CHAPTER I

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Present modern world is leading towards the 21st century which is hoped to bring a revolution in the human science and will make our human world free from all diseases with the help of vast development in the field of science and technology. But as much we go near towards fulfillment of our dream-world of healthy happy human beings that much we are rebounded back in the darkness of mystery with new diseases everytime. At present along with cancer the human race is facing a pandemic, of the most dreaded human disease AIDS (Acquired Immuno Deficiency Syndrome), which has put the human race under the threat of being wiped off. Worldwide, over 40 million people are infected and the count is increasing drastically with the every minute of the time, which proves to be the future of human world to be very threatful. In the past year there were 5 million new infectious and 3 million deaths from AIDS – nearly ten thousand deaths each day.

AIDS is not a disease in the usual meaning of the word. It is a polymorphous syndrome, characterized by repeated and rebellious infections of the opportunistic type which become serious in a patient only as a result of breakdown of his immune system. The spread of HIV (Human Immuno-deficiency Virus) all over the world cutting across the barriers of geographical, social, economic and ethnic distinctions poses global grave challenge for human beings.

The promoting agents to induce the AIDS are found to be very minute but highly hazardous. These agents are lavishly living in this world due to mismanagement in
human deeds. To induce an AIDS disease the interaction of three types of agents is necessary. Each of these three agents alone is hardly transmitted from person to person and harmless for immuno-competent persons.

A. By poisons of fungi (mycotoxins) of the types Aspergillus (Aflatoxin B) and Fusarium (fumonisins) and by virus of the Herpes group transient immunosuppressions are produced.

B. Because of that, HIV is able to establish itself and produce a permanent immunosuppression.

C. Because of that again, the agents of the typical AIDS disease are able to establish and cause illness.

An infection hardly ever occurs on contact with HIV. And even if a virus should nestle down in the body, this is by no means the starting point of an illness. Other factors would have to be added which reduce the body’s defense against retrovirus. Even research scientists talking of a natural outbreak of AIDS, state that HIV-infection generally is preceded by immuno suppression. First, the complementary system, among others, would have to be immobilized in order to allow the virus to become embedded and the disease to develop.

Aflatoxins damage the liver and have also been known to cause liver cancer along with prominent disorders in other vital organs such as kidney and also affects the reproductive organs and their functions on large scale. The toxin also suppresses the immune system, which means just like the AIDS virus or HIV, it makes a person susceptible to other health disorders or opportunistic infections.
There is, so far, no effective cure or vaccine available to combat these deadly diseases. Hence, we are left with only solution and that is global effort to prevent and check the future spread of this virus and to control the growth of deadly agents supporting the growth of this virus.

The main aim of the present investigation was to find out a possible and suitable ameliorative agent to reduce aflatoxin-induced toxicities, which should be easily available to all groups and race of human beings and which should also be eco-friendly so that like other synthetic drugs, it may not lead towards the danger of side-effects. Also we can turn again towards the lap of nature to get some boon and be saved from these diseases and its promoting agents ‘aflatoxin’.
AFLATOXIN

Aflatoxin research has a dramatic beginning. In 1960, one hundred thousand turkeys, in various poultry farms of England died in opisthotonous following earlier signs of anorexia, lethargy and muscular weakness. At post-mortem, subcutaneous haemorrhage and pale coloured liver with extensive necrosis and biliary proliferation were recorded (Sargeant et al., 1961). This mysterious disease was named as 'Turkey X disease'. Concurrent with loss of turkey poults, large scale losses of ducklings, patridges and pheasant poults were also reported (Asplin and Carnaghan, 1961; Blount, 1961). Investigations revealed that the common ingredient of feed in various poultry farms were incorporation of imported Brazilian peanut meal. In the ensuing months, clinical and pathological reports of the toxicity to poultry, cattles and pigs were rapidly accumulated and collated Coincidently in 1960 attention was drawn towards increasing incidence of hepatoma in hatchery reared trout in United States (Allcroft and Carnaghan, 1962). Occurrence of these incidences attracted many groups of scientists to work together for isolation and characterization of the compound present in the feed which was imported from Brazil.

