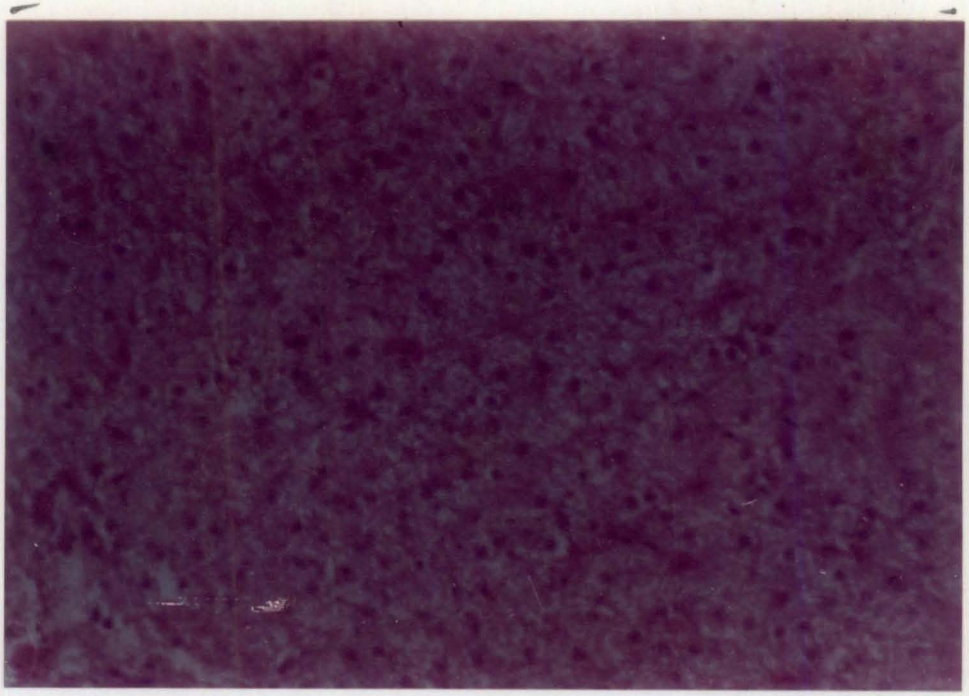


Fig.24: Section of the liver from the aflatoxin treated fish depicting hepatosis. (H&E 20X).

Fig.25: Section of the liver from the aflatoxin treated fish showing extensive hepatosis. (H&E 20x).

27 x 28



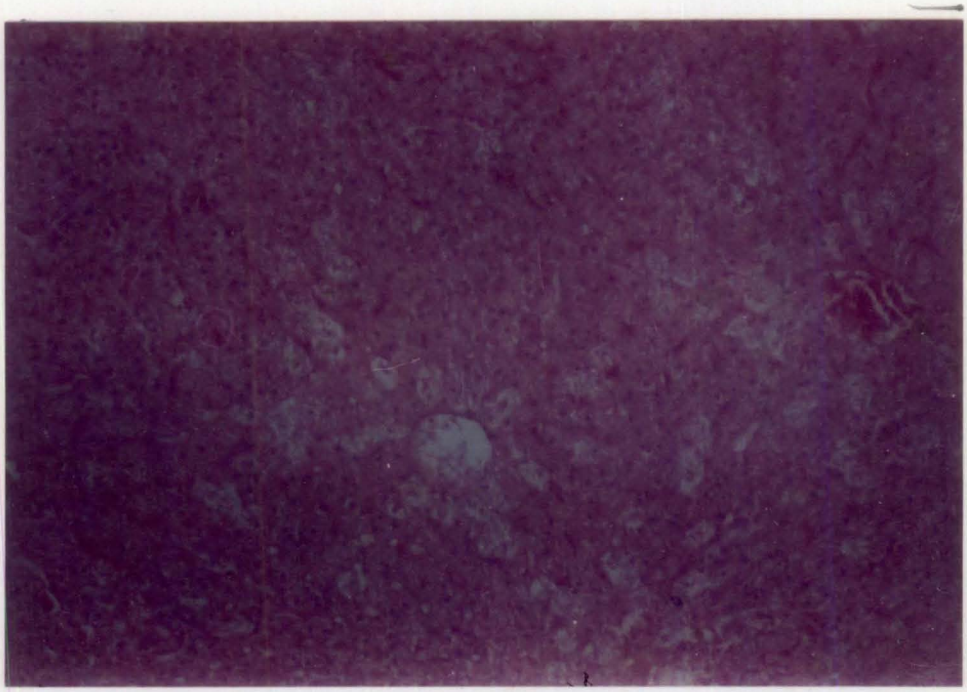
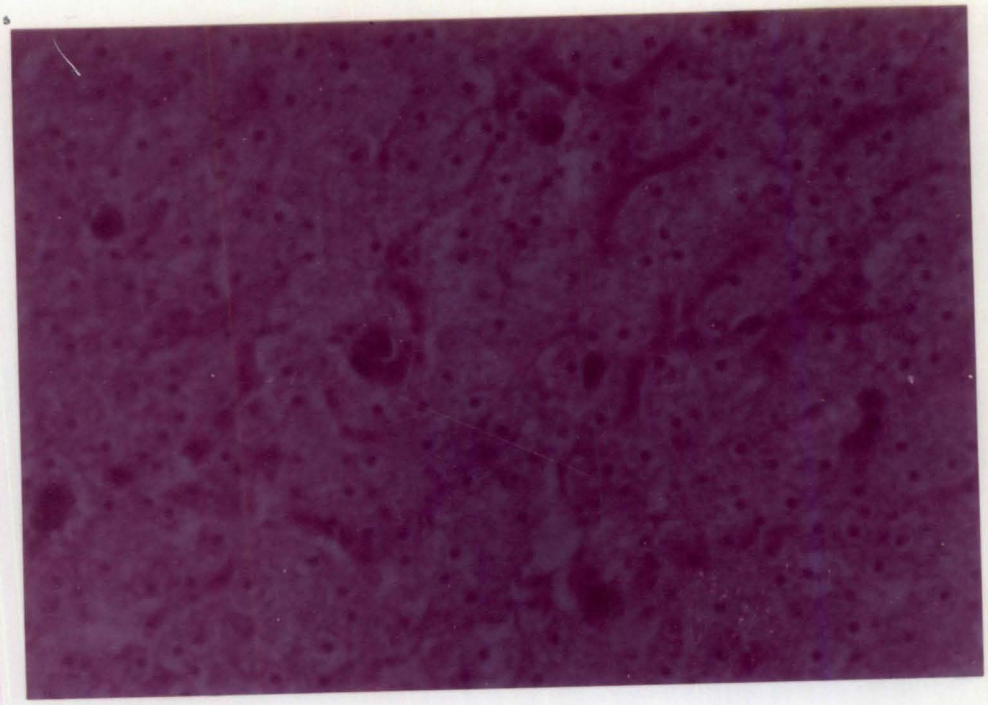


Fig.26: Section of the liver from the aflatoxin treated fish depicting proliferation of the stromal connective tissue (H&E 20X).

Fig.27: Section of the thymus from the aflatoxin treated fish depicting depletion of lymphocytes. (H&E 20X).

29830



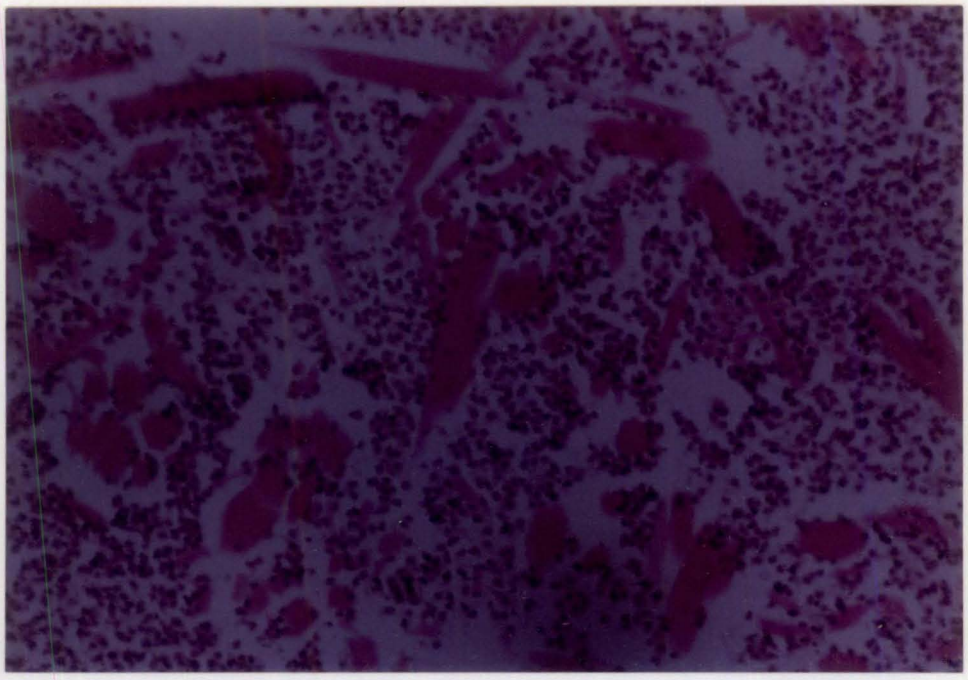
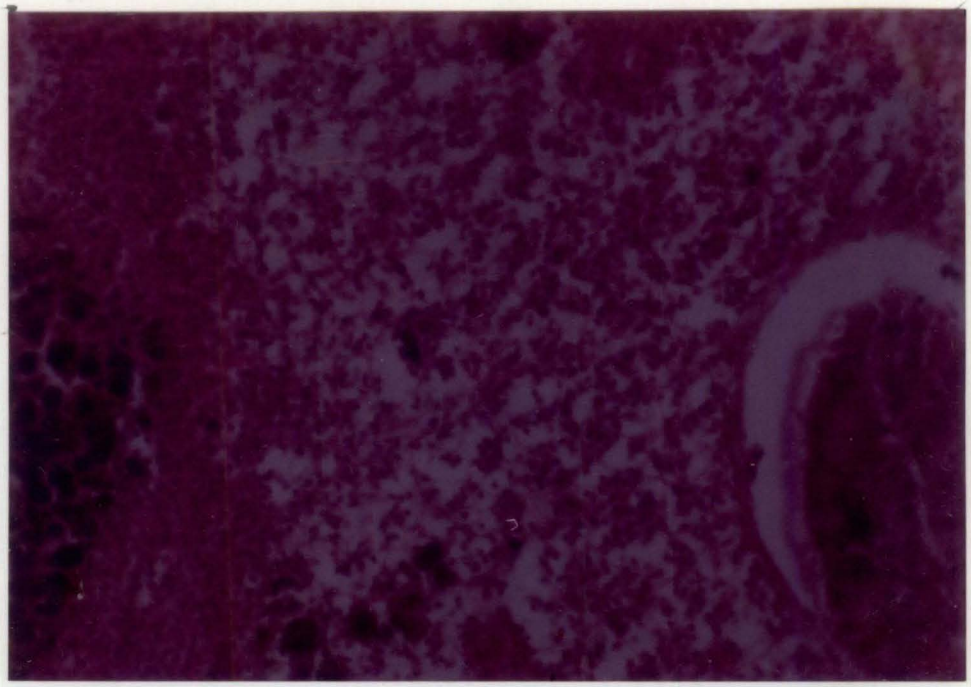


Fig.28: Section of the spleen from the aflatoxin treated fish depicting disruption of ellipsoids and depletion of cells in the parenchymal haemopoietic tissue (H&E 20X).

Fig.29: Section of the kidney from the aflatoxin treated fish showing depletion of the interstitial haemopoietic tissue (H&E 20X).

