BIOCHEMICAL AND HISTOPATHOLOGICAL EFFECTS OF AFLATOXIN ON *OREOCHROMIS MOSSAMBICUS* (PETERS)

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Certificate

This is to certify that the thesis entitled ***Biochemical and Histopathological Effects of Aflatoxin on Oreochromis mossambicus (PETERS)*** *is an authentic record of the research work carried out by Ms. Suchithra Varior under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of* ***Doctor Of Philosophy*** *in Biochemistry of Cochin University of Science and Technology, and no part thereof has been presented for the award of any other degree, diploma or associateship in any university.*

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Declaration

I hereby declare that the thesis entitled Biochemical and Histopathological Effects of Aflatoxin on Oreochromis mossambicus (Peters) is a genuine record of research work done by me under the supervision and guidance of Prof. Dr.Babu Philip, Head, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology. The work presented in this thesis has not been submitted for any other degree or diploma earlier.

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March 2003

SUCHITHRA VARIOR

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Suchithra Varior

LIST OF NOTATIONS AND ABBREVIATIONS

IUPAC	-	International Union of Pure and Applied Chemistry
LD_{50}	-	Lethal dose causing 50% mortality
TLC	-	Thin layer chromatography
HPLC	-	High pressure liquid chromatography
ELISA	-	Enzyme Linked ImmunoSorbent Assay
RIA	-	Radioimmunoassay
SDS	-	Sodium dodecyl sulfate
TEMED	-	NNNN Tetra methyl ethylenediamine
ALP	-	Alkaline phosphatase
ALT	-	Alanine transaminase
AST	-	Aspartate transaminase
ANOVA	-	Analysis of Variance
ATPase	-	Adenosine triphosphatase
IU	-	International Unit
LERA	-	Lysosomal enzyme release assay
LSD	-	Least significant difference
SOD	-	Superoxide dismutase.

CONTENTS

CHAPTER 1

Introduction

1.0 Introduction

Aquaculture today contributes substantially to the global fish production on account of the all round development during the past decades. A few decades ago, traditional tilapia farming depended mostly on extensive farming methods, where the fish obtained all their nutrition from the aquatic environments in which they were cultured. Nutrient input into the culture systems were limited to fertilizers, and agriculture and animal products or byproducts. In response to the increased cost of land and labor, as well as increased demand for fish, nowadays tilapia husbandry requires aquafarmers to stock fish at densities higher than could be supported by the natural productivity. Hence intensive culture of tilapia has gained popularity and nutritionally complete feeds have become a necessity. The use of artificial feeds in aquaculture systems has increased production and profits considerably (Alceste, 2000).

The production of aquafeeds has been widely recognized as one of the fastest expanding aquaculture industries in the world with annual growth rates in excess of 30% per year. It has been estimated (Tacon, 1997) that the total world production of manufactured compound animal feeds was about 560 million metric tonnes in 1995 of which aquatic feeds constituted 3% or 16.8 million metric tonnes. An estimated 60% of the total world aquafeed production was manufactured in Asia and Europe with a fourth produced for carp, tilapia, milkfish and other fish species feeding low on the food chain.

In tropical countries like India where aquaculture is still developing, it is common to observe pelleted feeds that are being produced with inappropriate procedures for bagging, transport and storage. These facts, in conjunction with the high levels of temperature and humidity in these areas

are probably the causes for the presence of fungal growth and the potential for aflatoxin production. Aflatoxins are a family of closely related heterocyclic compounds produced by some strains of the fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. The important feed ingredients like soybean meal, rice bran, groundnut oil cake and mustard oil cake at present considered as very valuable items in fish feeds may be contaminated by aflatoxins. During harvest and post harvest operations, the grain and oil seeds may not get dried up properly and these contain high undesirable amount of moisture, which makes them good media for the growth of moulds. Aflatoxin is a potent liver toxin and carcinogen, with aflatoxin B_1 being the most toxic compound (Ngethe *et al,* 1992) responsible for the major toxicity syndrome "aflatoxicosis".

Aquatic vertebrates of widely divergent taxa are known to suffer toxic effects of dietary aflatoxin. For example, dietary levels of AFB_1 at or below 25 ppb adversely affected the productive performance of ducklings (Ostrowski – Meissner, 1982 a,b); teleosts are also susceptible to AFB_1 toxicity. Rainbow trout has one of the highest sensitivities to aflatoxin of all animals. In this species, an intake of less than 1μg AFB_1 per kg diet can cause liver tumors, and the LD_{50} for aflatoxin in fish weighing 50g is 0.5 – 1.0 mg/kg of diet (Lovell, 1989). Warm water fishes are generally less sensitive to aflatoxin than cold-water species. Pathological responses to aflatoxin have been reported for channel catfish (Smith, 1982) and tilapia, *Sarotherodon spiluris* (Haller and Roberts, 1980).

The increasing awareness of the scale of aquatic environmental problems has focused attention on the urgent need for sensitive and precise diagnostic tools or biomarkers with a predictive capability for the assessment of toxic contaminant impact. Detection for changes in the amounts and/or

distribution of many cellular proteins and other constituents will enable pathological perturbations to be diagnosed at the very onset itself in otherwise healthy animals. Investigations of pathological alterations at the molecular and cellular levels also provide an improved understanding of the biomolecular disturbances induced by the toxic contaminants. The alteration in some of the biomarkers can thus act as an early warning system for the presence of critical levels of toxic pollutants. Histological, cytological and cytochemical responses observable from animal tissue sections form an important link between effects at the biochemical level and those measured in the whole organism. The tumour suppressor gene, p53 is a molecular genetic biomarker that can be exploited for monitoring carcinogens. Though these genes have been reported in fishes, the effects of various carcinogens on them are yet to be properly worked out in fishes. There is a lacuna regarding the molecular changes in nuclear proteins especially the tumor suppressing protein.

Of late attempts to develop a proper teleost model, which is available in abundance for the chemically induced carcinogenic studies, are underway. This is more relevant in the Indian context, where the tropical climatic conditions favour the development of organic pollutants in addition to the severe damage brought about in the aquatic environment by uncontrolled anthropogenic discharges. The fish *Oreochromis mossambicus* is an ideal teleost model, mainly due to its hardy nature, ease of rearing and maintenance, availability and also because it is one of the commonly cultured species in the south east asian countries.

Hence the present study was undertaken in *Oreochromis mossambicus* with the following objectives:

1. To document the **biochemical changes** mediated by aflatoxin.
2. To study the **effect of aflatoxin on the lipid peroxidation process.**
3. To document the **histopathological alterations** induced by aflatoxin.
4. To evaluate the **effect of aflatoxin on nuclear proteins and serum proteins with special reference to the tumor suppressor protein namely p53.**
5. To evaluate the **effects of aflatoxin on biological membranes, branchial Na^+K^+ATPase** and check for the possible retention **of aflatoxin in muscle.**

CHAPTER 2

Review of Literature

2.0. Review of literature

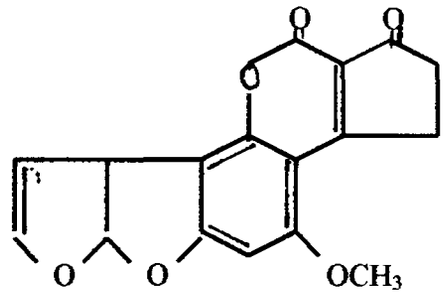
2.1. Historical review

Aflatoxins were first identified as etiological agents for animal disease in the early 1960's, following an outbreak of deaths of turkey in England and elsewhere. The disease, termed Turkey X disease because of the then unknown etiology was characterized by acute hepatic necrosis with bile duct hyperplasia, lethargy, loss of appetite and death. The cause of the effects was traced to Brazilian peanut meal that was used in a component of the poultry ration (Allcroft and Carnaghan, 1963a). The toxic factors in the peanut meal, namely aflatoxins were separated in to four distinct compounds: aflatoxin B_1, B_2, G_1 and G_2 (Nesbitt *et al*, 1962; Sargeant *et al*,1961).

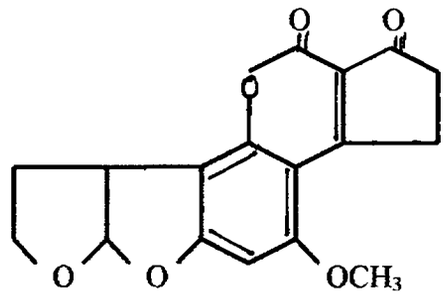
2.2. Structural diversity of Aflatoxins

Nearly all of the interest in aflatoxins has focused on AFB_1, primarily due to its extreme acute and chronic toxicity and its carcinogenic activity in animals, in addition to its potential effects in humans. The toxic factors isolated from feed were separated chromatographically into four distinct compounds: aflatoxins B_1, B_2, G_1 and G_2.The molecular formula indicated that aflatoxins B_2 and G_2 (AFB_2 and AFG_2) were dihydro derivatives of the parent AFB_1 and G_1 respectively (Asao *et al*, 1963;Chang *et al*, 1963; Cheung and Sim, 1964; Van der Merve *et al*, 1963; Van Dorp *et al*, 1963; Van Soest and Peerdeman, 1964). Aflatoxins contain a coumarin nucleus fused to a bifuran and either a pentanone (AFB_1 and B_2) or a six membered lactone (AFG_1 and G_2). AFB_1 and G_1 were more toxic to ducklings, rats, and fish than either AFB_2 or AFG_2 with AFB_1 being the most toxic (Wogan *et al*, 1971; Abedi and Scott, 1969). A similar pattern holds for its carcinogenic potency, $AFB_1 > AFG_1 > AFB_2$ (Wogan *et al*, 1971; Ayes *et al*, 1971).

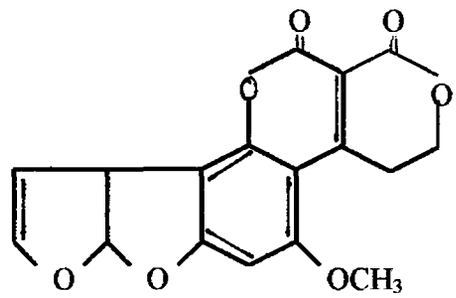
2.3 Structures of Aflatoxin B_1, B_2,$G_1$and G_2



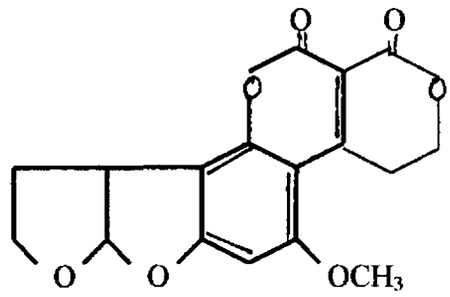
AFLATOXIN B_1



AFLATOXIN B_2



AFLATOXIN G_1



AFLATOXIN G_2

2.4. Biotransformation of Aflatoxin B_1

The major hydroxylated metabolite of AFB_1 formed by cytochromes P-450 are aflatoxin M_1 (AFM_1), aflatoxin P_1 (AFP_1), aflatoxin Q_1 (AFQ_1) and aflatoxin B_{2a} (AFB_{2a}). Additional metabolites, which are generally formed in smaller quantities depending on various conditions, include aflatoxicol M_1 and aflatoxicol H_1. These stable metabolites are considered to be detoxified relative to AFB_1, are more polar, and as such are more easily extractable. The cyclopentanol aflatoxicol (AFL) is not a product of oxidative metabolism, but rather a result of the reproductive metabolism of AFB_1 catalyzed by soluble NADPH - dependent reductases (Wong and Hsieh, 1978).

2.5. Biosynthesis of Aflatoxin

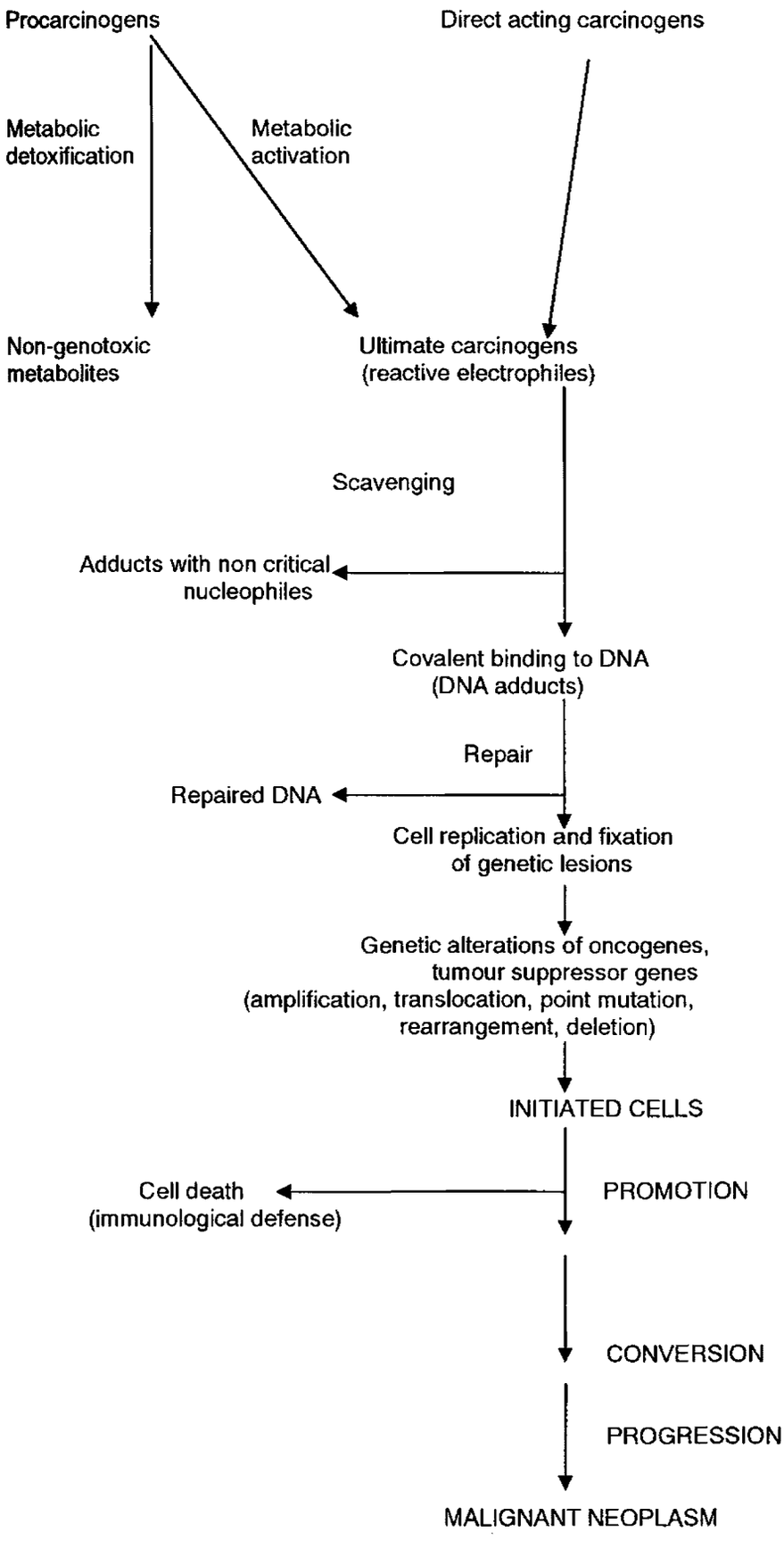
As is the case for many other toxic secondary metabolites produced by fungi, aflatoxins are synthesized by the polyketide route, wherein head-to-tail condensation of acetate units proceed via poly-β-keto-thiol ester intermediate (Applebaum and Marth,1981). In this biosynthetic pathway, the chain is initiated by acetyl coenzyme A, and malonyl COA is the source of additional carbon units (Money, 1976). Relative to other polyketide-derived mycotoxins, the synthesis of aflatoxins has been particularly difficult to elucidate. It is now known that aflatoxins are derived from a C20 polyketide (Smith and Moss, 1985).

2.6. Carcinogenicity of Aflatoxins

A requisite step in the toxic and carcinogenic action of AFB_1 is its conversion to one or more metabolites in the various tissues of exposed

animals. As is the case with other "procarcinogens", the majority of metabolic conversions of AFB_1 is catalyzed by cytochromes p450 which is a group of mixed - function oxidases present in the liver and other tissues. From a toxicological standpoint, the most important reputed toxic intermediate of AFB_1 is the AFB_1-2,3 epoxide (or the 8,9 epoxide by IUPAC nomenclature) which is thought to be the metabolite responsible for alkylation of cellular nucleic acids and subsequent carcinogenic and mutagenic activity. These epoxides bind either to cellular proteins resulting in cytotoxicity or to cellular DNA (N7- guanidine adducts) resulting in mutations of the p53 tumour suppressor genes and finally in preneoplastic lesions and hepatic cellular carcinoma. In the case of AFB_1, phase I biotransformation reactions facilitate bioactivation whereas phase II biotransformation reactions have proven to result in detoxification and excretion. AFB_1 - oxide can be inactivated by enzymatic conjugation with glutathione (GSH) (Degen and Newmann, 1978). Such conjugation has been shown to protect against the hepatocarcinogenic effects of AFB_1 (Degen and Newmann, 1981; Lotlikar *et al*, 1984). Only a small portion of administered AFB_1 will be present in the unmetabolized form in either the tissues or secretions of animals. Trout possess a complex but incompletely characterized array of cytochromes p450, transferases, and other enzymic systems for phase I and phase II procarcinogen metabolism. In general, trout exhibit only limited capacity for DNA repair, especially for removal of bulky DNA adducts. This factor, together with a high capacity for p450 bioactivation and negligible glutathione transferase - mediated detoxification of the epoxide, accounts for the exceptional sensitivity of trout to aflatoxin B_1 carcinogenesis (Bailey GS *et al*, 1996). Aniline hydroxylase and N-demethylase are enzymes responsible for modifying key structural features of aflatoxins. Like many other carcinogens it also acts as a nonspecific cell poison that exerts multiple effects on the structures and biochemically on susceptible cells (Swain and Singh, 1999).

2.7. Summary of events in a Carcinogenic process (Tanaka, 1997)



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 hydroxylase (mEH and cEH), aldehyde dehydrogenase (ALDH), DT diaphorase, gamma-glutamyl transferase (gamma GT), glutathione transferase (GST), uridine diphospho-glucuronyl transferase(UDGPT), and p450 IAI were measured, only aldehyde dehydrogenase and gamma-glutamyl transferase showed increase. Induction of aldehyde dehydrogenase, uridine diphosphoglucuronyl transferase and depression of cytochrome p450 IAI were also noticed. Hepatic accumulation of aflatoxin B_1 deferred in rainbow trout and tilapia (Ngethe *et al,* 1993).

The major target organ involved following chronic exposure of AFB_1 is the liver, but tumours of other organs appear, although these are less prevalent. As is the case with acute toxicity, there exist significant species differences with respect to susceptibility. The Mt. Shasta strain of rainbow trout is by far the most sensitive species of animal or fish to the hepatocarcinogenic effects of AFB_1 (Sinnhuber *et al*, 1977). Less than 1 ppb (parts per billion) in the diet will cause liver tumours in 20 months. The LD_{50} (dose causing death in 50% of the subjects) for aflatoxin in 50-gram rainbow trout is 500 to 1000 ppb. Signs of severe aflatoxicosis in rainbow trout are liver damage, pale gills and reduced red blood cell concentration. The use of rainbow trout in AFB_1 carcinogenesis studies grew out of observations of increased liver cancers in domesticated rainbow trout in many hatcheries in the U.S from 1957 to 1960. Since then, the rainbow trout has proven to be an attractive animal model for chemical carcinogenesis studies.

A diet containing 0.4ppb AFB_1 fed to trout over a 14 month period resulted in a 14% incidence of hepatocellular carcinomas (Lee *et al*, 1968).

When the dose was increased ten fold, a 60% incidence during the same time period was seen. In contrast, wild steelhead trout had an incidence of only 6% after 12 months on 8ppb AFB_1 (Sinnhuber *et al*, 1977).

Research carried out in Auburn University in 1991 revealed that all fishes are not sensitive to mycotoxins. Rainbow trouts are extremely sensitive but brook trouts, coho salmon are less sensitive to aflatoxin ingestion (Halver and Mitchell, 1967). In coho salmon, aflatoxin did not produce hepatoma but liver lesions were present. This included necrosis of hepatocytes and fatty change (Bruenger and Greuel, 1982). In tilapia culture aflatoxicosis was a major cause of losses (Roberts and Sommerville,1982). In carps, aflatoxin did neither produce any liver lesions nor any alteration in haematological values. There was no accumulation of aflatoxins in fish muscles. (Svobodova and Piskac,1982). Warm water fish are less sensitive to aflatoxin. The LD_{50} for channel catfish was found to be approximately 30 times that for rainbow trout. Pathological signs in channel catfish fed lethal dose of aflatoxin were death, liver damage and injury to the lining of the stomach, intestines, spleen, heart and kidney. Some authors also stated that channel catfish fingerlings showed a relatively low response when fed aflatoxin in doses upto 100mg/kg body weight (Ashley,1967).

2.8. Chronic aflatoxicosis

The response of trout to aflatoxicosis varies with size and duration of dose. A chronic response may arise from low prolonged dosages and usually results in a significant incidence of hepatoma, while acute response usually involves force feeding of single or repeated massive dosages of 15 or more mg/kg body weight in 25-50 g fish. These fish generally die within 8-10 days after exposure. Crude aflatoxin fed to rainbow trout at only 20 ppb resulted

in gross hepatoma in 44 of 117 fish after 12 months and in 50 of 88 fish in 16 months. Other trout fed crystalline aflatoxin B_1, the most toxic fraction, at 0.5, 2.0 or 8.0 ppb had gross tumours after 12 months in 37 of 116, 45 of 115 and 52 of 121 fish, respectively (Ashley *et al*, 1964; Halver, 1967).

2.9. Acute aflatoxicosis

Halver (1967) reported most rainbow trout force fed crude aflatoxin at 1, 3 or 5mg/kg body weight in single dose or 1mg/kg body weight daily for 5 days were moribund by day 10 and only six fish survived in the groups fed 1mg/kg body weight daily for 5 days. All fish had gross multiple haemorrhagic areas in liver and adjacent viscera. Moribund fish had dark skin, nearly white gills, indicative of severe anaemia, and were listless. Death usually occurred in less than 24 hours after symptoms appeared. Additional experiments using the more potent aflatoxin B_1 force fed to trout resulted in similar pathology and showed that B_1 is approximately 10 times more toxic than the crude aflatoxin previously used (Halver, 1969). Histopathologically, gills from acutely toxic fish had generalized edema and often the branchial vessels were greatly engorged with blood. Livers had varying degrees of pathological change, depending on total amount of aflatoxin ingested. Some had only slight hepatitis with scattered groups of hepatocytes whose nuclei were pycnotic, karyolytic or had chromatin margination. More severe toxic responses included varying degrees and amounts of hepatic necrosis with or without hyperemia and patches of haemorrhage.

Electron microscopy of classical trabecular hepatoma in rainbow trout was reported by Scarpelli *et al* (1963) and by scarpelli (1967). These authors observed highly developed endoplasmic reticulum and absence of glycogen within the neoplastic cell. The golgi complex was well developed in

neoplastic cells and was characterized by lamellae and vesicles associated with increased numbers of dense granules in some preparations. Peripherally, the cell was poorly organized and free ribosomes were increased. Plasma membranes were reticular and often dense material appeared to be passing into extracellular spaces. Microsomal incorporation of ^{14}C-lysine from trout hepatomas was greater than that from normal livers. A well-organized endoplasmic reticulum generously supplied with ribosomes occurred in most hepatomas. The evidence pointed out toward extensive protein synthesis and reduced level of lipid and glycogen material in tumour cells. Nucleic acids were formed in abundance and upon staining showed intense basophilia typical of neoplastic cells in trout hepatoma. Electrophoretic patterns of serum from normal and tumour bearing trout showed an increase in plasma protein components in hepatomatous fish (Snieszko *et al*, 1966).

The susceptibility of individual animals to aflatoxins varies considerably depending on species, age, sex, and nutrition. Once absorbed into the blood, AFB_1 binds avidly to plasma proteins and loosely to red blood cells (Kumagai *et al,* 1983; Luthy *et al*, 1980). Besides liver damage, aflatoxins cause decreased milk and egg production, recurrent infection as a result of immunity suppression (eg. Salmonellosis), in addition to embryo toxicity in animals consuming low dietary concentrations. While the young of a species are most susceptible, all ages are susceptible but in different degrees for different species. Clinical signs of aflatoxicosis in animals include gastrointestinal dysfunction, reduced reproductivity, reduced feed utilization and efficiency, anaemia, and jaundice. Nursing animals may be affected as a result of the conversion of aflatoxin B_1 to the metabolite aflatoxin M_1 excreted in the milk of dairy cattle. The induction of cancer has been extensively studied. AflatoxinB_1, aflatoxinM_1 and aflatoxinG_1 have been shown to cause various types of cancer in different animal species.

However, only aflatoxinB_1 is considered by the international agency for research on cancer (IARC) as having produced sufficient evidence of carcinogenicity in experimental animal to be identified as a carcinogen.

In a 12 month feeding study of rainbow trout, the hepatocarcinogenic activity of aflatoxin M_1 was roughly 25% that of the parent compound (Sinnhuber *et al*, 1974). Using a sensitive trout embryo exposure carcinogenesis assay, AFM_1 was nontumourogenic (Hendricks *et al*,1980). In hepatocytes isolated from rainbow trout, AFM1 formed DNA adducts at a level significantly less than that of AFB_1 (20% less), but interestingly, this binding activity was much higher than would be predicted from in vivo carcinogenesis studies (Loveland *et al,* 1988).

2.10. Biochemical Effects

Biochemically, aflatoxins can affect energy metabolism, carbohydrate and lipid metabolism. Aflatoxins may be considered as biosynthetic inhibitors both in vivo and in vitro, with large doses causing total inhibition of biological systems and lower doses affecting different metabolic systems (Moreau and Moss, 1969).

2.10 a. Energy metabolism

It has been shown that aflatoxin B_1, G_1 and M_1 inhibit oxygen uptake in whole tissues by acting on the electron transport chain system. They inhibit the activity of the enzyme adenosine triphosphatase to varying degrees, resulting in decreased production of adenosine triphosphate (Moss and Smith, 1985).

2.10 b. Carbohydrate and lipid metabolism

Several studies have also shown that hepatic glycogen levels are reduced due to aflatoxin action. This may be due to the effects of aflatoxin on (1) the inhibition of glycogenesis (2) depression of entry of glucose into liver cells, and (3) acceleration of glycogenolysis.

2.10 c. Nucleic acid and Protein metabolism

Aflatoxins may bind with DNA affecting its activity. Aflatoxin B_1 has been shown to bind more strongly with DNA than aflatoxins G_1 and G_2. Aflatoxin B_1 can also be converted to its epoxide form, which binds to DNA, preventing transcription (Clifford et al,1967; Swensen et al, 1977). It can also bind RNA inhibiting protein synthesis. Aflatoxin B_1 also forms an adduct with serum albumin in a dose dependent manner by binding to the lysine component of this protein, resulting in the formation of lyine – AFB_1, which has been used to assess the level of exposure of aflatoxin B_1 in humans (Sabbioni, 1990). Aflatoxin B_1 can also be converted to one of its metabolites, aflatoxin B_{2a} that react readily with free amino groups of functional proteins. Aflatoxin B_{2a} is not generally regarded as a mycotoxin and is believed to be in equilibrium with its dialdehyde, which reacts with the free amino groups to form schiffs bases, resulting in reduced enzyme activity (Moreau and Moss, 1979).

The level of AFB_1-DNA adducts formed in a species or a tissue is often an accurate indicator of susceptibility to the carcinogenic effects of AFB_1. For example, adduct levels in rainbow trout were found to be 7 to 56 times greater in rainbow trout than in coho salmon at various times following intraperitoneal injections of AFB_1, which correlated with data showing that

the former salmonid is clearly more sensitive to the hepatocarcinogenic effects of AFB_1 (Bailey et al, 1988). Activated AFB_1 binds exclusively with guanyl residues in DNA, and the AFB_1-N7-Gua adduct are by far the most predominant form. Although AFB_1 binds exclusively with guanyl residues, not all guanines in random sequences of double stranded DNA appear to be equally reactive, and the frequency of attack among guanyl sites can vary by ten fold or more (D Andrea and Haseltine, 1978; Misra *et al*, 1983; Muench *et al*, 1983). Not all damains in chromatin are equally accessible to AFB_1. Internucleosomal, or linker DNA is roughly five times as likely to become adducted with AFB_1 as is nucleosomal core DNA in rainbow trout liver, following intraperitoneal injection (Bailey *et al*, 1980).

More recent evidence indicates that the total level of DNA adduct formation by AFB1 (as well as by other chemical carcinogens) may not provide an accurate indicator of the alkylation potential as genetic "hot-spots", such as a proto-oncogene. Activation of proto-oncogenes in animal and human tumours and in cell transformation systems has been shown to involve specific mutations in base sequence, an event postulated to play a crutial role in the early stages of chemical carcinogenesis. Chang *et al* (1991) was the first to demonstrate *ras* gene activation by a known carcinogen in any fish species. Using PCR and oligonucleotide hybridization methods, a high proportion of the aflatoxin B_1 - initiated tumour DNAs showed evidence of activating point mutations in the trout *ras*-1 gene. Among these a predominant lesion was a GGA to GTA transversion in codon 12. This mutation is the most commonly found molecular lesion in rodent carcinogenesis models and many human tumours. Of the remaining mutant *ras* genotypes, two were codon 13 GGT to GTT transversions, and one was a codon 12 GGA to AGA transition.

Any discussion in multistage carcinogenesis is not complete without the mention of the role of tumour suppressor genes such as p53 and retinoblastoma (Rb) genes. In contrast to *ras* genes, such genes are responsible for the negative regulation of cell cycling. Unfourtunately, to date there are limited studies on fish tumour suppressor genes and their potential association with changes in *ras* (Krause *et al,* 1997; Bhaskaran *et al*,1999; Franklin *et al*, 2000). Studies have established that although fish tumour suppressor genes are conserved, a role in the etiology of feral fish tumours, with or without *ras* involvement has yet to be established. Cellular oncogenes and tumour suppressor genes from various species of fish have been isolated and to some extent characterized. Based on the structure, function and cellular location of their protein products several classes of oncogenes have been classified and those isolated from fish include ras, myc, src, erb-A, erb-B, Tu, etc of which much attention has focussed on *ras* gene. The antioncogene p53 has been confirmed in rainbow trout (Van Beneden and Ostrander, 1992).

A mutation in the p53 is emerging as the most common genetic change in human cancer. On the basis of the experiments conducted in vitro, aflatoxinB_1 specifically targets the third and not the second nucleotide of codon 249 (AGG) of the human p53 gene. A high frequency of mutations at a mutational "hotspot" (the third nucleotide of codon 249 in exon 7) has been found in p53 tumour suppressor genes in hepatocellular carcinomas. Thus mutation of the p53 gene could be related to exposure to a specific carcinogen and may be used as a marker for a specific carcinogen.

CHAPTER 3

Extraction and Estimation of Aflatoxin and its Incorporation into Fish Feed

3.0 Introduction

Fungi grow on pelleted feeds at relative humidities above 65%, moisture contents generally above 15% and temperatures that are specific to the fungal species. Most fungal growth occurs at temperatures above 25°C and relative humidities above 85%. Higher temperatures and moisture level favor increased growth. Fungal growth causes weight loss, encourages local rises in temperature and moisture content, off-flavour and discoloration and, perhaps worst of all, some common species produce aflatoxins which are known to be toxic and highly carcinogenic to a wide variety of animals, including some species of fish (New, 1987). Several biological, chemical and environmental factors affect the biosynthesis of aflatoxins. The biological factors include - strain variability, competing microflora and inoculum size. The chemical factors include - the type of substrate, type of nutrients and antifungal agents. The environmental factors include - temperature, water activity, atmosphere gases, light intensity and pH (Ellis *et al*, 1991).

3.1 Extraction

Two major purposes for the extraction steps are : 1) To transfer the toxin from the sample to a solvent effectively and 2) to partially remove the interference substances from the sample and to concentrate the toxins in a smaller volume that is manageable for subsequent analysis (Fun Chu,1991). Therefore, extraction procedures must be efficient, quantitative and must not alter or have any effect on aflatoxin. Early methods of extraction were based on defatting of sample prior to extraction. However, it has since been shown that aflatoxin extraction is not affected by the presence of lipid and that interfering substances, such as fats and pigments, are simpler and faster to remove from the extract than aflatoxins. Commodities with high lipid and

pigment content require a different treatment relative to those with a low content of these components. Most of the interfering substances are often soluble in the same solvents as aflatoxin, therefore, selective extraction or extensive purification methods are required to produce pure extracts. Therefore, the nature of the sample and properties of aflatoxin reflect the type of extraction procedure. Aflatoxins are soluble in slightly polar solvents and insoluble in completely non polar solvents. Practically all aflatoxin are extracted using mixture of organic solvents such as acetone, chloroform, or methanol in combination with small amounts of water (Bullerman, 1979). Aqueous solvents more easily penetrate hydrophilic tissues and enhance aflatoxin extraction (Moss and Smith, 1985). Characteristic fluorescence (Sargeant *et al*, 1961a) and absorption under long wave ultraviolet light (Vander Merwe and Fourie,1963) aid detection and estimation.

3.2 Detection of Aflatoxin

Analytical methods followed for the qualitative and quantitative estimation are TLC (Visual and fluorodensitometric), HPLC, ELISA and RIA. Visual TLC is the method of choice in the countries where other expensive instruments and the infrastructure for immunoassays are not available, though it is criticized for high degree of variation due to individual's acuity. Visual TLC estimation is simple and reliable, so long as the analyst ensures the validity of the method by acceptable recovery experiment. Background fluorescence should be considered, whenever fluorodensitometer is used. Immunoassays and HPLC methods, although sensitive are not readily applicable (Shantha, 1994). As Egmond and Wagstaffe, 1989 opined, it is more important to apply rigorous quality assurance to the measurement procedure than to rely blindly upon standardized and often archaic methods. Bearing this in mind, it became

imperative to use a method which is rapid, simple and accurate and this was made possible by employing a simple extraction and fluorescence test using microcolumns and the Velasco Fluorotoxinmeter. This part of the work namely the estimation of aflatoxin employing the Velasco Fluorotoxinmeter was carried out at Veterinary College, Kerala Agricultural University, Mannuthy, Kerala and this favour is deeply acknowledged.

3.3 Materials and methods

3.3.1 Subculture of the fungus, *Aspergillus flavus*

The culture of the fungus, *Aspergillus flavus*, MTCC No : 277 was obtained from Institute of Microbial Technology (IMTECH), Chandigarh. The fungus was maintained on CZAPEK YEAST EXTRACT AGAR as growth medium.

