Hepatic lesions associated with induced aflatoxicosis in the estuarine teleost *Etroplus suratensis* (Bloch, 1790)

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ABSTRACT

Aflatoxicosis is the disease caused due to ingestion or inhalation of aflatoxins, one of the most potent and dangerous groups of mycotoxins, which is produced by fungi of the genera *Aspergillus.* This fungus grows on feed as well as on feed ingredients used in aquaculture and the tropical humid climate is conducive for its growth and toxin production. The present study was undertaken to assess the susceptibility of a tropical estuarine teleost, *Etroplus suratensis* (Bloch, 1790) to this toxin by exposing the fishes to aflatoxin B_1 (AFB_1) incorporated feed at a rate of 0.4 mg AFB_1 kg^{-1} feed, over a period of 8 weeks. On termination of experimental feeding, gross changes such as pale liver with numerous black spots were observed in aflatoxin treated fishes. Progressive damages to the liver which ultimately led to the induction of hepatoma was demonstrated through histopathological and electron microscopic studies. The major histopathological changes such as shrunken pyknotic nuclei, formation of basophilic foci, polyhedral hepatocytes in different sizes, multiple nuclei and mitotic nuclei were observed. The ultrastructural changes such as appearance of electron dense inclusions in the nucleus, loss of structure of microvilli, fragmentation of endoplasmic reticulum, separation of desmosomes, loss of continuity of plasma-lemma and nuclear membranes were also evident. In the present study, susceptibility of the species to aflatoxicosis was demonstrated clearly and the resultant induction of hepatoma indicated the possibility of using this species as an animal model in studies on induction of hepatocarcinomas.

Keywords: Aflatoxin, *Aspergillus flavus, Etroplus suratensis,* Hepatocarcinoma, Liver pathology

Introduction

Among the mycotoxins, aflatoxins have been extensively studied in poultry and domestic animals. Aflatoxins are highly carcinogenic and immunosuppressive (Ottinger and Kaattari, 2000; Sahoo *et al.,* 2001) and can also severely damage liver tissue by causing dystrophy of hepatic tissue, fatty changes, necrosis, loss of architecture, proliferation of bile ductular cells/biliary epithelium and cirrhosis (Harshbarger and Clark, 1990; Kranz and Delhlefesen, 1990). Studies on aflatoxicosis have been mostly carried out to assess the effect of the toxin in humans and domestic animals. Among fishes, trouts have been extensively studied. Aflatoxins are implicated as the main causative factor for hepatocarcinomas in trout, guppy and tilapias (Haller and Roberts, 1980; Majeed *et al.,* 1984; Nunez *et al.,* 1991).

The tropical environment is conducive for the growth of aflatoxin-secreting fungi. Hence it is likely that aquaculture feed and feed ingredients may contain aflatoxins as these are very good substrates for the growth of fungi and can affect the health of fishes cultured in this area. The presence of aflatoxins in aquaculture feeds and feed ingredients has been well documented especially in developing countries (Abdelhamid *et al.*, 1998; Barbosa *et al.*, 2013). However, work on aflatoxicosis in tropical fishes is scanty and hence understanding the pathology of aflatoxicosis in tropical fish species would help in aquaculture health management strategies of these species. As different species including humans, poultry, swine and fish exhibit varying levels of mortality and morbidity upon aflatoxin exposure and the effects are largely species and dose-specific, more studies are needed to determine aflatoxin succesptibility of unevaluated species (Zychowski *et al.*, 2013). *Etroplus suratensis* (Bloch, 1790) or "pearlspot" is a euryhaline fish relished in many parts of India and is being cultivated in polyculture systems in India as it readily accepts artificial pellet feeds. It has been declared as the state fish of Kerala in southern India and is widely cultured in brackishwater ponds and cages. Chances for incidence of aflatoxicosis in *E. suratensis* are high owing to the use of artificial pellet feeds mostly

prepared from plant based ingredients. In this context, a study was undertaken to assess the susceptibility of *E. suratensis* to aflatoxin B$_1$ (AFB$_1$).