81832



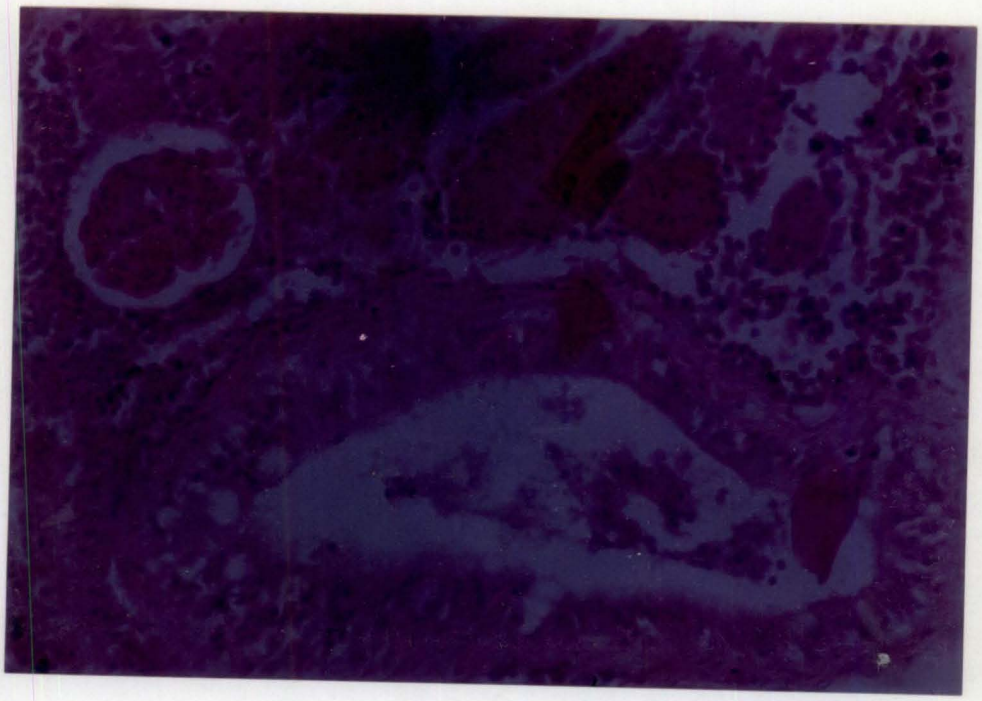


Fig.30: Section of the kidney from the aflatoxin treated fish depicting depletion of the haemopoietic tissue and nephosis of tubules (H&E 20X).

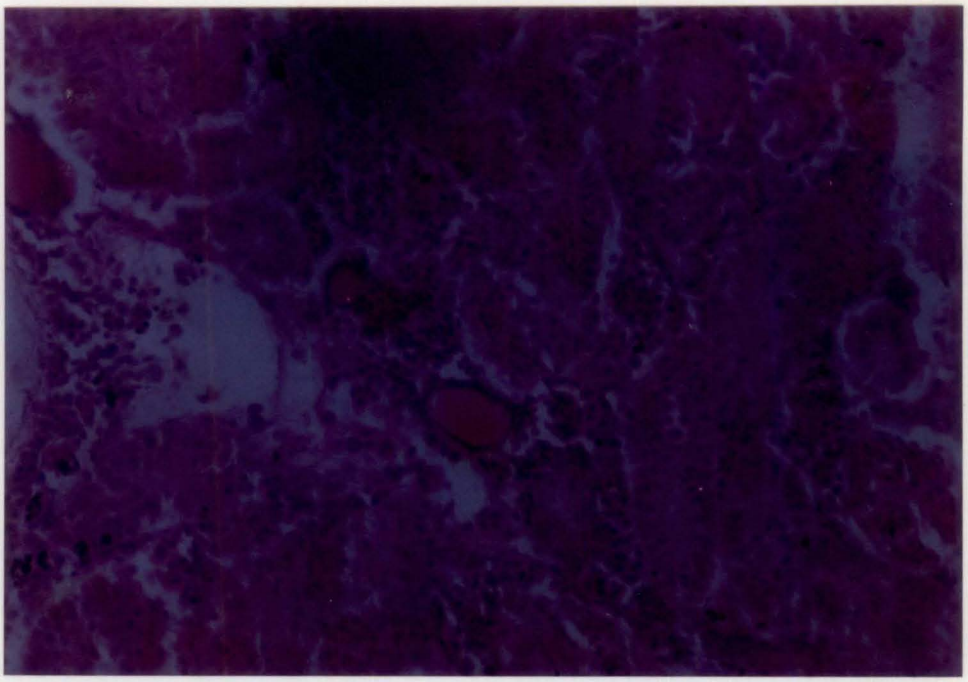


Fig.31: Electron micrograph of the liver from the fish treated with aflatoxin for two weeks. Note the proliferated and dilated endoplasmic reticulum and microvilli (15000X).

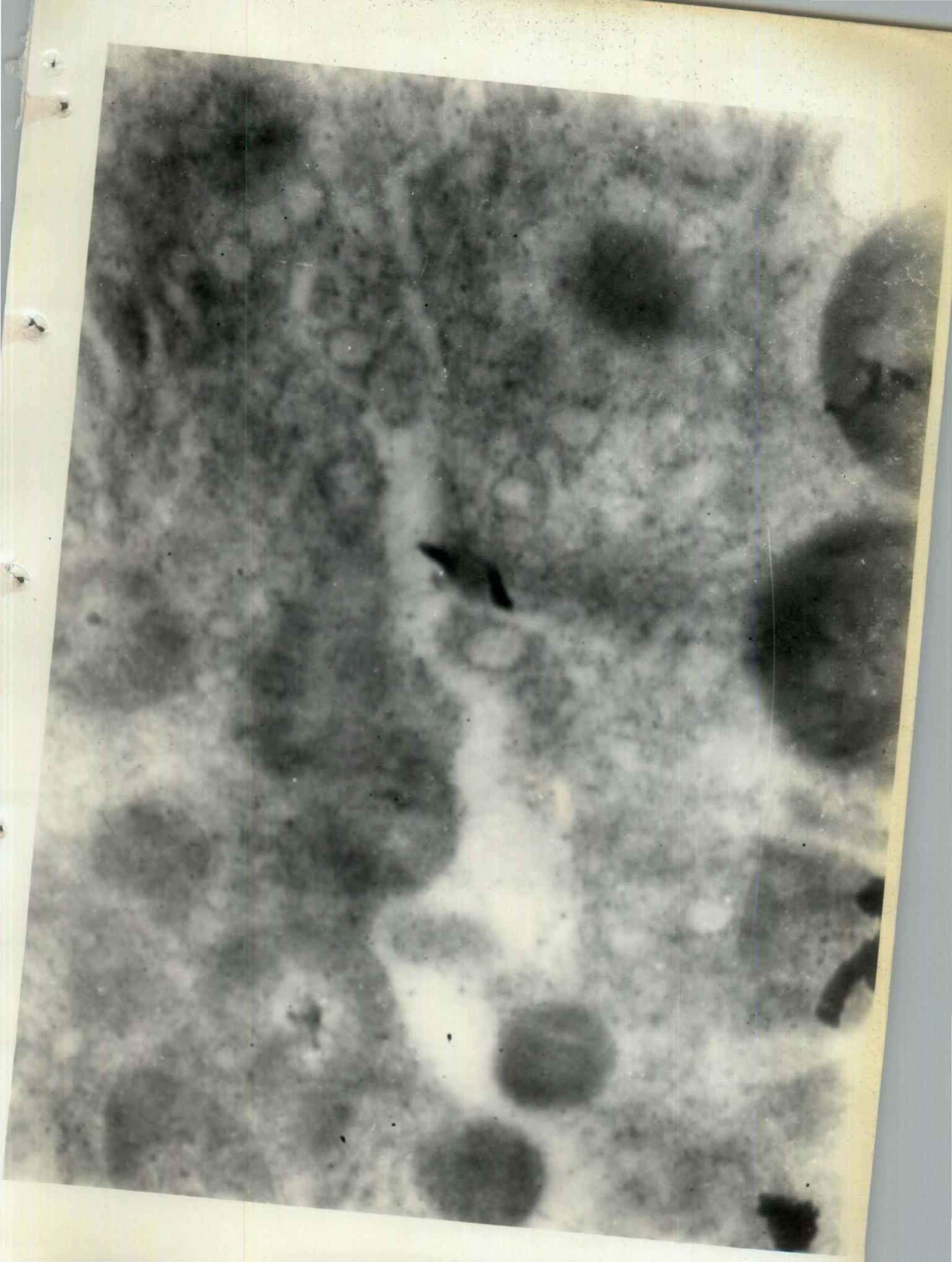


Fig.32: Electron micrograph of the liver from fish treated with the aflatoxin for two weeks. Note the irregular nuclei in hepatocytes (5000X).

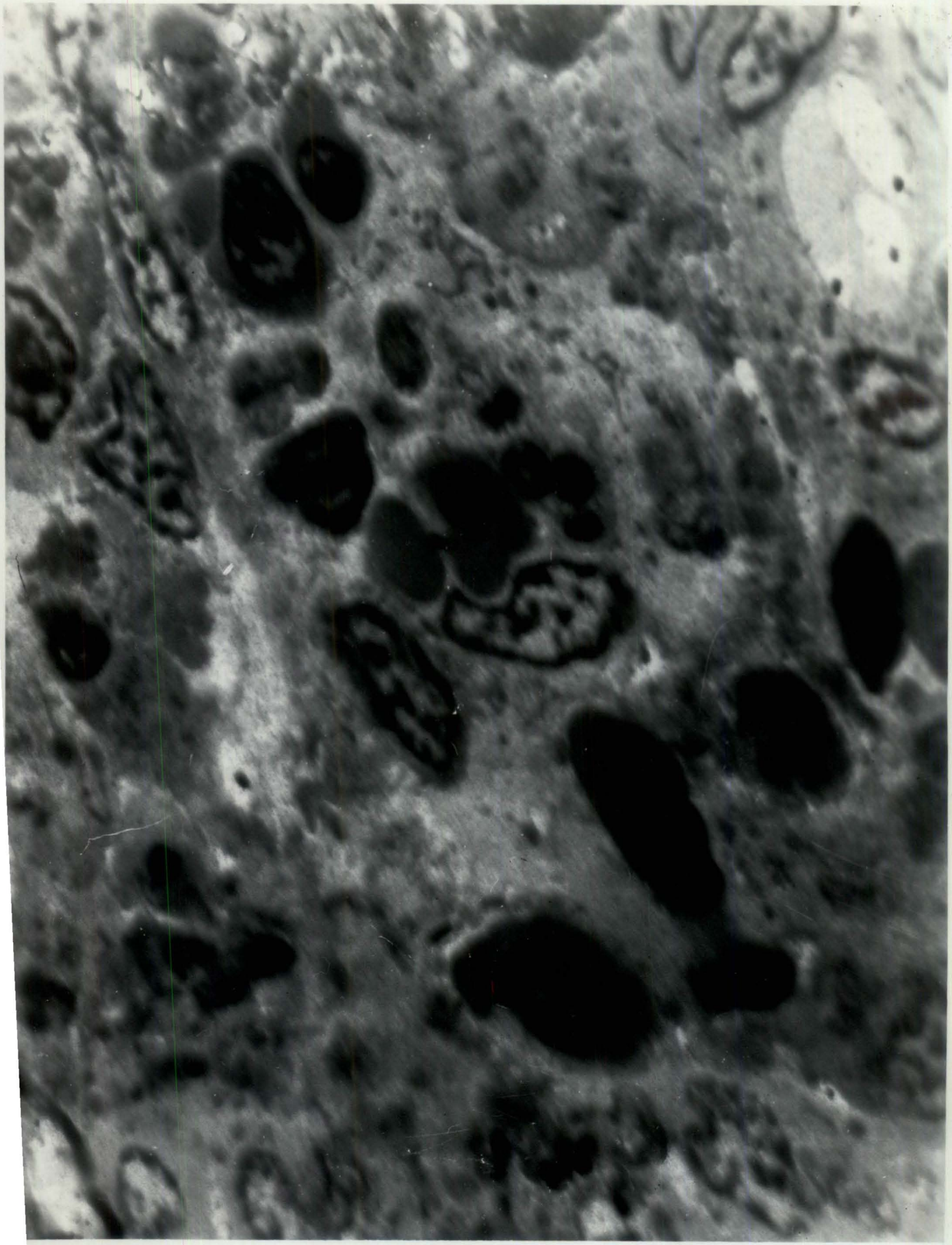


Fig.33: Electron micrograph of the liver from the aflatoxin treated fish showing loss of architecture of the hepatocytes. Note the intact erythrocyte (6000X).