Composition of Czapek Yeast Extract Agar

• Czapek Concentrate	10 ml
K_2HPO_4	1.0 g
Yeast extract	5.0 g
Sucrose	30 g
Agar	15 g
Distilled Water	1 L

- **Czapek concentrate**

$NaNO_3$	30 g
KCl	5 g

PLATE-1 ***ASPERGILLUS FLAVUS*** **CULTURED ON CZAPEK YEAST EXTRACT AGAR MEDIUM**

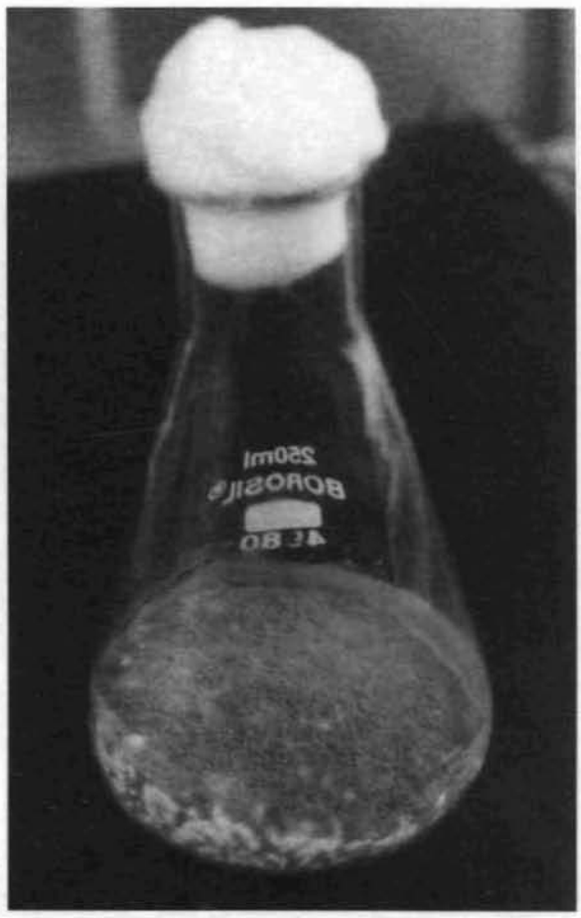


PLATE-2 **CULTURE OF** ***ASPERGILLUS FLAVUS*** **GROWN ON RICE GRAINS**

$MgSO_4.7H_2O$	5 g
$FeSO_4.7H_2O$	0.1 g
Distilled water	100 ml

Culture details

Growth condition	Aerobic
Temperature	30° C
Incubation time	7 Days
Subculture Frequency	30 Days

3.3.3 Extraction of Aflatoxin

A carbohydrate rich source namely, raw rice was used as the solid substrate for the growth of the fungus, *Aspergillus flavus*. A spore suspension was prepared by adding 4ml of normal saline to slant cultures of *Aspergillus flavus* and shaking it vigorously. This spore suspension was used for inoculation of the **autoclaved substrate.** This was then incubated in the dark for 7 days for the growth of the fungus.

Autoclaved Substrate

50 g of rice grain was taken in a 500ml conical flask, moistened with 10ml of Czapek concenterate and added a pinch of dextrose . Twenty nine such flasks were maintained .

3.3.4 Extraction protocol

Modified procedure of Pons *et al*, (1980) was used for the extraction of **aflatoxin.**

- To the flasks containing fungal culture grown on 50gm of rice grain, added 250 ml of acetone- water mixture (85:15) .
- Flasks were kept shaking at a moderate speed on a rotary shaker overnight.
- Sample extract was then filtered through an ordinary filter paper into a conical flask.
- To 125 ml of the filtrate in a separating funnel, added 20ml of 20% lead acetate and 50ml of distilled water.
- 50 ml of chloroform was then added and the separating funnel was shaken briskly for a few minutes.
- The lower chloroform layer was separated, decanted through anhydrous sodium sulphate and evaporated to dryness in a water bath.
- Residue dissolved in a known volume of chloroform.
- Quantification

3.3.5. Detection and Estimation of Aflatoxin using fluorotoxinmeter

Principle

Aflatoxin could be quickly determined in parts per billion by a simple extraction and fluorescence test using microcolumns and the Velasco Flurotoxinmeter (VFM). Aflatoxin is trapped in florisil of microcolumn. It is

excited by UV light and the emitted light is detected by photodetectors and recorded on scale set to ppb (parts per billion).

Procedure

Preparation of the micro-column

- One end of the column was plugged with glass wool.
- With the aid of a funnel and scoop, a layer of sand about 5 to 7 mm in depth was added.
- A layer of florisil was added to a depth of not more than 5 - 7 mm.
- A second layer of sand was added about 5-7 mm in depth.
- A layer of silica gel was added about 15 mm in depth.
- A layer of neutral alumina was added about 15 mm in depth.

Development and reading of the micro-column

The prepared column was wetted with chloroform by lowering bottom of the column into vial containing chloroform. Using a 1 ml syringe, transferred 1ml of the sample solution from vial into prewetted column and allowed to drain in for 2-5 minutes. Added 1 ml of chloroform to column and allowed to drain. Prepared simultaneously a similar column with 50 ng (nanogram) of standard Aflatoxin B_1, which corresponded to 20ppb standard column. Calibrated the VFM instrument by using both the blank column and 20 ppb standard column so as to read zero and 20ppb on the scale respectively. Now placed the sample column in calibrated VFM instrument and noted the reading of aflatoxin. Next the column was turned around 180 degrees and the second reading taken. The final aflatoxin reading was the average of the two.

3.3.6 Preparation of Fish Feed

3.3.6.a Control Diet

The ingredients for the preparation of control fish feed were similar to that of the commercial diets except that groundnut oil cake was replaced by coconut oil cake to eliminate the chance of occurrence of aflatoxin in the diet from the groundnut.

Ingredients	**Quantity incorporated as gram percentage**
Fish Meal	35
Soybean Flour	25
Coconut Oil cake	10
Tapioca starch	20
Gelatin binder	3
Vegetable oil	2
Fish oil	2
Mineral mix 0	1
Vitamin mix 00	2
0Ossopan granules TTK Pharma	
00Vit B1	10mg
B2	10mg
Pantothenic Acid	3mg
Nicotinamide	100mg
Calcium pantothenate	50mg
Folic acid	1500µg
Vitamin B12	15µg
Vitamin C	150µg

All the ingredients were powdered, sieved, blended and extruded through a kitchen noodle maker with a 3 mm die, dried at 45°C overnight and stored in airtight containers .

3.3.6.b Experimental diet

The experimental diet had the same composition as that of the control diet to which varying quantities of the toxin was added from the stock solution. Three experimental diets with 0.375ppm, 2.5ppm and 6ppm were prepared by adding the required quantities from the stock solution into the oil portion of the diet before blending and the chloroform was allowed to evaporate. The ingredients were mixed with water, extruded and then dried.

3.4 Results

The amount of aflatoxin extracted from 1450g of rice grain inoculated with the fungus was 2.7 mg and this was dissolved in 15ml of chloroform to obtain the **aflatoxin stock standard.**

3.5 Discussion

Amongst the chemical factors affecting aflatoxin synthesis and levels of aflatoxin production, the type of substrate used has a major influence on aflatoxin production. In the present study rice was chosen as substrate for production of aflatoxin since studies have shown that optimum aflatoxin production occurs on solid substrates rich in carbohydrates such as coconut, wheat, rice and cottonseed (Detroy *et al.* 1971).

Aflatoxin biosynthesis and level of production is influenced by the nutrient composition of the substrate. Simple sugars such as glucose, fructose and sucrose are the preferred carbon sources for aflatoxin biosynthesis by *Aspergillus flavus* (Davies and Diener,1968). In the present study, sucrose and glucose were used as carbon sources for the synthesis of aflatoxin.

Northolt *et al,* 1977 has reported that the optimum temperature range for fungal growth and aflatoxin production is 25^0c to 30^0c. In nature, temperatures are seldom constant due to seasonal variations through spontaneous heating in stored food commodities such as grain. As a result of temperature variation the yield of aflatoxin can vary considerably. However in this study, an attempt was made to maintain an almost optimum temperature range for fungal growth and aflatoxin production.

Light is essential for many mould species for the induction and completion of sporulation. It influences both the vegetative growth and aflatoxin production of toxigenic moulds in both liquid and solid media. With respect to species, the role of light may be either inhibitory or stimulatory due to photochemical effects on the medium (Carlile,1970). The type of substrate also affected photoresponses and aflatoxin production. Joffe and Lisker observed that aflatoxin production was completely inhibited in Czapek's medium in the presence of light. Bearing the above observation in mind, in the present study also the production of aflatoxin in Czapek's medium was carried out in an atmosphere devoid of light which was created in the lab with the help of thick black chart paper in order to avoid any chance of inhibition of aflatoxin production by light.

Methods of aflatoxin analysis are mainly based on the properties of aflatoxins (Pons and Goldblatt,1965). Among the equilibrium extraction

methods for primary extraction, the solvent system of acetone : water (70:30 or 65:35) to extract toxin from cottonseed, peanuts and other agricultural commodities, as suggested by Pons *et al.* 1966a or Stoloff *et al.*1966, is advantageous because neutral and polar lipids are relatively less soluble in acetone-water mixture and their interference during subsequent analytical steps, is thus avoided. Hence efficient defatting and aflatoxin extraction are conducted simultaneously. The presence of some water appears to facilitate removal of aflatoxin or the release of aflatoxin into the extracting solvent (Goldblatt,1971). Later, Pons *et al.*1980 modified the solvent system to 85:15 ratio of acetone: water in which neutral and polar lipids are not soluble. Hence in the present study, Pons modified solvent system of 85:15 ratio of acetone: water was used as the ideal solvent system believed to selectively extract aflatoxin.

As it was believed that a certain amount of moisture could facilitate the growth of the fungus and therefore the extraction of aflatoxin from it, in the present study, rice grain was prewetted with 10 ml of Czapek's concentrate instead of water. As supporting evidence it was reported that Lee, 1965 slurried defatted peanuts and peanut meal with tenfold excess water and extracted with chloroform by shaking for 30 min on a shaker. The advantage of prewetting the materials as reported by Lee was that almost pure aflatoxin extract could be obtained and hence this was adopted as the official method for the extraction of aflatoxin.

Precipitation of interfering substances, such as pigments, lipids and fatty acids can also be achieved using clarifying agents, such as lead acetate and in this study also a 20% lead acetate solution was used to precipitate the interfering substances.

The solubility of aflatoxins in organic solvents like chloroform, methanol, acetone, etc. helps their quantitative extraction from the commodities. Chloroform was used as the ideal organic solvent for the extraction of aflatoxin since wherever methanol is used, it undoubtedly extracts quantitatively but along with other substances. It also happens to be a good solvent for fat and pigment also (Pons and Goldblatt, 1965).

CHAPTER 4

Effects of Aflatoxin on the Lipid Peroxidation Process

4.0 Introduction

Chemical compounds and reactions capable of generating potential toxic oxygen species / free radicals are referred to as pro-oxidants. On the other hand, compounds and reactions disposing off these species, scavenging them, suppressing their formation or opposing their actions are called anti-oxidants. In a normal cell, there is an appropriate pro-oxidant: antioxidant balance. However, this balance can be shifted toward the pro-oxidant when production of oxygen species is increased or when levels of anti-oxidants are diminished. This state is called oxidative stress and can result in serious cell damage if the stress is massive or prolonged (Irshad and Chaudhuri, 2002).

The oxidant / free radicals are species with very short half-life, high reactivity and damaging activity towards biomolecules like proteins, DNA and lipids. Free radicals are formed by hemolytic cleavage of a covalent bond of a molecule, by the loss of a single electron from a normal molecule or by the addition of a single electron to a normal molecule. Most of the molecular oxygen consumed by aerobic cells during metabolism is reduced to water by using cytochrome oxidase in mitochondria. However, when oxygen is partially reduced it becomes "activated" and reacts readily with a variety of biomolecules. This partial reduction occurs in one-electron steps, by addition of one, two and four electrons to O_2, which leads to successive formation of reactive oxygen metabolites (ROMs). These are five possible species: $O_2^{\cdot-}$ (superoxide anions), $HO_2^{\cdot}$ (hydroperoxyl radical), peroxide ion (HO_2^-), H_2O_2 (hydrogen peroxide) and $^{\cdot}OH$ (hydroxyl radical).

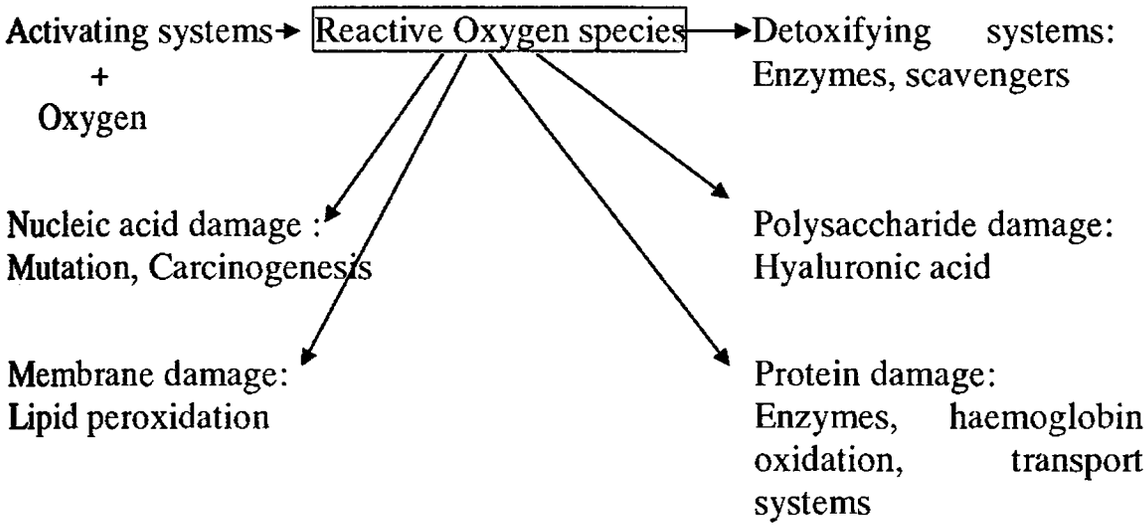
4.1 Source of Reactive Oxygen Species

The exogenous sources of Reactive Oxygen Species (ROS) include electromagnetic radiation, cosmic radiation, cigarette smoke, car exhaust, UV Light, Ozone (O_3) and low wavelength electromagnetic radiation. Similarly the endogenous sources of ROS are mitochondrial electron transport chain, respiratory burst by phagocytes, beta oxidation of fat in peroxisome, auto-oxidation of amino acids, catecholamines and hemoglobin. Superoxide anion radical (O_2) regulates metabolites capable of signaling and communicating important information to the cellular genetic machinery (Mc Cord, 2000). Hydroxyl radical is another damaging radical with a half-life of 10^{-5} sec and produced from H_2O_2 and O_2 by Haber – Weiss reaction (Beauchamp and Fridovich, 1970). Some HO may be produced from hypochlorous acid in phagocytic cells. Similarly, H_2O_2 is a relatively stable, poorly reactive non-radical oxygen species, which easily crosses cell membrane and attacks different rates by converting into HO. This is produced by dismutation of O_2 by superoxide dismutase (SOD) and finally meets many fates including its reduction to water. H_2O_2 is also involved in the generation of free radicals in presence of transitional metal ions.

4.2 Damage by Reactive Oxygen Species (ROS)

Even without pollution and xenobiotic metabolism there is a constant production of reactive oxygen species in all living cells. Due to the high reactivity of reactive oxygen species most components of cellular structure and functions are likely to be targets of oxidative damage (Kappus, 1987).

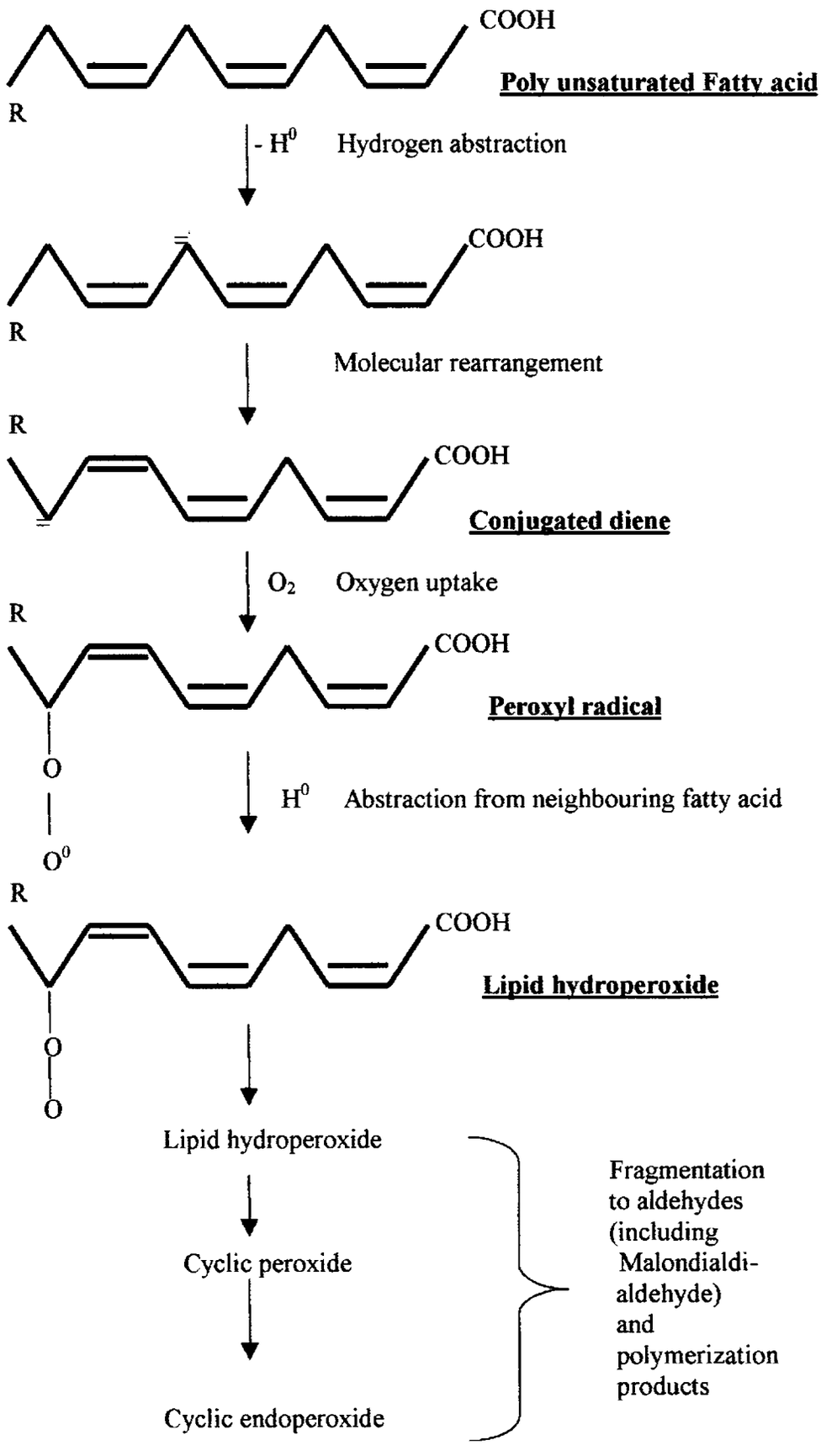
Generalized Scheme for Oxidative Injury to Macromolecules (after Jones, 1985)



4.3 The Lipid Peroxidation process

Cellular biomolecules like lipids are the most susceptible to oxidative damage. Reaction of ROS with lipids leads to the highly damaging reaction, lipid peroxidation. Singlet oxygen reacts with unsaturated fatty acids, forms lipid hydroperoxides that breaks down to several products of lipid peroxidation. (Thomas *et al,* 2002). The reaction sequence starts with a radical (eg. OH) which removes one proton from the hydrocarbon tail of the fatty acid leaving the radical of the acid. This radical undergoes isomerization and oxidation with molecular oxygen yielding a peroxy radical of the fatty acid. Peroxy radical removes protons from other molecules and become hydroperoxide. Since this proton may originate from another fatty acid, a new cycle is started and lipid peroxidation proceeds via a chain reaction, until the chain is interrupted by either the dimerisation of two radicals or until the proton is removed from a substance which forms relatively stable radicals with low reactivity (radical scavengers). Through this chain reaction, one initiating radical may lead to the peroxidation of hundreds of fatty acids. The resulting hydroperoxides are unstable and decomposed by chain cleavage to a very complex mixture of aldehydes, ketones, alkanes, carboxylic acids and polymerization products (Esterbauer *et al* , 1982). Hydroperoxides and decomposition products are toxic and may form fluorescent adducts with DNA (Fujimoto *et al* , 1984). The only mechanism, which produces malondialdehyde in biological systems, is lipid peroxidation. Malondialdehyde is not the major product of lipid peroxidation, but a typical degradation product.

Initiation and propagation reactions of lipid peroxidation



4.5 The Antioxidant Defense Mechanism

To counter the harmful effects of ROS, antioxidant defense mechanism operate to detoxify or scavenge these reactive oxygen species. The antioxidant system comprises of different types of functional components classified as first line, second line and third line defenses.

4.6 Preventive Antioxidants - First Line Defense

The first line defense comprises preventive antioxidants that act by quenching of O_2, decomposition of H_2O_2 and sequestration of metal ions. The antioxidants belonging to this category are enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase and non-enzymatic molecules like minerals and some proteins.

(a) Superoxide dismutase (SOD) (E.C. 1.15.1.1)

SOD mainly act by quenching of superoxide ($O_2^{\cdot -}$), an active oxygen radical, produced in different stages of aerobic metabolism (Meier *et al*, 1998; MacMillan-Crow *et al*, 1998; Yamakura *et al*, 1998; LiY *et al*, 1995; Kizaki *et al*, 1997)

$$2O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Different enzymes of SOD are described. The one present in the mitochondria is Mn^{2+} dependent, that present in the cytoplasm is independent. An extra cellular Cu-Zn dependent enzyme is also reported (Halliwell and Gatteridge, 1985; Fridovich, 1989).

(b) Catalase (E.C. 1.11.1.6)

Catalase is an extraneous enzyme, present in most of the cells and acts by catalyzing the decomposition of H_2O_2 to water and oxygen.

$$2H_2O_2 \longrightarrow 2H_2O+O2$$

Catalase is located almost exclusively in peroxisomes. Most purified catalases have been shown to consist of four protein subunits, each of which contains a haem (Fe(III) – protoporphyrin) group bound to its active site.

(c) Glutathione reductase (E.C. 1.6.4.2)

The function of this enzyme is to regenerate GSH, which has been converted to GSSG by oxidation and by thiol transfer reaction (Rana *et al*, 2002).

$$GSSG+NADPH+H^+ \longrightarrow 2GSH + NADP^+$$

The enzyme is a flavoprotein containing one mole of flavin adenine dinucleotide per enzyme subunit. It contains a cysteine moiety that undergoes reduction and oxidation during the catalytic cycle.

(d) Glutathione peroxidase (E.C. 1.11.1.9)

Glutathione peroxidases (GSH –PX) are selenoenzymes which catalyze the reduction of hydroperoxides at the expense of GSH (Flohe, 1989); Ursim *et al*, 1995). In this process, hydrogen peroxide is reduced to water whereas organic hydroperoxides are reduced to alcohol.

$$H_2O_2 + 2GSH \longrightarrow 2H_2O + GSSG$$

GSH –PX resides in the cytosol and mitochondrial matrix (Mills, 1960). The antioxidant minerals include Si, Mn, Cu and Zn and function primarily in the metalloenzymes.

4.7 Radical Scavenging Antioxidants – Second Line Defense

The antioxidants belonging to second line defense include glutathione (GSH), vitamin C, uric acid, albumin, bilirubin, vitamin E (mainly ∝-tocopherol) carotenoids, flavonoids and ubiquinol.

Glutathione (GSH)

Glutathione is a tripeptide (χ-glutamyl cysteinyl glycine) and its unique structure holds the key to its diverse functional activities. The multiple physiological and metabolic functions of GSH include thiol transfer reactions that protect cell membranes and proteins. These include thiol disulfide reactions that are involved in protein assembly, protein degradation

and catalysis. GSH participates in reactions that destroy H_2O_2, organic peroxides, free radicals and certain foreign compounds (Rana, 2002)

4.8 Repair and De-Novo Enzymes, Third Line Defense

Third line antioxidants are complex group of enzymes for repair of damaged DNA, damaged protein, oxidized lipids and peroxide and also to stop chain propagation of peroxyl lipid radical e.g. lipase, proteases, DNA repair enzymes, transferase, methionine sulphoxide reductase etc. (Henle and Linn, 1997)

4.9 Materials and Methods

4.9.1 Activity of Free Radical Scavenging Enzyme

4.9.1.a Estimation of Catalase (Maehly and Chance,1955)

Reagents

1. Phosphate buffer : 0.01 M pH 7.0
2. H_2O_2 : 30mM

The enzyme extracts were prepared by homogenizing the tissues in 0.01M phosphate buffer, pH 7.0 and centrifuging at 5000 rpm. The reaction mixture contained 0.01M phosphate buffer, 30mM hydrogen peroxide and the enzyme extract. The estimation was done spectrophotometrically by following the decrease in absorbance at 230nm. Specific activity was expressed in terms of international units/mg protein. 1IU = change in absorbance/min/extinction coefficient (0.021)

4.9.1.b Estimation of Superoxide dismutase (SOD) (Kakkar et al (1984))

Reagents

Sodium pyrophosphate buffer	: 0.052M (pH 8.3)
Tris-HCl Buffer	: 0.0025M (pH 7.4)
Phenazine methosulphate (PMS)	: 186μM
Nitro Blue tetrazolium (NBT)	:300μM
NaOH	:780μM
Glacial Acetic Acid	
n - butanol	

The tissues were homogenized in 0.33mM sucrose and subjected to differential centrifugation to get the cytosol fraction. This fraction was then dialysed against 0.0025M Tris-HCl buffer (pH 7.4) overnight before using for enzyme assay. Assay mixture contained 1.2ml of sodium pyrophosphate buffer, 0.1ml of PMS , 0.3 ml of NBT , 1.3ml of distilled water and 0.1 ml of the enzyme source. The tubes were kept 30°C for one minute. Reaction was initiated by the addition of NaOH incubated at 30°C for 90sec and the reaction stopped by addition of 1 ml of glacial acetic acid. Reaction mixture was shaken vigorously with 4.0ml of n- butanol. The mixture was allowed to stand for 10 min and centrifuged. The upper butanol layer was taken out. Color intensity of the chromogen in butanol was measured at 560nm against n-butanol blank. A system devoid of enzyme served as control. One unit of enzyme activity is defined as the enzyme concentration required to inhibit chromogen production by 50% in one minute under the assay conditions and specific activity expressed as units/mg protein

4.9.1.c. Estimation of Glutathione Reductase (Bergmeyer, 1974)

Reagents

Phosphate buffer : 0.067M (pH 6.6)
EDTA : 15mM (Ethylene diamine tetraacetic acid)
NADPH : 0.06%
GSSG : 1.15% (Oxidized glutathione)

Procedure

The tissues homogenates were prepared in phosphate buffer. The reaction mixture comprised of 1.6 ml of phosphate buffer, 0.1 ml of EDTA , 0.12 ml of NADPH, 0.12 ml of GSSG (oxidized glutathione)and 0.1ml of the enzyme source. The decrease in absorbance was noted for 3-5 minutes at 340nm. The controls were run with water instead of GSSG. Enzyme activity was expressed as units/mg protein. One unit was defined as the change in absorbance / minute.

4.9.2 Activity of Lipid Peroxidation Prodcucts

4.9.2.a Estimation of Malondialdehyde (MDA) (Niehaus and Samuelson, 1968)

Reagents

TCA -TBA -HCl reagent : 1.5% (w/v) TCA, 0.375% w/v TBA in 0.25 N HCl

TCA- Trichloroacetic acid ; TBA- Thiobarbituric acid

Preparation of Tissue Homogenate

The tissues were homogenized in 0.1 M tris-HCl buffer, pH 7.5 and allowed to stand for 5 minutes. The supernatant was used for the determination of lipid peroxidation products. 1 ml of the enzyme extract was combined with 2 ml of TCA-TBA-HCl reagent and mixed throughly. The solution was heated for 15 minutes in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 minutes. The absorbance of the sample was read at 535 nm against a blank that contained no enzyme extract. The extinction coefficient of MDA is 1.56×10^5 M^{-1} cm^{-1}.

4.9.2.b Estimation of Hydroperoxide (Mair and Hall, 1977)

Reagents

Potassium iodide (KI)	: 6 g KI in 5 ml distilled water
Cadmium acetate	: 0.5 % in distilled water
Tris – HCl buffer	: 0.1M(pH 7.5)

Procedure :

Liver, heart, muscle and kidney homogenates were prepared separately in Tris-HCl buffer. 1ml of the tissue homogenates were mixed thoroughly with 5ml of chloroform: methanol (2:1) followed by centrifugation at 1000g to separate the phases. 3ml of the lower chloroform layer was recovered using a syringe, transferred to a test tube and dried in a water bath set at 45°C. 1ml of acetic acid: chloroform (3:2) mixture followed by 0.05ml of KI

was quickly added and the tubes were stoppered and mixed. The tubes were placed in the dark at room temperature for exactly 5 min followed by the addition of 3ml of cadmium acetate. The solution was mixed and centrifuged at 1000g for 10 min. The absorbance of the upper phase was read at 353nm against a blank containing the complete assay mixture except the tissue homogenate. Molar extinction coefficient of hydroperoxide is 1.72 x $10^4 M^{-1} cm^{-1}$.

4.9.2.c Estimation of Conjugated Dienes (Retnagal & Ghoshal, 1966)

Procedure

Membrane lipids were extracted and taken to dryness as described for the iodometric assay for hydroperoxide. The lipid residue was dissolved in 1.5ml of cyclohexane and the absorbance at 233nm was determined against a cyclohexane blank. Molar extinction coefficient of conjugated dienes is 2.52 x $10^4 M^{-1} cm^{-1}$.

4.9.3 Activity of Antioxidants

4.9.3.1 Estimation of Glutathione (Patterson and Lazarov, 1955)

Alloxan	: 0.1M
Phosphate buffer	: 0.5M(pH7.5)
NaOH	: 0.5N
NaOH	: 1N

Procedure

Tissues homogenates were prepared in 0.5M phosphate buffer, pH 7.5. The reaction mixture containing 50 ml tissue homogenate, 50μl alloxan, 50μl phosphate buffer and 50μl NaOH (0.5N) was incubated at 25°C for 6 minutes. The reaction was stopped by the addition of 50μl of 1N NaOH. Absorbance was read at 305nm. A control tube was maintained with phosphate buffer instead of extract, the values were expressed as mg/100g tissue.

4.10 Results

The concentration of the lipid peroxidation products, the activities of the antioxidant enzymes and the level of the anti- oxidant in the different tissues subjected to different concentrations of aflatoxin for time periods of two weeks and six weeks is presented in tables 4.0 to 4.1 and figures 4.1 to 4.7 respectively. Results were statistically analyzed using ANOVA (analysis of variance) followed by LSD (Least significance difference) analysis.

Table 4. 0 Levels of Lipid Peroxidation Products in the different Aflatoxin exposed Tissues

Para-meter	Treatment						
		0.375ppm		2.5ppm		6ppm	
	Control	14days	42 days	14days	42 days	14days	42 days
Conjugated diene (mmoles/100g wet tissue)							
Liver	12.73±1.5	45.83±1.6	47.28 ±2.8	46.39±2.0	38.02±3.2	35.75±3.2	49.69±4.3
Heart	6.24±1.4	6.98±1.5	6.81±2.1	20.27±0.9	6.14±1.4	7.59±1.1	6.44±0.8
Muscle	13.63± 0.8	20.18±3.5	12.59±2.8	20.27±3.8	30.54±2.4	21.58±3.1	31.08±4.1
Kidney	8.15±1.6	9.31±3.7	11.50±4.2	15.39±2.6	43.83±1.9	10.57±2.8	41.40±3.3
Hydroperoxide (mmol/L)							
Liver	4.81±1.1	14.28±0.8	7.34±1.4	14.39±1.4	13.87±0.7	17.21±1.3	12.81±0.9
Heart	3.01±0.9	8.52±0.6	8.33±0.8	11.99±1.3	10.65±1.4	9.93±0.75	8.50±0.6
Muscle	1.76±0.41	3.12±0.3	2.74±0.54	8.25±0.34	3.48±0.77	9.75±0.75	3.53±0.59
Kidney	2.88±0.3	9.02±0.45	6.12±0.38	9.31±0.55	12.94±0.9	10.19±0.4	13.46±0.5
Malondialdehyde (mmoles/100g wet tissue)							
Liver	0.07± 0.02	1.42±0.21	0.25±0.09	1.00±0.05	0.17±0.06	0.54±0.1	0.73±0.2
Heart	0.01±0.01	1.15±0.03	0.12±0.02	0.59±0.03	0.10±0.04	1.45±0.06	0.24±0.11
Muscle	0.05±0.01	2.48±0.23	0.17±0.02	1.99±0.15	0.33±0.13	2.32±0.07	0.22±0.09
Kidney	0.02±0.01	1.24±0.04	1.06±0.03	2.62±0.05	1.08±0.02	0.89±0.01	0.77±0.03

Values are the mean ± SD of six separate experiments.

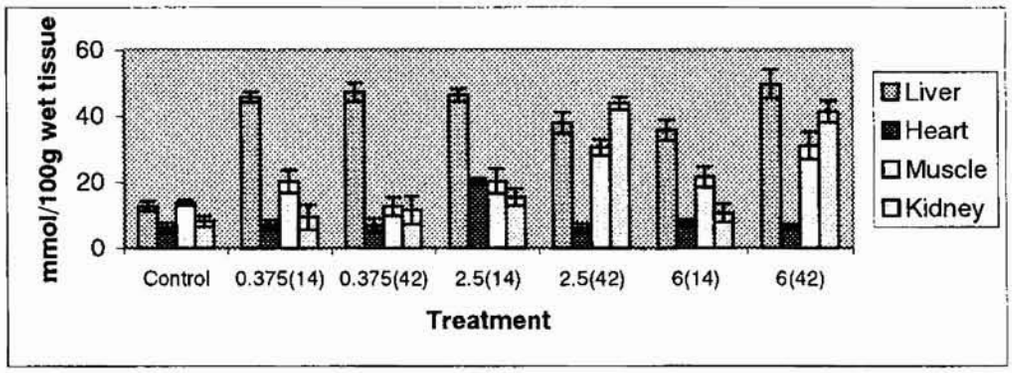


Fig 4.10 Levels of Conjugated Dienes in the various Aflatoxin exposed groups

Source of variation	SS	df	MS	F	
Total	7041.56	31			
Between concentrations	1297.46	3	432.488	4.99	p<0.01
Between tissues	3475.98	3	1158.66	13.38	p<0.01
Between days	189.385	1	189.385	2.19	NS
Error	2078.73	24	86.614		

SS - sum of squares, df - degrees of freedom, MS - mean of squares

Table 4. 0.a. ANOVA for Conjugated Dienes

There was a significant difference ($p<0.01$) between the different aflatoxin concentrations and the different tissues in the levels of conjugated dienes. A comparison between time periods did not reveal any significant change. Further comparison using LSD analysis revealed that there was significant difference seen between control and the different aflatoxin concentrations but a comparison between concentrations did not reveal any significant difference from one another. LSD value at 5% level was 9.604. Concentration 2.5ppm gave significantly higher value on comparison with the other concentrations. With the exception of the muscle and kidney, all

tissues differed significantly ($p<0.01$) from one another. Results of LSD analysis are presented in table 4.2. and table 4.3.