Materials and methods

Experimental fish

E. suratensis weighing 35 ± 5g were collected from the brackishwaters of Cochin Estuary (10° 2'N, 76° 14'E). These fishes were acclimatised to filtered brackishwater of 20‰ salinity in fiber reinforced plastic (FRP) tanks of 1000 l capacity, with thorough aeration and 50% daily water exchange. Water temperature and pH ranged from 26 to 28°C and 7.8 to 8.4 respectively throughout the experimental period. They were weaned to pellet feeds @ 3% of their body weight. The fishes were divided into two groups, each group comprising triplicates of ten fishes per tank. Thus each group had a total of thirty fishes held in 3 tanks. The first group received formulated feed having 38% crude protein free of aflatoxin which served as the control group. The second group (treatment group) also received formulated feed of same nutritional quality but incorporated with aflatoxin B$_1$ (AFB$_1$) at the rate of 0.4 mg kg^{-1} feed. The experimental feeding extended for a period of eight weeks. General activity of the fishes such as movement, feed intake and feed wastage if any were monitored throughout the experimental period. Six fishes from each group were sampled at the end of 2nd, 4th, 6th and 8th week and liver tissues were fixed for histological and electron microscopic evaluations.

Aspergillus flavus and Aflatoxin producton

Toxin producing strain of *Aspergillus flavus* was purchased from Central Food Technology Research Institute (CFTRI), Government of India, Mysore, India. Aflatoxin B$_1$ (AFB$_1$) was produced by the method of Shotwell *et al.* (1966), which is briefly described as follows: *A. flavus* was cultured in sterile conical flasks containing rice, which, was autoclaved at 121°C for 15 min and cooled. The rice was shaken in a rotary shaker and the growth of *A. flavus* was monitored. The toxin from the culture was extracted in chloroform as per the method of Pons and Golblatt (1969). The amount of aflatoxin B$_1$ (AFB$_1$) was quantified using standard (AFB$_1$) obtained from M/s SIGMA-ALDRICH, USA, at the laboratory of Spices Board, Ministry of Commerce and Industry, Government of India, Ernakulam, Kerala, India. AFB$_1$ was dissolved in chloroform and the chloroform solution of AFB$_1$ was added to the dough of feed mixture after gelatinisation at a rate of 0.4 mg AFB$_1$ kg^{-1} feed. The dough with the toxin was mixed thoroughly, made into pellets and air-dried. The control feed was prepared using the same proportions of feed ingredients and chloroform without AFB$_1$. This

was added to rule out the effect of chloroform to cause pathological changes.

Histopathology

Sampled fishes were killed and liver was dissected out immediately. The liver tissues sampled were scored several times with a sharp knife and immersed in 10% buffered formalin. The tissue samples were fixed in formalin for 18 to 24 h. Three millimeter thick pieces of liver tissue were dehydrated in ascending grades of ethyl alcohol, cleared in two changes of xylene and impregnated with molten paraffin (58°C). These tissue pieces were embedded in paraffin. The dehydration, clearing and paraffin impregnation were done in an automatic tissue processor (LEICA, Germany). Sections of 4 to 6 µ were cut in a semiautomatic rotary microtome (LEICA, Germany). These sections were deparaffinised in xylene and hydrated in descending grades of ethyl alcohol mixed with distilled water. The sections were stained by Harris Haematoxylin and Eosin (H&E) and observed under microscope.

Ultrastructural studies

Live fish was anaesthetised using clove oil, abdomen slit open and liver was exposed. Drops of chilled (4°C) 3% glutaraldehyde in cacodylate buffer of pH 7.3 was poured over the surface of liver tissue. One millimeter sized cubes of liver tissue were excised from the area where the fixative was poured and immersed in chilled 3% glutaradehyde in cacodylate buffer (pH 7.3). These pieces were kept in the fixative for 6 h at 4°C. After primary fixation, the tissue pieces were washed three times in chilled cacodylate buffer and left immersed in the buffer (pH 7.3) for 12 h under refrigeration. The tissues were post-fixed in 1% osmium tetroxide in the buffer for 1 h followed by three washings in cacodylate buffer. The tissues were dehydrated in ascending grades of acetone as per Dawes (1988) and embedded in Spurr's resin (Spurr, 1969). Ultrathin sections of 60-90 nm size were cut in an ultramicrotome (LKB Nova, LKB Products, Sweden) using glass knives. These sections were lifted on to the matted surface of copper grids (300 µ mesh size). The sections were stained with uranyl acetate (Watson, 1958) and lead citrate (Venable and Coggeshall, 1965), dried and observed under transmission electron microscope (HITACHI H-600, HITACHI Ltd., Japan).