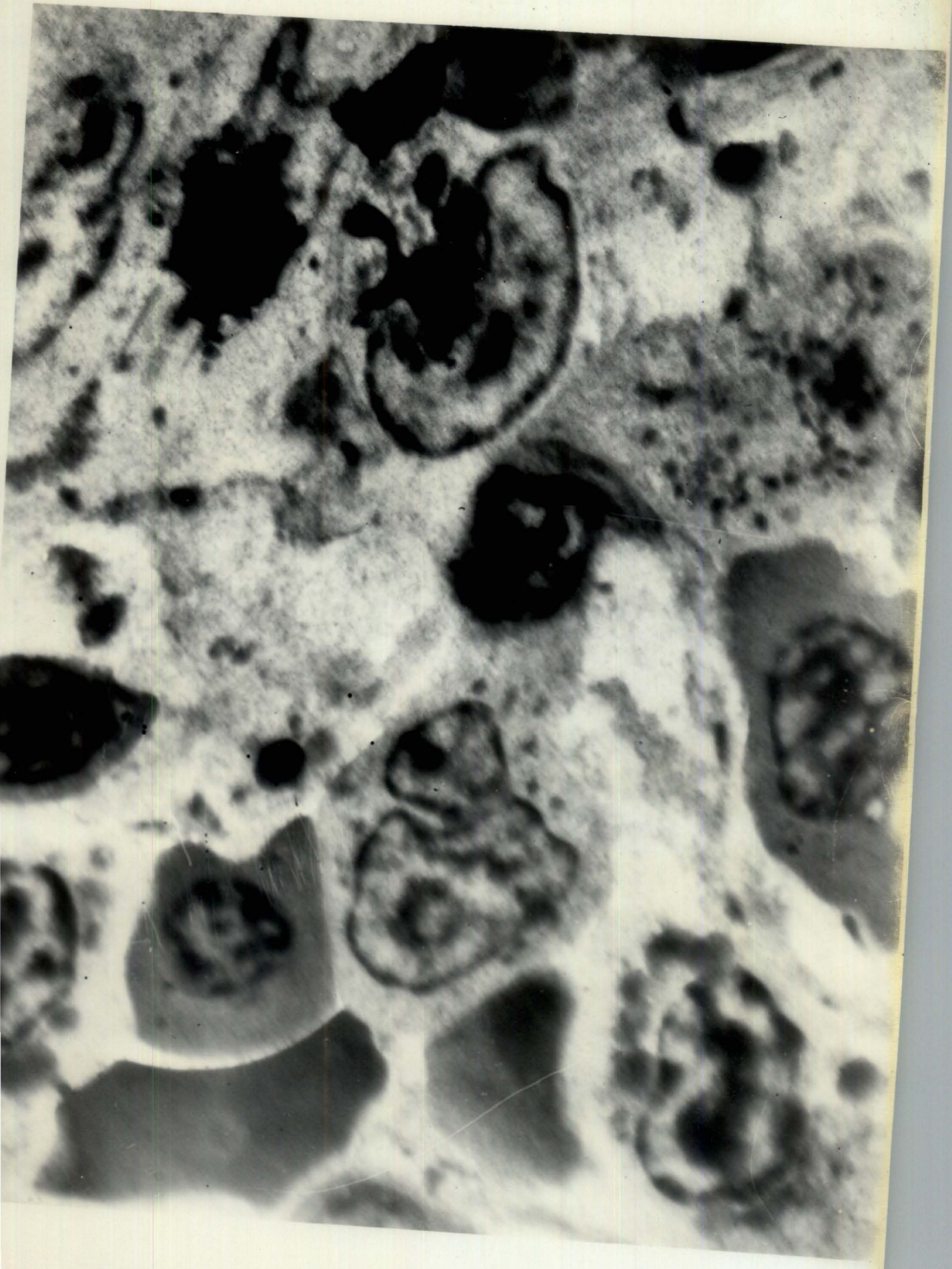


Fig.34: Electron micrograph of the liver from the aflatoxin treated fish showing accumulation of liposome in the hepatocytes (6000X).

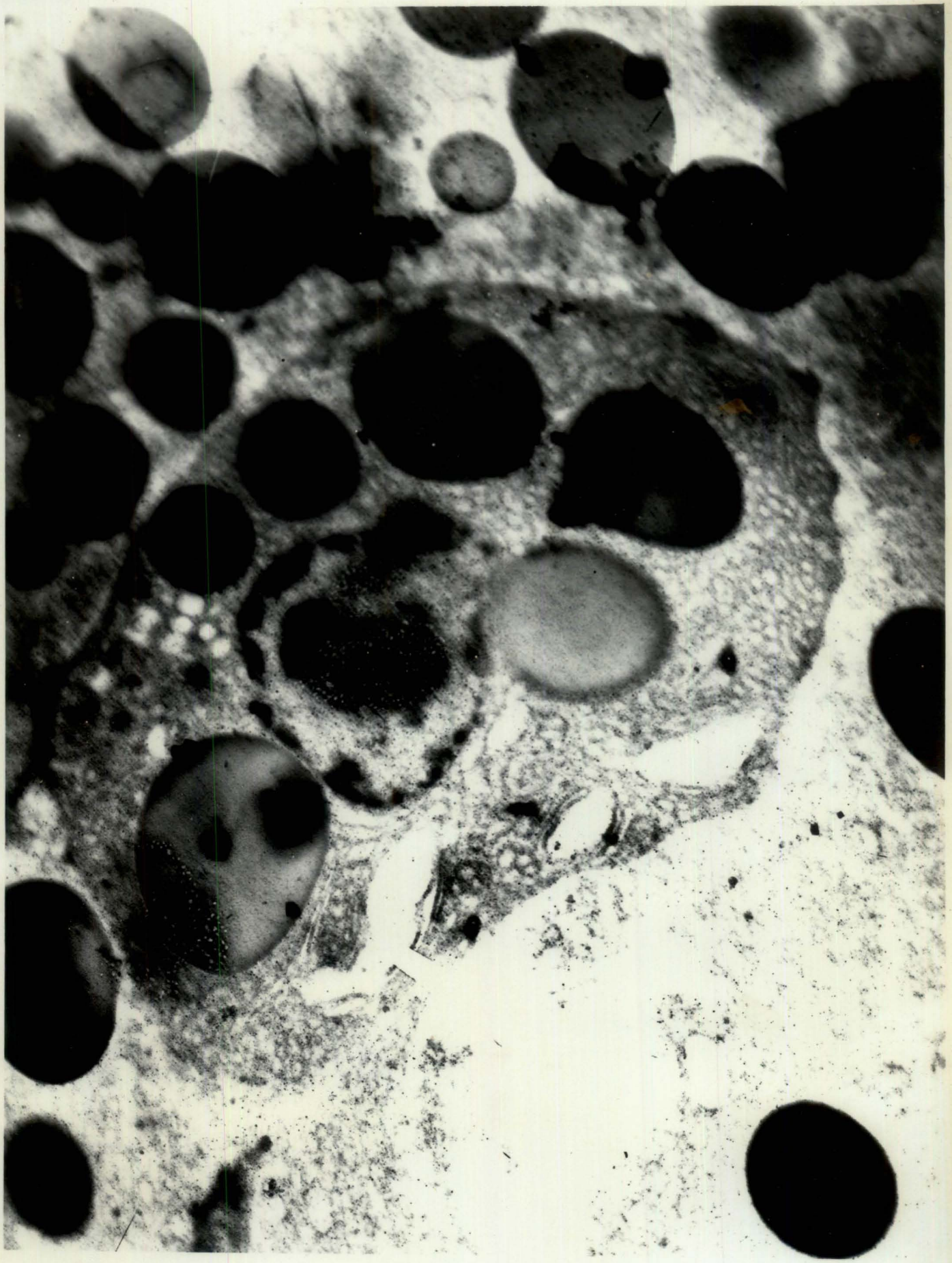


Fig.35: Electron micrograph of the thymus from fish treated with aflatoxin for two weeks (6000X).

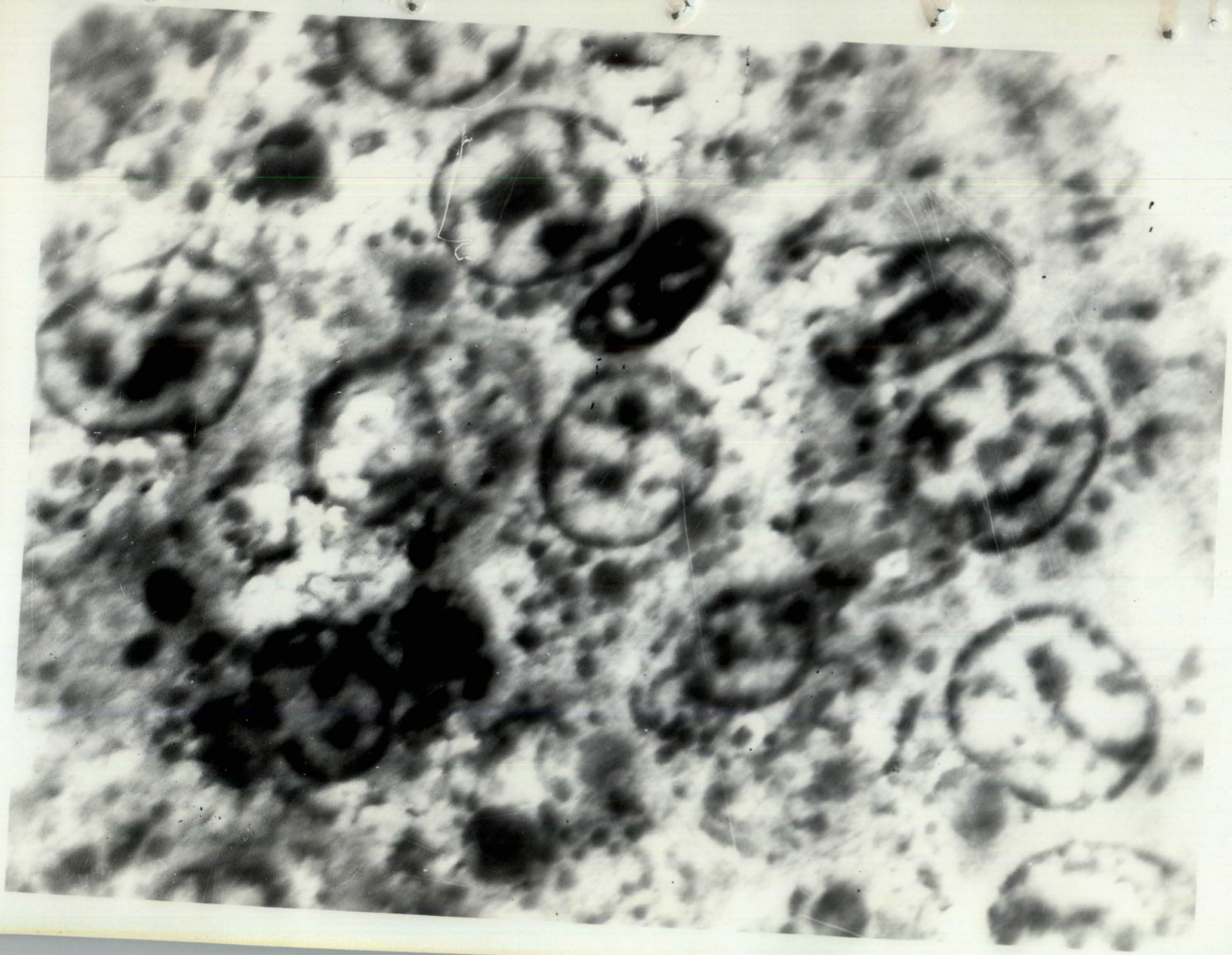


Fig.36: Electron micrograph of a thymic cell from fish treated with aflatoxin for eight weeks depicting complete loss of architecture of the cell and its organelles (6000X).