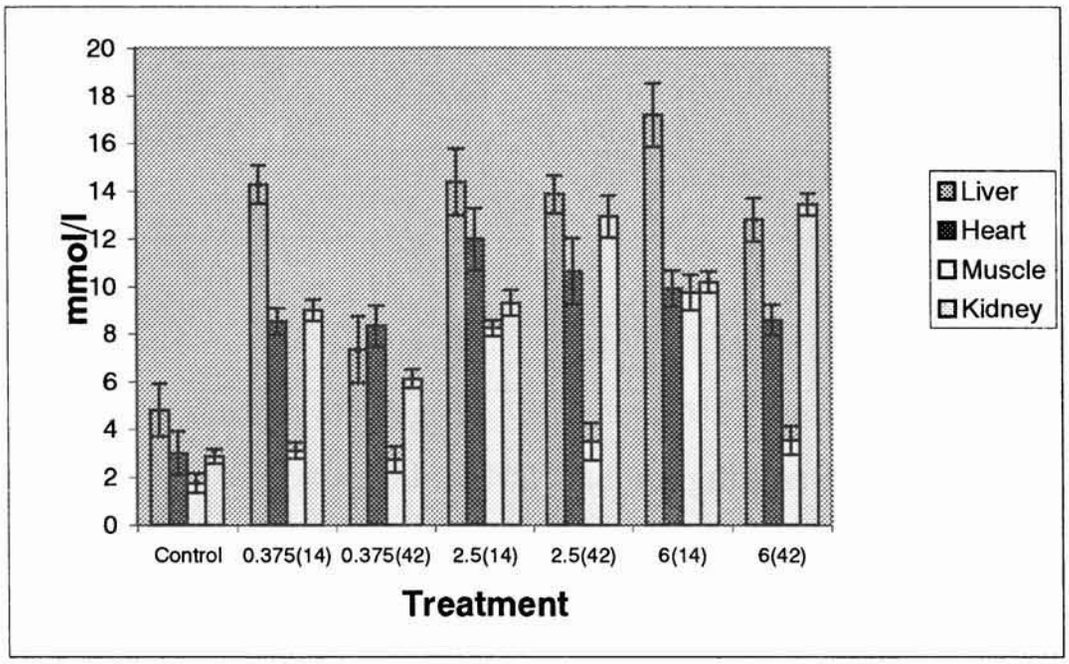


Fig 4.2. Levels of Hydroperoxide in the various Aflatoxin exposed groups

There was a significant difference ($p<0.01$) between the different aflatoxin concentrations and the different tissues in the levels of hydroperoxide. A comparison between time periods did not reveal any significant change. Further comparison using LSD analysis revealed that there was significant difference seen between control and the different concentrations but a comparison between concentrations did not reveal any significant difference from one another. LSD value at 5% level was 2.093. Concentration 6 ppm gave significantly higher values on comparison with the other concentrations. With the exception of the heart and kidney, all tissues differed significantly ($p<0.01$) from one another. Results of LSD analysis are presented in table 4.2. and table 4.3.

Source of variation	SS	df	MS	F	
Total	611.19	31			
Between concentrations	305.28	3	101.8	24.741	p<0.01
Between tissues	191.83	3	63.94	15.547	p<0.01
Between Days	15.365	1	15.37	3.736	NS
Error	98.718	24	4.113		

SS - sum of squares, df - degrees of freedom, MS - mean of squares

Table 4. 0.b. ANOVA for Hydroperoxides

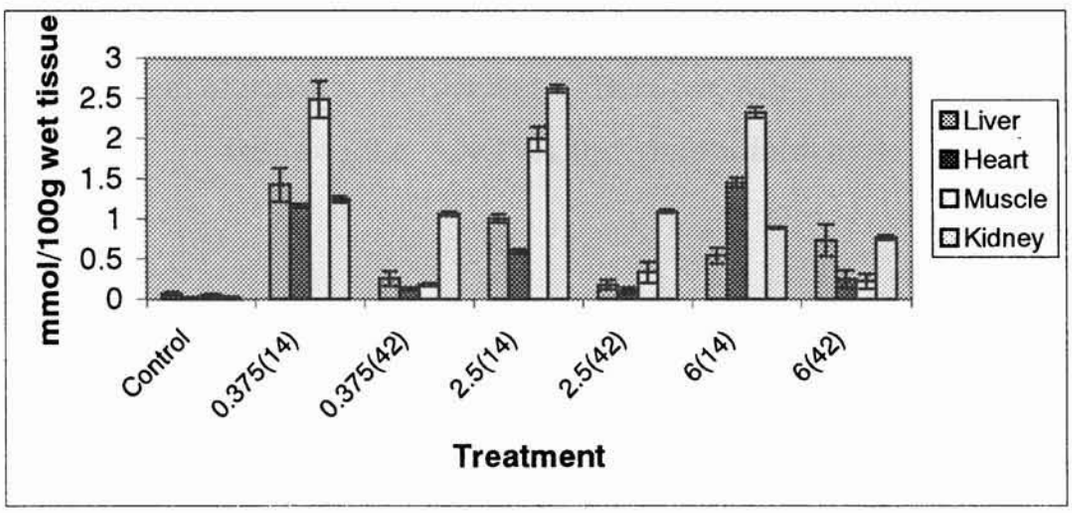


Fig 4. 3 Levels of Malondialdehyde in the various Aflatoxin exposed groups

There was a significant difference ($p<0.01$) between the different aflatoxin concentrations and the different time periods in the levels of malondialdehyde. A comparison between the different tissues did not reveal any significant change. Further comparison using LSD analysis revealed that there was significant difference ($p<0.01$) seen between control and the different concentrations but a comparison between concentrations did not reveal any significant difference from one another. LSD value at 5% level

was 0.559. Concentration 0.375 ppm gave significantly higher values on comparison with the other concentrations. The values at 14 days were significantly higher than the values at 42 days. Results of LSD analysis are presented in table 4.2. and table 4.3.

Source of variation	SS	df	MS	F	
Total	18.71	31			
Between concentrations	5.133	3	1.711	5.839	p<0.01
Between tissues	1.72	3	0.573	1.956	NS
Between days	4.828	1	4.828	16.478	p<0.01
Error	7.025	24	0.293		

. SS - sum of squares, df - degrees of freedom, MS - mean of squares

Table 4. 0.c ANOVA for Malondialdehyde

Table 4.1. Levels of Antioxidant enzymes in the different Aflatoxin exposed tissues

Parameter	Treatment						
		0.375ppm		2.5ppm		6ppm	
	Control	14days	42 days	14days	42 days	14days	42 days
Catalase (IU/mg protein)							
Liver	10.00 ±1.3	17.764 ±1.2	82.15 ±5.8	31.29 ±5.6	160.21±8.8	25.608 ±4.4	164.05 ±10.9
Heart	3.201 ±0.8	2.934 ±0.6	10.56±0.45	8.80 ±0.32	2.096 ±0.36	1.564 ±0.5	1.956 ±0.2
Muscle	3.353 ±0.02	3.467 ±0.03	3.085 ±0.4	11.22±0.67	17.99 ±0.98	32.27±4.56	37.218 ±1.67
Kidney	4.082 ±1.45	5.868 ±2.45	23.47±1.55	12.42±4.35	5.868 ±2.77	32.276 ±3.1	83.836 ±6.89
SOD (units/mg protein)							
Liver	12.63 ±0.33	21.87 ±1.09	16.53 ±2.32	18.50±0.98	20.65 ±1.43	16.33 ±1.89	10.78 ±2.11
Heart	14.38 ±0.98	16.08 ±0.45	21.31 ±1.22	8.144±1.45	18.27 ±0.94	7.60 ±0.22	20.89 ±1.2
Muscle	8.09 ±0.78	8.56 ±1.45	22.12 ±2.1	7.41 ±1.56	15.60 ±0.78	9.13 ±0.34	20.47 ±1.52
Kidney	10.69 ±1.22	10.86 ±1.34	7.73 ±0.67	10.57±1.37	4.41 ±2.2	9.99 ±0.73	7.04 ±1.59
GR $\times 10^{-3}$ (units/mg protein)							
Liver	3.8 ± 0.18	6.94 ± 0.34	9.75 ± 0.54	6.29 ± 0.20	6.66 ± 0.77	5.86 ± 0.56	7.61 ± 0.39
Heart	3.78 ± 0.50	5.48 ± 0.71	6.07 ± 0.32	8.09 ± 0.84	3.62 ± 1.21	7.39 ± 1.54	5.86 ± 0.76
Muscle	3.0 ± 1.1	8.09 ± 1.12	7.19 ± 1.32	2.69 ± 0.41	7.37 ± 1.08	3.28 ± 0.66	4.84 ± 0.76
Kidney	3.47 ± 0.98	4.38 ± 0.58	3.03 ± 0.91	2.39 ± 0.39	6.30 ± 1.56	3.33 ± 0.78	4.47 ± 0.54
Glutathione (nmole/100g wet tissue)							
Liver	1242.10±48.5	1301.4±62.8	2698.66 ±72.3	1978.43±69.4	2987.77 ±52.1	2350.31±40.4	3637.735 ±51.9
Heart	1816.29 ±58.3	1845.8 ±75.9	1941.08±82.1	2326.25±64.3	2355.31±52.6	2569.99±45.6	2279.150 ±58.8
Muscle	667.613±32.4	693.705 ±38.2	693.70S±37.8	759.326 ±43.5	818.697 ±38.5	801.979 ±49.1	859.788 ±49.2
Kidney	1413.97±69.6	1453.031 ±82.3	1515.37±91.5	1859.25±76.3	1885.45±56.7	2120.00 ±73.2	2442.00 ±58.3

Values are the mean ± SD of six separate experiments

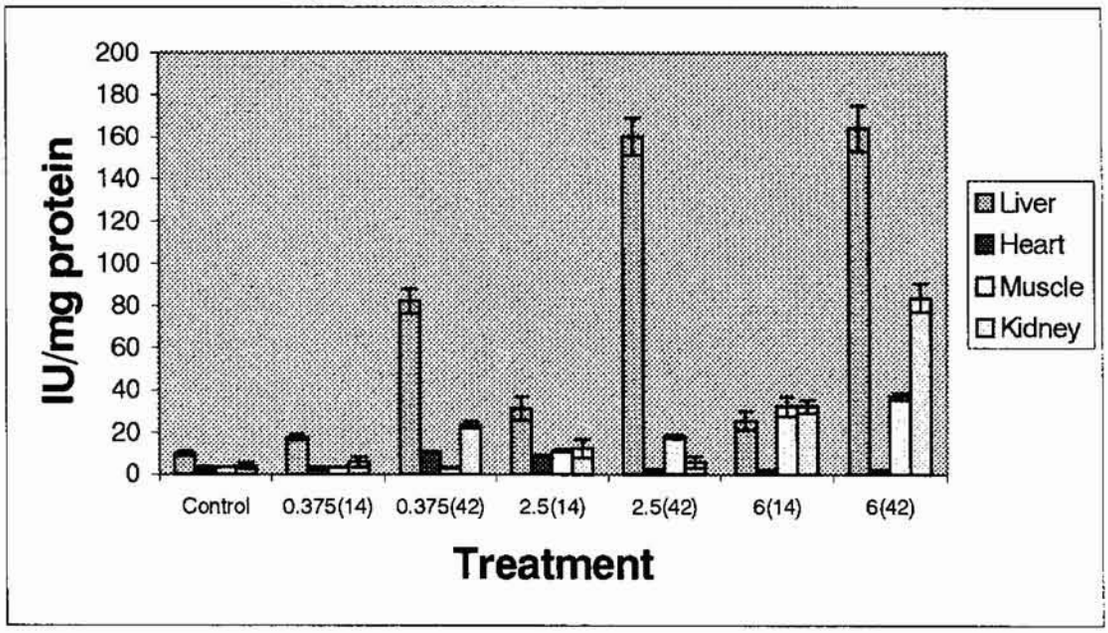


Fig 4.4 Levels of Catalase in the various aflatoxin exposed groups

There was a significant difference ($p<0.01$) between the different tissues and between the different time periods ($p<0.05$) in the levels of catalase. A comparison between the different concentrations did not reveal any significant change. Further comparison using LSD analysis was performed which revealed that the tissues differed significantly from one another. The liver tissue recorded significantly higher values than the other tissues The LSD value at 5% level was 34.199. The levels at 42 days were significantly higher than the values at 14 days. Results of LSD analysis are presented in table 4.2. and table 4.3.

LSD = Least significant difference

Source of Variation	SS	df	MS	F	
Total	52331.496	31			
Between concentrations	4981.047	3	1660.349	1.512	NS
Between tissues	15818.337	3	5272.779	4.802	p<0.01
Between days	5176.429	1	5176.429	4.714	P<0.05
Error	26355.591	24	1098.149		

SS - sum of squares, df - degrees of freedom, MS - mean of squares

NS = Not significant

Table 4.1a. ANOVA for Catalase

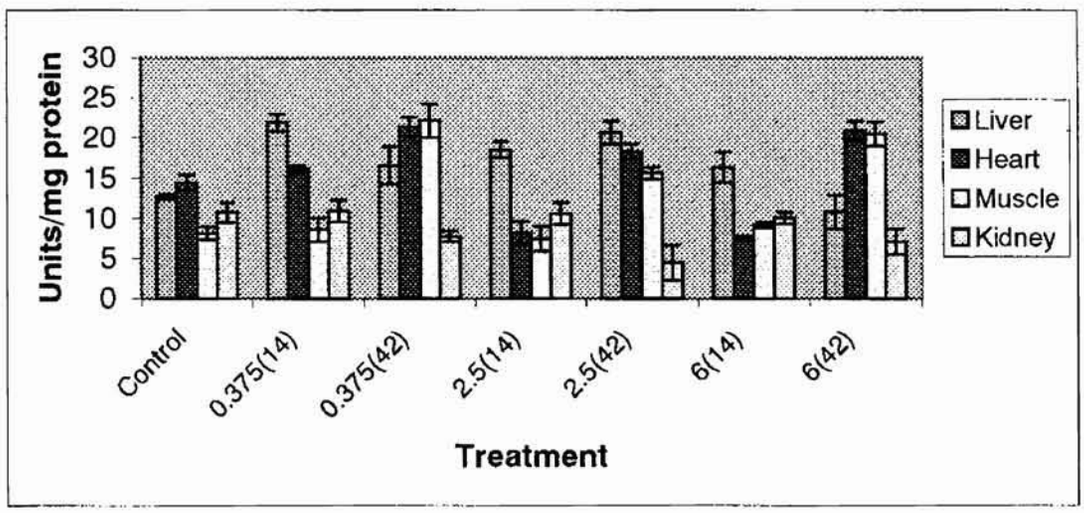


Fig 4.5. Levels of Superoxide dismutase in the various Aflatoxin exposed groups

There was a significant difference (p<0.05) between the different tissues in the levels of SOD. A comparison between the different concentrations and between time periods did not reveal any significant change. Subsequent comparison using LSD analysis was performed which revealed that only the tissues namely liver and kidney and also heart and muscle differed significantly from one another. The liver tissue recorded

significantly higher values than the other tissues The LSD value at 5% level was 4.58. Results of LSD analysis are presented in table 4.2. and table 4.3.

Source of Variation	SS	df	MS	F	
Total	847.16	31			
Between concentrations	73.83	3	24.61	1.25	NS
Between tissues	249.73	3	83.24	4.23	p<0.05
Between days	51.87	1	51.87	2.64	NS
Error		24	19.66		

SS - sum of squares, df - degrees of freedom, MS - mean of squares
NS: Not significant

Table 4.1b. ANOVA for Superoxide Dismutase

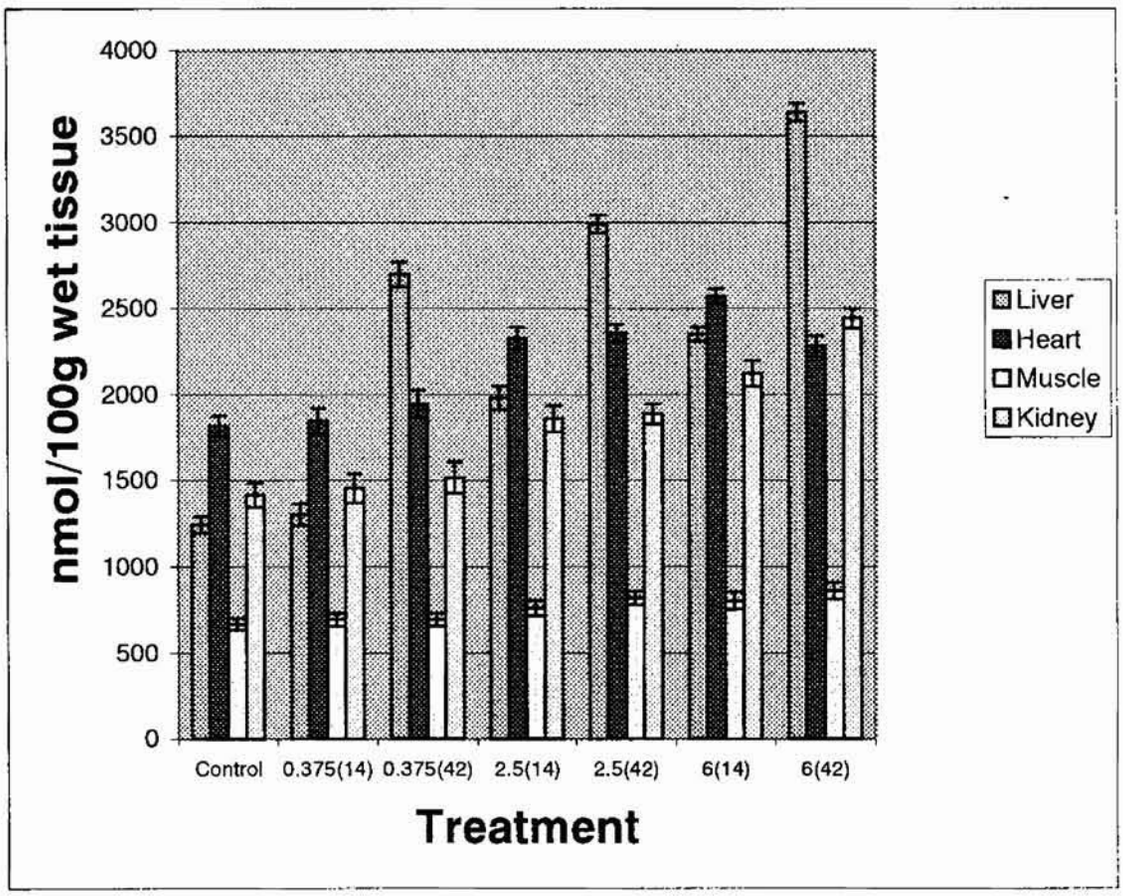


Fig 4.6 Levels of Glutathione in the various Aflatoxin exposed groups

There was a significant difference ($p<0.01$) between the different tissues and between the different concentrations ($p<0.01$) in the levels of glutathione. A comparison between the different time periods did not reveal any significant change. Further comparison using LSD analysis was performed which revealed that there was an overall significant difference seen between the different tissues. The liver tissue recorded significantly higher values than the other tissues The LSD value at 5% level was 188.983. All the concentrations differed significantly from one another. Results of LSD analysis are presented in table 4.2. and table 4.3.

Source of Variation	SS	df	MS	F	
Total	17885642.5	31			
Between concentrations	3375221.092	3	1125073.6	7.875	p<0.01
Between tissues	10567942	3	3522647.6	26.658	p<0.01
Between days	513884.068	1	513884.06	3.597	NS
Error	3428594.455	24	142858.102		

SS - sum of squares, df - degrees of freedom, MS - mean of squares

NS- Not significant

Table 4.1C. ANOVA for Glutathione

Table 4.2 Results of comparisons between the different Aflatoxin concentrations as obtained by LSD analysis

Treatments	Parameter	P value
Control vs 0.375ppm	Conjugated diene	P<0.01
	Hydroperoxide	P<0.01
	Malondialdehyde	P<0.01
	Catalase	NS
	SOD	NS
	GR	P<0.01
	GSH	P<0.01
Control vs 2.5ppm	Conjugated diene	P<0.01
	Hydroperoxide	P<0.01
	Malondialdehyde	P<0.01
	Catalase	NS
	SOD	NS
	GR	P<0.01
	GSH	P<0.01
Control vs 6 ppm	Conjugated diene	P<0.01
	Hydroperoxide	P<0.01
	Malondialdehyde	P<0.01
	Catalase	NS
	SOD	NS
	GR	NS
	GSH	P<0.01

NS- Not significant
GR- Glutathione reductase
GSH- Glutathione (reduced)
SOD- Superoxide dismutase

0.375ppm vs 2.5ppm	Conjugated diene	NS
	Hydroperoxide	P<0.01
	Malondialdehyde	NS
	Catalase	NS
	SOD	NS
	GR	P<0.01
	GSH	P<0.01
0.375ppm vs 6ppm	Conjugated diene	NS
	Hydroperoxide	P<0.01
	Malondialdehyde	NS
	Catalase	NS
	SOD	NS
	GR	P<0.01
	GSH	P<0.01
2.5ppm vs 6ppm	Conjugated diene	NS
	Hydroperoxide	NS
	Malondialdehyde	NS
	Catalase	NS
	SOD	NS
	GR	NS
	GSH	P<0.01

NS- Not significant
GR- Glutathione reductase
GSH- Glutathione (reduced)
SOD- Superoxide dismutase

Table 4.3. Results of comparisons between the different tissues as obtained by LSD analysis

Treatments	Parameter	P value
liver vs heart	Conjugated diene	P<0.01
	Hydroperoxide	P<0.01
	Malondialdehyde	NS
	Catalase	P<0.01
	SOD	NS
	GR	P<0.01
	GSH	NS
liver vs muscle	Conjugated diene	P<0.01
	Hydroperoxide	P<0.01
	Malondialdehyde	NS
	Catalase	P<0.01
	SOD	NS
	GR	NS
	GSH	P<0.01
liver vs kidney	Conjugated diene	P<0.01
	Hydroperoxide	P<0.01
	Malondialdehyde	NS
	Catalase	P<0.01
	SOD	P<0.05
	GR	P<0.01
	GSH	P<0.01

NS- Not significant
GR- Glutathione reductase
GSH- Glutathione (reduced)
SOD- Superoxide dismutase

heart vs muscle	Conjugated diene	P<0.01
	Hydroperoxide	P<0.01
	Malondialdehyde	NS
	Catalase	NS
	SOD	NS
	GR	NS
	GSH	P<0.01
heart vs kidney	Conjugated diene	P<0.01
	Hydroperoxide	NS
	Malondialdehyde	NS
	Catalase	NS
	SOD	P<0.05
	GR	P<0.01
	GSH	P<0.01
muscle vs kidney	Conjugated diene	NS
	Hydroperoxide	P<0.01
	Malondialdehyde	NS
	Catalase	NS
	SOD	NS
	GR	P<0.01
	GSH	P<0.01

NS- Not significant
GR- Glutathione reductase
GSH- Glutathione (reduced)
SOD- Superoxide dismutase

4.11 Discussion

The mechanism of action of mycotoxins on the cell is mediated through the production of free radicals and reactive oxygen species (ROS). In this study the results obtained reveal that the levels of lipid peroxidation products namely conjugated dienes and hydroperoxide were maximum in the liver when compared to other tissues and their levels increased consistently with increasing concentration of aflatoxin. Lipid hydroperoxides are prominent non-radical intermediates of lipid peroxidation whose identification can often provide valuable mechanistic information as to whether a primary reaction is mediated by oxy radicals (Thomas *et al,* 2002). Aflatoxins are important hepatocarcinogens and the hepatic tissues of the liver absorb these toxic substances from the blood stream and circulation. Aflatoxins, specifically aflatoxin B_1 is eventually secreted in the liver where it has been shown to be toxic to cells (Moreau and Moss, 1979). Aflatoxin in the liver is degraded in two phases by 1) - biotransformation to a more toxic product and 2) – detoxification to a less toxic and easily excretable product (Hodgsen and Levi, 1987).

The levels of malondialdehyde, the end product of lipid peroxidation process, which recorded a hike after 14 days of exposure to the toxin, was found to decline in the different tissues at the end of 42 days. It has been reported that lipid peroxidation and oxidative DNA damage are the manifestations of the aflatoxin B_1 induced toxicity. Supporting this, a significant increase in lipid peroxidation was reported in the liver of rat 72 hours after a single intraperitoneal dose of aflatoxin B_1 (Souza *et al,* 1999).

Verma and Nair have reported increased lipid peroxidation in the testes of aflatoxin treated mice. Aflatoxins can produce ROS by either direct

or indirect mechanisms (Halliwell, 1996). Oxidative stress can also occur when there is a decrease in the anitoxidant capacity of a cell (Trush and Kenslar, 1991). With regard to the antioxidant status of the animal in this study, results indicate that in the levels of the free radicals scavenging enzymes namely catalase, SOD and glutathione reductase, maximum increase was seen in the liver tissue followed by kidney in the case of catalase and followed by the heart in the case of SOD and glutathione reductase. Activity of catalase was found to increase with increase in the exposure period to the toxin and hence recorded increased concentration after a 42-day exposure. The level of the non-enzymatic antioxidant namely glutathione (GSH) was highest in liver followed by heart and kidney. The liver is very active in GSH biosynthesis and translocates GSH to the blood and to the bile. Kidney is active in removing GSH from blood plasma (Rana *et al,* 2002). It is also known that aflatoxins induce the formation of enzymes involved in ROS mechanism (Singh and Clausen, 1980). The levels of enzymatic antioxidants (SOD, glutathione peroxidase and catalase) and the non-enzymatic anti oxidants (Vitamin C, glutathione, Vitamin E) are the main determinants of the antioxidant defense mechanism of the cell. While SOD has been recognized to play an important role in the defense mechanism of the body against harmful effects of oxygen free radical in the biological systems, two related enzymes, glutathione peroxidase and catalase scavenge the dismutation of the superoxide radicals (Verma and Nair, 2000). Nakae *et al,* 1987 reported that killing of rat hepatocytes by aflatoxin B_1 was prevented by catalase, SOD, mannitol or deferoxamine. But on the contrary in the fish *Labeo rohita,* it was reported that AFB_1 produces a deficiency of all the antioxidant counter- parts (namely catalase, SOD, glutathione reductase, glutathione S transferase and glutathione) as was seen on the fourth day which was further aggravated on the tenth day suggesting that in these fishes coping mechanism has not set in even at the end of ten days, and detoxification and removal of AFB_1 has not been effected

(Madhusudhanan *et al*, 2000). But in this study, after a 42 day exposure to the toxin, a decrease seen in the malondialdehyde levels could be due to a rise seen in the levels of antioxidant enzymes right from 14 days to 42 days of exposure. In addition to this, the relative activities of detoxifying biotransformation pathways are also critical determinants of species susceptibity. The most important detoxification system is thought to be the glutathione S transferase - catalase conjugation of AFB_1. Glutathione S transferase (GST) comprises the family of cytosolic and microsomal enzymes that catalyze the conjugation of reduced glutathione (GSH) with compounds possessing an electrophilic centre. Conjugation of the electrophilic AFB_1-2,3 - epoxide with GSH provides an alternative to binding to nucleophilic sites in cellular macro molecules. Even though the level of the enzyme GST was not estimated in this study, an increase noticed in the glutathione level suggests a possibility of such a conjugation reaction mechanism to have been functional in the fish on exposure to the toxin.

From the foregoing results, it can be perceived that the fish namely *O mossambicus* on being subjected to aflatoxin stress for a prolonged period of 6 weeks was able protect itself from the abuse by the toxin with its existing antioxidant defense mechanism. The results obtained are supported by ANOVA and multiple comparison analysis of data according to the Least Significance Difference procedure.

CHAPTER 5

Histopathology

5.0. Introduction

Structural and functional integrity of cell organelles and cells is fundamental to the smooth orchestration of physiological functions in an organism. Histopathological techniques are rapid, sensitive, reliable and comparatively inexpensive tools for the assessment of stress response to xenobiotics. Cytological and histopathological alterations provide a direct record of stress effects. Cell damage is a result of persistent or irreversible biochemical and subcellular dysfunction caused by stress. Often stressed cells undergo inrreversible structural and biochemical changes, which result in alterations in the physiology of the animal. Assessment of histopathological manifestation provides insight into the degree of stress, susceptibility and adaptive capability of the stressed organism. The route that the toxicant takes during its metabolism often dictates the choice of organs for examining the effect of xenobiotics. Liver (being the major detoxifying organ directly receiving the materials from the intestine) and kidney (being the major excretory organ involved in xenobiotic excretion) were considered ideal for the study.

5.1. Materials and methods

Liver and kidney samples of 3mm thickness from specimens of tissues of control animals and of fishes exposed to aflatoxin for periods of two and six weeks were fixed in 10% formalin for twenty-four hours. The following protocol was used to make paraffin wax blocks for histopathological studies. Tissues were dehydrated in ascending grade of alcohol - 50% - 1/2 hour; 80% and 90%- 3/4th hour each. Tissues were given two changes in absolute alcohol of one hour each.

Cleared in xylene until tissues became translucent. Tissues were transferred to molten paraffin wax for one hour to remove xylene completely.

Embedded in paraffin wax of melting point 60°C - 62°C. Paraffin blocks were cut in a rotary microtome to prepare sections of thickness 4 to 6 microns.

5.2.. Staining technique followed with Haematoxylin-Eosin stain

1) Rinsed the slides twice in xylene: 5min each
2) Transferred slides to xylene: absolute alcohol mixture (1:1): 1min
3) Slides passed sequentially through absolute alcohol, 80% alcohol and 50% alcohol: 1 min each
4) Washed in running tap water: 5 min
5) Stained in haematoxylin: 2 min
6) Washed in running tap water: 5 min
7) Dipped once in acid: absolute alcohol mixture (0.5:100)
8) Washed in running tap water: 5 min
9) Stained in eosin: 1min.
10) Slides dehydrated sequentially through 50%, 80% and absolute alcohol: 1 min each
11) Dipped once in running tap water
12) Blotted slide on filter paper
13) Rinsed in xylene: 5 min
14) Rinsed in xylene: till clear
15) Mounted in D.P.X

5.3. Results

Several changes were observed in the liver and kidney tissues of animals exposed to different concentrations of aflatoxin and different time periods.

5.3.1. Liver

The liver of the control group that did not receive the toxin had normal structure. Hepatocytes were polygonal in shape having a distinctive central nucleus with densely staining chromatin margins and a prominent nucleolus. There was far less tendency for disposition of the hepatocytes in cords or lobules when compared to the mammalian liver. Sinusoids, which are irregularly distributed between the polygonal hepatocytes are lined by endothelial cells with very prominent nuclei. Biliary canaliculi were found in between hepatic cells as channels.

The fishes that were fed on the experimental feed containing aflatoxin were divided into three groups- GI, GII, and GIII. GI was subdivided into GIa and GIb. GI was designated as the experimental group that was exposed to 0.375 ppm of aflatoxin for 14 days (GIa) and 42 days (GIb). G II was subdivided into G IIa and G IIb. G II was designated as the experimental group that received 2.5 ppm of aflatoxin for 14 days (GIIa) and 42 days (GIIb). G III was designated as the experimental group that was exposed to 6ppm aflatoxin for 14 days (GIII a) and 42 days (GIIIb).