Results

Fish in treatment group and the control group did not elicit any behavioural changes with respect to general activity, swimming and feed intake. There was no incidence of the fishes going off the feed in both the groups. After two weeks of aflatoxin feeding, the fish

revealed pale livers. In fourth and eighth week of aflatoxin feeding, the liver tissue of aflatoxin treated fishes became pale in colour with high fragility. On termination of the experimental feeding at eighth week, the liver of treated fish showed numerous black spots and raised pale patches whereas the control group fishes did not exhibit any of these changes (Fig. 1, 2, 3 and 4).

Histological examination of liver tissues from treatment and control groups showed remarkable differences. Histological sections of liver from control

Fig. 1. *E. suratensis* from the control group exhibiting normal features of viscera and liver

Fig. 2. Liver of *E. suratensis* from the control group showing normal colour
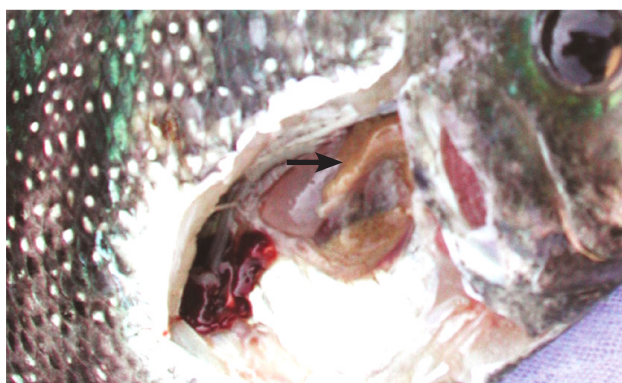
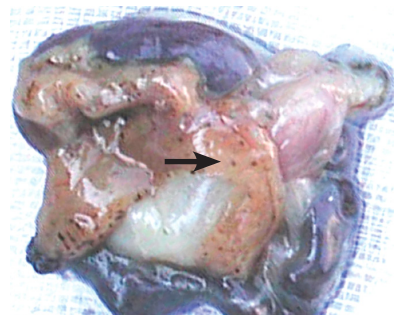Fig. 3. *E. suratensis* treated with aflatoxin exhibiting pale liver

Fig. 4. Liver of *E. suratensis* treated with aflatoxin for 8 weeks. Note the black spots on the surface (shown by arrow)

group fishes presented liver parenchyma with polyhedral plump hepatic cells with abundant foamy cytoplasm and vesicular nuclei. These cells were arranged in irregular cords forming tubular/glandular pattern. The pre-ductules, ductules and biliary canaliculi formed the lumen of the tubule. Single flat epithelial cells lined the pre-ductules, ductules and biliary canaliculi. The sinusoids joined the hepatic veins and biliary canaliculi joined to form larger bile ducts with complete columnar epithelial lining. Pancreatic acinar cells surrounded the hepatic veins. These cells appeared either columnar or pyramidal in shape. They surrounded blood vessels forming oval or round acini. The basal part of the cytoplasm of pancreatic cells took basophilic stain, while apical region took acidophilic stain (eosinophilia in Haematoxylin-Eosin staining) with numerous acidophilic granules (zymogen granules).

In aflatoxin fed fish, the liver histology demonstrated changes depending on the duration of aflatoxin exposure. The samples collected at the end of two weeks of aflatoxin exposure elicited initial stages of degenerative changes in hepatocytes. Initially hepatocytes had perinuclear vacuoles. Later on after four weeks of aflatoxin exposure, distinct vacuoles appeared in peripheral cytoplasm displacing nucleus to periphery. This indicated fatty degeneration of hepatocytes. Focal areas showed loss of cells and hepatocytes undergoing coagulative necrosis. Many hepatocytes had shrunken pyknotic nuclei. In hepatic parenchyma areas of biliary epithelial cell proliferation and fibroblast proliferation were seen (Fig. 5).