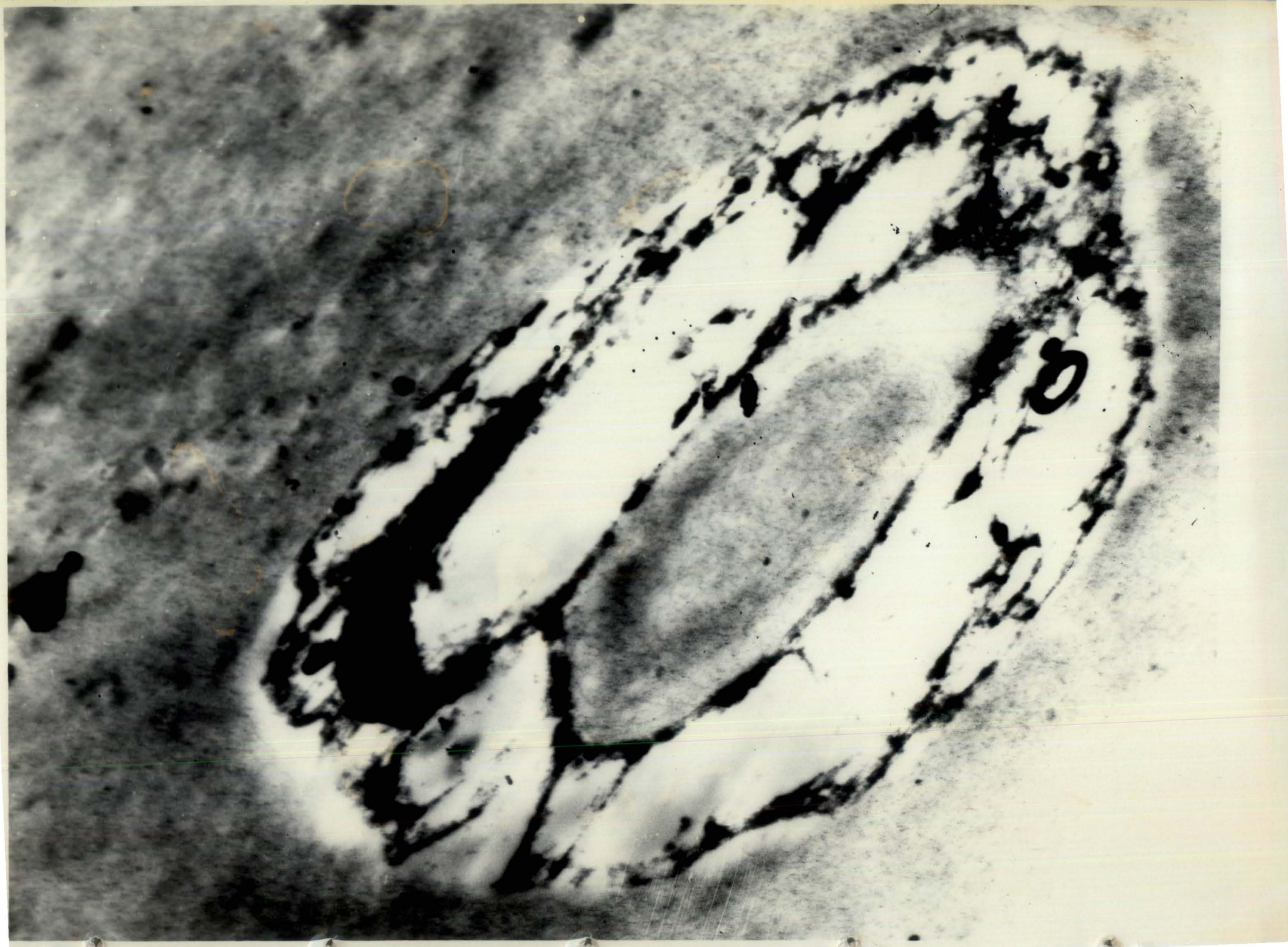


Fig.37: Electron micrograph of the kidney tubular cells of fish treated with aflatoxin B_1 for eight week. Note the degranulation of endoplasmic reticulum and dilatation of the cisternae of endoplasmic reticulum. Mitochondria are condensed. Autophagic veicles are also seen (6000X).

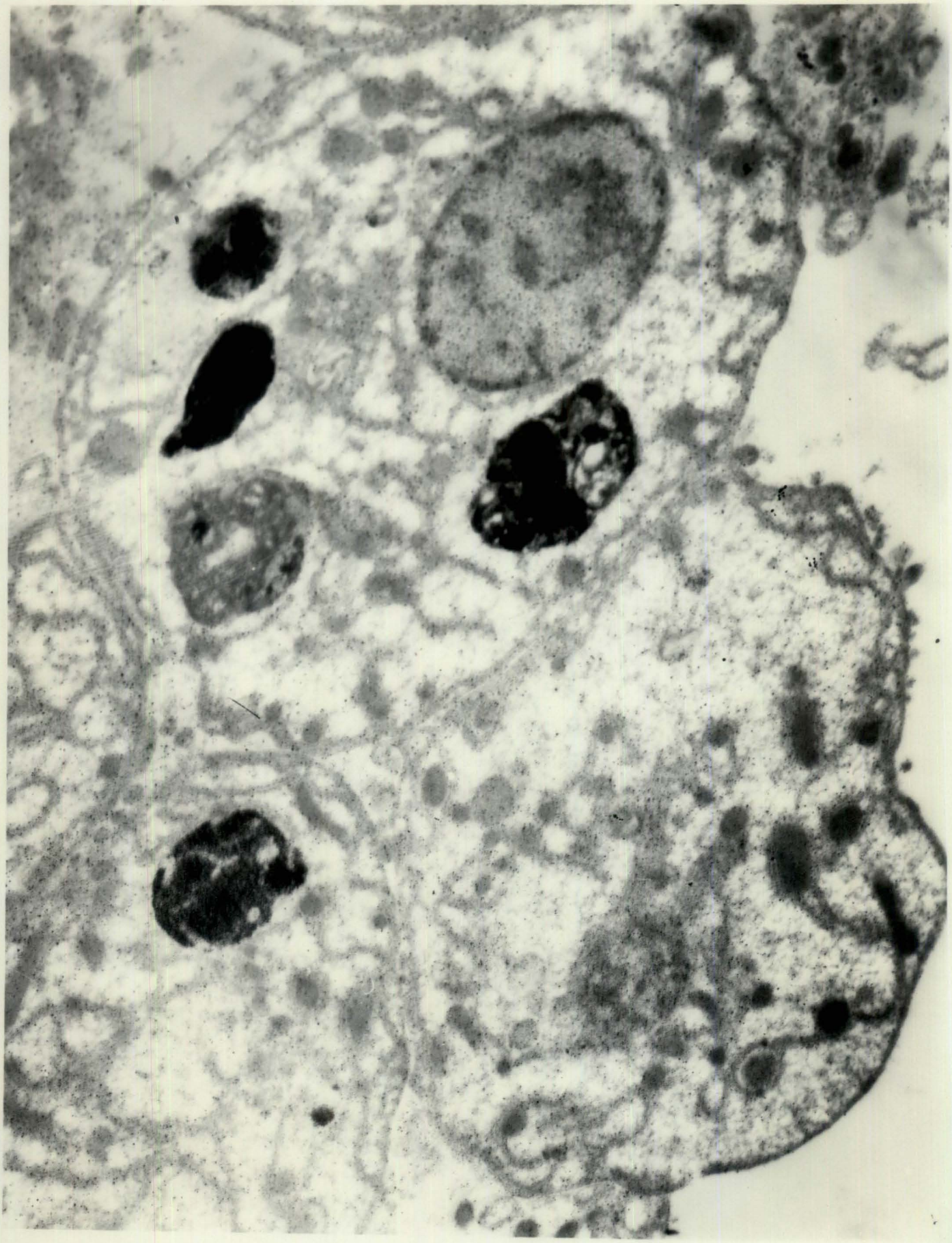


Fig.38: Electron micrograph of the kidney tubular epithelium from fish treated with aflatoxin for eight weeks. Note the separation of desmosomes, loss of microvilli, dilatation and vesiculation of endoplasmic reticulum cisternae (5000X).

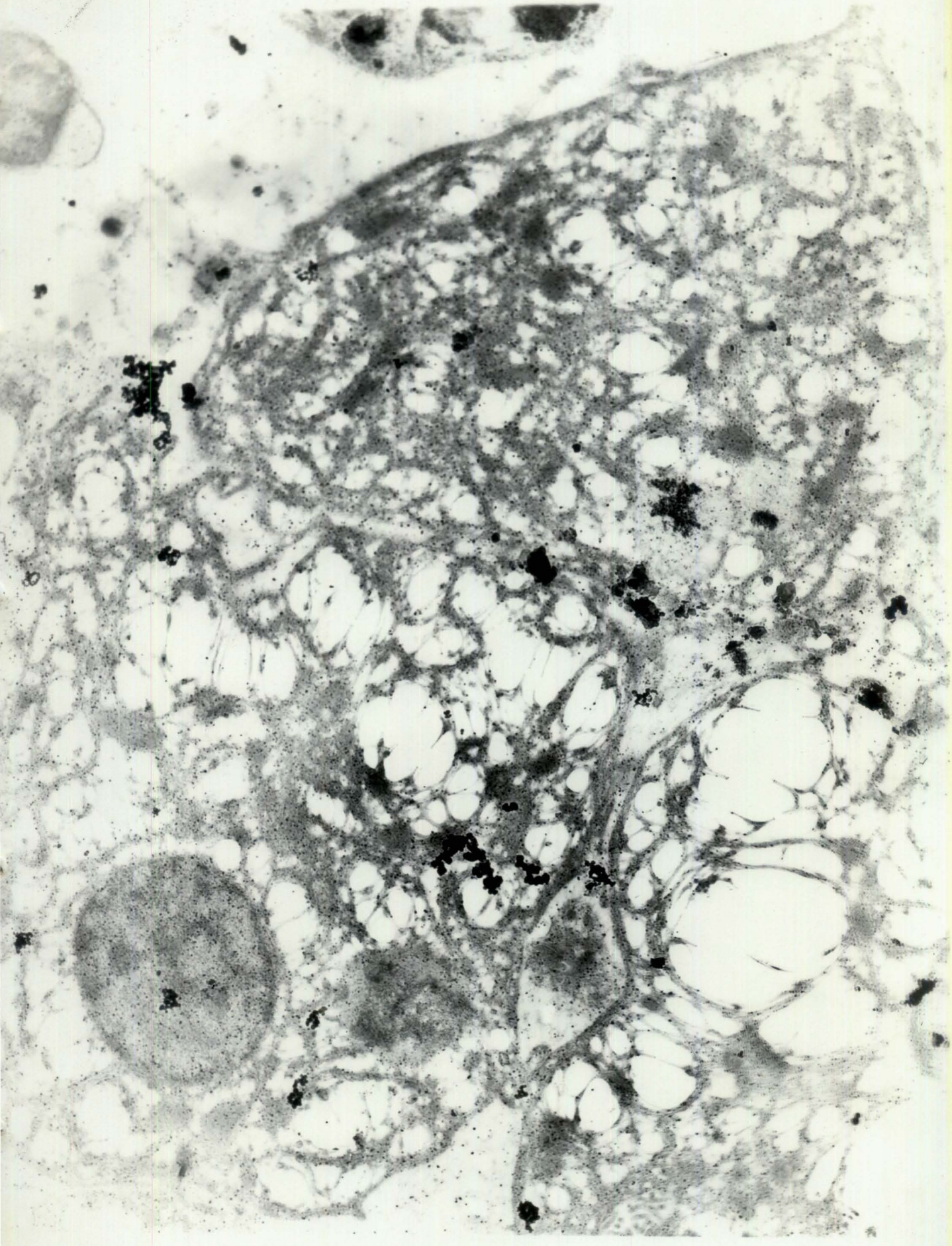


Fig.39: Electron micrograph of the kidney from aflatoxin treated fish showing condensation of mitochondria, fragmentation of ER and vesiculation of ER (6000X).