STRUCTURAL ALTERATIONS IN THE LIVER OF TILAPIA EXPOSED TO DIFFERENT CONCENTRATIONS OF AFLATOXIN

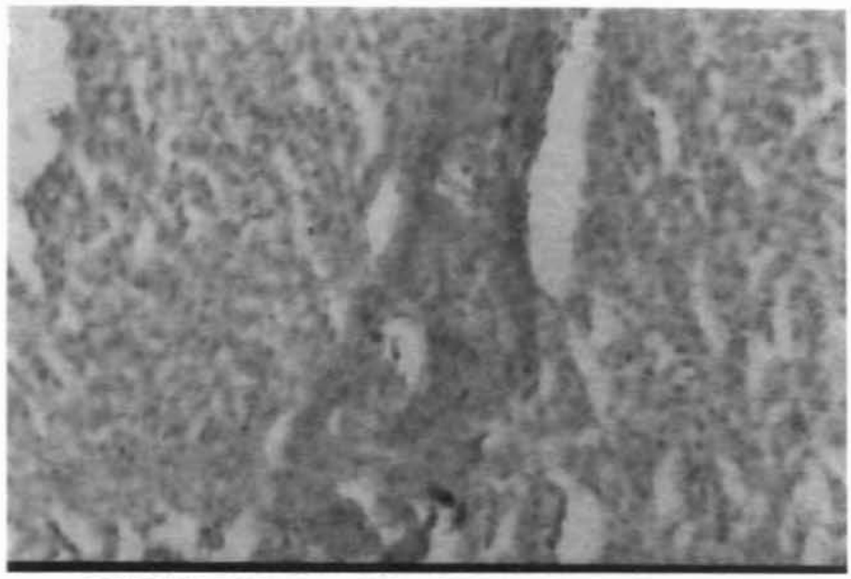


PLATE-3 MASSIVE PROLIFERATION OF BILIARY EPITHELIUM (40x)

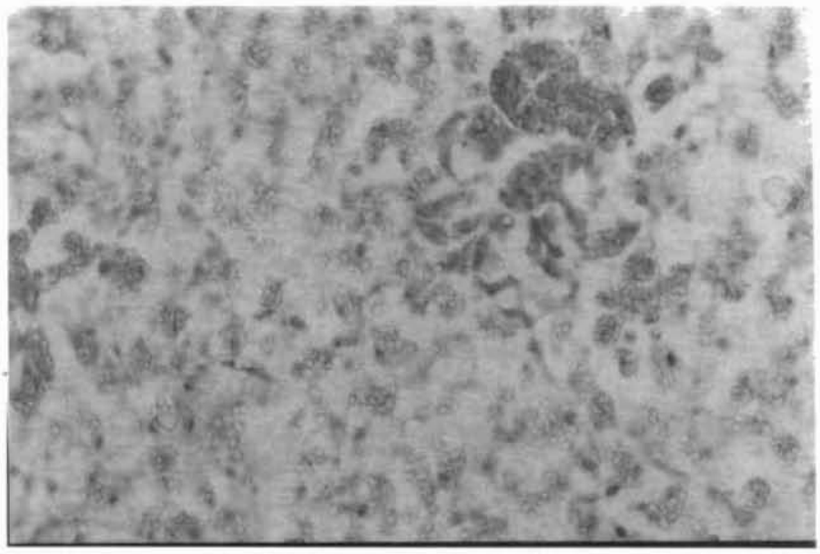


PLATE-4 LOSS OF ARCHITECTURE AND ACCUMULATION OF CEROID PIGMENTS (40x)

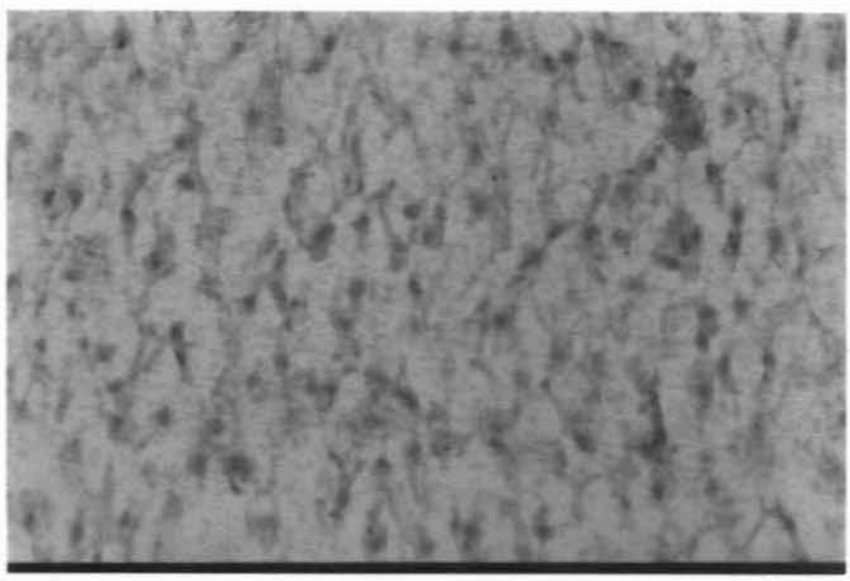


PLATE-5 AREA OF NECROSIS (40x)

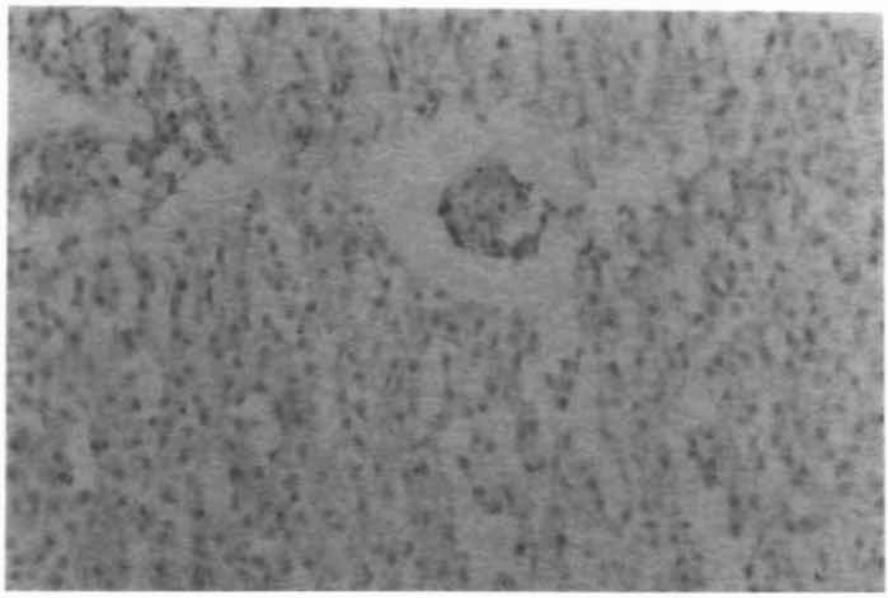


PLATE-6 FOCAL AREA OF NECROSIS (40x)

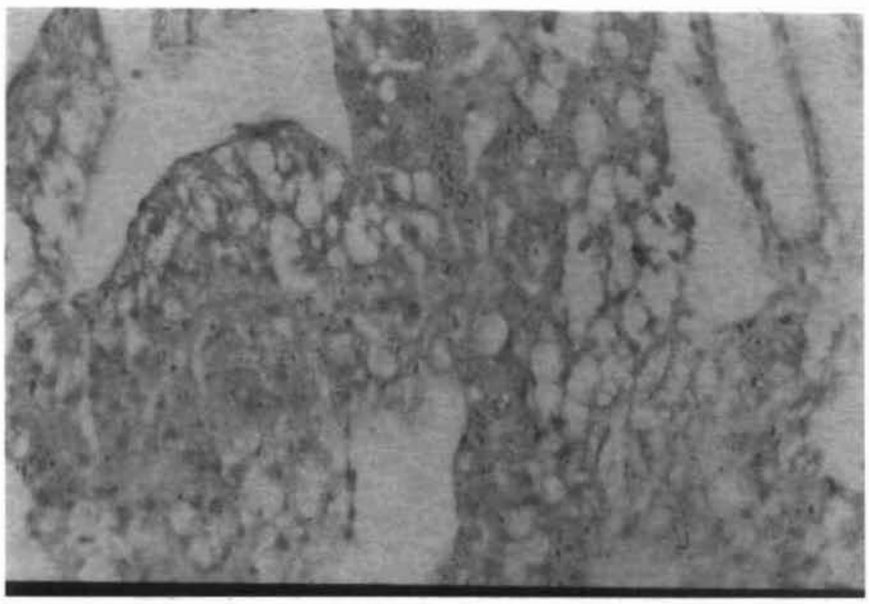


PLATE-7 AREAS OF FIBROSIS AND BILIARY EPITHELIAL PROLIFERATION (40x)

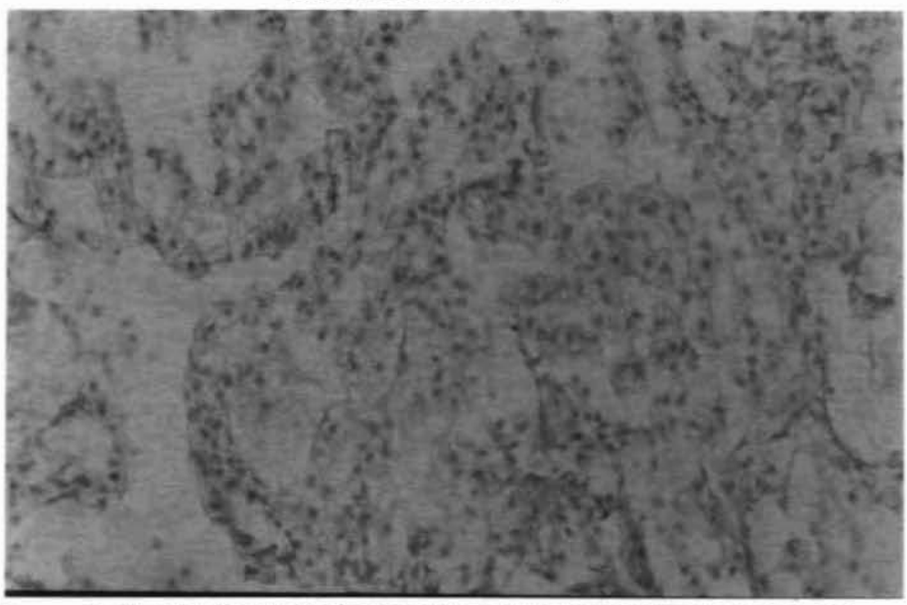


PLATE-8 DEGENERATIVE CHANGES IN THE HEPATOCYTES, LOSS OF ARCHITECTURE, PYKNOTIC NATURE OF HEPATIC NUCLEI (40x)

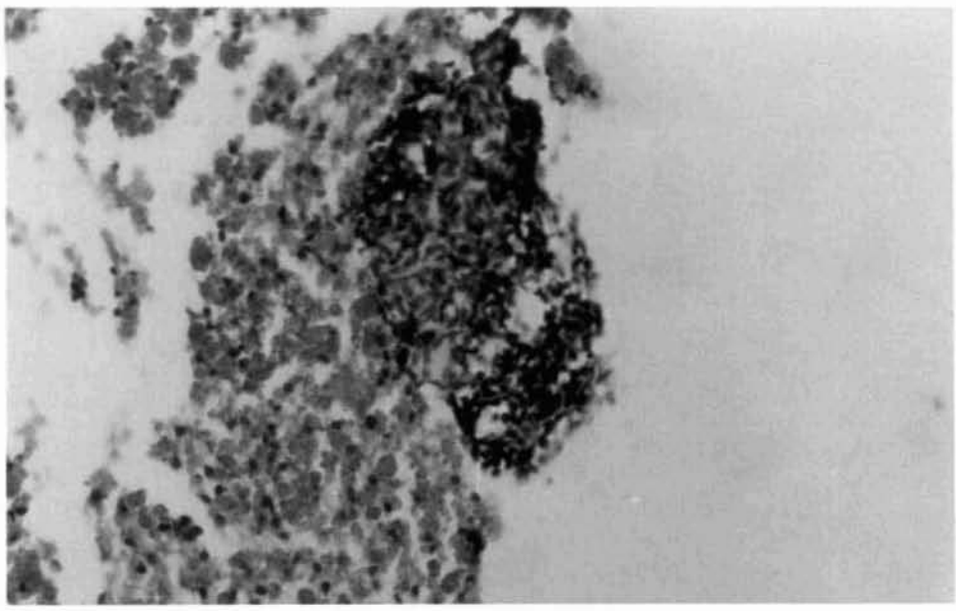


PLATE 8.5

Section of liver showing preneoplastic stage of tissue

Aflatoxin Dose (ppm)	0.375		2.5		6	
Group	GI		GII		GIII	
Subgroup	GIa	GIb	GIIa	GIIb	GIIIa	GIIIb
Period (weeks)	Two	Six	Two	Six	Two	Six

The liver sections of the group, GIa which received an aflatoxin dose of 0.375 ppm for two weeks showed the following changes :-

- Total loss of architecture
- Increase in the accumulation of ceroid pigments
- Loss of the glandular structure of the liver
- Necrosis of hepatic cells
- Biliary epithelial cell proliferation.

In GIb group the changes observed in the liver tissue were more severe. Proliferation of biliary epithelial cells and stromal connective tissue was discernable indicating a cirrhotic change. Regeneration of hepatic cells was also seen.

The group GIIa (aflatoxin dose of 2.5 ppm for 2 weeks) revealed several changes in the liver tissue. Loss of architecture of the hepatocytes was seen. The liver contained a large number of macrophages containing ceroid pigments. Necrosis of liver cells was noticed. There was general swelling and pyknosis of the hepatocyte nuclei. Biliary epithelium and connective tissue cells revealed proliferative changes.

The GIIb section of liver tissue showed complete necrosis of the hepatic cells. Hepatic parenchymal tissue showed disorganization. Cirrohotic changes were manifested by fibrosis of hepatic cords and biliary connective tissue.

In the liver sections of group GIIIa that was exposed to 6 ppm of aflatoxin for two weeks, several areas of hyperchromatic, hyperplastic, basophilic cellular proliferation were seen which may be preneoplastic lesions. Extensive biliary proliferation was seen together with necrosis, accumulation of ceroid pigments and loss of architecture. Similar changes were also observed in the liver of GIIIb.

5.3.2. Kidney

In the experimental group GIa, the kidney exhibited an increased cellularity in the glomeruli. A moderate loss of the epithelial cells of the tubules was seen.

In group GIb, the changes in the kidney were characterized by the vacuolation of the epithelial cells of the proximal convoluted tubules and distal convoluted tubules accompanied by a complete loss of the epithelial cells of the tubules. Other changes noticed were the degeneration of the epithelial cells and fibrosis of the glomerular tuft.

In GIIa, the glomeruli recorded increased cellularity and fibrosis of the glomerular tuft. Desquamation of the cells of the tubules was noticed.

TO DIFFERENT CONCENTRATIONS OF AFLATOXIN

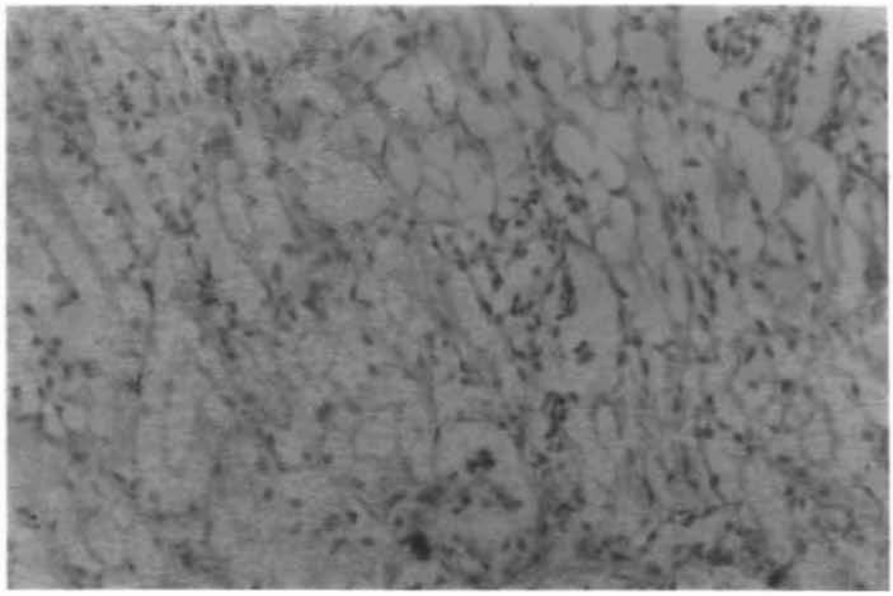


PLATE-9 SEVERE NECROSIS OF TUBULAR EPITHELIAL CELLS (40x)

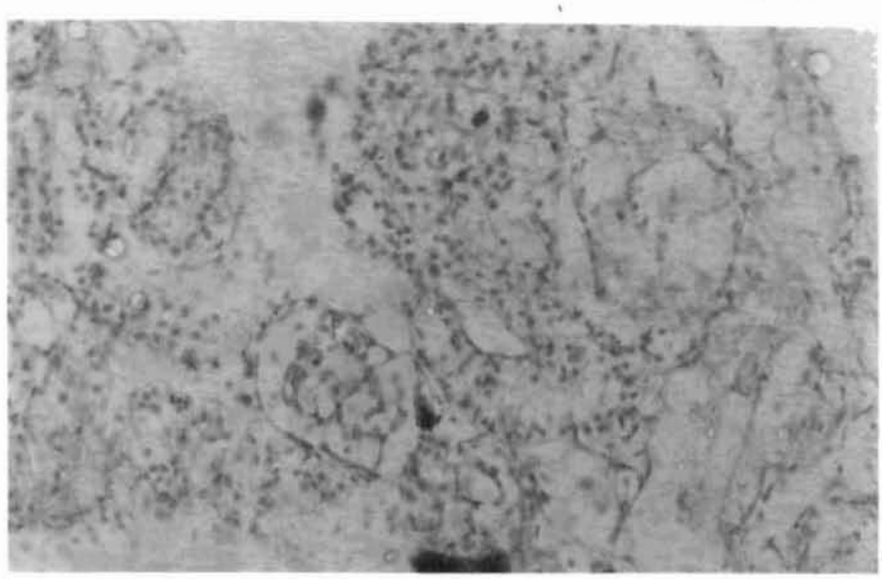


PLATE-10 TUBULAR EPITHELIAL NECROSIS AND THICKENING OF THE BOWMANS CAPSULE OF GLOMERULI (40x)

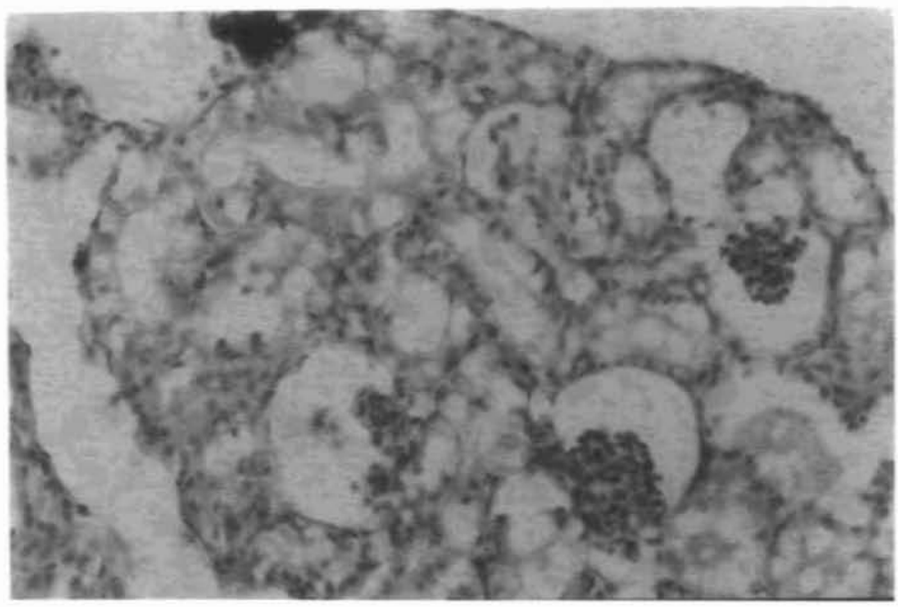


PLATE-11 SHRINKAGE OF GLOMERULI. NOTE THE VACUOLAR CHANGES IN THE TUBULAR EPITHELIAL CELLS (40x)

EXPOSED TO DIFFERENT CONCENTRATIONS OF AFLATOXIN

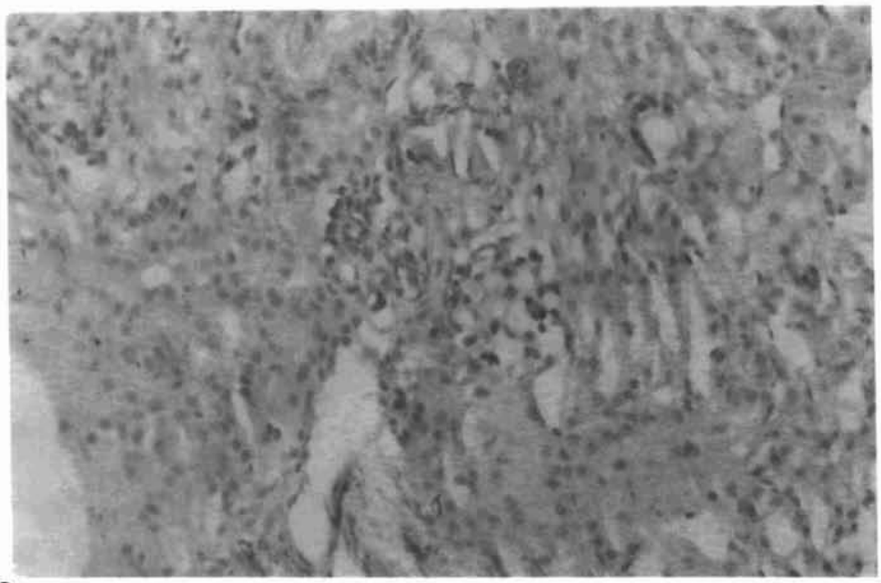


PLATE-12

INTER CAPILLARY THICKENING OF GLOMERULI
AND SCLEROTIC CHANGES IN THE GLOMERULI (40x)

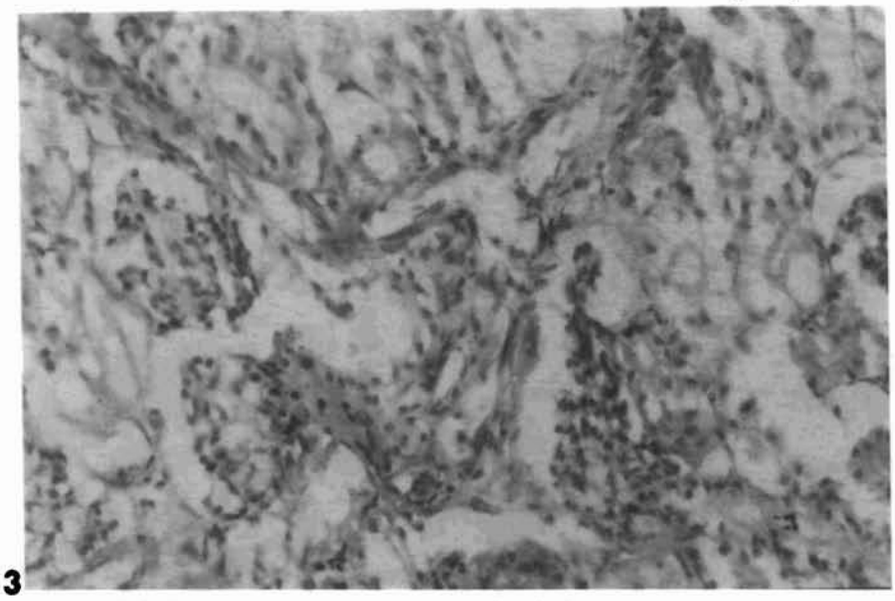


PLATE-13

FIBROSIS OF THE TUBULES, NECROSIS OF TUBULAR EPITHELIAL
CELLS AND SCLEROTIC CHANGES IN THE GLOMERULI (40x)

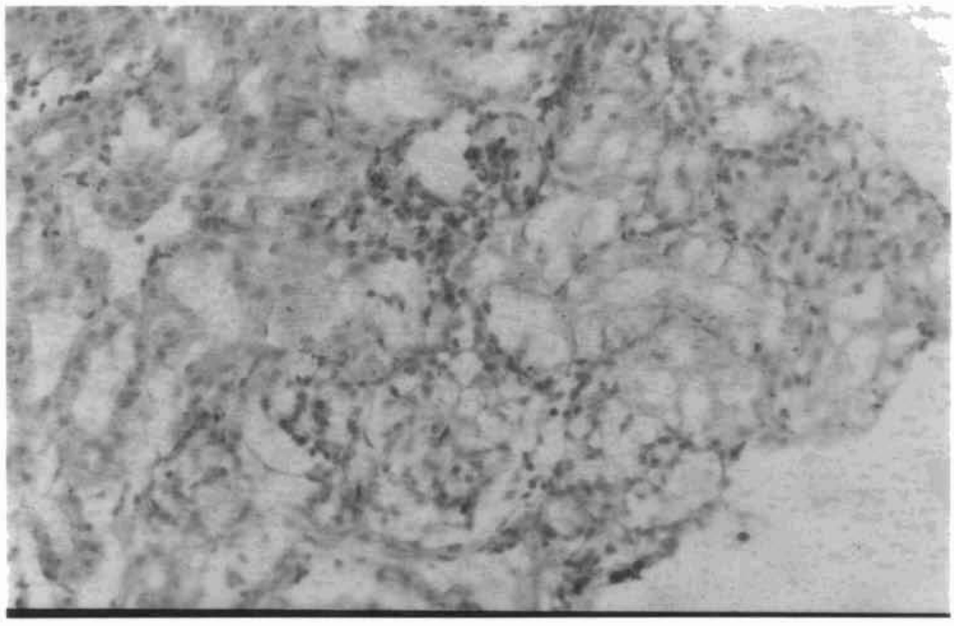


PLATE-14

GLOMERULAR CHANGES LIKE - THICKENING OF THE BOWMANS CAPSULE
AND INCREASED CELLULARITY IN THE GLOMERULI (40x)

In GIIb both proximal convoluted tubules and distal convoluted tubules revealed degenerative changes of the epithelial cells characterized by necrosis of tubular epithelial cells, vacuoles in epithelial cells and desquamation of cells. The glomeruli revealed hypercellularity and shrinkage. The Bowman's capsule showed thickening.

In G IIIa, tubular epithelial necrosis was extensive. Mild to moderate glomerular capillary thickening was apparent in many glomeruli.

In G IIIb, the glomeruli revealed shrinkage and sclerotic changes.

5.4. Discussion

Aflatoxin B1, a toxic product of the mould *Aspergillus* is the most potent of several mould metabolites known to produce recognizable liver abnormalities which precede neoplastic conditions. For several years, rainbow trout has been used as a carcinogenic model of hepatoma research and it is has been proved to be highly sensitive to hepatic carcinoma (Wood and Larson, 1961). The pathological changes induced by aflatoxin in the different organs of *Oreochromis niloticus* have already been documented. However, the present study is probably the first of its kind to highlight the pathological changes in the different organ tissues namely liver and kidney of *Oreochromis mossambicus*, the widely cultured tilapia species in India.

According to Post 1983, aflatoxins are absorbed from the diet in the alimentary canal and are passed to different organs. Liver being the primary target organ of metabolic action of aflatoxin, most reports is based on the compositional changes in liver tissue.

All experimental fish appeared normal and healthy after being fed on the experimental diets. This condition has been observed in rainbow trout affected with hepatic carcinoma (Wood and Larson, 1961). Jantrarotai *et al.* 1990 fed channel catfish 10 and 2.154 mg aflatoxin B1/kg diet and reported that the gross appearance and behaviour of all fish were normal.

Fish fed on the diet containing the highest dose of aflatoxin appeared to detect the aflatoxin contained in the diet and so did not take the feed completely for the first few days but later on began to gradually accept the entire feed.

Simon *et al.* 1967, Sinnhuber *et al.* 1968, Rogers and Newberne, 1969, Ashley 1970, Sato *et al.* 1973 and Ghittino 1976 have reported necrosis, fibrosis and bile ductular proliferation in advanced tumors induced by aflatoxin. Acute aflatoxicosis of the liver showing necrosis of the parenchymal cells was reported in rainbow trout force fed crude aflatoxin once at 5-mg/kg body weights. As necrosis developed, the damaged hepatocytes took up less stain until when necrosis was complete they were practically unstainable. Bile ductile proliferation in trout was occasionally evident (Ashley,1967b). Moderate to marked proliferation of bile duct was noticed in liver of *Labeo rohita* administered pure aflatoxin B1 intraperitoneally @ 0.75, 1.25 and 2.5 mg/kg of body weight. Bile duct proliferation (minimal trace) was also observed in the liver of the air breathing teleost, *Channa punctatus* administered aflatoxin intraperitoneally. In the present study, the liver of fish from all dietary treatments showed necrosis and biliary epithelial cell proliferation with damage being more extensive with increasing concentrations of aflatoxin in the diet.

Cirrhosis (a progressive liver disease resulting in liver failure), the outcome of prolonged hepatocellular injury, as manifested by fibrosis of hepatic cords and biliary connective tissue was observed in fish feeding on the lower doses of aflatoxin in the present study. Toxic liver cirrhosis was observed in the liver of the air breathing teleost *Channa punctatus* administered aflatoxin intraperitoneally. Hepatic Cirrhosis has also been reported in rainbow trout fed on aflatoxin-contaminated feed in addition to hepatoma (Lopez-Jimenez, 1983).

Loss of normal architecture of hepatocytes as cited by Sanchez *et al.*1994 in *Oreochromis niloticus* and by Verma, 1997 in *Channa punctatus* was observed in fish feeding on all the experimental doses of aflatoxin in the present study. Sanchez *et al.* 1994 and George KC,1997 have reported focal necrosis in the liver of *Oreochromis niloticus* and *Labeo rohita* respectively. In this study, focal necrosis was observed in the fish feeding on the highest dose of aflatoxin. Acute and extensive necrosis of liver cells may occur in toxic conditions but focal necrosis is more common..

An increase in the accumulation of ceroid pigments as documented by George KC in *Labeo rohita* was observed in the present study in the fish feeding on all experimental doses of the toxin. In this study, pyknosis of the hepatocyte nuclei, which reflects a state of degeneration of the cell, characterized by a very dark, shrunken nucleus and condensed chromatin was noticed in the fish consuming 2.5 mg aflatoxin per kg diet. Ashley, 1967b in trout liver, has cited similar observations.

In the present study, fish exposed to the highest dose of aflatoxin namely 6ppm for 6 weeks revealed hyperchromatic, hyperplastic and basophilic cellular proliferation that probably constitutes a preneoplastic

lesion, serving as precursors of hepatoma. Halver, 1969, has described hepatoma developing from basophilic patches. Chronic aflatoxicosis induced hepatoma when 200ppb crude or 0.5-8.0ppb crystalline aflatoxin B1, was fed to trout in the daily ration for 3-12 months. Salmon and catfish were refractive to aflatoxin – none had hepatoma after 2 years (Ashley, 1967a). The development of preneoplastic nodule in liver during subchronic aflatoxin toxicity in rohu, *Labeo rohita* has been described earlier (Svobodova *et al.1982).* The aforesaid evidences project the fact that aflatoxin exposure for a longer duration beyond six weeks could possibly induce hepatoma in *Oreochromis mossambicus*.

Ashley,1967 has reported necrotic changes in the kidney of Catfish and Coho salmon receiving 15 mg of aflatoxin B1/kg body weight. Murjani, Sahoo *et al.* 2001 and George KC, 1997 have also independently reported necrosis in the kidney of *Labeo rohita*. In this study also, necrosis of the tubular epithelial cells was observed in GIIb with necrosis becoming extensive in the fish consuming the highest dose of aflatoxin.

In the present study, an increased cellularity of the glomerular tuft was noticed in the kidney of fish exposed to the lower doses of aflatoxin and shrinkage of the glomeruli was observed in the fish exposed to the higher doses of aflatoxin. Supporting evidence has been provided by Sanchez *et al.*1994 who reported an increased cellularity of the glomeruli in *Oreochromis niloticus* together with the presence of a few shrunken glomeruli surrounded by widened Bowman's spaces. George KC has also reported similar changes in *Labeo rohita*..

Both the proximal convoluted tubules and the distal convoluted tubules in the present study revealed vacuolation and desquamation of the epithelial cells of the tubules. Similar changes were observed by George KC in the kidney of *Labeo rohita* exposed to aflatoxin for six weeks @ 0.4 mg /kg of feed. Mild to moderate glomerular capillary thickening and also sclerotic changes in the glomeruli as evidenced by George KC, 1997 in *Labeo rohita* was seen in the test animals subjected to the highest dose of aflatoxin.

The present study reports the appropriate dose and period of time aflatoxin induces chronicity and pathological changes in the tissues towards preneoplastic condition (in liver) in *Oreochromis mossambicus*. Aflatoxins are both acutely and chronically toxic to animals, including man. They produce four distinct effects: acute liver damage; liver cirrhosis; induction of tumours; and teratogenic effects (Stoloff, 1977). High temperature and humidity in the tropical countries promote the presence of favourable growth conditions for fungi. Unfortunately, because aquaculture is a relatively new technology, most farmers and aquaculturists are unaware of the deleterious effects of aflatoxin and therefore investigations into the exact nature and causes for mortality and reduced growth rate are hardly made. In addition to the alarming increase in deaths due to aflatoxicosis, an economical setback can incur through poor performance, lowered meat production, decreased resistance to infections and diseases and reproductive problems. Furthermore, most of the developing countries do not have facilities and regulations for controlling the quality of feed. Enforcement of these regulations is mandatory to bring about a revolutionary change in the fish health management practices and contribute substantially to the gross production statistics.

CHAPTER 6

Aflatoxin mediated Biochemical changes In *Oreochromis mossambicus*

6.0 Introduction

Of the energy resources available at the disposal of animals, carbohydrates form the chief constituent to be readily utilized. The carbohydrate metabolism is broadly divided into two segments (a) anaerobic segment or glycolysis which consists of breakdown of glycogen or glucose through Embden-Meyerhof pathway and (b) aerobic segment which consists of oxidation of pyruvate to acetyl CoA to be utilized through citric acid cycle. This is coupled by utilization and mobilization of reduced coenzymes (NADH or NADPH) for synthesis of ATP molecules through electron transport system coupled with oxidative phosphorylation (Lehninger, 1978). Besides synthesis of glycogen from glucose occurs not only through glycogenesis but also from non-carbohydrate precursors like amino acids, lactate and glycerol by gluconeogenesis. The above stamps constitute the main pathways of carbohydrate metabolism widely present in almost all the tissues of the vertebrates. Functionally, the operation of such complex biosynthetic and biodegradative steps in the carbohydrate metabolism certainly has a key role to play in supplying energy for cellular functions particularly under stress conditions like aflatoxin induced toxicemia. Glucose, the body's main source of carbohydrate energy is found stored as glycogen in both muscles and liver. When the need arises for carbohydrates energy, glycogen is converted to glucose in the serum (Melby,1974). Blood glucose concentration appears to be a sensitive, reliable indicator of environmental stress in fish. Glucose levels in blood and tissues may be used to indicate the toxicological significance of a substance in the aquatic environment (Elizoic et al, 1987)

Biochemically, aflatoxins can affect energy metabolism, carbohydrate and lipid metabolism, nucleic acid and protein metabolism (Ellis *et al.* 1991). Aflatoxins may be considered as inhibitors of biosynthetic processes both in vivo and in vitro, with large doses causing total inhibition of several biochemical systems and lower doses affecting different metabolic systems (Moreau *et al.* 1979).

Interestingly, the animals have the capacity to regulate and modulate the inherent diversions in their metabolism to meet the altered physiological or environmental conditions (Hoar, 1976). This is easily done to meet the energy demand under attenuated or imposed stress conditions to facilitate synthesis of extra energy to overcome such impeding situations. In the light of the above literature, some aspects namely total carbohydrate, glycogen and pyruvate of the carbohydrate metabolism in selected tissues were determined.

Free amino acids and certain types of proteins (like albumin) contribute to the maintenance of osmotic and acid base balance (Florkin and schoffeniels,1964; Robertson, 1965).

Aspartate aminotransferases (AST) and alanine aminotransferases (ALT) function as a link between carbohydrate and protein metabolism by catalyzing the interconversion of the strategic compounds like α-ketoglutarate and alanine to glutamic acid and pyruvic acid and aspartate and α-ketoglutaric acid to oxaloacetic acid and glutamic acid respectively (Knox and Greengard,1965; Watts and Watts,1974; Martin, Mayes, Rodwell, 1983). These enzymes are released into the circulatory system (serum) by cellular damage or destruction (Melby and Altman, 1977). AST is present in

both cytoplasm and mitochondria of cells. In cases involving mild tissue injury, the predominant form of AST is that from the cytoplasm, with a smaller amount coming from the mitochondria. Severe tissue damage results in more of the mitochondrial enzyme being released (Bergmeyer *et al,* 1985). Liver is rich in AST and ALT and changes in plasma levels of these enzymes may be indicative of liver dysfunction (Kapila,1999).

Even though fish are mostly ammonotelic, there are many reports on the excretion of urea by fish (Wekell and Brown, 1973). A full complement of urea cycle enzymes was reported to be present in the liver (Campbell,1961). Urea is the main end product of protein metabolism in the body. Removal of amino groups from amino acids, from which urea is formed takes place in the liver (Varley, 1988). The levels found circulating in sera are dependant on the rate of nitrogen metabolism and the ability of the kidney to remove the end products. Determination of blood urea is routinely used as an index of renal function (Melby, 1974)

Acid phosphatase and alkaline phosphatase are enzymes, which catalyze the hydrolysis of orthophosphoric acid esters at optimum pH levels below 7.0 and above 7.0 respectively (woodward, 1959). Alkaline phosphatase is involved in membrane transport and is a good indicator of stress in biological systems (Verma *et al.*1980).

The application of haematological and serological techniques have proved valuable for fishery biologists in assessing the health of fish and monitoring stress responses either due to fluctuations in environmental condition or due to sub lethal concentration of pollutants. Haematology concerns mainly investigations on cells present in the blood viz. red blood cells (RBC) count, haemoglobin concentration (Hb), packed cell volume

(PCV), white blood cells (WBC) count, mean corpuscular haemoglobin (MCH), mean corpuscular haemaglobin concentration (MCHC) and mean copuscular volume (MCV); whereas serology deals with the constituents in the fluid part of blood such as protein, enzymes, minerals, carbohydrates pigments, hormones, immune bodies e.t.c (Rajeev Kapila, 1999).

The serum proteins, composed of a non homogeneous mixture, may be classified according to the various physical and chemical properties. Basically the serum proteins are divided into two major fractions - albumin and globulin. Albumin and some of the globulins are synthesized in the liver. The proteins in plasma and sera are chiefly involved in nutrition, water distribution, acid-base balance, transport mechanism, immunity and enzymatic responses to specific metabolic needs. Serum protein concentrations can be used to monitor disease progress and general physiological status, as total protein levels tend to drop in diseased states. Sequential total protein analyses provide quantitative evidence of disease progression (Searcy *et al*, 1964).

LDH is responsible for the formation of lactic acid during glycolysis and its oxidation to pyruvate during respiration.
(LDH = Lactate dehydrogenase)

L - Lactate +NAD = Pyruvate+NADH

The serum LDH content is brought about by cellular damage or destruction. Normal levels found in serum are indicative of the body's routine destruction of senescent cells and their replacement. Abnormally high levels result from tissue damage, necrosis or trauma. (Altman, 1974).

Creatinine is the major waste product of creatine, which is closely linked to the energy metabolism of muscle and the inherent contractibility of muscle. Intracellular creatine receives high energy phosphorous from ATP to form the high energy creatine phosphate which is consumed during muscular contraction (Altman and Melby ,1974)

Cholesterol is an important precursor for steroid hormones, which are essential for homeostasis, normal reproduction and response to stressful situation.