Asplin and Carnaghan (1961) demonstrated that hot methanolic extract of toxic peanut meal, when partially purified by extraction with chloroform, produced proliferation of bile duct epithelium in 1 day old ducklings comparable to that seen when peanut meal itself was fed. By paper chromatographic method, Sargeant et al. (1961) isolated crystalline toxic material which had an Rf of 0.7 and gave blue fluorescence in UV light. On thin-layer chromatography (TLC), Nesbitt et al. (1962) resolved two fluorescent spots, one blue with an Rf of 0.6 and the other with lower Rf and with green
fluorescence. The fluorescent spots were designated as aflatoxin B and G. Later on, Hartley et al. (1963) isolated 4 fluorescent compounds on TLC using chloroform : methanol (98 : 2) and designated them as aflatoxin B₁, B₂, G₁ and G₂ in order of decreasing Rf values. The infrared and UV absorption spectra were similar indicating related structures; B₂ and G₂ were established as dihydroderivatives of B₁ and G₁. The structure of aflatoxins were elucidated by Asao et al. (1965). Allcroft and Carnaghan (1962, 1963) found that cows when fed with toxic groundnut meal, excreted a toxic factor in the milk which had the same biological effect in 1 day old ducklings as had aflatoxin B₁. De Iongh et al. (1964) showed by TLC that, this toxic factor, designated as milk toxin was a blue fluorescent compound with an Rf lower than that of aflatoxin B₁. Holzapfel et al. (1966) isolated two components from the urine of sheep fed with mixed aflatoxins, which were similar to the milk toxins and designated them as aflatoxin M₁ and M₂. Aflatoxin M₁ was shown to be hydroxylated AFB₁ (Fig. 1.1).

Aflatoxins (B₁, B₂, G₁, G₂) are highly substituted coumarin derivatives containing a fused dihydrofurofuran moiety. The B-toxins were characterized by the fusion of cyclopentenone ring to the lactone ring of the coumarin structure, whereas G-toxins contained an additional fused lactone ring. Aflatoxin B₁ (AFB₁) and to a lesser extent AFG₁ were responsible for the biological potency of aflatoxin-contaminated meals. These two toxins possessed an unsaturated bond at 2, 3 positions (8,9 position according to IUPAC nomenclature) on the terminal furan ring. Aflatoxin B₂ and AFG₂ are essentially biologically inactive unless these agents are first metabolically oxidized to AFB₁ and AFG₁ in vivo.
Fig. 1.1: Structure of Aflatoxins

AFLATOXIN B₁

AFLATOXIN B₂

AFLATOXIN G₁

AFLATOXIN G₂

AFLATOXIN M₁

AFLATOXIN M₂
Aflatoxins are soluble in methanol, chloroform and other organic solvents but are only sparingly soluble in water (10-30 µg/ml). These toxins strongly absorb UV light (362 nm) with extinction coefficients in methanol or ethanol varying from 17,000 for AFG2 up to 24,000 for AFB2. Though aflatoxins are quite stable in food and feed, they are rapidly inactivated by extremes of pH (less than 3 or more than 10), oxidizing agents or exposure to UV light in presence of oxygen (Pohland, 1993).

Aflatoxins are a group of closely related secondary toxic fungal metabolites produced by *Aspergillus flavus* and *A. parasiticus*, of relatively low molecular weight organic compounds characterized by their diversity, their frequent specificity with regard to the taxonomy of the producing organisms and their production during the stationary phase of the batch cultures. During early period of recognition it was considered as a part of storage flora of inadequate post-harvest storage and poor storage conditions during distribution of commodities such as maize, groundnuts, peanuts, barley etc. However, it has now become increasingly recognized that the contamination of some commodities especially peanuts and maize with aflatoxin is a far more complex phenomena which may involve infection and aflatoxin production in the field. Although contamination of crop in field is undoubtedly influenced by insect damage, *A. flavus* is also known to infect intact kernels of maize by colonizing and growth down the external silk (Bilgrami *et al.*, 1980; Sinha and Ranjan, 1989).

Aflatoxins can enter human and animal dietary systems by indirect or direct contamination. Indirect contamination of food/feed can occur when an ingredient of a process has previously been contaminated with toxin producing molds. Although the molds can be killed or removed during processing, aflatoxins often remain in the final
product. Contamination of cereals and oil seeds represents main point of entry of many 
mycotoxins into food chains. In direct contamination the product becomes infected with 
toxigenic molds with subsequent toxin production. Almost all foods and feeds are 
susceptible to moldy growth at some stage during their production, processing, transport 
and storage (Fig. 1.2).