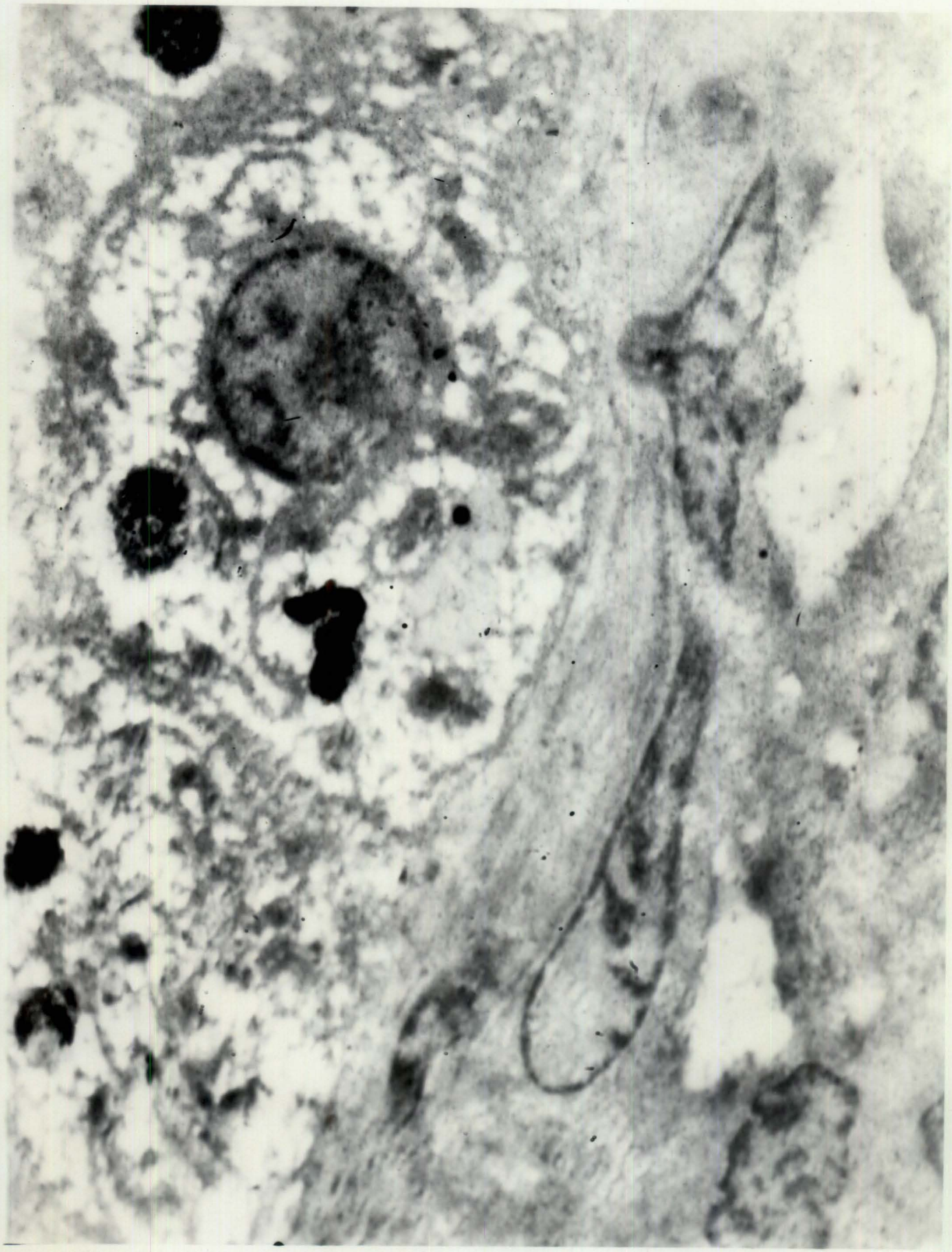
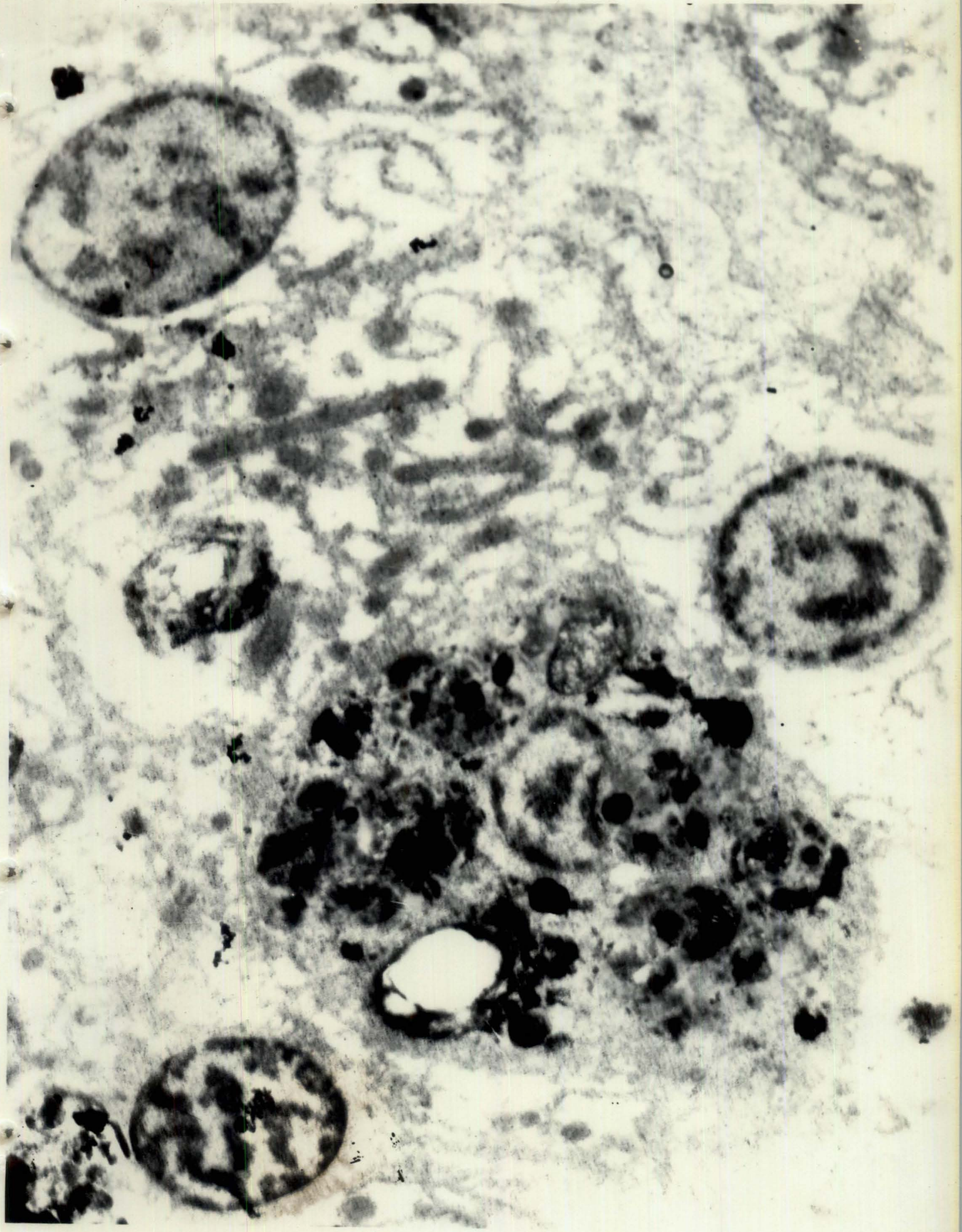


Fig.40: Electron micrograph of the kidney of fish treated with aflatoxin showing changes in the epithelial cells and interstitial cells. Note fragmentation of ER and condensation of mitochondria. Dilatation of ER can be seen. Nuclei contained heterochromatin clumps and perichromatin granules (6000X).



DISCUSSION

Chapter 6

DISCUSSION

There was reduction in feed consumption in the ducks and fishes when aflatoxin was fed. There was also reduction in the weight gain in the duck. There was fall in the body weight of the fishes. The mortality rate in the ducks and fishes increased as the feeding with aflatoxin was continued. Increase in the mortality and loss of weight were noticed in ducks, chicks, turkeys, pigs and cattle (Yoshida and Kamota, 1952; Asplin and Carnaghan, 1961; Chattopadhyay *et al.* 1985; Chen *et al.* 1985; Giambrone *et al.* 1985b; Dalvi, 1986; Colvin *et al.* 1989; Harvey *et al.* 1989; Mukit and Kwatra 1989; Roy *et al.* 1989; Neldon-Ortiz and Qureshi, 1992). The production system with intensive feeding there should be enhanced productivity and the presence of aflatoxin in the diet of ducks and fishes, therefore is going to be an important constraint in enhancing productivity. This, points out the need for analysing the feed, systematically for aflatoxin content.

In the ducks and fishes of the treatment groups there was high mortality rate. Similar observations were reported earlier in ducks, chicks, pigs and fish (Asplin and Carnaghan, 1961; Madhavan and Rao, 1966; Zaleski *et al.* 1979; Wales, 1979; Roberts and Sommerville, 1982). Dead ducks and fishes had haemorrhagic lesions. Ducks had

subcutaneous haemorrhages whereas in the dead fish internal haemorrhages were the main lesions. Chen *et al.* (1985) and Dalvi (1986) reported such haemorrhages in chicken and ducks due to aflatoxin contamination of the feed. In rainbow trout high levels of aflatoxin produced generalised haemorrhagic syndrome(Halver *et al.*, 1966;1969; Ashley, 1970 and Poppe *et al.*1985). Usually, the blood of fish clots very fast. In the later stages of aflatoxin exposure blood from many of these fishes failed to clot. Damage to the liver caused failure in the synthesis of fibrinogen, factors V, VII, VIII and IX. (Chatthopadhyay *et al.* 1985; Clark *et al.* 1986; Baker and Green, 1987). Such studies are not available in the case of fish. However, the damage to the liver and failure of the blood to clot indicated the mechanism operating in the fish might be very similar to higher vertebrates. Aflatoxin, should therefore be suspected, when haemorrhagic syndrome is seen in ducks and fish.