6.1 Experimental Design

Tilapia (*Oreochromis mossambicus*) collected from the culture ponds of Rice Research Institute, Vytilla were used for the study. They were acclimatized to the fresh water condition in the laboratory. The fishes were maintained in polypropylene tanks . The water was changed daily along with the faecal matter. Six healthy fishes of weight 10±3g, after a period of acclimatization for two weeks were transferred to each of the experimental tanks and allowed to acclimatize to these test containers for a week. During this period, fishes were maintained on a semipurified basal diet devoid of aflatoxin at the rate of 2% of the body weight. The basic physico-chemical parameters of water viz., dissolved oxygen (7 – 8 ppm), pH (7.0), temperature (25°C-30°C) and salinity (0 ppt) were maintained constant. Three experimental groups were fed on diets containing 0.375 ppm, 2.5 ppm and 6 ppm of crude aflatoxin for two time periods of four weeks and six weeks, while a fourth group fed on the semipurified diet formed the control. Fishes were deprived of food 24 hr before assay. They were sacrificed by pithing with a sharp needle and the tissues removed rapidly, blotted dry and weighed. Blood was drawn from the common cardinal vein in plastic syringes. This design was followed for the entire study

5.2 Materials and methods

5.2.1 Estimation of pyruvate (Friedemann and Haugen, 1943)

Reagents

1. 10% Trichloroacetic acid (TCA)
2. 2,4 - Di nitro phenyl hydrazine (0.1%) (DNPH)
3. 2.5N NaOH
4. Pyruvate Stock standard: Dissolved 125mg of sodium pyruvate in 0.1N H_2SO_4 and diluted to 100ml with 0.1N H_2SO_4. 1 ml of this solution contains 1.25mg of sodium pyruvate.

Working Standard: Diluted 5ml of Stock to 100ml such that 1ml of it contains 0.0625 mg of sodium pyruvate.

Procedure

Liver, heart, muscle and kidney tissue homogenates were prepared separately in 10% TCA and centrifuged at 1000g for 15 min. To 2ml of the supernatant, 0.5ml of 0.1% 2,4 di nitro phenyl hydrazine was added and the tubes were kept for 5 min at room temperature and 3ml of 2.5N NaOH solution was added. After 10 min the colour was read in a spectrophotometer at 540 nm against a reagent blank. The blank consisted of 2ml of 10% TCA, 0.5ml of 0.1% 2,4 DNPH and 3ml of 2.5N NaOH solution. The standard graph was prepared by using sodium pyruvate. The values were expressed as μ moles of pyruvate /g wet wt of tissue.

6.2.2 Estimation of Urea (Natelson, 1971)

Reagents

1. 15% Perchloric acid (PCA)
2. Di acetyl monoxime (2%): Dissolved 2g of di acetyl monoxime in 60ml of distilled water and then added 2 ml of glacial acetic acid. Shaken well to dissolve with slight warming and made up to 100ml with distilled water.
3. Acid mixture: To 150ml of 85% phosphoric acid added 140ml of distilled water. Mixed well and then added 50ml of concentrated sulphuric acid slowly whilst mixing.
4. Urea stock standard: 250 mg %.
   Working Standard: Dilute the stock one to hundred times to give solution containing 0.025mg Urea/ml.
5. Sodium tungstate (10 %)

Procedure

Liver, heart, muscle and kidney tissue homogenate were prepared separately in 15% perchloric acid and centrifuged at 1000g for 15 minutes. Washed 0.1 ml of blood into 3.3 ml of water and added 0.8 ml of 10% sodium tungstate and 0.3 ml of 2/3 N H_2SO_4. Mixed well and centrifuged at 1000g for 15 minutes. To 0.5ml of the supernatant, 1.0ml of the acid mixture (3 parts of phosphoric acid and one part of Analar sulphuric acid) was added and mixed well. To this, 0.5ml of 2% di acetyl monoxime was added and kept for boiling in a water bath for 15 min. After cooling the tubes, the colour was read in a spectrophotometer against a reagent blank.

The blank consisted of 0.5ml of PCA, 1.0ml of acid mixture and 0.5ml of 2% di acetyl monoxime which received the same treatment as that of the samples. The tissue and blood urea values were expressed as μ moles of Urea /g wet wt of tissue and mg/100ml respectively.

6.2.3 Estimation of Alanine Transaminase (ALT) (Mohun and Cook, 1957)

Reagents

1. Buffered substrate: (0.1M Phosphate buffer,pH 7.4; 0.2M DL- alanine; 2mM 2-oxoglutarate). Dissolved 1.5g of di potassium hydrogen phosphate, 0.20g of potassium di hydrogen phosphate, 0.030g of 2-oxoglutarate and 1.78g of DL- alanine in distilled water and made up to 100ml. Adjust pH to 7.4 with 0.4N NaOH.
2. 2,4 - Di nitro phenyl hydrazine (DNPH): 1mmol/L in 1N HCl
3. NaOH : 0.4N
4. Pyruvate stock standard: 2mmol/L
   Working standard 1 in 20 dilution of the stock standard.

Procedure

The liver, heart, muscle and kidney tissue homogenates were prepared separately in 0.33M sucrose solution (10%) and centrifuged in a refrigerated centrifuge for 15 min at 1000g. The supernatant obtained was used as the enzyme source. Pipetted out 1ml of buffered substrate into two test tubes labeled "test" and "control ". Added 0.2ml of the enzyme / serum to the tube labeled "test" and incubated the tubes at 37°C for 30 min. After incubation, 0.2ml of the enzyme / serum was added to the control tube. 1ml of 2,4

DNPH reagent was added to both the tubes and kept at room temperature for 20 min. The reaction was stopped by the addition of 10ml of 0.4N NaOH, vortexed and kept at room temperature for 5 min .The absorbance was measured at 520nm in a spectrophotometer. The values of tissue and serum ALT were expressed as units/min/mg protein and units/L respectively.

6.2.4 Estimation of Aspartate Transaminase (AST) (Mohun and Cook, 1957)

Reagents

1. Buffered substrate : (0.1M Phosphate buffer, pH- 7.4; 1M aspartic acid; 2mM 2-Oxoglutarate). Dissolved 1.5g of di potassium hydrogen phosphate, 0.20g of potassium di hydrogen phosphate, 0.030g of 2-oxoglutarate and 1.57g of L-aspartate mono sodium salt (or 1.32g L-aspartic acid) in 70 ml distilled water. Adjusted pH to 7.4 with 0.4N NaOH and diluted to 100ml with distilled water.
2. 2, 4- Di nitro phenyl hydrazine (DNPH): 1mmol/L in 1 N HCL.
3. NaOH: 0.4N
4. Pyruvate standard: 2mmol/L
   Working standard : 1in 20 dilution of the stock standard.

Procedure

The liver, heart, muscle and kidney tissue homogenates were prepared separately in 0.33M sucrose solution (10%) and centrifuged in a refrigerated centrifuge for 15 min at 1000g. The supernatant obtained was used as the enzyme source. Pipetted out 1ml of buffered substrate into two test tubes labeled "test" and "control ". Added 0.2ml of the enzyme/ serum to the tube labeled "test" and incubated the tubes at 37°C for 60 minutes. After

incubation, 0.2ml of the enzyme / serum was added to the control tube. 1ml of 2,4 -DNPH reagent was added to both the tubes and kept at room temperature for 20 min. The reaction was stopped by the addition of 10ml of 0.4N NaOH, vortexed and kept at room temperature for 5 min .The absorbance was measured at 520nm in a spectrophotometer. The values of tissue and serum AST were expressed as units/min/mg protein and units/L respectively.

6.2.5 Estimation of Alkaline Phosphatase (Colowick , 1957)

The procedure followed was the same as that of acid phosphatase except that sodium carbonate - bicarbonate buffer (100mmol/L) (pH 10) was used. The values were expressed as mg of p-nitro phenol formed /min/mg protein.

6.2.6 Estimation of Free Amino acids (Moore and Stein,1954)

Reagents

1. 80% ethanol
2. Ninhydrin reagent: Dissolved 2 g of ninhydrin in 25ml of methyl cellosolve. Mixed this with 25ml of 0.2M acetate buffer of pH 5.5. Stored in an amber coloured bottle.
3. 50% ethanol
4. Tyrosine standard: Dissolved 500 mg of tyrosine in 100ml of distilled water.

   Working standard: Diluted 1 to 20 using distilled water.

Procedure

Liver, heart muscle and kidney homogenates were prepared separately in 80% ethanol and centrifuged at 15,000g in a refrigerated centrifuge. 0.5ml of supernatant was taken in different tubes and the volume was made up to 4ml in each of the tubes. 1.0ml of ninhydrin reagent was added and the tubes were kept in a boiling water bath for 15 min. The tubes were then cooled and 1ml of 50% ethanol was added. The colour developed was read in a spectrophotometer at 550nm.The values were expressed as μ moles of tyrosine/g wet wt of tissue.

6.2.7 Estimation of Proteins (Lowry *et al.* 1951)

Reagents

1. NaOH : 0.1 N
2. Na_2CO_3 (2%) in 0.1 N NaOH
3. $CuSO_4$ (0.5%)
4. Sodium potassium tartrate solution (1%)
5. Alkaline copper reagent : A mixture of 50 ml 2% Na_2CO_3 solution and 0.5 ml of each of $CuSO_4$ solution and sodium potassium tartrate solution.
6. Folin's phenol reagent - 1:1 dilution with distilled water
7. Standard protein solution: 100 mg % in 0.1 NaOH

Procedure

Pipetted out 0.2 ml of serum or extract to the test tube and added 1ml of NaOH solution and 5 ml of alkaline copper reagent. Shaken well and kept

the mixture for 10 min. After 10 min, 0.5 ml of Folin's phenol reagent was added and mixed well. The mixture was kept for another 30 minutes. The absorbance was measured at 500 nm, in a spectrophotometer. The system devoid of sample was used as the blank.

6.2.8 Estimation of Serum Total Protein, Albumin and Globulin- Biuret method

Reagents

1) 28% sodium sulphite solution
2) Biuret reagent
3) Ether, Analar
4) Standard BSA - 600mg of BSA/ 100ml

Procedure

A) Total Protein

Pipetted 1.9 ml of 0.9% saline to a test tube. To this added 0.1 ml of serum. Mixed by inversion. To this solution added 5 ml of Biuret reagent.

B) Albumin

Precipitation of globulin (Preparation of Albumin)

Pipetted 5.7 ml of 28 % Na_2SO_3 solution into a centrifuge tube and to this added 0.3 ml of serum. Rotated the tube gently between the palms. 3 ml of ether was added and the tube was gently shaken upside down for about 20

times. Waited for 10 minutes till a "globulin button" formed at the interphase of ether saline. Centrifuged for at least 10 minutes to complete the process of "globulin button formation" and hardening it. After centrifuging, tilted the tube and inserted a pipette into the clear solution below the globulin layer. Precipitate should not be disturbed. Pipetted 2 ml of this and added to 5 ml of the biuret reagent.

0.2 ml to 1.4 ml of standard was pipetted out into clean dry tubes and made up to 2 ml with 0.9 % saline. Added 5 ml of Biuret reagent. All the tubes were shaken well and placed in a water bath at 37°C for 10 min. Allowed to cool to room temperature for 5 minutes and then read the absorbance at 555 nm. The difference between total protein and albumin gives globulin. The values were expressed as g/dl.

6.2.9 Estimation of Serum Lactate Dehydrogenase (Spectrophotometric method of Wroblewski and La Due, 1955)

Reagents

1. Phosphate buffer (IM) pH 7.4: Dissolved 136g of potassium hydrogen phosphate and 33g of KOH in 1L of water and adjusted the pH to 7.4.
2. Phosphate buffer for use (0.1M) pH 7.4: Diluted the stock buffer 1 to 10ml.
3. Reduced Nicotinamide adenine dinucleotide: 2.5 mg per ml phosphate buffer.
4. Sodium pyruvate: 1mg/ml phosphate buffer

Procedure

To a spectrophotometric cell of 1cm light pathlength, 2.5 ml of 0.1M phosphate buffer, 0.2ml of NADH and 0.1 ml of serum was added and mixed well by covering the mouth of the cuvette with the help of a spectrophotometer cap and was set to zero absorbance. Incubated the mixture for 20 min at room temperature. If the reading remained constant for 5 minutes during incubation it meant that incubation was sufficient. When the spectrophotometer showed steady reading i.e. (when pyruvate present in the serum was consumed completely) added 0.2 ml of pyruvate without taking out the cuvette from the compartment, mixed it properly with the cap and immediately started the timer. Read the absorbance every minute beginning one minute after the start of the reaction and continuing for 6 min at 340nm. The values were expressed as units/L.

5.2.10 Estimation of Blood Glucose (Method of Marks using O –Toluidine)

Reagents

1) 0.9 % NaCl solution

2) 5% $ZnSO_4.7H_2O$ solution

3) 0.3N NaOH

4) 1 % O-Toluidine solution in absolute ethanol

5) "Fermcozyme": a stable liquid preparation of glucose oxidase containing 750 units /ml

6) 0.15M Acetate buffer (pH 5): Added approximately 3 volumes of 0.15M acetic acid to 7 volumes of 0.15M sodium acetate (17.7g $CH_3COONa2H_2O$/L) and adjusted to pH 5.

7) Peroxidase: 20 mg/100ml of acetate buffer.

8. Glucose oxidase reagent. Added 0.5ml of Fermcozyme to about 80 ml of acetate buffer. To this added 5 ml of the peroxidase solution, mixed and then added 1 ml of o-tolidine. This was then made up to 100 ml with buffer and keep in the refrigerator in a dark bottle.

   Standard glucose solutions: Prepared a solution containing 100mg glucose/100ml saturated benzoic acid and diluted with the same to obtain solutions containing 2.5, 5.0, 7.5 and 10 mg / 100ml that are equivalent to 50, 100, 150 and 200 mg glucose/100ml.

Procedure

Shortly before use added 0.4 ml of 5 % $ZnSO_4.7H_2O$ solution and 0.4 ml of 0.3N NaOH to 1.1 ml of 0.9 % NaCl and to this 0.1 ml blood was added. Mixed well, centrifuged and separated the supernatant as soon as possible. Transferred 1 ml of this to a test tube and into two other tubes measured 1 ml of water as blank and 1 ml of standard glucose solution. Added 3 ml glucose oxidase reagent to each at half minute intervals, mixed gently for not more than ten seconds, and read the colour developed exactly ten min later at 625 nm or using an orange filter. The values were expressed as mg glucose /dl.

6.2.11 Estimation of Alkaline Phosphatase (Kind and King,1954)

Reagents

1. Substrate: Di sodium phenol phosphate (10 mmol/l). Dissolved 2.18 g (2.541 g dihydrate) in water and make up to 1 L. It was quickly brought to boil, cooled, added a little chloroform and kept in the refrigerator.

2. Buffer: Sodium carbonate- bicarbonate buffer (100 mmol/l). Dissolved 6.36g anhydrous sodium carbonate and 3.36g sodium bicarbonate in water and make up to one L.
3. Buffered substrate: Mixed equal volumes of substrate and buffer. This had a pH of 10.
4. Stock phenol standard : 100 mg% in 0.1 N HCl
5. Working standard 1mg%. Dilute stock 1-100 using 0.1 N HCl
6. Sodium hydroxide 0.5N (20g/L)
7. Sodium bicarbonate : 0.5N (42g/L)
8. 4-aminoantipyrine : 6g/ L in water
9. Potassium ferricyanide : 24g/ L in water.

Procedure

Pipetted out 2 ml of buffered substrate into each of the two tubes marked "test" and "control" and incubated for a few minutes at 37°C. Then added 0.1 ml serum to the "test". Again incubated at 37°C for 15 minutes after which the tubes were removed from the water bath. Added 0.8 ml of NaOH and 1.2 ml of $NaHCO_3$ to both the tubes. Then added 0.1 ml serum to the "control". This was followed by the addition of 1 ml of 4 aminoantipyrine and 1 ml of potassium ferricyanide to both the tubes. Read the absorbance at 520 nm. For standard, pipetted out 1 ml of working standard and 1.1 ml of buffer. Added NaOH and $NaHCO_3$, 4-amino antipyrine and potassium ferricyanide as in the "test" and read. For blank pipetted out 1 ml of distilled water and 1.1 ml buffer and proceeded as above. The values were expressed as units/L.

6.2.12 Estimation of Acid Phosphatase (Kind and King,1954)

The technique is the same as that of alkaline phosphatase except that citric acid - sodium citrate buffer (pH 4.9) was used for preparing the buffered substrate. Incubation was for 1 h and in developing the colour with 4- amino antipyrine, it was necessary to add 1.0ml of 0.5N NaOH and1.0 ml of 0.5N $NaHCO_3$ to bring the pH to 10.2, that required for colour development. The values were expressed as units/L.

6.2.13 Estimation of Serum Creatinine (Alkaline picrate method)

Reagents

1. Standard picric acid solution: Taken 13 g picric acid in 1L of water. Allowed the excess picric acid to remain in contact with the water shaking occassionally. Filtered and stored it in a polyethylene bottle.
2. 0.75N NaOH
3. Stock Creatinine standard (150 mg%): Dissolved 150 mg of creatinine in 100ml HCl (100mmol/L)

   Working standard : Diluted stock standard solution 10 times before use.

Procedure

Deprotenization of sample : 0.5ml of serum was added to 3ml of picric acid and added 0.5ml distilled water to make up the volume to 4ml. Mixed well and kept in a boiling water bath for exactly one min. Cooled immediately under tap water and centrifuged. 2.0ml of the supernatant was

added to 0.5ml of 0.75N NaOH, mixed well and allowed to stand at room temperature for exactly 20 min. Measured the absorbance immediately at 520nm. 0.5ml of standard and blank solution was added to 1.5ml of picric acid and 0.5ml NaOH. The values were expressed as mg/dl.

6.2.14 Estimation of Serum Cholesterol (Zak's method)

Reagents

1. Stock ferric chloride reagent: 840mg of pure dry ferric chloride was weighed and dissolved in 100ml of glacial acetic acid.
2. Ferric Chloride precipitating reagent: 10ml of stock ferric chloride reagent was placed in a 100ml standard flask and made up to the mark with pure glacial acetic acid.
3. Ferric chloride diluting reagent: 8.5 ml of the stock ferric chloride was diluted to 100ml with pure glacial acetic acid in a 100ml standard flask.
4. Standard Cholesterol solution: 100mg of pure dry cholesterol was placed in a clean dry 100ml standard flask and dissolved in glacial acetic acid. Made up to the mark with pure glacial acetic acid.
5. Working standard: 10ml of the stock standard was placed in a 100ml standard flask containing 0.85 ml of ferric chloride stock reagent and made up to the mark with pure glacial acetic acid. 1ml of this solution contains 100µg of cholesterol.

Procedure

0.5 - 2.5 ml of working cholesterol solution was pipetted out into clean dry test tubes. The total volume of each tube was made up to 5 ml with ferric chloride diluting reagent. To 0.1 ml of the serum added 4.9 ml of ferric

chloride precipitating reagent and mixed well. Allowed to stand for a while and centrifuged. Transferred 2.5ml of the clear supernatant into a dry test tube and added 2.5 ml of ferric chloride diluting reagent. Mixed well. Tubes were kept in cold water and to each tube added 4 ml of conc H_2SO_4 drop by drop. The solution was mixed well .The tubes were allowed to come to room temperature. A blank was also simultaneously prepared by taking 5 ml diluting reagent and 4 ml of cone H_2SO_4. After 30 min, the intensity of the colour developed was read at 540 nm against the blank. The values were expressed as mg/dl.

6.2.15 Estimation of Triglycerides (method of Van Handel and Zilversmit)

Reagents

1. Chloroform
2. Florisil
3. Ethanolic KOH (0.4%) : 2 g KOH dissolved in 100 ml of ethanol. This was then diluted 5 times with ethanol
4. H_2SO_4 (0.2 N)
5. Sodium metaperiodate (0.05M)
6. Sodium arsenite (0.5 M)
7. Chromotropic acid : 2 g chromotropic acid (or 2.24g sodium salt) was dissolved in 200 ml distilled water. 600 ml of Conc.H_2SO_4 was added slowly to 300ml of distilled water which was chilled on ice. This chilled and diluted acid was then added to the chromotropic acid solution (0.05 mg/ml)

Procedure

2g of florisil was taken in a glass stoppered tube and 3 ml of chloroform was added. 0.2 ml of serum was layered on top of florisil and mixed. More chloroform was then added to this to a total of 10 ml. It was then stoppered and was shaken intermittently for about 10 minutes. After filtration, 1 ml was pipetted out into each of 3 tubes. 1 ml of the working standard of glycerol was pipetted out into each of 3 tubes. The solvent was evaporated at 60-70°C. Then 0.5ml of ethanolic KOH was added to 2 out of 3 tubes (saponified sample) and 0.5 ml of ethanol was added to the third tube (unsaponified sample). The tubes were closed and kept at 60-70°C for 15 minutes. To each tube, 0.5 ml of 0.2N H_2SO_4 was added and then placed in a gently boiling water bath for about 15 minutes to remove alcohol. They were then cooled to room temperature, 0.1 ml sodium metaperiodate was added to each tube and kept for 10 minutes. 0.1 ml sodium arsenite solution was then added. An yellow colour of iodine appeared and vanished within a few minutes. To each tube, 5 ml of chromotropic acid was then added and mixed. The tubes were closed and heated in a boiling water bath for 30 minutes. They were then cooled and the absorbance was read at 570 nm. The values are expressed as mg/dl.

6.2.16 Estimation of HDL and LDL Cholesterol (Phosphotungstate precipitation method)

Reagents

1) Phosphotungstate : Dissolved 4 g of phosphotungstic acid in appropriately 50 ml of water. Added 16 ml of 1N NaOH with stirring and made up the volume to 100 ml with water.

2) $MgCl_2$ (2M) : Dissolved 40.7 g of $MgCl_2.6H_2O$ in water and made up to 100 ml.

Procedure

Added 0.1 ml of phosphotungstate to 1 ml of serum in a centrifuge tube and vortex mixed immediately. Add 0.025 ml of $MgCl_2$ solution and again vortex mixed immediately. Centrifuged at 1500 g for 30 min. The supernatant which contained HDL was analyzed for cholesterol by the Zak's method to obtain HDL cholesterol. The difference between total cholesterol and HDL cholesterol gave LDL + VLDL cholesterol. The values are expressed as mg/dl.

6.2.17 Estimation of Haemoglobin (Cyanmethaemoglobin method)

Reagents

1) Drabkins diluent solution : Mixed 1g of sodium bicarbonate, 0.05 g of potassium cyanide (carefully) and 0.2g of potassium ferricyanide in 1L of distilled water. The solution was preserved in a dark bottle and preferably under cold storage.
2) Cyanmethaemoglobin standard : 60mg/ml (obtained commercially).

Procedure

0.02ml blood was mixed with 5 ml of Drabkins diluent solution and allowed to stand for 5 min for the formation of Cyanmethaemoglobin. Absorbance was measured against a reagent blank which consisted of 5 ml of

diluent solution. Using a commercial cyanmethaemoglobin standard, a standard calibration curve was prepared from which the values of haemoglobin could be read directly as g/dl.

6.2.18 Determination of Packed Cell Volume (PCV)- (Microhaematocrit Centrifugation method)

Procedure

Blood collected in EDTA was allowed to run about 1/2 to 3/4 th the length of an heparinized even bored capillary tubes and the tubes were sealed on the opposite end using sealing wax. The tubes were then transferred to a high speed microhaematocrit centrifuge and placed in the grooves of the capillary head. They were centrifuged for 15 min at 11,000 rpm. PCV was measured directly on a microhaematocrit reader associated with the centrifuge as volume percent.

6.2.19 Red Blood Cell Count

Reagents

Heyem's Solution (diluting Fluid)

Procedure

The blood was drawn up to 0.5 mark in the RBC pipette and along with this, the diluting fluid was drawn up to the 101 mark. The pipette was shaken thoroughly and charged the diluted blood into the counting chamber after discarding one or two drops . The solution was allowed to settle for a

minute and then counting was done under high power microscope. The number of cells in the four corner groups and one central group of 16 squares was counted. If the dilution of blood were one to 200, then the total number of cells found in the five groups of one square is multiplied by 10,000 in order to express the number of cellsin millions per cubic mm of blood.

6.2.20 Calculation of RBC Constants

Based on the results of the tests which measured total RBC, Hb and PCV, several calculations have been derived which give quantitative information about the RBC. These values are called RBC constants.

1) **Mean Corpuscular Volume (MCV)**

MCV, the mean corpuscular volume is the volume of the average cell or the average cell volume of all the RBC's

$$MCV = \frac{PCV\% \text{ (haematocrit)}}{RBC \text{ in million /mm3}} \times 10 \text{ expressed in } \mu 3$$

2) **Mean Corpuscular Haemoglobin (MCH)**

MCH is the amount of Hb in the average RBC or average amount of Hb per cell in all the red cells

$$MCH = \frac{Hb(g/dl)}{RBC \text{ in million/mm3}} \times 10 \text{ expressed in pg}$$

3) Mean Corpuscular Hb Concentration (MCHC)

MCHC is the portion of the average RBC containing Hb or the concentration in the average cell .

Hb = haemoglobin

$$MCHC = \frac{Hb(g/dl)}{PCV\ (\%)} \times 100 \text{ expressed in } \%$$

6.3 Results

The results of the various biochemical parameters are presented in the following tables and figures respectively.

rameter	Treatment						
		0.375ppm		2.5ppm		6ppm	
	Control	14days	42 days	14days	42 days	14days	42 days
AST(units/min/mg protein)							
Liver	1.37 ±0.11	1.36 ±0.09	4.86 ±0.24	1.56 ±0.21	5.52 ±o.3	2.41 ±0.06	10.81 ±0.3
Heart	2.07 ±0.28	2.00 ±0.34	4.25 ±0.15	4.23 ±0.27	6.95 ±0.33	4.07 ±0.35	15.79±0.41
Muscle	2.34 ±0.34	1.32 ±0.23	3.44 ±0.42	1.65 ±0.34	4.37 ±0.54	3.43 ±0.33	10.69 ±0.55
Kidney	1.43 ±0.32	3.69 ±0.23	2.72 ±0.45	2.39 ±0.5	5.73 ±0.76	3.77 ±0.32	10.67 ±0.5
ALT(units/min/mg protein)							
Liver	5.63 ±*1.04*	8.17± 0.84	12.78±0.9	9.29 ±1.15	19.8±1..27	12.9 ±1.79	26.73 ±1.8
Heart	1.95 ±*0.71*	1.36 ±0.52	2.80 ±0.48	1.48 ±0.4	2.79 ±0.6	1.59 ±0.7	6.04 ±1.8
Muscle	*2.40 ±0.75*	1.08±0.5	1.41 ±0.52	2.48 ±0.62	4.45 ±0.8	2.72 ±0.6	10.96 ±2.1
Kidney	3.47 ±*0.31*	2.47±0.6	7.44 ±0.8	5.50 ±0.7	15.64 ±1.4	15.09 ±1.4	53.44 ±3.9
ruvate (μ moles of pyruvate/g wet wt tissue)							
Liver	5.45 ± 0.08	2.05 ±0.29	3.50 ±0.45	4.09 ±0.23	3.41 ±0.46	6.82 ±0.43	1.36 ±0.34
Heart	3.08 ±0.28	2.73 ±0.08	1.21 ±0,33	2.05 ±0.44	1.02 ±0.53	2.72 ±0.11	0.68 ±0.37

Values are the mean ± SD of six separate experiments

Table 6.1 Levels of AST, ALT and Pyruvate in aflatoxin dosed tissues

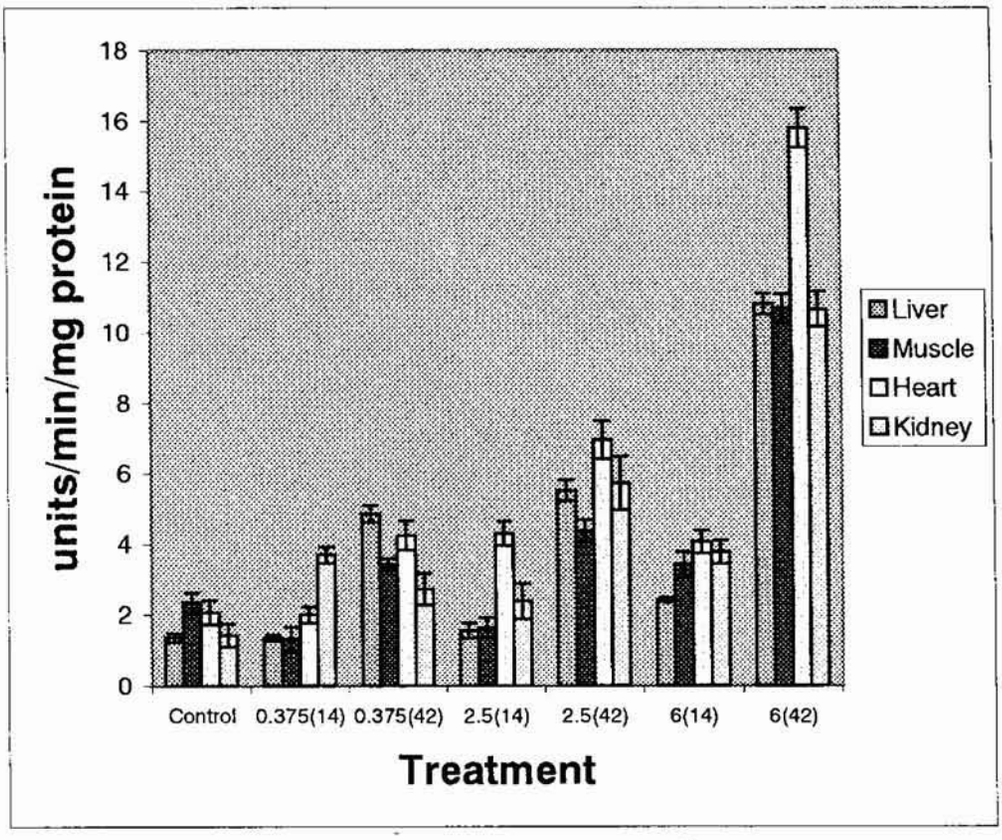


Fig 6.1 Concentration of AST in the various tissues exposed to different aflatoxin doses

Three way ANOVA revealed that a significant difference ($p<0.01$) was seen between concentrations and between days in the levels of **AST** in the control and aflatoxin exposed groups. A comparison between tissues did not reveal any significant change. Further comparisons by LSD analysis revealed that no significant difference was observed between control and 0.375ppm and also between 0.375ppm and 2.5ppm. All other comparisons gave significant results. LSD at 5% level was 2.17. Concentration 6ppm gave significantly higher values when compared with the other concentrations. The values at 42 days were significantly higher than the values at 14 days.

Source of Variation	SS	df	MS	F	
Total	366.106	31			
Between concentrations	156.613	3	52.204	11.75	P<0.01
Between Tissues	12.367	3	4.122	<1	N.S.
Between Days	90.462	1	90.462	20.36	P<0.01
Error	106.664	24	4.444		

SS-sum of squares, df-degrees of freedom, MS-mean of squares

N.S = Not significant

Table 6.1a ANOVA for AST

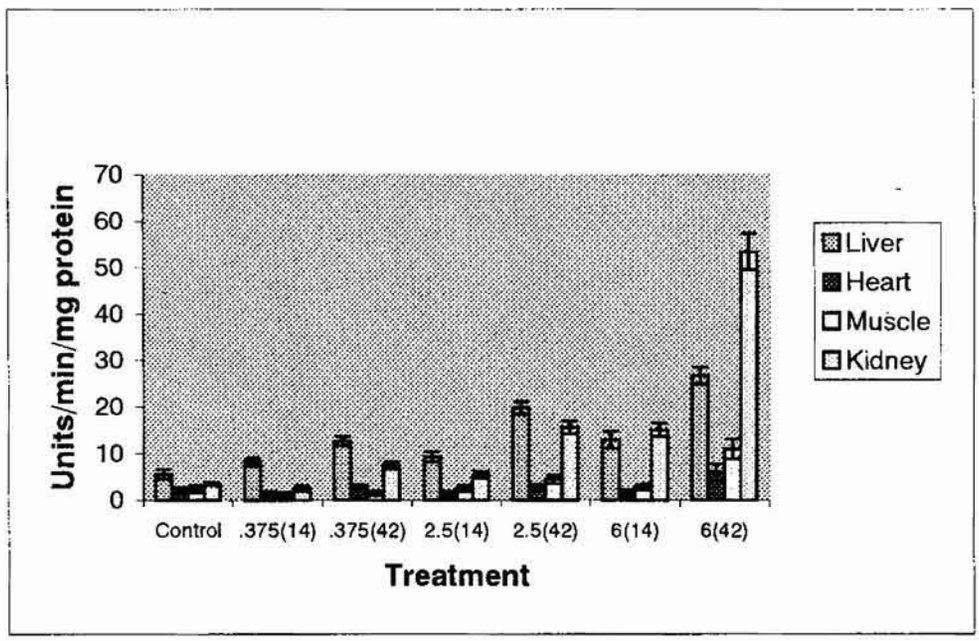


Fig 6.2 Concentration of ALT in the various tissues exposed to different aflatoxin concentrations

Three way ANOVA revealed that a significant difference ($p<0.05$) was seen between concentrations, between time periods and between tissues in the levels of **ALT** in the control and aflatoxin exposed groups. Further comparisons by LSD analysis revealed that no significant difference was observed between control and 0.375ppm, control and 2.5ppm and also between 0.375ppm and 2.5ppm. LSD at 5% level was 7.84. Concentration 6ppm gave

significantly higher values when compared with the other concentrations. The values at 42 days were significantly ($p<0.05$) higher than the values at 14 days. Comparison between tissues showed significant variation ($p<0.05$) in the enzyme levels. The kidney gave significantly greater values than the other tissues. No significant change was noticed on comparison between liver and kidney and also between heart and muscle tissue.