As the aflatoxin exposure progressed to sixth week, the livers showed extensive damage. The hepatic tubules lost their architecture due to disruption of their structure. The pancreatic acini underwent severe necrosis. Eosinophilic foci of hepatocytes appeared in parenchyma. Proliferation of biliary epithelium increased. This led to formation of new biliary ductules and bile ducts along with fibrous tissue. Basophilic oval preductular epithelial cells arose from proliferating bile ductules. There was also accumulation of mononuclear cells around proliferating
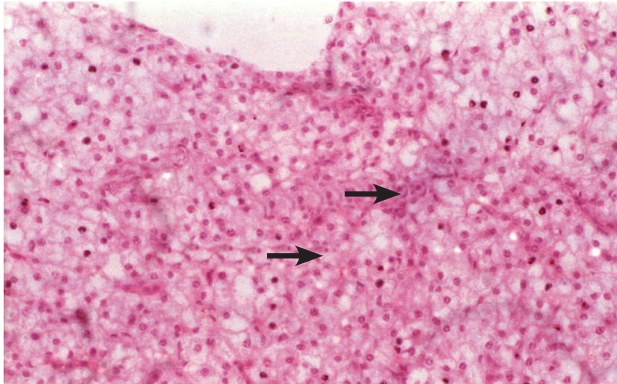
Fig. 5. Section of the liver of aflatoxin treated *E. suratensis* eliciting biliary epithelial proliferation (arrow) and pyknotic nuclei of hepatocytes. Many hepatocytes are vacuolated. H&E; 200 X

bile ducts. Formation of basophilic foci of hepatocytes were evident in many areas (Fig. 6). Pancreatic tissue underwent necrosis followed by hyperplasia and metaplasia.
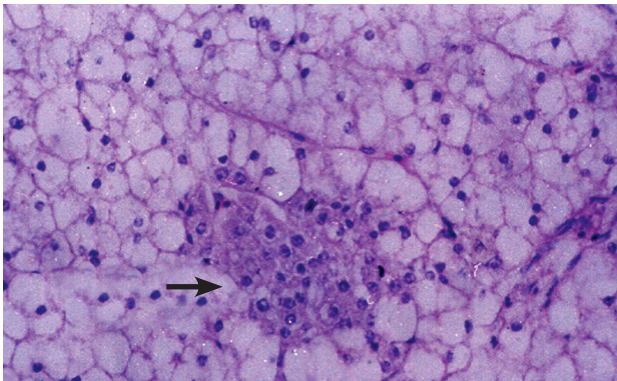

Fig. 6. Section of the liver of aflatoxin treated *E. suratensis* showing foci of basophilic regenerating cells (arrow). Note the other cells showing vacuolation. H&E; 400 X

In the livers of three fish, which were exposed to aflatoxin for eight weeks, massive proliferation of hepatocytes was evident. In two cases, the proliferating hepatocytes had cellular and nuclear hypertrophy. Hepatic tissue in general lost its tubular pattern. The cytoplasm of cells appeared basophilic. Hepatocytes were polyhedral, with different sizes and many had multiple nuclei. Mitotic nuclei were numerous. These cells invaded the parenchyma of liver, which showed degenerating and necrotic hepatocytes. Areas of biliary tissue growth also occurred along with this proliferating hepatocytes (Fig. 7). In one case, the proliferating cells appeared highly basophilic and pleiomorphic. These cells had prominent nucleoli and were found arranged in lose collection. These
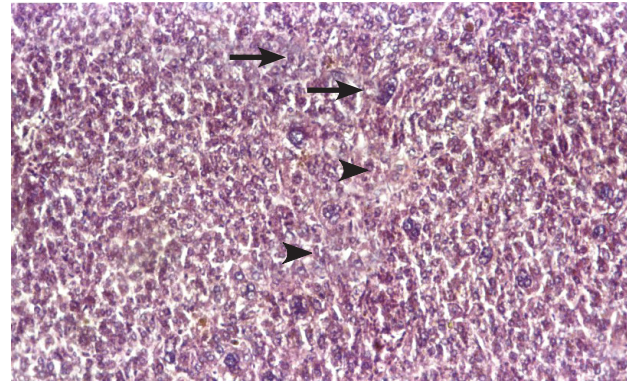