There was reduction in the body weight of fishes. The experimental group of fishes were reluctant to take aflatoxin containing feed. More pronounced body weight loss was observed in the treatment groups. According to Svobodova and Piskac (1982) and Svobodova *et al.* (1980) aflatoxin had no effect on feed conversion ratio growth rate and general condition of carps. Jantrarotoi *et al.* (1990); Jantrarotoi and Lovell (1990); and Chavez-Sanchez *et al.* (1994) had noticed depression in the weight, growth response and feed intake in channel catfish and Nile tilapia

(*Oreochromis niloticus)* which received different levels of aflatoxin in the feed. Pronounced weight loss and refusal to feed observed in the present study could be attributed to aflatoxin as reported in channel cat fish and nile tilapea.

Total erythrocyte count revealed a time dependent decreasing trend in aflatoxin fed ducks and fishes. The lowest values were observed in ducks and fishes which received aflatoxin for six weeks and eight weeks. Erythrocyte sedimentation rate in the ducks and fishes did not show much change. However, packed cell volume and haemoglobin values indicated decreasing trend as exposure to aflatoxin continued. Several authors had indicated that packed cell volume, haemoglobin values and erythrocyte count were decreased in chicken, calves, ducks and rabbits; when they received aflatoxin (Chattopadhyay *et al.* 1985; Wyatt *et al.* 1985; Dalvi, 1986; Doerr *et al.* 1987; Huff *et al.* 1988a&b; Raval and Varma 1992; Verma and Raval, 1992). There was clear evidence of anemia in both ducks and fishes following aflatoxin intake.

In fishes only very limited studies have been conducted on the effect of mycotoxin on haematological values. Svobodova and Piskac (1980) and Svobodova *et al.* (1982) were of the opinion that haematological values of carps were not affected by aflatoxin, where as Al-sabti (1985 & 1986) reported increased micronuclei formation in erythrocytes of carp. Plumb *et*

al. (1986); Jantrarotoi & Lovell (1990), Jantrarotoi *et al.* (1990) reported that Channel cat fish which were treated with various levels of aflatoxin B_1 had low erythrocyte count. *L. rohita* belong to Cyprinidae (carp family) and in the present study erythrocytes count were low when they were fed with aflatoxin B1. This finding was further supported by the severe necrosis of the haemopoietic organs of the anterior kidney and spleen observed in fishes which received aflatoxin for longer duration. The difference in the results obtained by various authors could be attributed to the difference in metabolic path ways of aflatoxin in different species. Further studies are needed to confirm the present observations.

Like the erythrocyte count, the total leucocyte count also decreased in ducks and fishes when they were given aflatoxin. This decrease was proportional to the duration of toxin feeding. Aflatoxin caused lymphoid necrosis and lymphopenia in chicken, pigs, cattle and ducks (Harvey *et al.* 1988; Ghosh *et al.* 1990; Balakrishnan, 1992; Potchinsky and Bloom, 1993; Raisuddin *et al.* 1993; Sharma, 1993). Contrary to the above cited reports Neldon-Ortiz and Qureshi(1992) observed that there was increase in the number of lymphocytes and monocytes of chick embryo when they were treated with aflatoxin.

In fishes, decrease in the trend in total leucocyte count was recorded as the duration of feeding of aflatoxin containing feed continued.

Jantrarotoi *et al.* (1990) reported that high doses of aflatoxin B_1 resulted in reduction in total leucocyte count in the channel cat fish. Subchronic toxicity caused increase in the total leucocyte count of channel cat fish and Indian cat fish *Claria batrachus* (Parashari and Saxena, 1983; Jantrarotoi *et al.* 1990). In the present study in both ducks and fishes aflatoxin produced depression in the leucocyte count, this could be correlated with the general necrosis observed in the lymphoid tissues. The leucopenia induced by aflatoxin will lead to immunosuppression and consequent increase in susceptibility to infection.

The change in the total serum protein level in ducks and fishes in response to aflatoxin differed. In ducks the total serum protein levels in the experimental group did not alter much. However, the albumin level fell in proportion to the duration of aflatoxin feeding. Decrease in the albumin was accompanied by relative increase in the globulin fractions. There was significant alterations of A:G ratio in the experimental group.

Serum from fishes of the treatment group revealed proportionate reduction in the total protein and albumin as the duration of aflatoxin feeding continued. The globulin values of the different groups in the fish did not show any significant variation. The elecrophoretic pattern of the serum protein in the fish indicated reduction in the albumin

fractions of the treatment group. This selective reduction in the albumin level without change in the globulin level is an unusual finding in the fish.

Several workers have reported hypoproteinemia, hypoglobulinemia and decrease in the serum albumin during aflatoxicosis in ducks, chicken, rabbits and pigs.(Brown and Abram, 1965;Baker and Green, 1987; Singh *et al.*1987; Huff *et al.* 1988a&b; Harvey *et al.*1988). In the case of fishes both total protein values and albumin values indicated a decreasing trend. There were no earlier studies on the protein and albumin values of fishes in aflatoxicosis. There was severe damage to the hepatic cells as observed by histological and electronmicroscopic studies and this can be attributed for these changes.

There was, therefore, variation in the plasma protein profile in ducks and fishes in aflatoxicosis and this could be attributed to the differential biological response in these species.

Phagocytic index is a major indicator of non-specific immunity and it is intimately linked to the antibody response. In both ducks and fishes in the treatment groups there was severe depression of phagocytosis. In both species, different methods were adopted to asses the phagocytic ability. In ducks we adopted colloidal carbon clearance test where as in fishes

invitro phagocytic studies on macrophages isolated from the peritoneum and blood were carried out. Killed yeast particles were used as inert particles. Carbon clearance at fifteen minutes was dull in ducks of the experimental group compared to the fast clearance obtained in the control ducks. The macrophages from the control group of fishes ingested more yeast particles per cell as well as more number of cells participated in the phagocytic process. The macrophages from the treatment group could take only less number of yeast particles per cell and large number of cells did not take part in the phagocytic process. There was thirty percent reduction in the phagocytic index. These observations gave proof to the fact that there was reduction in CMI response in aflatoxicosis.

A number of workers reported severe depression of phagocytosis in birds and mammals which were exposed to aflatoxin (Chang and Hamilton, 1979; Kadian *et al.* 1988; Neldon-Ortiz and Qureshi, 1992; Balakrishnan 1992; Raisuddin *et al.* 1994; Cusumano *et al.* 1995). The results of the present study were in agreement with the observations cited above. However, no such work was carried out in fishes in connection with aflatoxicosis. Hence, the present study has clarified that fish phagocytes probably behaved like avian phagocytes and were suppressed by aflatoxin.

Assessment of the cell-mediated immunity (CMI) in ducks was carried out by intra dermal injection of PHA-M, leucocyte migration inhibition test and also by ANAE reaction of peripheral leucocytes. PHA-M sensitivity test showed moderate variation between the control and treatment group of ducks. The treatment group showed moderate depression in skin sensitivity.

Many authors are of the view that CMI reactions are adversely affected by aflatoxin. These reactions included delayed hyper sensitivity, graft versus reaction, number of ANAE positive cells and skin response to PHA (Pier *et al.*1972; Michael *et al.* 1973; Giambrone *et al.* 1978; Richard *et al.*1983; Bodine *et al.* 1984; McLoughlin *et al.*1984, Vishalakshan *et al.*1984' Giambrone *et al.* 1985; Reddy *et al.* 1987; Ghosh *et al.* 1990; Pier 1992; Balakrishnan, 1992; Van Heughten *et al.* 1994). In the present work, the treatment group of ducks revealed moderate depression of CMI confirming the views of these earlier workers. The intra dermal skin sensitivity test against PHA-M and counts of ANAE positive lumphocyte showed moderate depression in the experimental group of ducks compared to the control group of ducks. Though the amplitude of these reactions did not indicate statistical significance still it revealed a trend towards immuno suppression by aflatoxin. The immunotoxic effect of aflatoxin might have been significant if the duration and the dose adopted were more.

In fishes also skin sensitivity response to PHA showed very moderate depression. In the case of treatment groups leucocyte migration inhibition test did not reveal any variation. Rijkers *et al.* (1980); Anderson *et al.* (1982 & 1984); Blazer and Wolke (1984); Li and Lovell (1985) were of the opinion that fish immune responses were highly influenced by external factors such as pollutants, toxins and nutritional factors. The role of immuno suppressants in the CMI response has not been understood properly in fishes. However, there was suppression of CMI in the present study as evidenced by skin sensitivity reactions. There has been no reports on the CMI response in fishes in aflatoxicosis. This appears to be the first report.

Humoral antibody response of ducks against sheep RBC(SRBC) showed significant difference between the control and treatment groups. In the control group minimum haemagglutination titre was 64(1:64). In the treatment group minimum heamagglutination titre was 8(1:8). Maximum titre for control group was 256 while that of treatment group was 64. This clearly demonstrated that the immunological response of the ducks was significantly affected. It appeared that the humoral response was more significantly affected than the CMI response in aflatoxicosis.

In mammals and birds aflatoxin was reported to produce poor response to bacterial, viral infections and vaccinations. It was reported that aflatoxin formed binding with RNA polymerase and blocked the synthesis of immunoglobulins. It was also noticed that aflatoxin produced low levels of serum globulins in ducks. (Brown and Abram, 1965; Clifford and Rees, 1967; Lafarge and Frayssinet, 1970, Pier and Heddleston, 1970; Thaxton and Hamilton, 1971, Edds *et al.* 1974; Tung *et al*, 1975; Miller *et al.* 1984; Reddy *et al.*1983; Gopalkrishnan Nair, 1986; Marshaly *et al.* 1986; Okuda *et al.* 1988; Rao, 1988; Sharma, 1988; Uchida *et al.* 1988; Virdi *et al.* 1989; Padmanabhan, 1989; Balakrishnan, 1992;).

A number of workers are of the view that immuno-supression observed in aflatoxicosis was not due to the poor production of antibody but due to poor interferon generation and complement factor production (Pier et al 1971; 1972; Stewart *et al.* 1985; Panangala *et al.* 1986). In a number of studies it was noticed that aflatoxin B_1 affected generation of antibody producing cells. The toxin caused lethal effects on B lymphocytes. This process was found reflected in the weight and size of the lymphoid organs especially the bursa of *Fabricius* (Paul *et al.* 1977; Giambrone *et al.* 1978; Campbell *et al.* 1983; Pier, 1986; Rao *et al.* 1990; Potchinsky and Bloom, 1993).

The size of the bursa of *Fabricius* was much reduced in a number of ducks. There was severe oedema of the bursa of *Fabricius*. Histological and ultrastructural observations did reveal severe loss and necrosis of lymphoblasts. The immunotoxic effect of aflatoxin on the bursa was very significant and this can be an important contributing factor in causing immuno suppression.

In fishes the haemagglutination titre was very similar to that of the ducks. Lowest titre observed in the control group fishes was 128 (1:128) and highest reached 512 (1:512). In the aflatoxin treated fishes the titre did not cross 32 and minimum titre was 4. This observation clearly demonstrated the immuno suppressive effect of aflatoxin. The histological and ultra structural studies of the lymphoid organs indicated loss of blast cells, severe necrotic and degenerative changes accounting for the immuno suppression. The antibody production in the fish is highly dependent on environmental, nutritional and toxic factors (Anderson *et al.* 1984; Blazer and Wolke, 1984; Li and Lovell, 1985). The role of mycotoxins in modulating the immune response in the fish has not been investigated thoroughly. The study carried out by Arkoosh and Kaattari (1987) on the rainbow trout showed that aflatoxin B_1 depressed the anamenestic antibody response. They were of the view that AFB_1 affected the generation of memory cells. The primary response was not affected. The observation made in the study also agreed with the above findings.