Source of Variation	SS	df	MS	F	
Total	3295.8	31			
Between concentrations	796.33	3	265.44	4.605	$p<0.05$
Between Tissues	802.93	3	267.64	4.643	$p<0.05$
Between Days	313.05	1	313.05	5.431	$p<0.05$
Error	1383.5	24	57.645		

-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.1b ANOVA for ALT

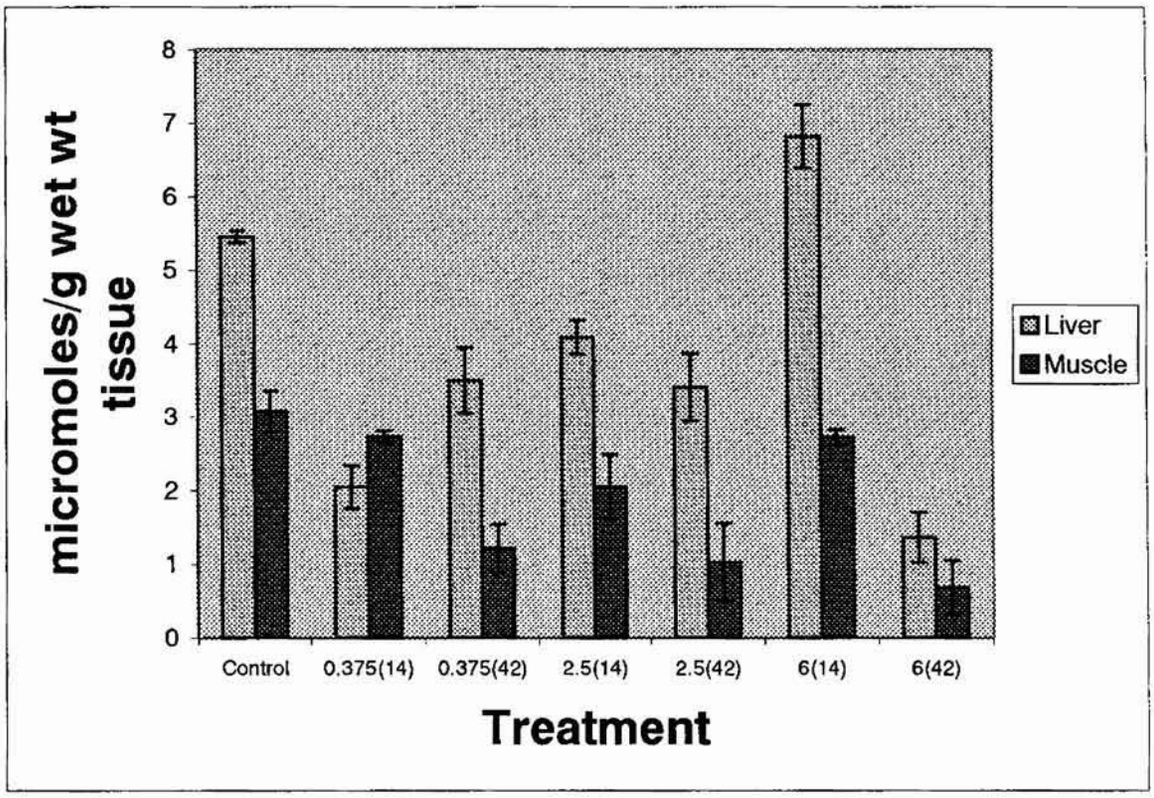


Fig 6.3 Concentration of Pyruvate in the various tissues exposed to different aflatoxin doses

Parameter	Treatment			
	Control	**0.375ppm**	**2.5ppm**	**6ppm**
Urea (μ moles of urea/wet wt tissue)				
Liver	7. ±1.1	11.66±1.8	10.0±0.4	19.18 ±1.99
Heart	5.0±2.43	30.0 ±1.98	25.0±1.65	40.0 ±0.89
Muscle	2.5±0.43	2.08 ±0.89	3.75±1.23	4.17 ±0.21
Kidney	10. ±2.88	12.5 ±3.22	22.9±1.57	15.0 ± 2.45
Free amino acids (μ moles of tyrosine/g wet wt tissue)				
Liver	27.5±3.4	32.29±4.6	32.03±6.1	82.79 ±2.3
Heart	27.8±5.8	37.09±4.3	52.98±2.9	54.08 ±3.9
Muscle	4.4±1.1	12.14±3.1	*18.76±5.3*	45.26 ±4.7
Kidney	4.4±1.4	12.14±4.5	39.73±7.3	54.64 ±4.4
Alkaline phosphatase (mg of pnp formed/min/mg protein)				
Liver	3.48±0.9	6.41±1.5	6.69±2.4	6.71 ±1.4
Heart	4.82±1.5	5.87±2.6	4.95±0.8	7.59 ±2.6
Muscle	0.29±0.02	0.47±0.01	0.35±0.03	0.61±0.04
Kidney	9.55±2.8	12.11±3.5	19.7±3.5	26.99 ±2.2

Values are the mean ± SD of six separate experiments
Pnp – para nitro phenol

Table 6.2 Levels of Urea, Free amino acids and Alkalinephosphatase in the aflatoxin exposed groups

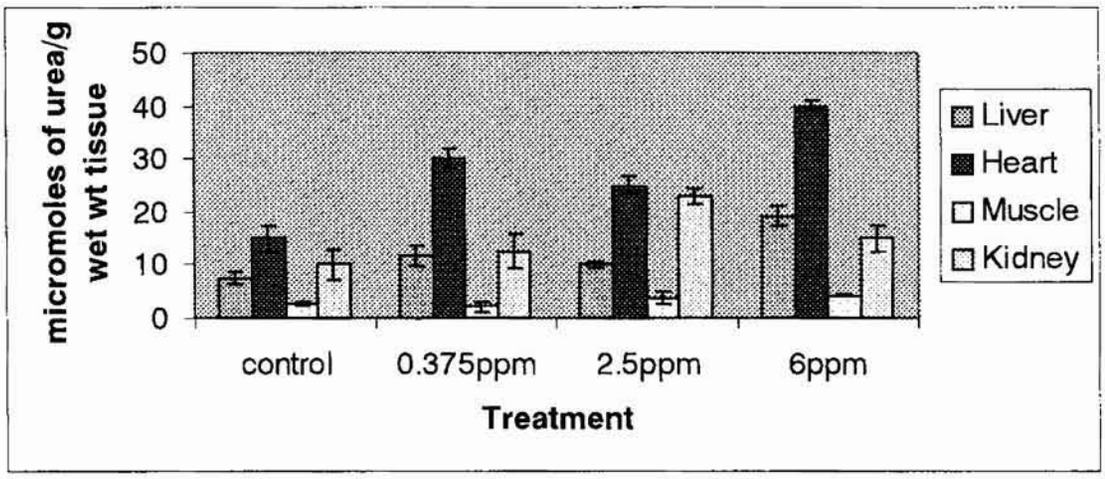


Fig 6.4 Concentration of Urea in the various tissues exposed to different aflatoxin doses

Two way ANOVA revealed that a significant difference ($p<0.01$) was seen between tissues in the levels of **urea** in the aflatoxin exposed groups. Further comparisons by LSD analysis revealed that significant difference was observed between the different tissues with the exception of liver and kidney. LSD at 5% level was 8.56. No significant change was noticed on comparison between concentrations.

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	239.888	3	79.9626	2.790492	0.101604	3.862539
Columns	1218.321	3	406.1071	14.17209	0.000932	3.862539
Error	257.8987	9	28.65541			
Total	1716.108	15				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.2a ANOVA for Urea

G8502

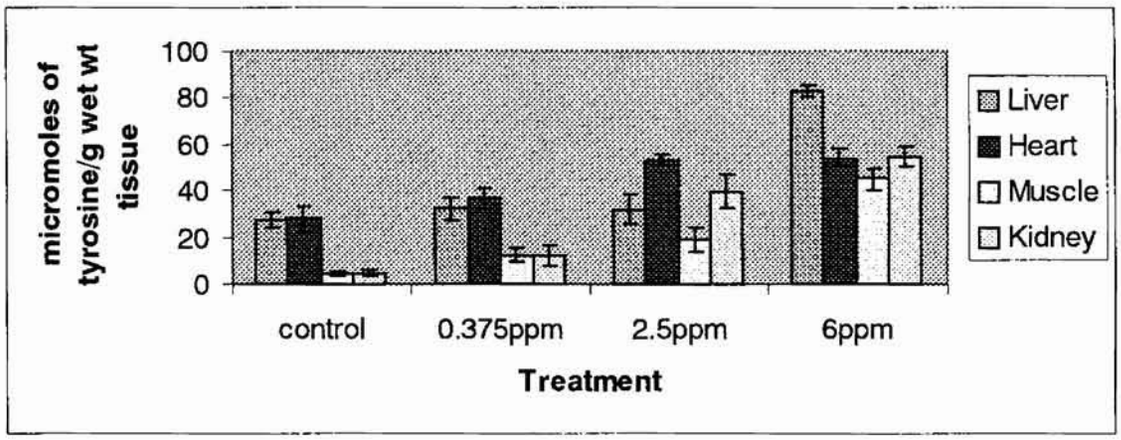


Fig 6.5 Concentration of Free amino acids in the various tissues exposed to different aflatoxin doses

Two way ANOVA revealed that a significant difference ($p<0.05$) was seen between tissues and between concentrations ($p<0.01$) in the levels of **free amino acids** in the aflatoxin exposed groups. Further comparisons by LSD analysis revealed that significant difference was observed between the different tissues with the exception of liver and heart, heart and kidney, and muscle and kidney. LSD at 5% level was 15.58. Significant change was noticed on comparison between concentrations. Comparisons between control and 0.375ppm and between 0.375ppm and 2.5ppm did not show significant change.

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	4285.809	3	1428.603	15.04683	0.000749	3.862539
Columns	1620.61	3	540.2033	5.689717	0.018279	3.862539
Error	854.4941	9	94.94379			
Total	6760.913	15				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.2b ANOVA for Free amino acids

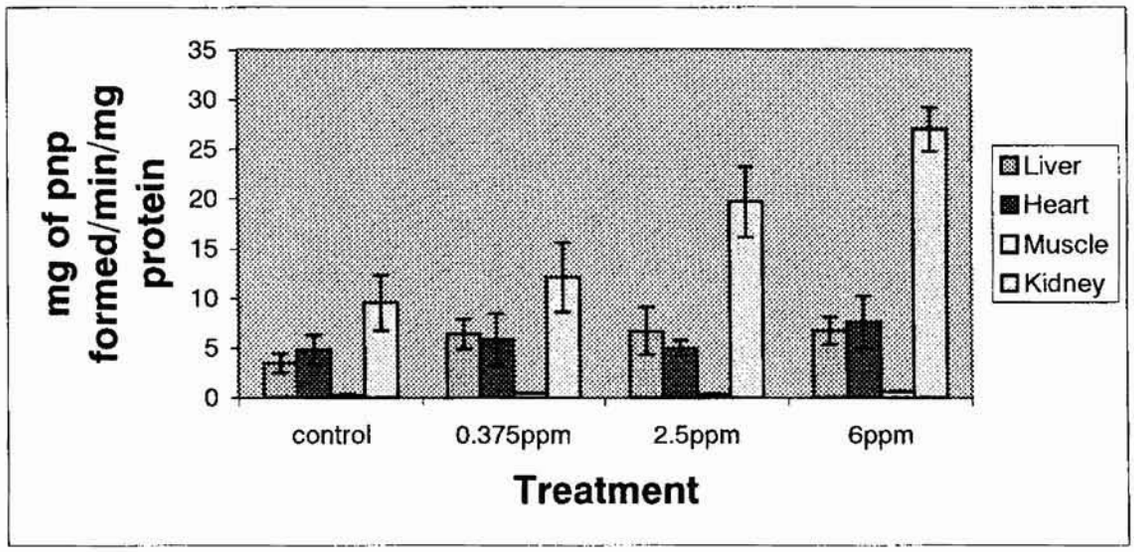


Fig 6.6 Concentration of Alkaline phosphatase in the various tissues exposed to different aflatoxin doses

Two way ANOVA revealed that a significant difference ($p<0.01$) was seen between tissues in the levels of **alkaline phosphatase** in the aflatoxin exposed groups. Further comparisons by LSD analysis revealed that kidney gave significantly higher values when compared with other tissues. Significantly lower value was observed in muscle. LSD at 5% level was 6.79 and 3.94 respectively. There was no significant difference between concentrations at 5% level.

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	77.27958	3	25.75986	1.904813	0.199336	3.862539
Columns	589.7889	3	196.5963	14.53731	0.00085	3.862539
Error	121.7121	9	13.52356			
Total	788.7805	15				

SS-sum of squares,df-degrees of freedom,MS-mean of squares

Table 6.2c ANOVA for Alkaline phosphatase

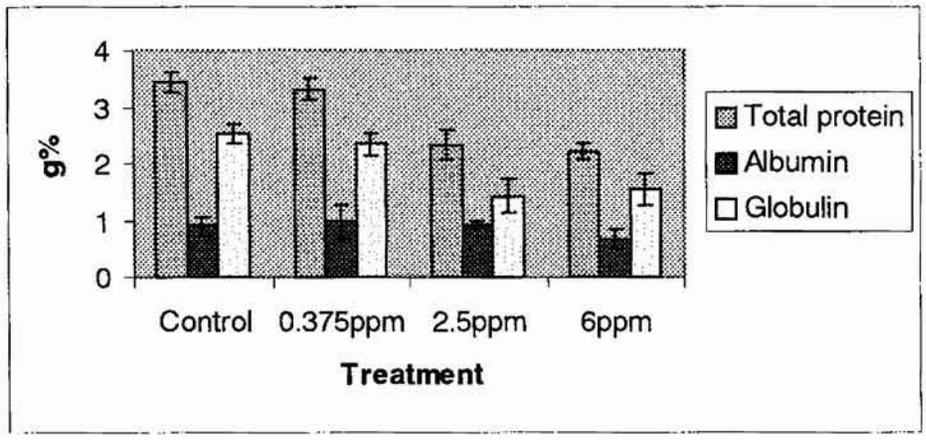


Fig 6.7 Concentration of Total proteins in serum of fish exposed to different aflatoxin doses

One way ANOVA showed significant variation ($p<0.01$) in the concentration of **serum protein** ($p<0.01$), **albumin** ($p<0.05$) and **globulin** ($p<0.01$) between control and the different aflatoxin doses. LSD values at 5% level were 0.24, 0.225 and 0.295 respectively. In the case of total proteins ,no significant difference was noticed between control and o.375ppm. No significant difference was noticed in the levels of albumin between control and o.375ppm, control and 2.5ppm and 0.375ppm and 2.5ppm.In the case of globulin significant difference was not noticed between control and 0.375ppm, 0.375ppm and 2.5ppm and between 0.375ppm and 6ppm.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	8.2104	3	2.7368	70.9996	7.71E-11	3.098392
Within Groups	0.770933	20	0.038546			
Total	8.981333	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.3 ANOVA for Total proteins

urce of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.3394	3	0.113133333	3.214319538	0.04487	3.098392654
Within Groups	0.703933333	20	0.035196667			
Total	1.043333333	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.3.1 ANOVA for SerumAlbumin

urce of Variation	SS	df	MS	F	P-value	F crit
Between Groups	6.611933333	3	2.203977778	35.27305059	3.52E-08	3.098392654
Within Groups	1.249666667	20	0.062483333			
Total	7.8616	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.3.2 ANOVA for Serum Globulin

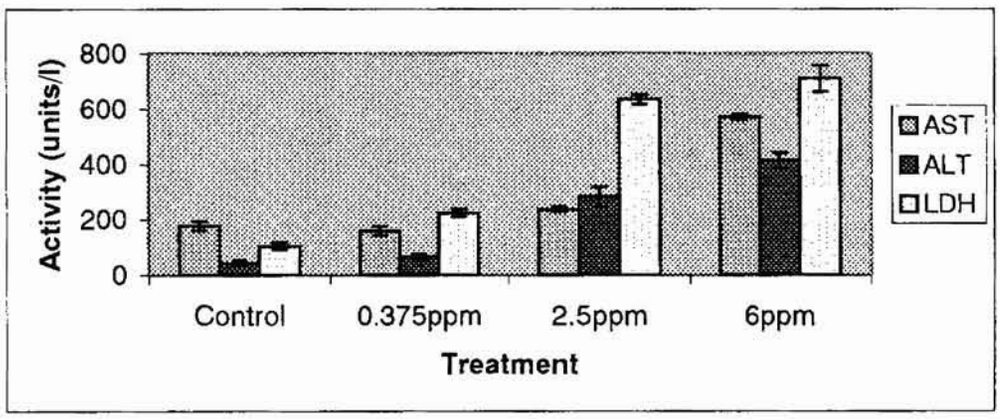


Fig 6.8 Concentration of AST, ALT and LDH in serum of fish exposed to different aflatoxin doses

Significant variation ($p<0.01$) was noticed in the levels of the **serum** enzymes namely **AST, ALT and LDH (lactate dehydrogenase)** as revealed

by one way ANOVA. LSD values at 5% level were 15.59, 28.58 and 32.43. LSD analysis showed that the different concentrations of aflatoxin differed significantly from one another in the levels. Both AST and LDH differed significantly between concentrations.In the case of ALT, significant difference was not noticed between control and 0.375ppm.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	664525.6	3	221508.5	1321.461	3.75E-23	3.098393
Within Groups	3352.48	20	167.624			
Total	667878.1	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.4 ANOVA for AST

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	569428.5	3	189809.5	337.0383	2.76E-17	3.098393
Within Groups	11263.38	20	563.169			
Total	580691.9	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.4a ANOVA for ALT

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1602822	3	534274	736.9002	1.24E-20	3.098393
Within Groups	14500.58	20	725.029			
Total	1617323	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.4b ANOVA for LDH

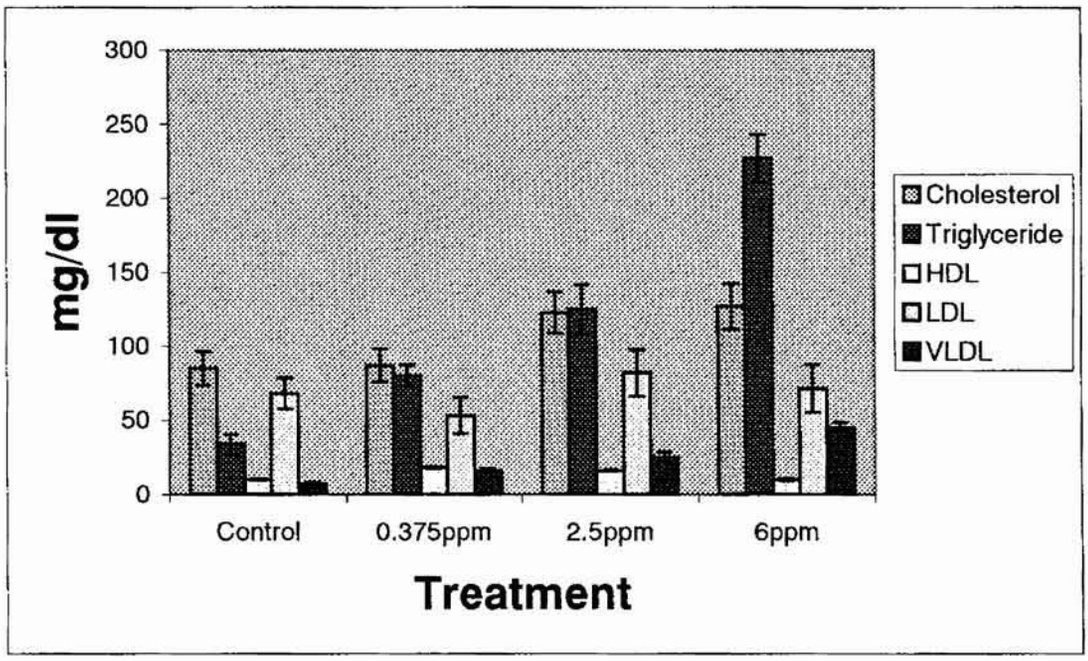


Fig 6.9 Lipid Profile of serum of fish exposed to different aflatoxin doses

The one way ANOVA of the serum lipid profile revealed significant variation ($p<0.01$) in the levels of **cholesterol, triglycerides, HDL, LDL** and **VLDL**. LSD values at 5% level were 15.88, 15.20, 0.69, 15.31.and 0.17 respectively.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	9173.711	3	3057.904	17.58338	7.9E-06	3.098393
Within Groups	3478.175	20	173.9087			
Total	12651.89	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.5 ANOVA for Serum Cholesterol

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	122560.5	3	40853.5	256.4505	3.99E-16	3.098393
Within Groups	3186.073	20	159.3037			
Total	125746.6	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.5a ANOVA for Serum Triglycerides

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	306.05	3	102.0167	308.8567	6.5E-17	3.098393
Within Groups	6.606083	20	0.330304			
Total	312.6561	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.5b ANOVA for HDL Cholesterol

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2428.858	3	809.6194	5.01291	0.009411	3.098393
Within Groups	3230.137	20	161.5069			
Total	5658.995	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.5c ANOVA for LDL Cholesterol

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	4901.04	3	1633.68	254.3453	4.32E-16	3.098393
Within Groups	128.4616	20	6.42308			
Total	5029.502	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.5d ANOVA for VLDL

Treatment	Parameter	P value
Control vs 0.375ppm	Cholesterol	NS
	Triglyerides	P<0.01
	HDL	P<0.01
	LDL	NS
	VLDL	P<0.01
Control vs 2.5ppm	Cholesterol	P<0.01
	Triglyerides	P<0.01
	HDL	P<0.01
	LDL	P<0.01
	VLDL	P<0.01
Control vs 6.0ppm	Cholesterol	P<0.01
	Triglyerides	P<0.01
	HDL	NS
	LDL	NS
	VLDL	P<0.01
0.375ppm vs 2.5ppm	Cholesterol	P<0.01
	Triglyerides	P<0.01
	HDL	P<0.01
	LDL	P<0.01
	VLDL	P<0.01
0.375ppm vs 6.0ppm	Cholesterol	P<0.01
	Triglyerides	P<0.01
	HDL	P<0.01
	LDL	P<0.01
	VLDL	P<0.01
2.5ppm vs 6ppm	Cholesterol	NS
	Triglyerides	P<0.01
	HDL	P<0.01
	LDL	NS
	VLDL	P<0.01

Table 6.5e LSD analysis of Lipid profile of serum

NS - Not significant

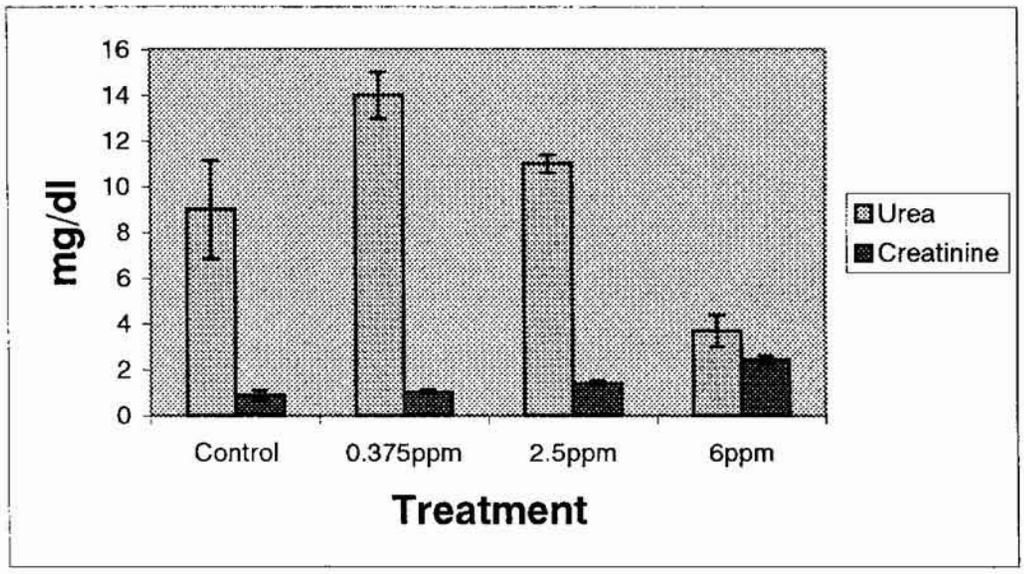


Fig 6.10 Urea and Creatinine in serum of fish exposed to different aflatoxin doses

Significant variation ($p<0.01$) was noticed in the levels of the **blood urea** between concentrations as revealed by one-way ANOVA but the levels of **serum creatinine** showed no significant change between concentrations. LSD values at 5% level were 1.5 in the case of blood urea. LSD analysis showed that the different concentrations of aflatoxin differed significantly from one another in the levels of blood urea.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	338.205	3	112.735	72.96764	6.01E-11	3.098393
Within Groups	30.9	20	1.545			
Total	369.105	23				

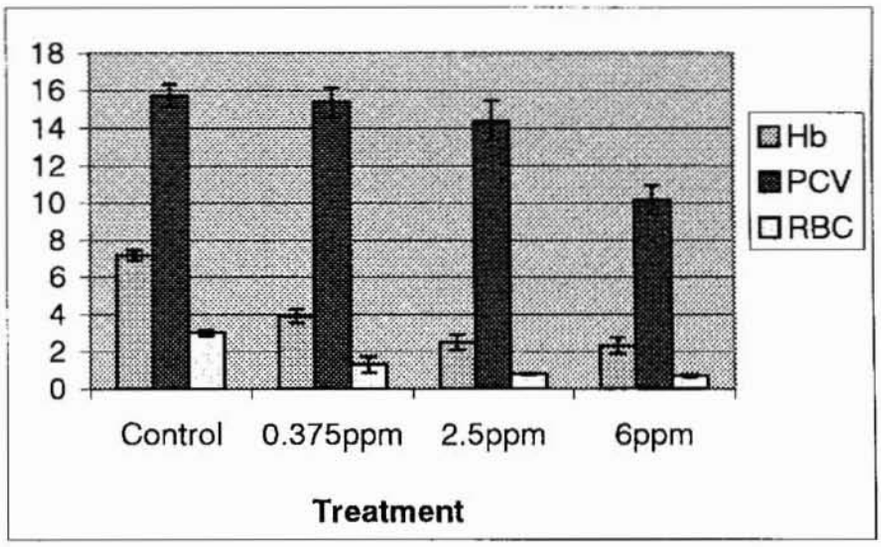
SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.6 ANOVA for Blood Urea

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	8.894346	3	2.964782	120.0762	5.85E-13	3.098393
Within Groups	0.493817	20	0.024691			
Total	9.388163	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.6a ANOVA for Serum Creatinine



Hb-Haemoglobin, PCV-Packed Cell Volume, RBC-Red Blood Cell Count

Fig 6.11 Concentration of Hb, PCV and RBC in fish exposed to aflatoxins

The one way ANOVA of the haematological parameters namely **haemoglobin** and the **erythrocyte count** revealed significant variation ($p<0.01$). LSD values at 5% level were 0.47 and 24.38 respectively. No significant difference was seen between concentrations in the levels of **PCV** (packed cell volume). Significant difference was not seen between 2.5ppm and 6ppm in the case of haemoglobin.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	92.325	3	30.775	199.8377	4.48E-15	3.098393
Within Groups	3.08	20	0.154			
Total	95.405	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.7 ANOVA for Haemoglobin

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	19.085	3	6.361667	66.97431	1.31E-10	3.098393
Within Groups	1.899733	20	0.094987			
Total	20.98473	23				

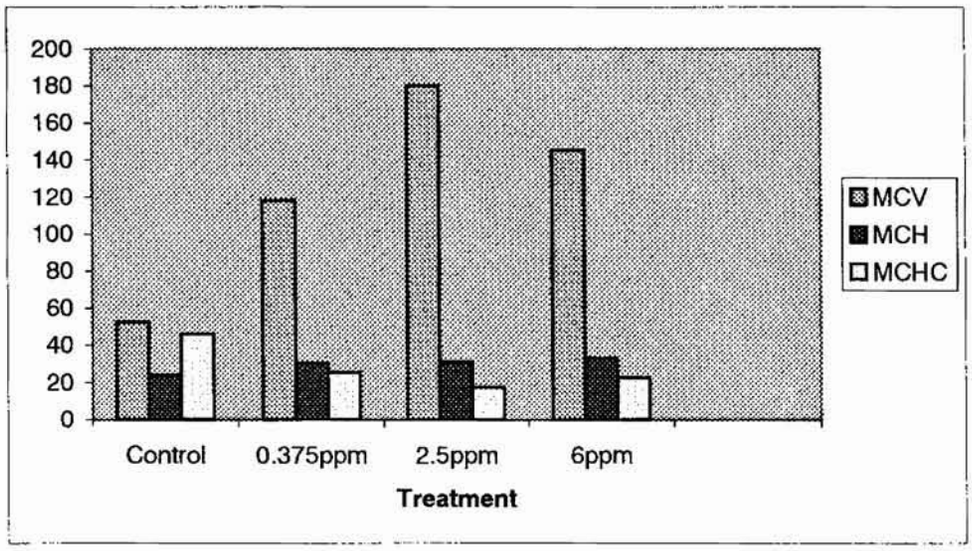
SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.7a ANOVA for Red Blood Cell Count

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	601.386	3	200.462	0.489016	0.69381	3.098393
Within Groups	8198.583	20	409.9292			
Total	8799.969	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.7b ANOVA for Packed Cell Volume



MCV-mean corpuscular volume (μ3), MCH- mean corpuscular haemoglobin(pg), MCHC-mean corpuscular haemoglobin concentration (%)

Fig 6.12 MCV, MCH and MCHC in fish exposed to aflatoxins

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	53053.56	3	17684.52	32.48492	6.97E-08	3.098393
Within Groups	10887.83	20	544.3917			
Total	63941.4	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.7c ANOVA for MCV

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	335.9083	3	111.9694	2.187195	0.121213	3.098393
Within Groups	1023.863	20	51.19316			
Total	1359.772	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.7d ANOVA for MCH

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2763.003	3	921.0009	48.177	2.46E-09	3.098393
Within Groups	382.3405	20	19.11702			
Total	3145.343	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 6.7a ANOVA for MCHC

The one way ANOVA of the haematological parameters namely MCV, MCH and MCHC revealed significant variation ($p<0.01$). LSD values at 5% level were 28.1, 8.61 and 5.26 respectively.

6.4 Discussion

Aflatoxins may be considered as biosynthetic inhibitors both in vivo and in vitro, with large doses causing total inhibition of biochemical systems and lower doses affecting different metabolic systems. In the present study the level of ALT was found to be higher in the liver tissue followed by kidney, muscle and heart suggesting the hepatic tissue to be very efficient in utilizing amino acids for metabolic purposes. In the case of AST maximum activity was found in the muscle, followed by heart, kidney and liver. Another noteworthy feature is that the ALT activity is found to be relatively higher than AST activity in the liver and kidney in the control fish suggesting that pyruvate contribution is slightly more than oxaloacetate formation in these tissues. In the liver and kidney tissues, ALT predominates over AST where the feeding of amino acids into the energy cycle is more through alanine-pyruvate pathway representing anaerobic tendency of the tissues. Normally under stress, to cope with the energy demands and to make up for the high decrease in tissue glycogen levels the amino acids of the glycogenic

type seem to take an active role to act as precursors of carbohydrates metabolism by being fed into the TCA cycle through transaminase reaction. The levels of serum ALT and serum AST were found to be increasing with increasing dosage of aflatoxin. Similar reports have been made in Nile tilapia (Saber N A, 1999), in ducklings (Soni *et al*, 1991)and cockerels (Jacob *et al*, 1994). The observed increase in ALT may be due to the partial necrosis of hepatocytes.

The total free amino acid content showed a significant increase in the tissues of fish exposed to aflatoxin. The tissue specific increase in the different combination of aflatoxin was as follows: Liver>Heart >Kidney > muscle. The increased free amino acid level suggests tissue damage probably due to the increased proteolytic activity under aflatoxin stress. However, the elevated levels of free amino acids can be utilized for energy production by feeding them into the TCA cycle through amino transferase reaction. The increase in the levels of free amino acids on aflatoxin stress may also be attributed to the synthesis of amino acids in addition to their elevation by protein hydrolysis. A third possibility for increased free amino acid level might be their increase due to transamination and amination of keto acids. The stress felt on the liver may be due to its unique status as a centre of detoxification.

An increase in the level of glucose in blood was seen with increasing concentration of the toxin which may indicate breakdown of glycogen to glucose and its mobilization to other tissues to meet the energy crisis. The increase in the glucose level in blood may also be due to decrease in the glycogen synthesizing potentiality of tissues as a result of cellular damage as is evident in the necrosis of the liver on histopathological examination in this study.

A significant decrease in the levels of pyruvate with increasing aflatoxin concentration in both liver and muscle tissue may be due to the role played by pyruvate as a precursor for many metabolic products or due to its conversion to lactate. A concomitant increase in the lactate dehydrogenase activity in the serum indicates a shift towards anaerobiosis i.e. the pyruvate to lactate conversion is favoured thereby implying that the environment for pyruvate oxidation through Krebs cycle is not favorable similar results were obtained in Nile tilapia (Saber,1995).

Serum from the aflatoxin dosed fishes revealed a significant reduction in the total protein, globulin and albumin levels with increasing damage. The decrease in the serum protein level could be correlated with severe damage of hepatocytes as indicated by histopathological studies. Similar observations were observed in Nile tilapia by Saber,1995. The total protein levels were found to be decreased in the aflatoxin fed farm animals and cockerels, but the protein expressions were elevated (Jacob et al,1994).

CHAPTER 7

Electrophoretic Analysis of Aflatoxin Stress in *Oreochromis mossambicus*

7.0 Introduction

Advances in molecular genetics have lead to marked progress in the understanding of the genetic basis of cancer which is a complex, multistep process and controlled by two sets of genes : oncogenes and tumour suppressor genes. While the focus of oncogene research for the past decade has been on mammalian systems, a recent movement has been made toward the development of alternative model systems for the study of both human and environmental health. Of particular interest are the aquatic organisms. These animals are currently being developed as models for better understanding of normal and aberrant functioning of oncogene and suppressor gene products. While numerous studies have reported the chemical induction of tumours in fish, very little is known about the molecular basis of carcinogenesis in these animals (Hoover, 1984). It was not until 1986 that the first oncogenes from fish were cloned and sequenced from the goldfish and the rainbow trout (Nemoto *et al.* 1986 and Van Beneden *et al.*1986). Since then, however, interest in fish as model systems for molecular studies has risen exponentially.

While the role of cellular oncogenes in normal cells is as dominant, positive regulators of growth and differentiation, the tumour suppressor genes (also termed "recessive oncogenes") act as negative regulators of cell proliferation (Van Beneden *et al,* 1994). Tumour suppressor genes enable a cell to respond in the proper context to growth inhibitory signals. Inactivation of these genes leads to the unregulated growth characteristic of a malignant neoplasm.

Among the many biomarkers the study of the altered structure, function and expression of the tumour suppressor gene p53 and its gene product protein (57KDa) is being ideally used as a molecular level marker to monitor the carcinogens in human and other mammals. In humans and other mammals, the p53 is a 53 KDa phosphoprotein encoded by a gene of 20kb localized on the short arm of chromosome 17 (Soussi, 1990). This act as a check point blocking the cell cycle under adverse conditions like DNA damage. This does not permit cell division till the damaged DNA is repaired. If the repair is not possible it orders for the destruction of the cell termed "apoptosis" (Gupta and Singh, 1995). When the DNA is damaged by a carcinogenic agent, the p53 gene gets activated, transcribing the gene product viz 53 KDa protein which in turn regulate the cell cycle. Hence the p53 gene expression can serve as a genetic biomarker to monitor the abuse caused by the carcinogen. The p53 has been reported to be actually a 57KDa protein in trout as against the 53 KDa in human and other mammalian species (Soussi, 1990). Expression of p53 gene has been detected in fish cell lines EPC (epithelioma papillosum cyprini) and CHSE 14 (Chinook head salmon embryo -14) (Smith *et al*, 1988).

7.a Apoptosis

Apoptosis is a physiological and pathological process of cell deletion that functions as an essential mechanism of normal tissue homeostasis and it may play a critical role in relation to disease status (Kerr, Wyllie and Currie, 1972; Columbano, 1995; Wertz and Hanley, 1996). The removal of physiologically irrelevant cells by apoptosis is part of the normal development and physiological regulation of multicellular organisms (Kerr *et al.* 1992).Cells undergoing apoptosis in normal as well as neoplastic cells

present a distinctive sequence of morphological and biochemical changes. In nucleus, these changes include condensation of the nuclear content into clumps of heterochromatin and finally packaging of the nuclear fragments into multiple membrane enclosed apoptotic bodies. These apoptotic bodies were phagocytosed from tissue without any inflammatory changes (Babu and Padikkala, 1996).

7.b Genes Controlling Apoptosis

Apoptosis results from active gene directed processes and it often requires RNA and protein synthesis (Barres et al. 1992). Recently several genes implicated in apoptosis of different cell types have been identified. These include p53, c-myc, bcl2, p35, nur -77 bak and rpr.

7.1 Materials and methods

7.1.1 Study of serum protein profile

7.1.1a Collection of blood samples

The sampling was done from both treated and control fishes. The samples were collected every two weeks and six weeks. The blood collection for serum protein analysis was done using a sterile non-heparinized syringe (22 gauge needle).

7.1.1b Processing of blood for serum protein analysis

Blood collected without the anticoagulant was kept at room temperature for about an hour. The partially clotted blood was kept inside the

refrigerator for some time to ensure the complete shrinkage of the blood cells, which increased the yield of serum. Later the samples were subjected to centrifugation for ten minutes at 4000 rpm. The serum was collected carefully into small polypropylene tubes and stored at -20^{0}C until used for electrophoretic analysis.