Fig. 7. Section of the liver of aflatoxin treated *E. suratensis* depicting neoplastic transformation of hepatocytes. Note the alteration in architecture of liver parenchyma, multinucleated hepatocytes (arrows) and proliferation of biliary tissue (arrow head). The pleomorphic nature of hepatocytes is also seen. H&E; 400 X

cells did not have any polar orientation and appeared to be anaplastic cells. They did not form the usual hepatic cords/tubules. These cells also invaded the parenchyma and caused degeneration and necrosis. The pancreatic tissue showed necrosis, hyperplasia and metaplasia (Fig. 8, 9 and 10).

Ultrastructural studies of liver showed significant changes in aflatoxin fed fish. The hepatocytes of control fish had rich and abundant profile of smooth and rough endoplasmic reticulum (ER) interspersing the cytoplasmic matrix arranged in parallel arrays. The matrix cytoplasm also contained abundant free ribosomes arranged in rosettes (polyribosomes). In many areas of cytoplasm there were lakes of electron dense granular deposits of glycogen. Well developed golgi apparatus was evident. Mitochondria were found dispersed in between ER as numerous oblong structures with internal membranes
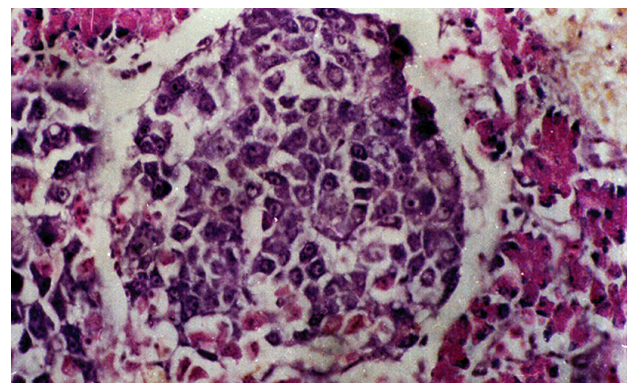

Fig. 8. Section of the liver of aflatoxin treated *E. suratensis* showing proliferation of anaplastic pleomorphic cells indicating induction of carcinoma. H&E; 400 X
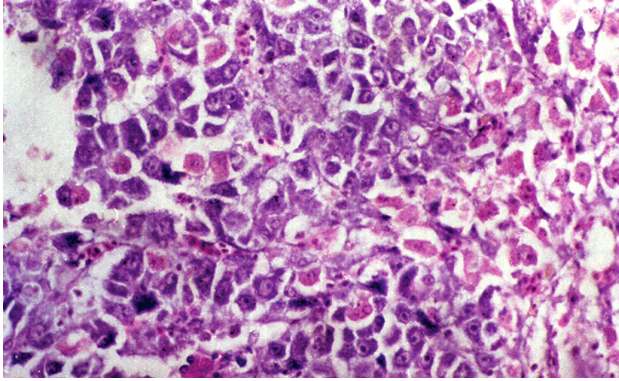
Fig. 9. Liver section from *E. suratensis* fed aflatoxin showing growth of pleomorphic polyhedral anaplastic cells. Note the normal hepatocyes in between the growing neoplastic cells. H&E; 400 X
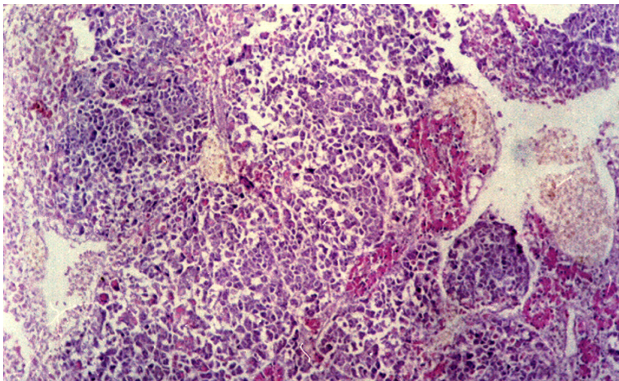