Histopathological studies made in the case of ducks revealed that aflatoxic hepatosis was characterised by necrosis, biliary epithelial hyperplasia and fatty degeneration of hepatocytes. These changes were proportional to the duration of exposure. The ultra structural studies of the hepatocytes also revealed retrograde changes. These included degranulation of RER, appearance of electron dense lipid droplets, proliferation of SER and more heterochromatin clumps in the nucleus. The smooth endoplasmic reticulum underwent fragmentation. The desmosomes were separated. The histological changes such as hepatosis, hepatoma, bileduct epithelial hyperplasia, adenomatous changes, fatty degeneration of the hepatocytes and necrosis of parechymal cells were the common findings when the ducks were fed with aflatoxin containing feed. (Yoshida and Kamota,1952; Asplin and Carnaghan, 1961; Armbrecht and Fitzhugh, 1964; Butler, 1964; Newberene *et al.* 1964; Yadagiri, 1970; Dalvi, 1986; Mukit and Kwatra, 1989; Roy *et al.* 1989; Balakrishnan, 1992). These hepatic changes were also observed in chicken, quails, pigs, rabbits and cattle (Chattopadhyay *et al.* 1985; Chen *et al.* 1985; Tapia and Seawright, 1985; Borisova *et al.* 1987; Cook *et al.* 1989. Hall *et al.*1989; Van-Halderen *et al.* 1989; Krishna *et al.* 1991; Rogers *et al.* 1991)

The ultrastructural studies revealed changes that supported the observation in the serum protein values and histological changes. The

proliferation of smooth endoplasmic reticulum indicated attempts of detoxification. SER is concerned with biotransformation of xenobiotics. It was reported that when increased demand for biotranformation was put on proliferation of these membranes, occurred (Zweifach, 1965; LaVia and Hill, 1971; Brandle and Gabbiani, 1983; Cheville, 1983). Fragmentation of the endoplasmic reticulum and degranulation of the RER were the result of peroxidation of unsaturated lipids in the membranes, by free radicals generated. Formation of folds, blebs and whorls led to separation from the junctional sites. Disintegration of membrane structure led to osmotic swelling. Lysosomes became more prominent for autodigestion of degenerate components (Cheville, 1983; Thomson, 1984). Cytocrome, p-450 monooxygenases were mostly involved in production of epoxides, which are located in the SER. Glucuronosyl Glutathione Sulpho-tranferases were found in the liver endoplasmic reticulum. Therefore, the endoplasmic reticulum was considered as one of the major site of xenobiotic action (Garfinkel, 1958; Klingenberg, 1958; Morgenstern *et al*, 1984; Morgenstern and De Pierre, 1985). The appearance of increased amount of heterochromatin and perichromatin granules in the nucleus indicated suppression of protein synthesis in the cell. This was further evidenced by degranulation of endoplasmic reticulum (Porter and Bonneville, 1964; Scarpelli and Trump, 1964; Stenger, 1970; La Via and Hill, 1971; Trump *et al.* 1973; Thomson, 1984). The suppression of protein synthesis was reflected in the serum albumin values in the present investigation.

The liver of the fish *L.rohita* of the experimental groups revealed extensive histological changes. This included fatty change in the hepatocytes, and focal necrosis to extensive necrosis of the parenchyma. Ceroid bearing macrophages increased in the liver. Diffuse infiltration of leucocytes was also evident.

No information on the pathological changes induced by aflatoxin in Indian major carps like the *L. rohita* is available. This observation appears to be the first. The changes observed were generally similar to those seen in ducks and other fishes. Svobodova and Piskac, (1982); and Svobodova *et al.* (1982) were of the opinion that aflatoxin did not have any effect in the carp livers. Al-Sabti (1985 and 1986) did notice some pathological effect on the erythrocytes of carps. Majority of the aflatoxin related work was conducted on trouts because high incidence of hepatomas occurred in the rainbow trout due to aflatoxin contamination of the feed. Rain-bow trout was considered to be an animal model for carcinogenic studies. (Ashley and Halver, 1961; Halver *et al.* 1962; Halver, 1969; Ashley, 1970; Sinnhuber *et al.* 1977; Wales, 1979; Hendrick *et al.* 1979; Rasmussen *et al.* 1986; Nunez *et al.* 1990; 1991; Ngethe *et al.* 1993; Sarcione and Black, 1994;). Other than trout, Tilapia was considered to be susceptible to aflatoxin. Among the fishes in tilapia multiple range of neoplasms were produced (Haller and Roberts, 1980). However, Tereza *et al.* (1987) reported occurrence of

hepatosis in tilapia due to mycotoxin contamination of the feed. Bruenger (1982); Bruenger and Greuel (1982) observed that aflatoxin did not produce hepatoma in the salmon (Coho salmon) instead it produced degenerative and necrotic lesions in the liver. Studies conducted on acute and subacute aflatoxicosis in channel cat fish revealed necrotic foci in livers (Jantrarotoi and Lovell, 1990; Jantrarotoi *et al.*1990). Chavez-Sanchez *et al.* (1994) studied the effect of aflatoxin on *Oreochromis niloticus* and found that the toxin produced fatty liver, nuclear hypertrophy and cellular atrophy and leucocytic infiltration in the liver. In the present investigation degenerative and necrotic changes in the hepatocytes were the main findings and these changes were highly remarkable. This investigation, has therefore indicated that *L.rohita* is very much susceptible to aflatoxin. Hepatosis was the predominent finding. The carcinogenic effect has to be assessed by still long term experiment.

The ultra structural changes in the liver of fishes were mild initially but later it became very severe. Initial changes included degranulation of the RER, increase in the profile of SER and appearance of numerous electronlucent bags. These electronlucent bags had dense core and were probably peroxisomes. Later severe changes were noticed in the architecture of organelles. The nucleus was displaced to the periphery of the cells with development of vescicles in the ER. Nunez *et al.* (1991) described pleiomorphic nuceli, large nucleoli, dilated endoplasmic

reticulum, increased numbers of lysosomes in rainbow trouts fed with aflatoxin. Proliferation and dilation of the RER were the striking feature of hepatocytes in rainbow trouts exposed to aflatoxin (Scarpelli *et al.* 1963). Stehr *et al.* (1988) observed that the RER cisternae were greatly dilated in the hepatic neoplasm of the fish *Parophrys vetulus*. In the present study RER was severely dilated in the hepatocytes of fishes exposed to AFB_1 for six and eight weeks. This suggested that the hepatocytes of *L.rohita* may also undergo neoplastic transformation on prolonged exposure to aflatoxin.

The nuclear envelope appeared dilated and nuclei were pleomorphic. Patches of heterochromatin and increase in perichromatin granules were noticed in the treatment groups. These findings were in agreement with the observation of Nunez *et al.* (1991). Arnold *et al.* (1995) observed that in endosulfan and disulfoton toxicity of the rainbow trout the RER underwent progressive loss of structural integrity and vesiculated at high doses. Nuclear envelope underwent dilation and irregular contours developed; perichromatin granules appeared in the nucleus. At lower doses RER showed transient proliferation. The changes observed in the hepatocytes were similar to those seen in pesticide toxicity mentioned above. The xenobiotic action of aflatoxin on cells generally gave non-specific type of response, therefore no specific conclusions could be made (Stenger, 1970). In the present study, the degenerative hepatopathy was more severe in fishes when compared to the ducks as evidenced by electron micrographs. The

histological and ultra-structural studies, has confirmed the very high susceptibility of the Indian carp, *L.rohita* to aflatoxin.

The thymus in the treatment group of ducks revealed degenerative and necrotic changes. Severe necrosis occurred in the ducks receiving aflatoxin for longer duration. The ultrastructural studies gave further support to the histological observations. The lymphoblasts revealed increased heterochromatin in the nucleus and moderate vesiculation of the endoplasmic reticulum initially in the treatment groups.

As the exposure time increased many of the follicles did not have lymphoblasts and the presence of nucleus with large amount of heterochromatin indicated lysed cells. These findings clarified that there was lymphocytolysis induced by the toxin. Balakrishnan (1992) observed thymus dependent lymphocytopenia in ducklings fed aflatoxin. He also noticed suppressed T-cell reaction against dinitro-chlorobenzene and PHA-M. In aflatoxicosis, involution of the thymus and poor CMI response were noticed by many workers in different domestic animals and birds (Pier *et al.* 1972; Giambrone *et al.* 1978; Chang and Hamilton, 1979; Reddy *et al.* 1983; Richard *et al.* 1983; Bodine *et al.* 1984' McLoughlin *et al.* 1984; Vishalakshan *et al.* 1984; Sinha and Arora, 1985; Giambrone *et al.* 1985a&b; 1988; Raisuddin *et al.* 1993; Sharma, 1993). This investigation clearly indicated lymphocytolysis in the thymus of ducks.

In the control group the thymus of the fish revealed small area of lymphoid cells. However, in the treatment groups the thymus revealed only sparse lymphoid cells. The reason for the absence of lymphoid cells in the treatment group was clarified by the electron microscopic studies. Initially the changes in the lymphoblasts were mild, the nuclear chromatin changed to more condensed heterochromatin. Lysosomes and autophagic vaccuoles became apparent. The most remarkable changes were noticed in later stages. The lymphocytes lost their structure. The cytoplasm contained large vesicles and nucleus lost its contents. Electron dense materials were seen in the endoplasmic reticulum cisternae. There has been only very limited work on the effect of aflatoxin on the thymus of fish. Hence the present work assumes great significance. The observations have indicated severe adverse effect of aflatoxin on the thymus of fish and this perhaps will lead to immuno suppression.

The spleen of the duck in the experimental group revealed on histological examination reduction in the lymphoid tissue. The electron micrographs revealed severe degenerative changes in the lymphocytes as in the thymus. The nucleus revealed increase in the heterochromatin and ER developed vesicles. Similar changes were also observed in the spleen of fishes both histologically and electron microscopically. Necrosis of the

spleen in association with aflatoxicosis was reported in the duck (Balakrishnan, 1992).

The changes in the bursa of *Fabricius* in the duck were characterised by necrosis and degenerative changes in the lymphoid tissue. These histological findings were further supported by the extensive nuclear and cytoplasmic degenerative changes observed under the electron microscope. Necrosis of the lymphoid follicles and decrease in the size of the bursa of Fabricius were noticed in the ducks, chicken and quails (Campbell *et al.* 1983; Padmanabhan, 1984; Pier, 1986; Rao *et al.* 1990; Balakrishnan, 1992). The immunotoxic effect of aflatoxin on the lymphoid organs of the ducks and the fish appear to be similar and comparable.

The haemopoietic tissue of the kidney as well as the anterior kidney of the fish revealed degenerative and necrotic changes. Electron microscopic pictures also revealed extensive changes in the cell organelles. Aflatoxin was reported to be immunotoxic and caused extensive necrosis of the lymphoblasts in mammals and birds (Thaxton and Hamilton, 1971; Paul *et al.* 1977; Giambrone *et al.* 1978; Campbell, 1983; Pier, 1986; Padmanabhan, 1989; Rao *et al.* 1990; Potchinsky and Bloom, 1993). The recent work on aflatoxicosis in the fish was mostly confined to the liver lesions, and there was no available published reports on the immunotoxicity. However, the present investigation has clearly

demonstrated that aflatoxin caused immunotoxicity in the fish and the pathogenesis was clarified by histological and electron microscopic studies.