7.1.1c Study of nucleoprotein profile

i) Isolation of nucleoproteins from liver nuclei

One gram of freshly dissected out liver tissues from fish exposed to aflatoxin for six week. Aflatoxin exposed specimens were washed in 0.9% normal saline and blotted dry on a filter paper.

ii) Reagents for isolation

i. **Tissue homogenizing solution**

0.25M Sucrose - 8.55g

0.0018M Calcium chloride - 0.02g

The mixture was dissolved in minimum water and made up to 100ml.

ii. **Gradient**

0.034M Sucrose - 11.6g

0.00018M Calcium chloride - 0.002g

The mixture was dissolved in minimum water and made up to 100 ml.

iii. **Nuclear pellet dissolving solution**

0.025M Sucrose - 4.275g

0.00018M Calcium chloride - 0.001g

The mixture was dissolved in minimum water and made up to 50 ml.

The filter-dried liver sample was finely minced in a cavity block and transferred to a glass homogenizer. The homogenization was done in 10 ml of tissue homogenizing buffer under ice-cold conditions. 2.5ml of the cell free homogenate was carefully layered over 5ml of sucrose gradient solution. The preparation was centrifuged at 3000 rpm for 10 min at 4°C in a refrigerated centrifuge, increasing the speed slowly without disturbing the gradient. The gradient solution was discarded and the pelleted nuclear fraction was saved.

The nuclear pellet isolated from one gram tissue was dissolved in 1.2 ml of 10% SDS prepared in 0.00015M $CaCl_2$ solution. The preparation was kept for 10 minutes at room temperature for the lysis of the nuclear membrane and denaturation of the nuclear proteins. The prepared sample was stored under refrigerated condition until used for electrophoretic analysis.

7.1.2 Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis (SDS PAGE page)

Principle

SDS is an anionic detergent which binds strongly to the protein and denatures it. The number of SDS molecules bound to the polypeptide chain is approximately half the number of amino acid residues in that chain. The protein SDS complex carries a net negative charge, hence move towards the anode and the separation is based on the size of the protein.

7.1.2a Standardization of SDS PAGE

The basic methodology adopted was as described by Laemmli et al (1970) with some modification.

The percentage of separating gel is a critical parameter in all electrophoretic separions using discontinuous system of buffer alongwith a stacking gel. Separating gels of 12.5%, 11.5% and 11% were tried to choose an ideal percentage which gives a better electrophoretic separation. Finally a separating gel of 11.5% concentration, prepared from a 30% stock of acrylamide and bisacrylamide monomers were selected along with a stacking gel of 6%. The concentration of protein samples to be loaded on the gel was also standardized to get an ideal resolution.

7.1.2b Reagents for electrophoresis

Stock acrylamide solution (30%)

Acrylamide - 29.1g

Bisacrylamide - 0.8g

The mixture was dissolved in minimum quantity of water and made up to 100ml using distilled water. The mixture was filtered using Whatman No.1 filter paper and stored in amber coloured bottles in a refrigerator.

Gel buffers

a. Separating Gel Buffer

1.5M Tris - 18.17g

SDS - 0.4g

The pH was adjusted to 8.8 using 2M HCl and the solution was made up to 100 ml using distilled water .

b. Stacking gel buffer

0.5M Tris - 6.05g

SDS - 0.04g

The pH was adjusted to 6.8 using 2M HCl and the solution was made up to 100 ml using distilled water.

c. Electrode Buffer

Tris	-	3g
Glycine	-	14.4g
SDS	-	1g

Adjusted Tris pH to 8.6 with HCl and added SDS. Made up to 1000ml using distilled water.

d. Polymerizing Agent

Ammonium per sulphate - 10% (freshly prepared).
(APS)

e. 10% SDS Solution

10g SDS in 100ml distilled water.

Composition of 11.5% gel

i. Separating gel

Acrylamide and Bisacrylamide	-	11.5ml
Separating gel buffer	-	6ml
Water	-	12.7ml
10%SDS	-	300ul
TEMED (N N N'N' Tetra methyl ethylene diamine)	-	30ul
APS	-	100ul

ii. Stacking gel

Acrylamide and Bisacrylamide	-	2ml
Stacking gel buffer	-	2.5ml
Water	-	5.4ml
10%SDS	-	100ul
TEMED	-	10ul
APS	-	40ul

The separating gel components were mixed gently and poured into the prepared cassette. Few drops of butanol were over layered to prevent meniscus formation and the gel was left undisturbed to set for 30 minutes. After polymerisation of the separating gel, the overlaying butanol was removed and the cassette was washed with double distilled water and dried. The prepared stacking gel mixture was then poured over the separating gel. The comb was placed in the stacking gel and allowed to set for 30 minutes.

After the gel had solidified the comb was removed without distorting the shape of the well. The gel was carefully set on the electrophoretic apparatus after removing the clips, bottom spacers etc with the plate having the "U" shape cut facing the upper tank using the clamps and screws provided. The electrode buffer was added to the tanks and care was taken to prevent entrapment of air bubbles at the bottom of the gel. The electrodes were then connected to the power pack.

7.1.2c Preparation of samples for loading

Stocks of sample buffer with SDS and without SDS (for serum protein) and without SDS (for nuclear proteins) were prepared.

Sample buffer without SDS (10ml)

Glycerol	-	2ml
(mercaptoethanol	-	1ml
Stacking gel buffer	-	1.8ml
Bromophenol blue	-	1ml (0.5%)
Sample buffer with SDS (10 ml)		
Glycerol	-	2ml
(mercaptoethanol	-	1ml
Stacking gel buffer	-	1.8ml
Bromophenol blue	-	0.6ml (0.5%)
SDS	-	1ml (10%)

Serum and nuclear proteins isolated and stored at -20^{0}C were brought to room temperature and further processed as follows to load into the gel.

i. Nuclear protein samples

Sixty micro litres of the nuclear protein samples were mixed with equal volume of sample buffer without SDS and boiled for three minutes to ensure complete interaction between proteins and SDS, centrifuged at 10,000 rpm for 10 minutes and the supernatant was used for loading.

ii. Serum

Ten micro litres of the sample were mixed with 90µl of distilled water. 50µl of this mixture was then mixed with 50µl of sample buffer with SDS and boiled for 5 minutes.

iii. Marker

Ten micro litres of the SDS protein molecular weight marker from GENEI Bangalore was mixed with 60µl of sample buffer with 2% SDS and boiled for 1 minute.

7.1.2d Sample application and electrophoresis

The prepared samples were applied into the wells of the stacking gel and layered with running buffer in order to avoid disturbance to the sample. A constant voltage of 60 volts was applied until the dye front crossed the stacking gel and it was increased to 140 volts and electrophoresis was continued until the dye front reached the bottom of the gel.

7.1.3 Staining the gels

Immediately after the completion of electrophoresis, the gels were carefully separated from the trays into plastic trays and washed in tap water to remove excess SDS. After staining the gels for two hours in Coomassie Brilliant Blue R 250, the excess stains were washed off and the gels were immersed in destainer.

Stain(500ml)

Coomassie Brilliant Blue R 250	-	0.75g
Methanol	-	230ml
Acetic acid	-	40ml
Distilled water	-	230ml

Destainer (freshly prepared)

Methanol	-	25ml
Acetic acid	-	35ml
Distilled water	-	440ml

7.1.4 Determination of molecular weights

Molecular weights of standard SDS PAGE molecular markers used were 97.4kDa, 68kDa, 43kDa, 29kDa and 14.3kDa.. Rf values of the standard markers were calculated by using the following formula.

Rf = Solute front /Dye front

Using these Rf values calculated for standard markers, a graph was drawn with Rf and $\log_{10}$ of the molecular weights of the standard proteins on a semi-log graph. The Rf values of unknown samples were calculated and extrapolated on a standard graph to determine the molecular weight.

7.2 Isolation of DNA from whole blood

A rapid, non-enzymatic protocol for the isolation of nuclear DNA of fishes from the whole blood was standardized. Basic methodology followed was as described by Debomoy and Bill (1993). Modifications made were in the volume of non-ionic detergent, ionic detergent and saturated sodium chloride. Double the volume of SDS (10%) and saturated sodium chloride was needed for the isolation of intact nuclear DNA from the blood collected. Lysis with SDS for additional 10 min at 55^0C was also needed.

The qualitative integrity of the isolated nuclear DNA was checked by agarose gel electrophoresis. 0.6% agarose gel in Tris EDTA Boric acid (TEB) pH 8.0 was run at constant voltage of 30 volts. The gels were stained in ethidium bromide (1μg/ml) and observed on U.V. transilluminator and photographed.

7.2.1 Reagents for isolation

1) 15% EDTA
2) Low salt buffer (pH 7.6)
   10mM Tris
   10mM KCl
   2mM EDTA
   4mM Magnesium chloride

The reagents were dissolved in minimum water and made upto 1200ml with double distilled water.

3) 10% SDS

4) 6 M Sodium chloride

5) Ethanol

6) Tris EDTA

10mM Tris (pH 8.0)

1mM EDTA

7.3 Results

7.3.1 Protein profile

The profile of serum proteins and nuclear proteins as resolved by SDS page analysis of samples from both control as well as those exposed to different levels of aflatoxin B_1 (AFB_1) are presented below. Remarkable variations were observed in the expression of proteins in the animals exposed to AFB_1.

a) Serum proteins

Of the 21 serum proteins detected by SDS page, only a few showed marked changes in their expression on exposure to aflatoxin B_1 .From the figures it is evident that the general expression of all the serum proteins above the molecular weight 97.4 KDa were weak in all the experimental groups exposed to 14 days of aflatoxin in comparison with control. Their expression was however intensified by the 42nd day of aflatoxin exposure. On the 42 nd day, proteins of molecular weight 25 KDa, 29 KDa and 57 KDa were over expressed in the test groups when compared with control.

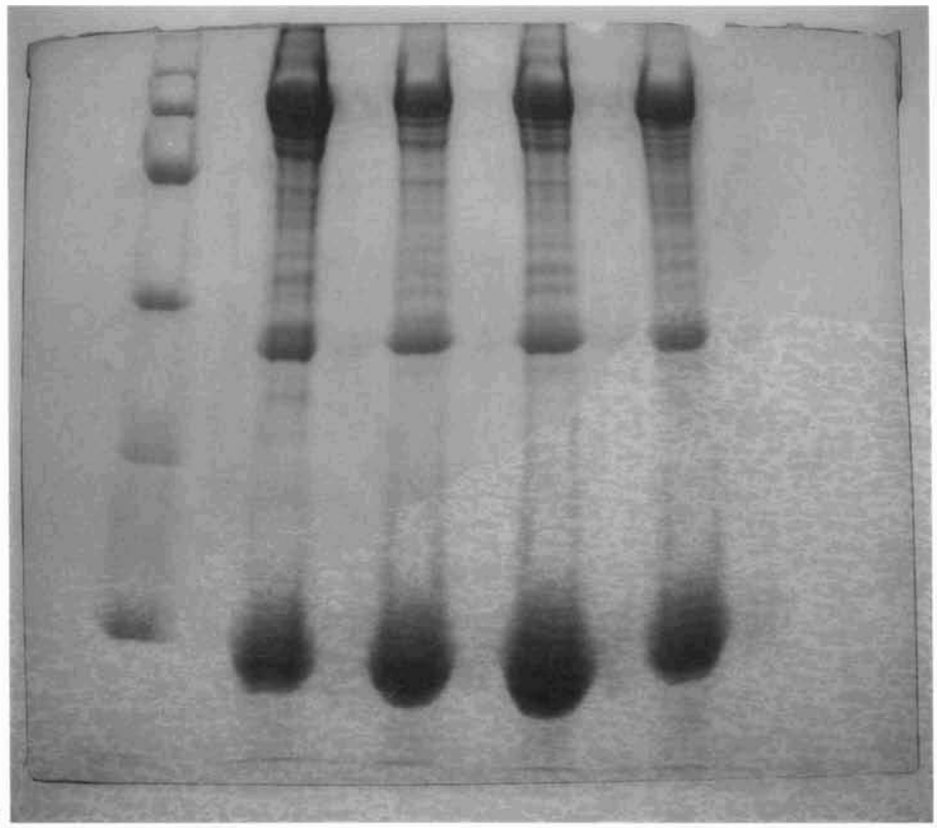


PLATE-15

SDS PAGE ANALYSIS OF SERUM PROTEINS - 14TH DAY

LANE 1- MOLECULAR WEIGHT MARKER; LANE 2 - CONTROL;

LANE 3 - 0.375 PPM; LANE 4 - 2.5 PPM; LANE 5 - 6 PPM

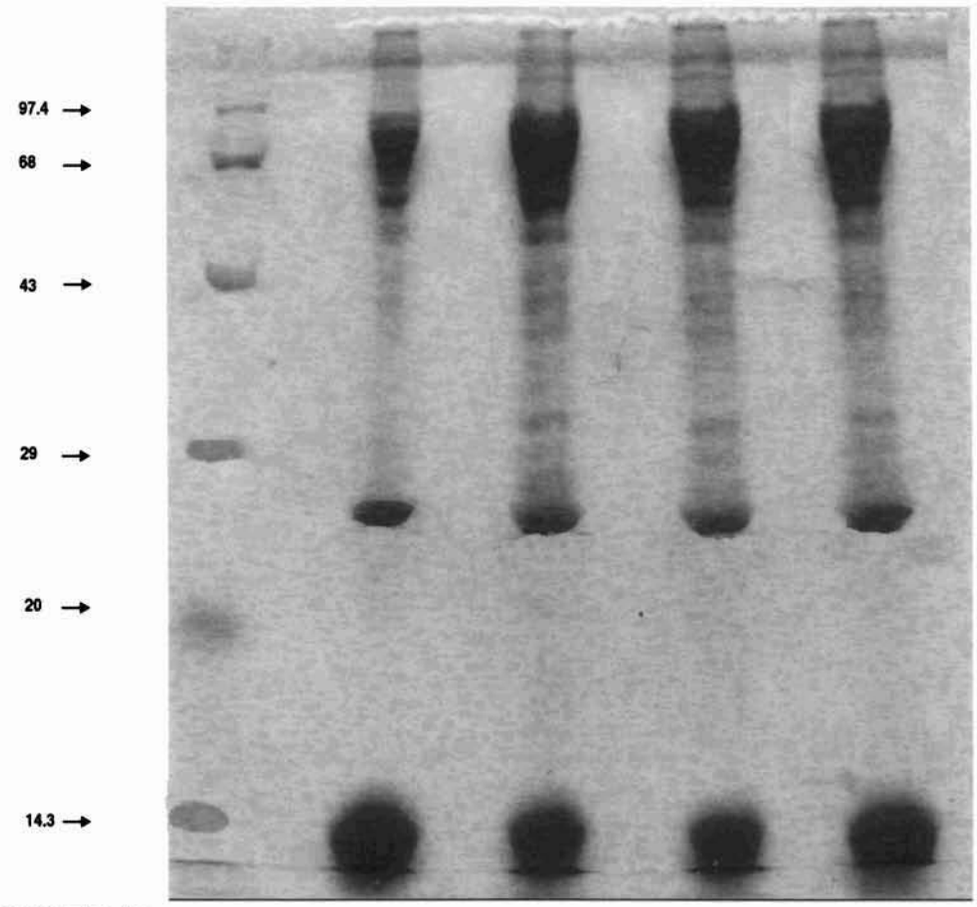


PLATE-16

SDS PAGE ANALYSIS OF SERUM PROTEINS - 42ND DAY

LANE 1- MOLECULAR WEIGHT MARKER; LANE 2 - CONTROL;

LANE 3 - 0.375 PPM: LANE 4 - 2.5 PPM: LANE 5 - 6 PPM

b) Serum Protein (Non SDS Page)

A specific protein with medium molecular weight between cathode and anode is specifically overexpressed in animals fed with higher doses of the carcinogen.

c) Nuclear Protein

16 different nuclear proteins with molecular weight ranging from 14 to 100 KDa were detected in the control animals .Five out of the 16 nuclear proteins detected in the control animals showed variation in the intensity of expression in the experimental groups namely those receiving 2.5 ppm and 6 ppm of aflatoxin for 42 days.

d) DNA Integrity

Genomic DNA isolated from the experimental groups when resolved through agarose gel electrophoresis and subsequent ethidium bromide staining revealed that genomic DNA was intact without signs of apoptosis.

7.4 Discussion

The initial effects of a toxic substance could be at cellular and sub-cellular levels of liver, which is the central metabolic organ, where detoxification processes occur. The nucleoproteins isolated from the liver nuclei of aflatoxin B_1 exposed fishes showed remarkable changes, suggesting that this might be due to the genotoxicity of aflatoxin, as most of the nucleoproteins are the translation products of the nuclear DNA. Since AFB_1

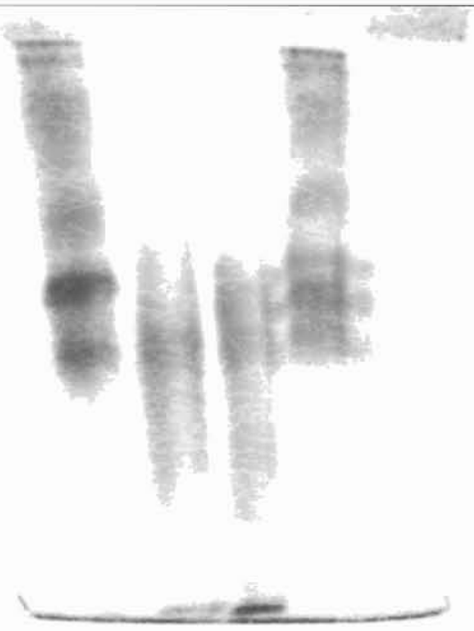


PLATE-17

NON-SDS PAGE ANALYSIS OF SERUM PROTEINS

LANE 1- CONTROL; LANE 2 - 0.375 PPM; LANE 3- 2.5 PPM; LANE 4- 6 PPM

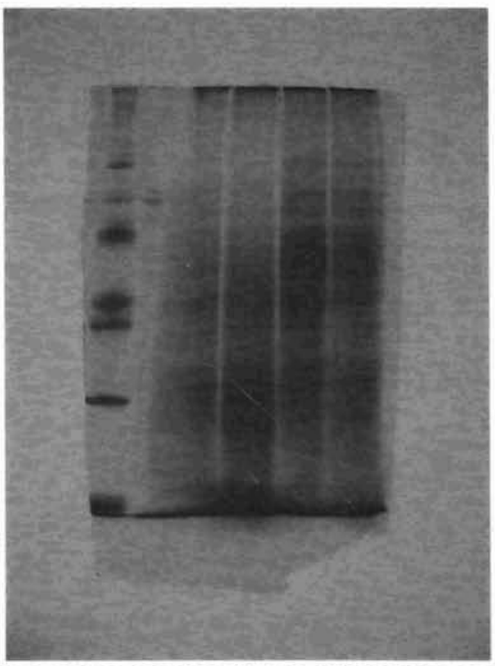


PLATE-18

SDS PAGE ANALYSIS OF NUCLEAR PROTEINS

LANE 1- MOLECULAR WEIGHT MARKER; LANE 2 - 6 PPM; LANE 3- 2.5 PPM; LANE 4- 0.375 PPM; LANE 5- CONTROL

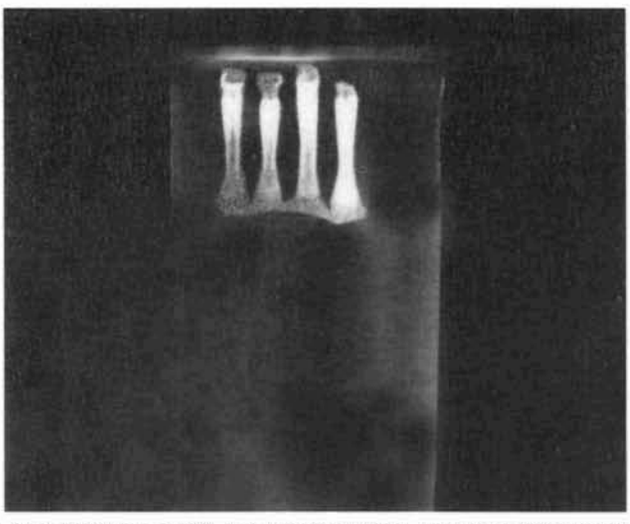


PLATE-19

DNA SEPARATED BY AGAROSE GEL ELECTROPHORESIS

LANE 1- CONTROL ; LANE 2 -0.375 PPM; LANE 3- 2.5 PPM; LANE 4- 6 PPM

is one of the most potent carcinogens capable of inducing DNA damages, the accompanying changes in translated proteins are to be expected. Many of the effects of aflatoxin is related to their reaction with nuclear proteins so that they interfere with protein formation and maintenance of cellular integrity (Patterson, 1976).

The enhancement in the nucleoprotein and serum protein expression in the test groups in comparison with the control may be due to the activation of the genes coding for the nuclear proteins under the insult of AFB_1 for enhanced rate of transcription and translation of gene products. As most of the nuclear proteins are the translation products of genomic DNA having a regulatory role, this type of a change in nuclear protein is to be expected. Since AFB_1 is a potent carcinogen, activation of oncogenes and / antioncogenes, elevated levels of aflatoxin - DNA adduct formation etc also can lead to an alteration in nuclear processes (Sato and Omura, 1978; Bendetal,1984). The elevated levels of serum proteins were reported in rainbow trout with hepatocarcinoma (Scarpelli,1969).

The tumour suppressor gene and the qualitative changes in its expression under the influence of AFB_1 were also investigated by SDS PAGE analysis. Two prominent tumor suppressor genes in fish are p53 and retinoblastoma. Normally the tumour suppressor gene, especially the p53 gene acts as a molecular guardian, monitoring the effects of the carcinogens like AFB_1 in DNA. Whenever a carcinogenic insult occurs on DNA, the p53 will express itself more strongly and will suspend the DNA replication and allow the system to repair the damage. But if the system fails to repair the damage of DNA, p53 will mediate for another process namely 'apoptosis' or 'programmed cell death' - which involves the cleavage of DNA into

fragments of similar size. This can be detected by isolating the DNA and analyzing it on an agarose gel.

A 57 KDa protein has been detected by SDS PAGE analysis in rainbow trout. This was identified as the product of trout tumour suppressor gene (p53) from its ability to bind to SV 40 large T antigen (Soussi *et al.*1990).

Analysis of the nuclear and serum protein profile observed in the present study in this reported background information, the 57 KDa protein of tilapia, showing differential expression on comparison with the control can be concluded to be the product of tumour suppressor gene p53. The elevated levels of this tumour suppressor gene encoded protein 57 KDa, in the treated sample are also indirect evidence suggesting the possible genetic damage of the genomic DNA by aflatoxin.

The finding of the 57 KDa protein and assuming this to be the p53 analogue in humans is based on earlier reports and consistent changes observed. Further immunological characterization of this protein using specific monoclonal antibody to p53 is to be performed.

The action of p53 is to suppress the developing tumours by breaking down or cleaving the nuclear material, by a process known as programmed cell death or "apoptosis". Hence by apoptosis the tumour cell DNA will be cleaved in to different fragments thereby causing cell death. Another positive indication of apoptosis noticed in this study is that of a hike in the level of the enzymes ALT and alkaline phosphatase. This finding was seen in mice along with apoptotic body formation by cytotoxic stimuli (Babu and Padikkala). It is with this idea of apoptosis process that the DNA is isolated

to see the ladder like DNA fragmentation as a result of apoptosis. But the pattern of DNA obtained by doing agarose gel electrophoresis showed no sign of apoptosis in spite of the other positive indications seen above. Another characteristic feature as detected by histopathology is the presence of cells with shrunken nucleus but in this study histopathological analysis did notreveal any cell with apoptosis. But the preneoplastic state of the liver as detected by histopathological evidence in this study lead us to assume that the pathways mediated by p53 leading to apoptosis are in action and further continuous exposure of the toxicant may bring about apoptosis.

CHAPTER 8

Effect of Aflatoxin on Biological Membranes, Na^+K^+ ATPase and Studies on Muscle Retention

8.0 Studies on the Lysosomal Membrane stability

8A. Introduction

Many toxic substances or their metabolites result in cell injury by reacting primarily with biological membranes. The lysosomal membrane is often a target of injury by xenobiotics (or their metabolites) in addition to its role in sequestration (Moore, 1985). One of the fundamental biochemical properties of lysosomes is the structure linked latency of many of their hydrolytic degradative enzymes like acid phosphatase, β-glucuronidase etc which is a direct consequence of the impermeability of the lysosomal membrane to many substrates as well as the internal membrane bound nature of many of the enzymes which renders them inactive. However, the membrane stability or fragility of the lysosomes can be altered under certain physiological and pathological conditions (Bitensky *et al*, 1973) which activate and in some instances release the previously bound enzymes. The latency of lysosomal enzymes (lysosomal stability) has therefore been used in both vertebrates and invertebrates as a measure of the condition of the lysosomes in response to a variety of stressors (Allison and Mallucci, 1964; Gahan, 1965; Allison, 1969; Gabrielescu, 1970).

Lysosomes are the subcellular organelles involved in the degradation of food taken up by endocytosis into the cell and principally in the regulation of macromolecular half-life (Moore, 1982). Lysosomes have an acid environment, which is maintained by a membrane Mg^{2+} ATPase dependant H^{+} ion proton pump (Okhuma *et al*, 1982). Release of the degradative hydrolytic enzymes from the lysosomal compartment into the cytosol that

may result from destabilization of the lysosomal membrane (Baccino, 1970) may also involve increased lysosomal fusion with other intracellular vacuoles leading to the formation of pathologically enlarged lysosomes. The consequences of these lysosomal changes would be increased autolytic activity leading to atrophy.

8B Materials and methods

The experimental design was the same as that mentioned in chapter 6 .The liver tissue of the control and aflatoxin treated group subjected to a six week aflatoxin stress was used for the experiment.

i) Fractionation of fish liver

Fishes were caught from the tank just before the experiment without giving them any stress. They were pithed, dissected and the liver rapidly removed and washed free of blood in ice cold isotonic sucrose (0.33M), blotted dry and weighed. Liver was homogenized in isotonic sucrose in cold to give a 10% homogenate which was centrifuged at 600 g for 10 min in a high speed refrigerated centrifuge. The sediment of nuclei and cell debris (nuclear fraction) was separated .The supernatant was again centrifuged in cold at 15,000g for 30 min. The lysosome rich 15,000g sediment (lysosomal fraction) and the supernatant (soluble fraction) as well as the nuclear fraction were used for the determination of enzyme activities. The lysosomal fraction and the nuclear fraction were resuspended in citrate buffer containing 0.2% Brij-35. The 15,000g supernatant was diluted with an

equal volume of double strength buffer. The acid phosphatase enzyme activity was determined in all these fractions (Plummer, 1987)

ii) Determination of the Release of Acid Phosphatase from the Lysosomal fraction – Lysosomal Enzyme Release Assay (in vivo studies)

Liver tissue from the control fishes and the six-week aflatoxin exposed groups were homogenized in isotonic sucrose and the lysosomal fraction was obtained as described above. The lysosomal pellet thus obtained was washed, centrifuged at 15,000g for 10 min and again resuspended in sucrose. A definite volume of this suspension was incubated at room temperature and aliquots were withdrawn at various time intervals of 10,15 and 30 min. Both the control and test aliquots were centrifuged at 15,000g for 30 min, to separate the unbroken lysosomes and the acid phosphatase activity in the supernatant was determined.

iii) Determination of enzyme activity (Colowick, 1957)

Reagents

1) Substrate: 400mg of p-nitro phenol phosphate (di sodium salt) was dissolved in 100ml of distilled water to obtain a solution of about 10.8M.The degraded product, p-nitro phenol gives a yellow colour in alkaline medium. The reagent was stored in a plastic bottle below 4°C

2) Buffer : Citrate buffer (0.05M) of pH 4.8

3) NaOH : 0.1N

4) Stock p-nitro phenol standard: Dissolve 100mg of p-nitro phenol in 100ml of distilled water.

Procedure

0.5ml of the substrate and 0.5ml of buffer were pipetted out into two tubes marked "test" and "control" and incubated at room temperature for three minutes. Added 0.1ml of the enzyme to the "test". Incubated the mixture for 30 min at room temperature. The blank consisted of 0.5ml of substrate, 0.5ml of citrate buffer and 0.1ml of the enzyme added after incubation. 4 ml of 0.1N NaOH was added to the tubes to stop the reaction after incubation. The colour developed was read against the blank at 410nm. The values were expressed as mg of p-nitro phenol formed/min/mg protein. Protein was estimated by the method of Lowry *et al* (1951).

iv) Statistical analysis

Statistical analysis of results was done by ANOVA followed by Least Significant Difference (LSD) (Zar,1999).

8C Results

1) Subcellular Activity of Acid Phosphatase

The activity of acid phosphatase in the different subcellular fractions of liver is given in table 8.0 and fig 8.0.

Group	Nuclear Fraction	Lysosomal Fraction	Soluble Fraction	Ratio Lysosomal/Soluble (LSI)
Control	6.8±0.35	12.08±1.29	6.3±0.68	1.917
0.375 ppm	13.379±0.28	4.01±1.08	4.5±1.4	0.891
2.5 ppm	15.033±1.7	6.714±0.53	7.545±1.5	0.889
6 ppm	8.1661±0.85	5.392±0.57	9.703±1.49	0.555

Values are the mean ± SD of six separate experiments

Table 8.0 Activity of Acid phosphatase in the different subcellular fractions of liver in the aflatoxin treated groups

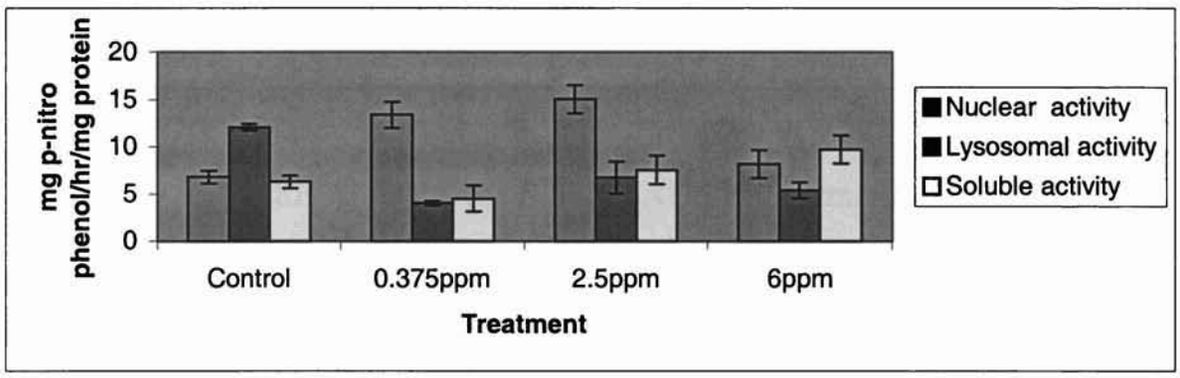


Fig 8.0 Activity of subcellular acid phosphatase in the aflatoxin exposed groups

One way Analysis of variance (ANOVA) revealed an overall significant change ($P<0.01$) in the enzyme activity in the nuclear fraction, (Table 8A), soluble fraction (Table 8B) and lysosomal fractions (Table 8C) in the test groups. Subsequent comparison between different concentrations was done by least significant difference (LSD) analysis, the results of which are presented below in table 8D.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	284.7486	3	94.9162	99.36788107	3.47E-12	3.098392654
Within Groups	19.104	20	0.9552			
Total	303.8526	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 8A ANOVA for Nuclear Acid Phosphatase

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	85.00605	3	28.33541	6.488644	011.24E-05	3.098392654
Within Groups	34.36953	20	1.71848			
Total	119.375623					

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 8B ANOVA for Soluble Acid Phosphatase

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	224.4354	3	74.8118	87.0997796	1.18E-11	3.098392654
Within Groups	17.17842	20	0.85892			
Total	241.613923					

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 8C ANOVA for Lysosomal Acid Phosphatase

Groups	P-Value		
	Nuclear	Lysosomal	Soluble
Control vs 0.375ppm	P<0.01	P<0.01	P<0.01
Control vs 2.5ppm	P<0.01	P<0.01	P<0.01
Control vs 6ppm	P<0.01	P<0.01	P<0.01
0.375ppm vs 2.5ppm	P<0.01	P<0.01	P<0.01
0.375ppm vs 6ppm	P<0.01	P<0.01	P<0.01
2.5ppm vs 6ppm	P<0.01	P<0.01	P<0.01

Table 8D LSD analysis for Subcellular Acid Phosphatase activity

The LSD (least significant difference) values of the nuclear, soluble and lysosomal acid phosphatase fractions were 1.18, 1.58 and 1.12 respectively. A significant increase (P<0.01) was observed in nuclear and soluble acid phosphatase activities in all the three-aflatoxin concentrations when compared to the control. A significant decrease was noticed on the other hand in lysosomal acid phosphatase activity of the aflatoxin exposed groups when compared to the control. Comparison between the various aflatoxin concentrations revealed no significant difference in the soluble acid phosphatase activity between control and 2.5ppm but significant difference (P<0.01) was noticed between control and the other aflatoxin concentrations

and also between concentrations. In the case of nuclear and lysosomal acid phosphatase activity, significant differences ($P<0.01$) were noticed between control and the different aflatoxin concentrations and also between concentrations. The lysosomal acid phosphatase activity in the aflatoxin dosed groups was lower than that of control unlike the soluble acid phosphatase activity which recorded an increase. This finding in fact indicate that aflatoxin cause damage to the lysosomal membrane. The lysosomal stability index (LSI) that is the ratio of the lysosomal acid phosphatase activity to the soluble acid phosphatase activity was the least (0.56) in the highest concentration (6ppm) of aflatoxin followed by 2.5 ppm (0.88) and 0.375 ppm. The LSI of the control group was 1.92. This result reflects the damage inflicted on the lysosomal membrane with increasing concentration of aflatoxin.

2) Lysosomal enzyme release assay (*in vivo*)

Time (min)	Control	0.375ppm	2.5ppm	6ppm
0	0.521±1.44 (4.31)	0.53±1.1 (4.38)	0.904±1.4 (7.48)	1.261±1.56 (10.44)
15	0.698±1.56 (5.77)	0.733±0.78 (6.06)	1.222±0.89 (10.11)	3.915±1.61 (32.41)
30	0.741±1.99 (6.13)	0.764±0.57 (6.32)	1.247±0.76 (10.32)	4.271±1.44 (35.36)

Values within brackets represent acid phosphatase release as % of total activity

Values are the mean SD ± of six separate experiments

Table 8a Percentage release of Acid Phosphatase from lysosomes

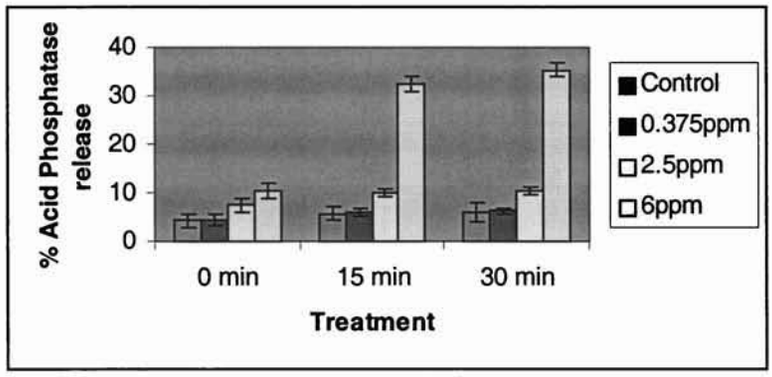


Fig 8a Percentage release of Acid Phosphatase from lysosomes (*in vivo*)

Two way ANOVA revealed that release of acid phosphatase did not differ significanty with time. A significant difference ($p<0.05$) was observed between the different aflatoxin concentrations with respect to the release of acid phosphatase.