Fig. 10. Section of the liver from aflatoxin treated *E. suratensis* showing massive growth of pleomorphic anaplastic cells into liver parenchyma replacing most of the normal hepatocytes. Only islands of normal hepatocytes are seen. H&E; 400 X
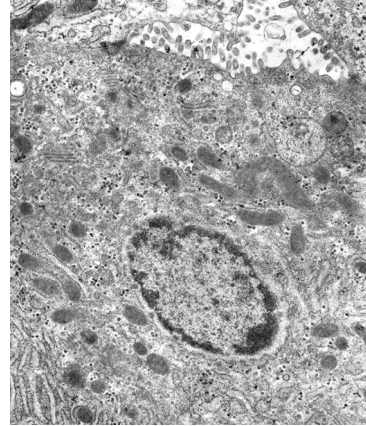

Fig. 11. Electron micrograph depicting the structure of hepatocytes in control fish (*E. suratensis*). Note the abundant RER, large number of mitochondria, presence of glycogen deposits and nucleus having abundant euchromatin and moderate amount of heterochromatin adhering to the nuclear membrane. 5000 X
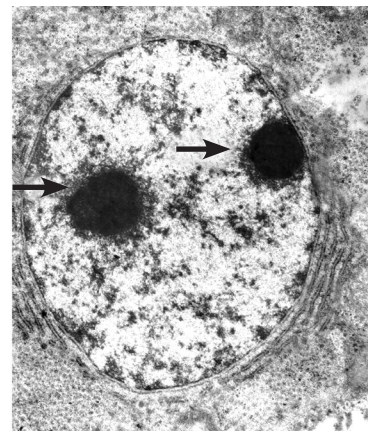

Fig. 12. Electronmicrograph of the nucleus of a hepatocyte from aflatoxin treated *E. suratensis*. Note the presence of electron dense inclusions (arrows) and loss of ribosomes from endoplasmic reticulum at the periphery of the nucleus. Free ribosomes in the cytoplasmic matrix are also seen. 15000 X

thrown into projections. The nucleus of hepatocytes appeared spherical with abundant electron-lucent euchromatin and well developed nucleolus (Fig. 11). The cell membranes facing blood sinuses (Space of Disse) and bile ductules formed finger like evaginations (microvilli). The endothelial cells lining the sinuses had irregular shaped nucleus and scanty cytoplasm.

In aflatoxin fed fish, the hepatocytes underwent degenerative changes leading to destruction of the structure of cells. Initially nucleus revealed extensive changes and subsequently lost their oval/round structure, assumed irregular contour and further electron dense inclusions appeared in the nucleus (Fig. 12). The euchromatin got reduced, while heterochromatin increased and there was also appearance of chromatin and perichromatin granules.

As the nuclear changes progressed, the cytoplasm showed changes in organelles. Initially the smooth endoplasmic reticulum (SER) proliferated forming whirls of SER. The rough endoplasmic reticulum (RER) lost ribosomes and this was followed by fragmentation of SER and RER. Meanwhile the mitochondria lost their structure, many appeared swollen, with disappearing cristae and granules. Subsequently the ER dilated forming large vesicles containing electron-lucent matrix. Vacuoles also appeared in the cytoplasm. Numerous electron dense bodies were seen. The microvilli of hepatocytes lost

their structure, desmosomes got separated and plasma-lemma as well as nuclear membranes lost their continuity (Fig. 13). Autophagosomes containing degenerating organelles were seen in hepatocytes of fish treated with aflatoxin for more than six weeks (Fig. 14).
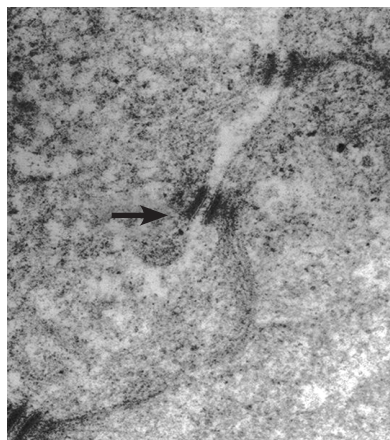