The present investigation showed general nephrotic changes in the kidneys. The epithelial cells of the proximal convoluted tubules lost their brush broader and became granular and swollen. The parietal cells lining the Bowman's Capsule became more columnar and hypertrophied. Desquamation of the epithelial cells, interstitial haemorrhages, periglomerular fibrosis, glomerular shrinkage and mesenchymal proliferation were noticed. However, such changes were not reported in ducks by earlier workers. The above mentioned changes were noticed in cattle, pigs, turkeys, dogs and guinea pigs (Asplin and Carnaghan, 1961; Siller and Ostler, 1961; Allcroft and Carnaghan, 1963; Butler, 1966; Newberne *et al.* 1966; Madhavan and Rao, 1967; Chaffee *et al.* 1969).

In fishes, the excretory kidney on histological examination showed extensive degenerative and necrotic changes. The ultrastructural studies also supported the findings. There was severe damage to the tubular epithelial cells. Initially these cells revealed swelling and loss of microvilli. Dilatation of ER was evident. Loss of ribosomes from the ER was another finding. As exposure time increased separation of cells and destruction of desmosomes were seen. Mitochondria revealed extensive degenerative changes and dilatation of the ER led to formation of vesicles. All these

changes indicated the progressive pathological changes induced by aflatoxin. The published reports on the effects of aflatoxin on the kidney of fishes are very scanty. According to Walker (1987) the renal tissue of the fish would be at a major toxicologic risk; since kidneys received blood flow from both the renal portal venous system and renal arteries. Kidney excreted metabolites of various xenobiotics to which fish had been exposed (Pritchard and Renfro, 1982). There were reports about the presence of xenobiotic metabolizing enzymes like Arylhydrocarbon hydroxylase (AHH); cytochrome p.450 monoxygenase in the kidneys of various species of fishes (Bend *et al.* 1973; Lindstrom-Seppa *et al.* 1981; Payne *et al.* 1984; Stegeman *et al.* 1979; Williams *et al.* 1986). According to Hinton (1993), lack of adequate histopathological studies on the kidneys probably contributed to the failure of recording toxicologic lesions in this important organ. This investigation has clearly brought to light that aflatoxin has severe adverse effect on teleost kidney leading to necrosis of excretory and haemopoietic tissue. The histological and ultrastructural studies categorically established that aflatoxin has more adverse effect on the kidney of the fish than in the duck. The dichotomous blood supply to the organ explains this pronounced effect.

Cardiac tissue in the duck and fish revealed only minor degenerative changes. Such changes were reported in Turkey by Austwick (1978).

The brain tissue of the fish and the duck indicated comparable degenerative changes in the treatment groups. These changes included focal encephalomalacia, chromatolysis, neuronal vacuolation, neuronophagia, satellitosis and gliosis. These were non-specific type of changes. Such changes were noticed in association with toxicological conditions in mammals, birds and fishes (Jones and Hunt, 1983; Ferguson, 1989 and Hinton *et al.* 1991).

The comparative studies on the pathological changes in ducks and fishes in experimental aflatoxicosis has brought to light several interesting findings. These included the haematological changes, growth pattern, protein changes in serum, immunological effects, hepatoxicity, renal toxicity and lymphoid toxicity.

There was significant reduction in the growth profile of ducks. The haemorrhagic lesions in the ducks were more pronounced when compared to fishes. Fishes showed depression in the growth rate, and had anorexia and mild internal haemorrhages. The response of these two species were more or less identical. However, refusal of the feed in the case of fish was more pronounced.

In both ducks and fishes anaemia was produced along with leukopenia. The haematological picture could be correlated with the general necrosis observed in the haemopoietic organs like the anterior kidney, spleen, thymus and bursa of Fabricius. The severe reduction in the leukocyte counts of ducks and fishes would certainly affect their resistance against diseases and make them more susceptible for infection.

The total serum protein values in the duck did not show much variation between the control and experimental groups. However, severe fall in the albumin values was recorded in ducks treated with aflatoxin. It was seen that fall in the albumin values were accompanied by increase in certain fractions of globulins. The fall in albumin fractions could be correlated with the degranulation of RER in the hepatocytes of ducks.

The total serum protein and albumin values showed significant dose dependent decrease in fishes. The decrease in the serum protein could be correlated with severe damage of hepatocytes as indicated by histopathological studies and electronmicroscopic studies.

Phagocytic index in both ducks and fishes were lowered in aflatoxin treated groups. This would affect the non-specific immunity and resistance, since the macrophages are involved in antigen processing and antigen

presentation to immunocompetent cells. The decreased activity of these cells would certainly affect the specific immune reactions.

The CMI response in both ducks and fishes showed only moderate variations in aflatoxicosis. There was general depression of the CMI in both ducks and fishes fed aflatoxin.

Apparently, the humoral antibody response in both fish and duck indicated immunosupression due to aflatoxicosis.

Histopathological and ultrastructural studies of the liver in duck showed severe damage to the hepatocytes accompanied by the proliferation of the biliary epithelium. The liver of fishes revealed more severe hepatosis than in the ducks and this clarified that the fish liver is more sensitive. The histopathological and electronmicroscopic studies of the lymphoid organs revealed comparable degenerative and necrotic changes in the lymphoblasts of the ducks and fishes. This clarified the immunotoxic effect of aflatoxin in ducks and fishes.

In the kidney of the fish, the immuno toxic effect of aflatoxin was more severe and extensive when compared to the duck.

The duck and the fish were very much susceptible to the aflatoxin. However, generally in ducks severe lesions were observed in the liver and lymphoid organs and in fishes severe lesions were seen in the liver, kidney and the lymphoid organs.

From this investigation, the pathological effects of aflatoxin in ducks and fishes were brought to light, their significance was clarified and the pattern of biological changes in these two species was delineated.

Chapter 7

SUMMARY

1. The pathological effects of aflatoxin on the ducklings and juvenile rohu fishes were studied by exposing them to sublethal toxic levels of aflatoxin B_1 and sacrificing them at different time intervals. Both the ducks and the fishes were given aflatoxin B_1 (M/s.SIGMA Chemicals). They were sacrificed at 2^{nd}, 4^{th}, 6^{th} and 8^{th} weeks.

2. There was reduction in feed intake in both ducks and fishes when they were given aflatoxin B_1.

3. Dietary aflatoxin B_1 reduced growth rate in ducks and fishes, this was evident from the poor weight gain observed in the treatment groups.

4. Mortality was observed in experimental group of ducks and fishes. In the dead ducks there was subcutaneous haemorrhages and hepatosis while in the dead fishes internal haemorrhage was more prominent.

5. In fishes which were exposed to aflatoxin there was failure of blood to clot. The liver appeared pale in these fishes and the gall bladder was enlarged with bile.

6. There was anemia and leucopnia in ducks and fishes directly proportional to the aflatoxin exposure time.

7. Serum protein pattern indicated changes in aflatoxin treated ducks and fishes. Though in ducks, the total serum protein values did not show much variation between different groups, there was severe fall in albumin values and significant variation in different globulin fractions and albumin/globulin ratio. In aflatoxin treated fishes, there was fall in total serum protein values as well as albumin values.

8. For the first time it was demonstrated that there was severe depression of the phagocytic activity in the aflatoxin treated group of ducks and fishes.

9. By elegant appropriate experimental methodology, it was clarified that there was severe depression of the cell-mediated and humoral immunity in experimental group of ducks and fishes. The immunotoxic effect of aflatoxin was therefore established.

10. Histological studies of the liver in ducks revealed focal to extensive necrosis of hepatocytes, fatty degeneration of hepatocytes, biliary epithelial proliferation and fibrosis.

11. Histopathological studies of the liver in experimental fishes showed fatty change and necrosis of hepatocytes. However, biliary hyperplasia and fibrosis were not significant.

12. Histopathological studies of the thymus, bursa of *Fabricius* and the spleen of the duck showed shrinkage and atrophy of the lymphoid follicles, necrosis of lymphoblasts and haemorrhage.

13. In the fishes, the thymus, kidney and spleen revealed necrosis and loss of haemopoietic elements in the experimental group.

14. The kidney in the experimental group of ducks and fishes revealed nephrotic changes characterized by tubular epithelial degeneration and necrosis. Parietal epithelial cell proliferation in the Bowman's capsule and mesenchymal cell proliferation in the glomeruli were also noticed in the ducks. In the fishes nephrosis was much more pronounced when compared to the ducks.

15. Ultrastructural studies of the liver of experimental group of ducks and fishes revealed degranulation of RER, proliferation of SER and fragmentation and dilatation of both RER and SER. Mitochondrial swelling and separation from desmosomes were also seen. Nuceli

became irregular in shape and there was increase in heterochromatin. The changes were comparable in both ducks and fishes.

16. Ultrastructural studies of the lymphoid organs of the duck indicated severe damage to lymphoblasts as evidenced by dilatation of RER, nuclear invagination and increase in heterochromatin.

17. The electron microscopic studies of the kidney of the experimental group of fishes showed loss of microvilli in the epithelial cells, degranulation of RER, swelling of the mitochondria and dilatation of ER and nuclear envelope. In the interstitial tissue, the cells developed several vesicles in the endoplasmic reitculum. Several multivesicular bodies and myelin figures were seen in the interstitial cells.

18. It was demonstrated that duck and fishes (*L.rohita*) are very sensitive to aflatoxin and the pathological changes were generally comparable. The hepatotoxic and immunotoxic effects of aflatoxin in these species were brought to light and the possible increased susceptibility to infection was indicated. The need for screening the feed samples for aflatoxin was stressed and prescribing a safe level of aflatoxin in the feed was indicated.

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COMPARATIVE PATHOLOGY OF AFLATOXICOSIS IN THE DUCK AND FISH WITH SPECIAL REFERENCE TO THE IMMUNE SYSTEM

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Abstract

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Abstract

Pathological responses of ducklings and juvenile Indian carps *Labeo rohita* in experimental aflatoxicosis were studied. Both the ducklings and fishes were given sublethal toxic levels of aflatoxin B_1. The ducklings and fishes were exposed to aflatoxin B_1 for 2, 4, 6 and 8 weeks respectively and they were sacrificed at the end of the experiment and samples for haematological, serum protein profile, histological and ultrastructural investigations were collected. Pathological changes were studied with special reference to the humoral and cell-mediated immune responses.

Among the haematological parameters, total erythrocyte count, total leucocyte count and packed cell volume were found to be reduced in fishes. The reduction was directly proportional to the duration of aflatoxin treatment.

In the fishes, there was moderate fall in the total proteins and the albumin. The electrophoretic picture of the serum of the fishes also showed fall in the albumin fractions. In the ducks there was severe reduction in the albumin level and this was reflected in the electrophoretic picture as well as A:G ratio.

In both the ducks and the fishes humoral immune response was suppressed due to aflatoxin treatment. Antibody titres against sheep RBC fell in the experimental groups of the ducks and the fishes. The cell-mediated immunity was assessed in the ducks and the fishes by intra-dermal PHA-M response, count of ANAE positive lymphocytes in the peripheral blood and leucocyte migration inhibition test. In the ducks and the fishes of the aflatoxin treated groups, there was significant fall in the skin sensitivity response and also in the number of ANAE positive lymphocyte. These findings clearly established that CMI was suppressed by aflatoxin.

The histological and electron microscopic studies revealed severe degenerative and necrotic changes in the hepatocytes in the ducks and fishes. However, hepatosis was more severe in the fishes whereas in the ducks biliary proliferation was a prominent feature. The hepatic changes could be correlated with the changes in the plasma protein profile in the duck and the fish. The lymphoid organs of the duck and the fish (thymus, spleen, bursa of *Fabricius* and anterior kidney) showed extensive necrosis and degenerative changes. These changes clearly established the reason for the immunosuppression and low leucocytic counts observed in the aflatoxin fed ducks and fishes. The kidneys of the duck and the fish exhibited nephrotic changes. Degenerative changes were also observed in

the brain and the heart of the two species. The pathologic changes due to aflatoxicosis were comparable in the ducks and fishes.