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	2.137552	2	1.068776	1.886238	0.231441	5.143249
Columns	12.62259	3	4.207529	7.425693	0.019157	4.757055
Error	3.399707	6	0.566618			
Total	18.15985	11				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 8b Two way ANOVA for Lysosomal enzyme release

Subsequent LSD analysis reflected significant differences ($p<0.05$) between concentrations control and 6ppm, 0.375ppm and 6ppm, and 2.5ppm and 6ppm in the release of acid phosphatase.

Groups	P value
Control vs 0.375ppm	NS
Control vs 2.5ppm	NS
Control vs 6ppm	P<0.05
0.375ppm vs 2.5ppm	NS
0.375ppm vs 6ppm	P<0.05
2.5ppm vs 6ppm	P<0.05

NS-not significant

Table 8c LSD analysis for lysosomal enzyme release

8D Discussion

A decrease in the activity of lysosomal acid phosphatase was noticed with increasing concentration of the toxin when compared with the control. It has been reported that acid phosphatase is very important for tissue reorganization and tissue repair. Intracellulary, acid phosphatase activity is restricted to the lysosomes. Increased acid phosphatase activity in the testis of aflatoxin – treated mice was reported to be due to an increase in the leakage of the enzyme (Verma and Nair, 2001). The liver is the primary target of aflatoxin action and becomes infiltrated with fatty deposits when sufficiently high levels of aflatoxin are administered. Breakdown of lysosomes and the release of degradative lysosomal enzymes into the surrounding tissues is previously implicated in the hepatic necrosis and haemorrhage consistently observed with acutely toxic doses of AFB_1

(Shank R C, 1981). Histopathological examination of the liver section of the aflatoxin exposed groups in this study revealed areas of massive necrosis. This could be one of the reasons for the labilisation of the lysosomal membrane leading to release of the latent marker enzyme namely acid phosphatase. This thereby explains the fact that there was a decrease seen in the levels of the enzyme with increasing damage inflicted on the membrane with increasing concentration of the toxin due to leakage of the enzyme into the soluble fraction.

Results of lysosomal enzyme release assay, in vivo, indicate a significant increase in the release of this enzyme with increasing time. This suggests that with increasing time of exposure to the toxin, the degree of labilisation of the membrane also increased. Normally cellular membranes are selectively permeable; hence allow only certain solutes to pass through. The utility is lost due to lipid peroxidation whose products modify the physical characteristics of biological membranes. Incorporation of lipid hydroperoxide, changes the physical structure of the membrane by decreasing the fluidity and increasing permeability. When free fatty acids get damaged, membrane conformation is lost and may lead to gaps in the membrane. It can also cause cross-links between two fatty acids, fatty acid and proteins etc. This can eventually lead to change in membrane properties and loss of its bound enzymes (Thomas *et al*, 2002).

From the foregoing results it is evident that aflatoxin causes rupture of the lysosomal membrane thereby damaging it.

8.1 Studies on the Erythrocyte Membrane Stability

8.1A Introduction

Erythrocytes have been choice objects of inquiry in studies of membranes because of their ready availability and relative simplicity. They lack organelles and thus have only a single membrane, the plasma membrane. Nearly all of the cytoplasmic contents of these cells can be released by osmotic haemolysis to give ghosts, which are quite pure plasma membranes. Earlier reports on the effects of aflatoxin on the erythrocyte membrane are not available and therefore this study was envisaged to elucidate the effects of aflatoxin on the erythrocyte membrane.

8.1B Materials and Methods (for RBC membrane stability)

O*rechromis mossambicus* of size 10 $\pm$ 3g were used for the experiment.

a) Blood collection in fish

Blood was drawn from the common cardinal vein in plastic syringes containing citrate as the anticoagulant (Michael *et al*, 1994). Fresh solutions of saline - isotonic (0.85%) and hypotonic (0.5%) were prepared.

Stock RBC suspensions were prepared after washing of the cells thrice with isotonic saline. Different volumes of the suspension were then mixed with distilled water to haemolyse the cells and centrifuged at 1000g for 5 min . The absorbance of the supernatant was read at 540 nm against

distilled water blank. The dilution giving a suitable absorbance for 100% haemolysis was selected. Also a suitable volume of blood giving a suitable absorbance for 100% haemolysis was noted.

The experiment was carried out with each of three concentrations of aflatoxin as described below.

1) To 0.1 ml of the stock RBC suspension in a centrifuge tube, 5ml of isotonic saline was added and incubated for 30 min at room temperature. It was then centrifuged at 1000g for 5 min. The absorbance of the supernatant was read at 540 nm. This gave the absorbance of the " blank" (B)
2) To 0.1ml of the stock RBC suspension in three centrifuge tubes, 4.5 ml of distilled water was added and incubated for 30 min at room temperature. To this 0.5 ml of the aflatoxin stock solution was added (such that the final concentration of aflatoxin in each of the tubes were 0.375 ppm, 2.5 ppm and 6 ppm respectively). It was then centrifuged at 1000g for 5 min and the absorbance of the supernatant was read at 540 nm. This gave the absorbance corresponding to 100% haemolysis (H).
3) To 0.1 ml of the stock RBC suspension in three centrifuge tubes, 4ml of hypotonic saline and 0.5ml of distilled water was added and incubated for 30 min at room temperature. To this 0.5 ml of the aflatoxin stock solution was added (such that the final concentration of aflatoxin in each of the tubes were 0.375 pm, 2.5 ppm and 6 ppm respectively). It was then centrifuged at 1000g for 5 min and the absorbance of the supernatant was read at 540 nm. This gave the absorbance of the control (C).

4) To 0.1ml of the stock RBC suspension in three centrifuge tubes, 4ml of hypotonic saline and 0.5ml of the aflatoxin stock solution was added (such that the final concentration of aflatoxin in each of the tubes were 0.375ppm, 2.5ppm and 6ppm respectively) and incubated for 30 min at room temperature. To this, 0.5ml of distilled water was added and centrifuged at 1000g for 5 min. The absorbance of the supernatant was read at 540 nm. This gave the absorbance corresponding to the "test" (T)

Calculation

% Haemolysis in the control (X) = C-B/H-B x 100

% Haemolysis in the test (Y) = T-B/H-B x 100

% Labilisation by test = Y-X/X x 100

8.1C Results

The results are presented in Table 8.1A and Fig 8.1A.

Treatment	Percentage Haemolysis
Control	0
0.375ppm	14.24± 1.84
2.5ppm	55.20± 3.05
6ppm	94.74± 6.37

Values are the mean SD ± of six separate experiments

Table 8.1A Percentage Haemolysis of RBC membrane

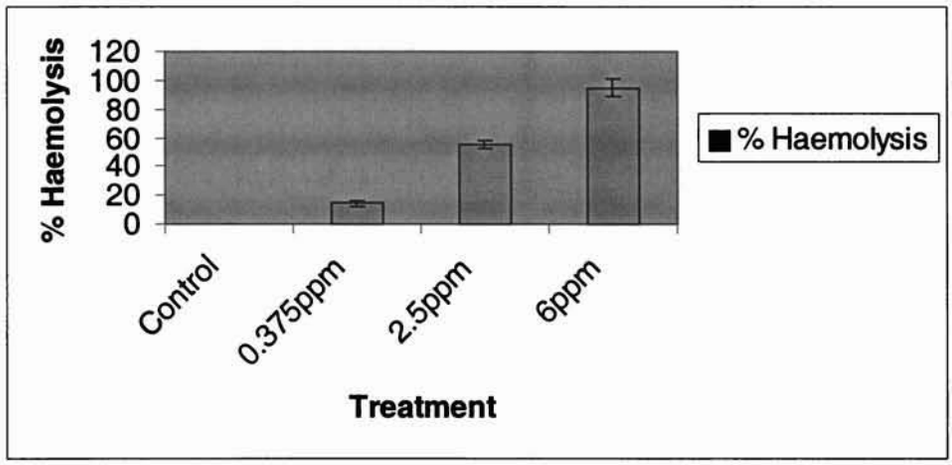


Fig 8.1A Percentage Haemolysis of RBC membrane

In vitro studies on the erythrocyte membrane stability revealed that exposure to aflatoxin had a labilising effect on the erythrocyte membrane (Table 8.1A and Fig 8.1 A). The highest concentration namely 6ppm gave the maximum percentage inhibition (94.74%) followed by 2.5ppm and 0.375ppm, which gave percentage inhibitions of 55.20% and 14.24%.

One-way analysis of variance revealed that there was significant difference ($p<0.01$) between the different aflatoxin concentrations with respect to their haemolytic activity.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	32535.32	3	10845.11	814.9466	4.56E-21	3.098393
Within Groups	266.155	20	13.30775			
Total	32801.47	23				

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Table 8.1B ANOVA for RBC membrane stability

Subsequent LSD analysis (Table 8.1C) revealed that the three different concentrations of aflatoxin differed significantly ($p<0.01$) from each other and from the control.

Groups	P value
Control vs 0.375ppm	P<0.01
Control vs 2.5ppm	P<0.01
Control vs 6ppm	P<0.01
0.375ppm vs 2.5ppm	P<0.01
0.375ppm vs 6ppm	P<0.01
2.5ppm vs 6ppm	P<0.01

Table 8.1C LSD analysis for RBC membrane stability

8.1D Discussion

The results obtained in study indicate that the degree of damage to the erythrocyte membrane is directly proportional to the increasing concentration of aflatoxin. One of the possible reasons for the labilisation of the erythrocyte membrane on exposure to aflatoxin is that of the role played by peroxidation of the membrane lipids, which increase the membrane permeability thereby decreasing its fluidity. The release of haemoglobin has been used as the criteria for evaluating the haemolytic effect when exposed to aflatoxin.

8.2 Studies on Branchial ATPases

8.2A Introduction

Generally, ATPase activity is associated with the active transport system, which is responsible for the extrusion of Na^+ from animal cells and the accumulation of K^+ within these cells (Schwartz). This enzyme is, therefore, fundamental to such function as the generation of membrane potentials and the regulation of cell volume and electrolyte composition. The presence of ATPase (ATP phosphohydrolase, EC 3.6.1.3) was first demonstrated in the crab's myelinated nerves, which are activated by Na^+, Mg^{2+} and K^+ (Skou, 1958). Subsequent studies revealed the presence of Na^+K^+ATPase in a wide variety of animal tissues (Bonting, 1970). Na^+K^+ATPase plays a significant role in both, whole body ion regulation and cellular water balance in marine animals (Towle, 1981). Pollutants have been shown to inhibit Na^+K^+ATPase activity in a variety of marine

organisms (Haya and Waiwood, 1983) by disrupting energy producing metabolic pathways (Watson and Beamish,1981;Verma *et al*,1978). Harris and Bayliss (1988) reported that potential Na^+ pumping capacity of the gills was highly correlated with gill Na^+K^+ATPase specific activities and more closely correlated with enzyme activity than Na^+ gradient. Most animal cells have a high concentration of K^+ and low concentration of Na^+ relative to the external medium. These tonic gradients are generated by a specific transport system that is called the Na^+ K^+ pump because the movement of these ions is linked. The active transport of Na^+ and K^+ is of great physiological significance. Indeed, more that a third of the ATP consumed by a resting animal is used to pump these ions. The Na^+ K^+ gradient in animal cells control cell volume, renders nerve and muscle cells electrically excitable and drives the active transport of sugars and amino acids.

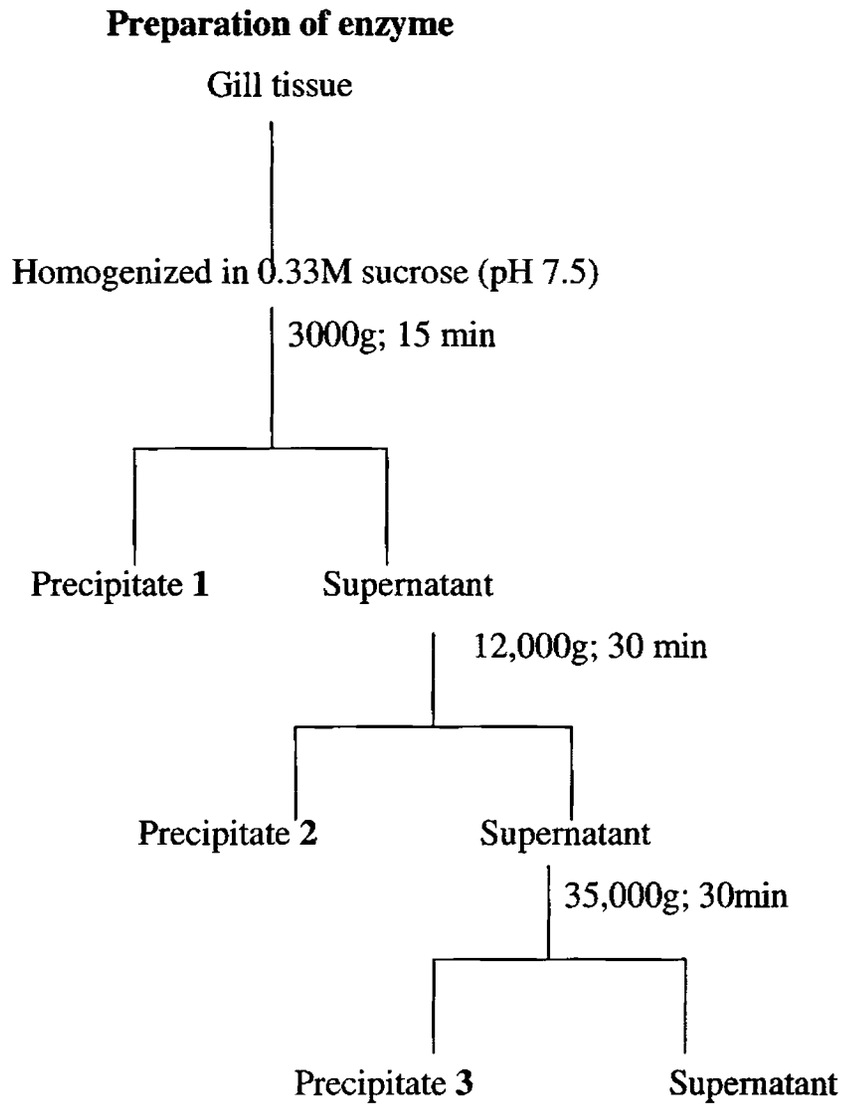
8.2B Materials and methods (for branchial ATPase determination)

The experimental design was the same as that mentioned in chapter 6.The gill tissue of the control and aflatoxin treated groups subjected to two weeks and six weeks of aflatoxin stress was dissected out, washed with 0.33M sucrose (the pH being adjusted to 7.5 with Tris-HCl buffer) and then cut into small pieces. The following procedure was carried out at 4°C.

a) Preparation of the enzyme

The gill pieces were homogenized in 10 volumes of the above mentioned 0.33M sucrose. Cell fractionation of the gill homogenate was done according to the modified method of Davis (1970). Fractionation procedure by centrifugation of the gill tissue is schematically shown below.

b) Fractionation of Gill Na^+ K^+ ATPase



The precipitate **3** thus obtained which corresponds to the heavy microsomal fraction of Davis (1970) was suspended in 0.33 M sucrose and used as the enzyme solution.

c) Assay of the enzyme activity

Na^+K^+activated, Mg^{2+} dependant ATPase (Total ATPase) was determined by using the reaction mixture containing 60 mM NaCl, 20 mM KCl, 2mM $MgCl_2$, 30mM Tris-HCl (pH 7.5) and 2.5 mM Tris ATP. The Mg^{2+}ATPase activity was measured by substituting 0.33 M sucrose in place of NaCl and KCl. The reaction mixture was incubated at 37°C for 15 min,

The Na^+K^+ATPase was calculated in terms of the difference between total ATPase and Mg^{2+}ATPase values. After incubation, 2ml of 10% trichloroacetic acid (TCA) was added to the reaction mixture and the supernatant was separated off by centrifugation at 1300g for 10 min. The inorganic phosphate (Pi) liberated from ATP was measured by the method of Fiske–Subbarow (1925). The specific activity of Na^+K^+ATPase was defined as micromoles of Pi/mg of enzyme protein/h. The protein was determined by the method of Lowry *et al* (1951) using bovine serum albumin as standard.

8.2C Results

The results are presented in Table 8.2a and Fig 8.2a.

Treatment	Period of exposure to aflatoxin	
	14days	42days
Control	24.48 ± 2.13	18.76 ± 1.54
0.375ppm	19.47 ± 1.87	15.41 ± 1.76
2.5ppm	13.62 ±2.04	9.08 ± 2.11
6.0ppm	12.32 ± 1.65	8.32 ± 1.74

Activity of Na^+k^+ATPase is expressed as micromoles of inorganic phosphate liberated/h/mg protein

Values are the mean SD ± of six separate experiments

Table 8.2a Activity of Na^+K^+ATPase in the aflatoxin exposed groups

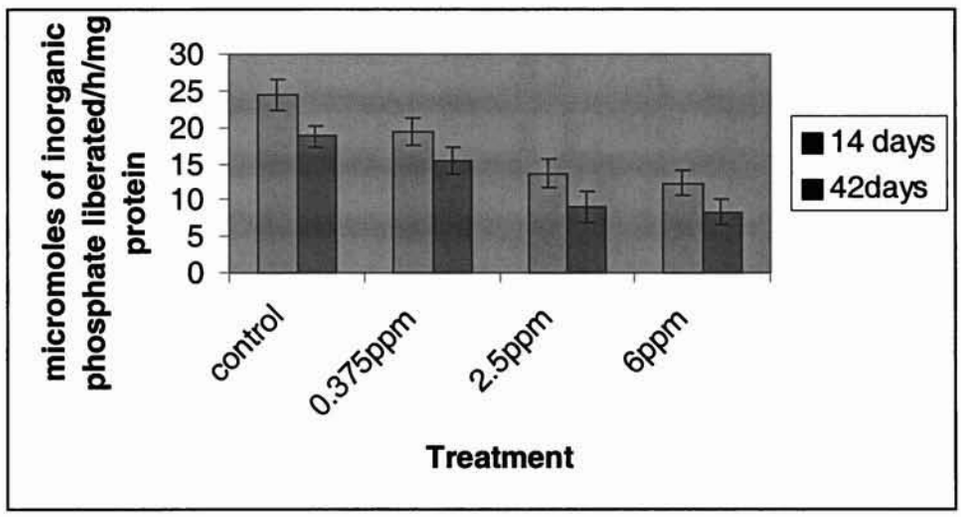


Fig 8.2a Activity of Na^+K^+ATPase in the aflatoxin exposed groups

Source of Variation	SS	Df	MS	F	P-value	F crit
Rows	169.756	3	56.58535	177.7399	0.000709	9.276619
Columns	41.93906	1	41.93906	131.7345	0.00142	10.12796
Error	0.955081	3	0.31836			
Total	212.6502	7				

SS - sum of squares, df - degrees of freedom, MS - mean of squares

Table 8.3b ANOVA for Na^+K^+ATPase

Two way analysis of variance (Table 8.3b) revealed that there was significant difference ($p<0.01$) between the different aflatoxin concentrations and the different time periods in the activity of Na^+K^+ATPase.

Treatment	P value
Control vs o.375ppm	P<0.01
control vs 2.5ppm	P<0.01
Control vs 6ppm	P<0.01
0.375ppm vs 2.5ppm	P<0.01
0.375ppm vs 6ppm	P<0.01
2.5ppm vs 6ppm	NS

NS- Not Significant

Table 8.2c LSD analysis for Na^+K^+ATPase

Subsequent LSD analysis (Table 8.2c) revealed that the three different concentrations of aflatoxin differed significantly ($p<0.01$) from each other and from the control with the exception of concentration 2.5ppm and 6ppm. Significant difference was also noticed between time periods, with the enzyme activity at 14 days being greater than the enzyme activity at 42 days.

Treatment	Period of exposure to aflatoxin	
	14days	42days
Control	18.76 ± 1.33	18.76 ± 1.89
0.375ppm	16.87 ± 1.43	14.92 ± 1.78
2.5ppm	14.52 ± 2.1	13.84 ± 1.55
6.0ppm	11.23 ± 2.15	10.74 ± 1.34

Activity of Mg^{2+}ATPase is expressed as micromoles of inorganic phosphate liberated/h/mg protein

Values are the mean SD ± of six separate experiments

Table 8.2d Activity of Mg^{2+}ATPase in the aflatoxin exposed groups

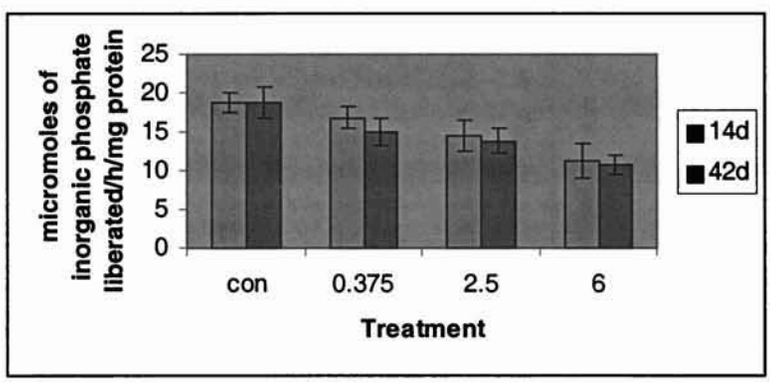


Fig 8.2d Activity of Mg^{2+}ATPase in the aflatoxin exposed groups

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	63.4463	3	21.14877	61.25934	0.003439	9.276619
Columns	1.2168	1	1.2168	3.524573	0.157101	10.12796
Error	1.0357	3	0.345233			
Total	65.6988	7				

SS - sum of squares, df - degrees of freedom, MS - mean of squares

Table 8.2e ANOVA for Mg^{2+} ATPase

Two-way analysis of variance (Table 8.3e) revealed that there was significant difference ($p<0.01$) between the different aflatoxin concentrations but not between different time periods in the activity of Mg^{2+}ATPase.

Treatment	P value
Control vs o.375ppm	P<0.01
control vs 2.5ppm	P<0.01
Control vs 6ppm	P<0.01
0.375ppm vs 2.5ppm	P<0.01
0.375ppm vs 6ppm	P<0.01
2.5ppm vs 6ppm	P<0.01

Table 8.2f LSD analysis for Mg^{2+} ATPase

Subsequent LSD analysis (Table 8.2f) revealed that the three different concentrations of aflatoxin differed significantly ($p<0.01$) from each other

and from the control. Significant difference was not noticed between time periods. The LSD at 5% level was 1.328.

8.2D Discussion

In this study, a decrease was noticed in the activities of both Na^+K^+ATPase and Mg^{2+}ATPase. In aflatoxin treated rats also, a fall in the ATPase activity was noticed. It is possible that this hepatotoxic agent inhibits the sodium pump by interfering with sodium dependent phosphokinase step in the reaction sequence of ATPase as explained for methanethiol induced hepatic injury by Foster *et al* (1994). It has been reported that aflatoxicosis causes accumulation of intracellular calcium, which is known to cause mitochondrial dysfunction and reduce ATP generation (Verma R J *et al*, 1994). Reduced aerobic oxidation and ATP generation could be responsible for the reductions in the ATPase activity. Roy (1968) reported mitochondrial swelling during aflatoxicosis. This has been reported in testis of rat exposed to aflatoxin. Increased calcium can result in opening of the mitochondrial PT (proton translocation) pore resulting in the collapse of the proton gradient across the inner membrane and consequent uncoupling of oxidative phosphorylation (Green D R and Reed J C, 1998). This also results in increase in generation of reactive oxygen species.

8.3 Studies On Muscle Retention

8.3A Introduction

Aflatoxin can contaminate animal feeds before and during harvest, or because of improper storage. The presence of aflatoxin residues or toxic metabolites in animal tissues can end up in food for human use. Various studies have proved that aquatic animals are sensitive to carcinogenic stimuli and can serve as indicators of carcinogenic hazards to man. It has also been shown that livestock products like meat, milk and eggs do contain aflatoxin metabolites in the form of residues (Rajan, 1995). Therefore, it is certain that human beings are getting exposed to aflatoxin by consuming cultured fish that are contaminated by these toxins. The International Agency for Research in Cancer (IARC) reported in 1976 that liver cancer incidence and aflatoxin intake provide circumstantial evidence on the role of aflatoxin in cancer production. By 1987, IARC concluded that aflatoxin is a probable human carcinogen (Rajan, 1995). Researches have been conducted to obtain tissue distribution pattern of aflatoxin B_1 in domesticated livestock. Tissue distribution pattern of aflatoxin B_1 was first examined in sheep (Allocraft *et al*, 1966; Nabney *et al*, 1967; Stoloff *et al*, 1971). It was also observed in the liver, kidney, blood and muscle tissues (1 to 4 ppb AFB_1) of growing pigs fed with 400ppb AFB_1 diet for 4 weeks (Jacobson, 1978). Reports from South east Asia prove the occurrence of aflatoxin contamination in some fish products too. In a survey of Thailand foods, aflatoxin was detected in 5 percent of dried fish samples at an average concentration of 166 µg /kg (FAO, 1979). In Indonesia, samples from salty fish contained AFB_1 at an average level of AFB1 as 5µg/kg (Shank *et al,* 1972). But in *Oreochromis*

mossambicus, the effects of aflatoxin and its relevance to human health are not available.

8.3B Materials and Methods (for muscle retention studies)

The experimental set up was the same as that mentioned in section 6.1 of chapter 6. The muscle tissues of the six-week aflatoxin exposed groups were used for the study. Muscle tissue was chosen as the study material for aflatoxin retention studies as this forms the major portion of the tissues that end up as food for human use. The muscle tissue was cut up in to small pieces and kept in acetone under refrigeration until extraction. The extraction protocol was the same as that mentioned in section 3.3.4 of chapter 3.

a) Estimation of aflatoxins by HPLC

HPLC can rapidly screen aflatoxins with ultraviolet/visible detection. Reverse phase chromatography in conjunction with UV/Vis detection at 365nm can monitor aflatoxins.

b) Reverse phase chromatography with UV/Vis detection of aflatoxins

Column: Waters Nova-Pak C_{18}, 3.9mm 15cm

Mobile phase:$CH_3OH/CH_3COOH/H_2O$, 20/20/60

Flow rate: 1.0 ml/min

Detection: Waters 440 Absorbance at 365nm

c) Pre- column derivatiztion

Pre-column derivatization offers two major benefits: the ability to tailor the chemistry of the native analyte so that it can be separated with different chromatographic conditions, and the flexibility to be able to choose between different detection schemes to either increase sensitivity or specificity. Pre-column derivatization of aflatoxins with a strong mineral acid is a particularly good technique. Aflatoxins $B_1$and G_1 are derivatized to their hemiacetals, B_{2a} and G_{2a}, in the presence of water and strong mineral acid., These derivatives fluoresce more strongly and chromatograph differently than their parent compounds.

d) Pre-column derivatization of aflatoxin standards

Trifluoroacetic acid (TFA) is the most commonly used mineral acid for this pre-column reaction. The excellent sensitivity can be attributed to not only the derivatization procedure but also a special aflatoxin lamp and filter kit for Waters 420 AC fluorescence detector.

e) Pre-column derivatization of 1.0ppb aflatoxin standards

Column : Waters Nova-Pak C_{18}, 8.0mm 10cm

Radial Pak cartridge

Mobile phase : CH_3OH/CH_3CN/5.0 % CH3COOH, 14/14/72

Flow rate : 2.5 ml/min

Detection : Waters 420 AC fluorescence, Excitation 365nm, Emission 425nm with aflatoxin lamp

e) Post column derivatization

References to post-column derivatization for increased analytical sensitivity to aflatoxins have more recently appeared in the literature. Water saturated with iodine is the post-column reagent most often cited. This post-column technique derivatizes the sample on-line, which minimizes sample preparation and is potentially more reproducible.

Although the post-column derivatization technique for aflatoxin analysis requires additional component hardware, its sensitivity is excellent, it improves reproducibility, and it minimizes sample preparation.

f) **Statistical analysis**

Statistical analysis of results was done by ANOVA followed by Least Significant Difference (LSD) (Zar, 1996).

8.3C Results

The results are presented in table 8.3a and table 8.3b.

Concentration of aflatoxin (ppm)	Retention values in muscle (ppm) after a 6 week exposure to aflatoxin
0.375	0.336
2.5	0.380
6	0.396

Table 8.3a Retention of aflatoxin in muscle tissue

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.012237	2	0.006118	182.4569	2.97E-11	3.682317
Within Groups	0.000503	15	3.35E-05			
Total	0.01274	17				

Table 8.3b ANOVA for muscle retention of aflatoxin

One way ANOVA revealed that the different concentrations of aflatoxin differed significantly ($p<0.01$) in the retention of aflatoxin in the muscle. LSD at 5% level is 0.0069. LSD analysis revealed that all the concentrations of aflatoxin differed between each other in the retention of aflatoxin in the muscle.

Treatment	P value
0.375ppm vs 2.5ppm	P<0.01
0.375ppm vs 6ppm	P<0.01
2.5ppm vs 6ppm	P<0.01

Table 8.3c LSD analysis for muscle retention

Discussion

The foregoing results imply that aflatoxin residue was detected in the fish muscle even with the lowest level of the toxin administered. As supporting evidence it has been reported that when Nile tilapia fingerlings were fed on diets containing 50ppb, 100ppb and 200ppb of aflatoxin B_1, even with the lowest level fed, the residue was detected in the fish body in higher amounts than that reported in other species (El-Banna *et al*, 1992). It can therefore be concluded that AFB_1 residue showed a cumulative effect relative to the levels of aflatoxin administered. This is not a positive sign and thereby substantiates the urgency in imposing strict regulations governing the testing of food products for aflatoxin.

9.0 Summary

The present study was undertaken to evaluate the changes induced by aflatoxin in the teleost, *Oreochromis mossambicus* through different approaches like biochemistry, histopathology and molecular biology. Diets supplemented with different levels of aflatoxin B_1 (0.375ppm, 2.5ppm and 6ppm/kg body weight) were fed to the experimental groups for time periods of two weeks and six weeks. The salient findings are listed below.

- Rice was used as the substrate for aflatoxin production and the amount of aflatoxin extracted from 1450g of rice grain was 2.7mg.
- Fish fed on the diet containing the highest dose of aflatoxin namely 6ppm refused to rake the experimental diet for the first few days but later on began to gradually accept the entire feed.
- The biochemical parameters namely alanine transaminase, aspartate transaminase, alkaline phosphatase and free amino acids were found to increase in the aflatoxin treated groups.
- The concentration of pyruvate in the liver and muscle were found to decrease after a six-week aflatoxin stress.
- The lipid peroxidation products namely conjugated dienes, hydroperoxide and malondialdehyde recorded an increase in level up to two weeks after which they registered a decrease in concentration up to six weeks.
- The antioxidant enzymes namely catalase, superoxide dismutase and glutathione reductase showed an increase in their levels with the duration of exposure, the rate being significantly high after six weeks. The antioxidant, glutathione recorded the same trend as above.
- The levels of cholesterol, triglycerides, LDL cholesterol and VLDL were found to increase in the aflatoxin exposed groups where as the levels of

HDL cholesterol which recorded an initial increase was found to decrease in the highest dose namely 6ppm.

- The haemoglobin and erythrocyte count values indicated a decrease in proportion to the aflatoxin exposure. The decrease in the packed cell volume was not significant.
- Histopathological damages to the liver and kidney tissues intensified with increase in concentration and duration. The histopathological changes observed in the liver were extensive to focal necrosis, biliary proliferation, loss of architecture and preneoplastic stage of the liver tissue after a six week exposure. Important changes observed in the
- kidney were severe necrosis of tubular epithelial cells, thickening of the bowmans capsule and shrinkage of the glomeruli.
- Levels of enzymes in serum namely aspartate transaminase, alanine transaminase, lactate dehydrogenase, acid phosphatase and alkaline phosphatase; and other parameters like blood urea, creatinine and glucose also corroborated the damaging effects of aflatoxin to the animal.
- Significant reduction in the concentration of serum protein, albumin and globulin were noticed with increase in concentration as well as duration of exposure to aflatoxin.
- We strongly suspect the induction of prominent tumour suppressor genes namely p53 from the expression of the gene product viz 57Kda nucleoprotein analogous to the one reported from rainbow trout.
- Exposure to aflatoxinB_1 for six weeks did not induce apoptosis.
- Exposure to aflatoxin caused labilisation of the lysosomal and erythrocyte membranes together with an inhibition of Na^+K^+ATPase and Mg^{2+}ATPase.

- Aflatoxin residues could be detected in the muscle tissue even with the lowest level of aflatoxin administered.

9.1 Conclusion

While considering the results of this study from different approaches like histopathology, biochemical methods and molecular biology, it is evident that the animal is trying its level best to counteract the effect of the toxicant. It uses its various detoxifying enzymatic mechanisms to defend against the xenobiotic entered. This is evident from the elevated level of the antioxidant enzymes at the end of 14 days of exposure. Similarly the antioncogenes or tumour suppressor genes are also showing their expression as revealed by the presence of the protein with molecular weight 57KDa. The 57 KDa proteins are reported to be the analog of tumour suppressor gene p53 in higher animals. The presence of this protein is detected in serum and nuclear proteins after an exposure of 42 days. The same proteins were not expressed in serum after 14 days of exposure. This indicates that the animal is first attempting to nullify the effect of the toxicant by the enzymatic machinery as evident from the hike of the antioxidant enzymes after 14 days of exposure. The presence of the preneoplastic stage of the tissues is detected after a 42-day exposure to the toxin by histopathological means. It is at this juncture that the tumour suppressor gene p53 is initiating its action. This must be the reason for observing the presence of antioncogene in serum and nucleus after 42 days of continuous exposure. Hence we can assume that at 42 days the animal is using its molecular guardians like p53 to suppress the preneoplastic stage of the tissues. The action of p53 is to suppress the developing tumours by breaking down or cleaving the nuclear material by a process known as programmed cell death or apoptosis. Hence by apoptosis the tumour cell DNA will be cleaved into

different fragments thereby causing cell death. The role of p53 is to initiate a pathway resulting in apoptosis. Another positive indication of apoptosis observed in the study is the hike in the level of enzymes like ALT and alkaline phosphatase. Many studies have reported an increase in the level of these enzymes while the pathways of apoptosis are in action. It is with this idea of apoptosis process that the DNA is isolated to see the ladder like DNA fragmentation as a result of apoptosis. But the pattern of DNA isolated after separation by agarose gel electrophoresis indicate no sign of apoptosis. Even though there is a positive indication of apoptosis by the presence of other factors like the p53 gene expression and hike in the levels of ALT and alkaline phosphatase, the characteristic pattern of apoptosis is not observed. Another characteristic feature of apoptosis as detected by histopathology is the presence of shrinkage in the nucleus. The analysis of the histopathological sections in the study did not reveal any signs of cells with apoptosis. Hence both the molecular biology and the hisopathological results are not showing symptoms of apoptosis whereas the biochemical techniques indicate the initiating process of apoptosis. Hence it may be assumed that the pathways mediated by p53 leading to apoptosis are in action and further continuous exposure to the toxicant will definitely bring about apoptosis. Another possibility is that always the mechanism of tumour suppressor gene action may not always be successful in totally eradicating the effect of the toxicant. This is because of the fact that the p53 gene itself can be mutated. There are several reports of p53 mutation, which will ultimately act as an oncogene deviating from its normal role as antioncogene. Hence the results of this study indicate the potential threat of this toxicant to the fish especially when subjected to prolonged exposure beyond six weeks.

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