Fig. 13. Electron micrograph of hepatocytes from *E. suratensis* exposed to aflatoxin showing loss of contact between cells indicated by the separation of desmosomes (arrow) and loss of integrity of cell membranes. Disassembly of microtubules is also evident. 60,000 X
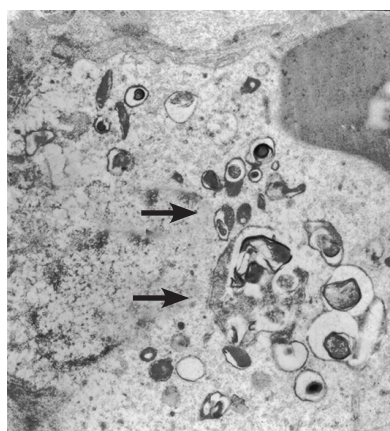

Fig. 14. Hepatocyte from aflatoxin exposed *E. suratensis* depicting different stages of autophagic vacuoles, multivesicular bodies and formation of lipofuchsin. 10,000 X

Discussion

E. suratensis is a table fish relished in many parts of India and is being cultured in polyculture systems in these areas. Groundnut oil cake and rice bran are used as feed in many fish farms. As the present study did not reveal any behavioural changes in aflatoxin fed fish, it can be inferred that, the effect of aflatoxin in this fish remains masked unless histological examination of liver is done. The fish feed ingredients as well as fish feed used in many parts of India contain undesirable levels of aflatoxin (Kalaimani *et al.,* 1998; Begum and Samajpati, 2000). Hence mass mortalities in aquaculture systems due to prolonged exposure to the toxin can be averted only by adopting strict feed storage protocols to prevent contamination by aflatoxins.

The aflatoxin containing feed during the initial stages of exposure caused dystrophic changes in liver parenchyma. This was evident from vacuolar degenerations and accumulation of neutral lipid in hepatocytes. Vacuolar degeneration is an indication of the failure of ion pumps. Swelling of cells is an important common response to cellular injuries. Focal coagulative necrosis and loss of architecture of the hepatic parenchyma were also noticed in the present study. Coagulative necrosis in liver has been identified as a biomarker in a number of toxicological studies in fish (Pitot, 1988). Coagulative necrosis, fatty degenerations, leucocytes infiltration and other dystrophic changes have also been observed in association with aflatoxicosis in tilapia, coho salmon and channel cat fish (Bruenger, 1982; Jantrarotoi and Lovell, 1990; Chavez *et al.,* 1994). In *Labeo rohita,* feeding of aflatoxin incorporated diet led to severe liver lesions like fatty change, coagulative necrosis and fibrosis (George, 1998).

Increase in biliary epithelium as well as stromal tissue were evident initially. Most of the carcinogens induce biliary ductular proliferation in liver (Ward and Vlahakis, 1978). This begins as proliferation of primitive oval cells that arise from ductular cells. These cells are bipotent stem cells, which can differentiate into either hepatocytes or biliary epithelium and they are considered to be cytotoxicant-resistant candidates for the origin of hepatocellular and biliary neoplasm (Sell, 1990; Shiojiri *et al.,* 1991). Nunez *et al.* (1990) demonstrated in aflatoxin induced lesions of rainbow trout, the emergence of small basophilic cells, which appeared to emerge from the central region of the hepatic lobules, the location of biliary preductular cells. Proliferation of preductular cells and biliary epithelium increased at the later stages of exposure and these lesions occupied major portion of liver parenchyma. There was accumulation of mononuclear cells around proliferating bile ductules. Proliferation of biliary passageways occurs as a chronic response to liver injury and this is closely associated with extensive fibroblast proliferation and increase in connective tissue (Hinton and Lauren, 1990). Foci of hepatocytes with eosinophilia and basophilia were noticed in a number of livers. Such tinctorially altered foci occur in carcinogen exposure (Hinton *et al.,* 1992). These foci are believed to be precursors of hepatic neoplasm. Though several such foci develop, only a few progresses to actual neoplasm.

Hence, such foci are considered as preneoplastic lesions (Maronpot *et al.,* 1989; Popp and Goldsworthy, 1989). It is generally viewed that, such foci progress to persistent neoplastic nodules or adenomas and thence to hepatocellular carcinoma (Bannasch *et al.,* 1989; Maronpot *et al.,* 1989). Hepatocellular carcinoma often develops as a result of exposure to aflatoxin in fish. This has been noticed in trouts (Majeed *et al.,* 1984; Nunez *et al.,* 1991). In the treatment group of the present study, some fish after eighth week showed hepatic changes similar to neoplasia. In two cases, massive proliferation of hepatocytes, pleomorphic nature of cells, multinucleated hepatocytes and marked enlargement of cytoplasm of cells indicated a neoplastic transformation. Histological changes observed in the liver tissues were closely similar to hepatocellular carcinomas described elsewhere (Majeed *et al.,* 1984; Nunez *et al.,* 1991). In another case, the cells appeared highly anaplastic and pleomorphic with basophilic cytoplasm. The nuclei contained prominent nuceolus and the cells were arranged in lose collections without any polar orientation. These cells invaded the liver parenchyma causing pressure atrophy and necrosis of normal parenchyma. It gave a picture of highly anaplastic neoplasm. The picture is very similar to hepatocellular carcinoma of fish described by Vogelbein *et al.* (1990) and Myers *et al.* (1987).

Induction of tumor in *E. suratensis* as observed in the present study indicates that aflatoxin B$_1$ (AFB$_1$) is a genotoxin for this species. Studies in the field of genetic toxicology have thrown some light on the formation of DNA adducts. Chemical carcinogens are metabolised to reactive species that bind covalently to DNA (Miller and Miller, 1981). Any chemical that forms DNA adduct has either carcinogenic or mutagenic potential (de Serres, 1988). AFB$_1$-DNA adduct in fish has been found to persist and this persistence may be a factor for induction of neoplasm in liver (Bailey *et al.,* 1988). The present study did not include the estimation and evaluation of DNA adducts. However, the nuclear changes noticed under electron microscopy, such as reduction of euchromatin, accumulation of electron dense inclusion, appearance of chromatin granules and change in morphology of nucleus can be linked to the DNA changes induced by aflatoxin.

Trouts exposed to aflatoxin have revealed proliferation and dilatation of endoplasmic reticulum (ER) (Scarpelli *et al.,* 1963). Hepatocytes are the site of detoxification of xenobiotics and the organelles involved in detoxification process are smooth endoplasmic reticulum (SER). Proliferation of SER could be attributed to increased demand for biotransformation of toxins (Cheville, 1983; Arnold *et al.,* 1995). Increased volume of cytoplasm

in hepatocytes is also due to the proliferation of ER (Hinton, 1993). Nucleus was displaced to the periphery of cells due to the development of vesicles from severe dilatation of ER. Increase in the number of lysosomes was also evident. Such changes are in conformity with the changes observed in rainbow trout in aflatoxin exposure and pesticide toxicity (Nunez *et al.,* 1991; Arnold *et al.,* 1995). Fragmentation and de-granulation of RER indicate lipid peroxidation of membrane system by free radical generation. This may result in separation of desmosomes. Evidence of autophagy, multivesicular bodies and mitochondrial changes, all point towards peroxidation induced cell injury (Cheville, 1983). Intense ultrastructural changes were noticed in aflatoxin treated *E. suratensis* in the present study. All these clearly indicate that, aflatoxin has adverse effect on liver tissue of pearl spot.

Among fishes, trouts are found to be comparatively more sensitive to aflatoxins (Nunez *et al.,* 1990; 1991; Ngethe *et al.,* 1993; Sarcione and Black, 1994) and among tropical fishes tilapia is found to be susceptible (Haller and Roberts, 1980) whereas carps are less sensitive (Svobodova and Piskae, 1980; Svobodova *et al*., 1982). While comparing these results with the present study, it can be inferred that, *E. suratensis* is more sensitive to aflatoxin than other tropical species such as carps and tilapia. Presently studies on aflatoxicosis in fish are mainly concentrated on trouts, owing to the high sensitivity of the fish for carcinoma induction on exposure to aflatoxins. Results of the present study clearly indicated susceptibility of *E. suratensis* to aflatoxicosis and possibility of liver carcinoma induction in the species. Hence, there is much scope for using this species in carcinogenicity studies and the possibility of using this fish as an animal model for studies on hepatocarcinomas needs to be further explored.

Acknowledgements

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