For farm animals or human beings whose diet largely contain plant products, 
aflatoxin may be produced directly by growth of molds on animal feed or human food. 
Illness caused by consuming such contaminated food/feed-stuffs are conveniently 
referred as ‘primary aflatoxicosis’. It is also possible that toxicosis may occur as a result 
of ingestion of animal products such as milk, cheese (Govaris et al., 2001; Oruc and 
Sonal, 2001; Aycicek et al., 2002), meat (Hoogenboom et al., 2001) or eggs (Oliveira et 
al., 2002; 2003; Bintvihok et al., 2002; Kim, M.J. et al., 2003; Sur and Celik, 2003) 
which have not themselves been contaminated by moldy growth. Illness arising from 
such sources are referred as ‘secondary aflatoxicosis’ (Fig. 1.2).

Although natural occurrence of aflatoxin in agricultural products have been 
reported from many countries and on vast array of crops, the levels of aflatoxins detected 
vary greatly from area to area. Higher quantities of aflatoxins were recorded in 
commodities from tropical and subtropical countries where environmental conditions are 
more congenial for moldy growth and toxin production. In a survey of peanut products in 
North America, 19% of 1416 samples examined were contaminated with an average of 1 
µg/kg, whereas, in Thailand 49% of 216 samples contained AFB₁ at an average level of 
424 µg/kg (Shank et al., 1972a). As much as 260 µg aflatoxin/kg was found in the 
sample of oat in Sweden which was contaminated with A. flavus. Very severe
Fig. 1.2: Factors influencing the occurrence of mycotoxins in human food or animal feed.
contamination may sometimes occur. In parts of India 100% of maize samples have been found contaminated with aflatoxin in the range of 6,250-15,600 µg/kg (Krishnamachari et al., 1975). Caldas et al. (2002) reported 1,706 mg/kg of total aflatoxin in peanuts in Brazil.

PHARMACOKINETICS

Route of administration

In general, human beings and mammals are exposed to aflatoxin through food/feed-stuffs, milk, meat and eggs. Consumption of aflatoxin in many parts of the world varies from 0 to 30,000 ng/kg/day. In most areas consumption varies from 10 to 200 ng/kg/day (Denning, 1987). Aflatoxins have been found in human cord blood and apparently can enter the developing fetus in humans and animals (Appelgren and Arora, 1983; Denning et al., 1990; Hsieh and Hsieh, 1993; Abdulrazzaq et al., 2002). It may be secreted in breast milk up to 3 to 84 ng/l (Wild et al., 1988; Hendrickse, 1991). If a 5 kg baby was to drink half a litre of contaminated breast milk in 24 h, the child’s consumption could be as high as 8 ng/kg/24 h (Autrup et al., 1991; Patel et al., 1992). In addition, it may also be present in infant formula (Aksit et al., 1997) and dairy products (Srivastava et al., 2001; Thirumala-Devi et al., 2002) (Fig. 1.3).

Also, farmers and farm workers may be exposed to potentially hazardous concentration of AFB1 particularly during bin cleaning and animal feeding in enclosed chambers (Selim et al., 1998; Nuntharatanapong et al., 2001). Hendrickse et al. (1989) also reported aflatoxin B1 contamination in heroin samples. Intravenous heroin users thus risk direct systemic exposure to aflatoxin B1. An important difference between this kind
Fig. 1.3: Routes of absorption, distribution and excretion of aflatoxins in the body.
of exposure and that from contaminated food is that after absorption from the gut some or all of the aflatoxin in food is detoxified by the liver which does not occur in intravenous exposure (Fig. 1.3).

**Distribution**

Marvan and his colleagues (1983) have experimentally studied distribution of AFB1 in goslings and chickens and according to AFB1 concentration various organs and tissues were categorized as follows: gonads; parenchymatous organs—liver and kidney; lymphopoietic organs—spleen, bursa cloacalis, thymus; followed by the endocrine glands and muscles; lungs have low concentrations and in brain, the lowest. In Chinese hamsters, Petr and his colleagues (1995) have shown that after a single intraperitoneal dose of 0.1 mg AFB1/kg body weight free aflatoxin B1 has been detected in blood, liver, kidney and testis from min upto 8-10 h after injection. The measured serum level of AFB1 in one study rose from 33.6 to 218 pg/ml after a meal (Hendrickse, 1991) (Fig. 1.3).

**Metabolism**

Once inside, aflatoxin undergoes enzymatic conversion by the microsomal mixed function oxidase (MFO) primarily present in the liver, but probably also present in the lungs, kidneys and elsewhere. MFO can detoxify xenobiotics by hydroxylation to enhance their excretion. But some compounds get converted by MFO to more reactive, more electrophilic species and are therefore capable of binding to macromolecules. Thus this later process can cause some compounds to be altered to the point, where they can...
interfere with normal cell functions often resulting in toxic or carcinogenic response. There are two types of biotransformations - phase-1 and phase-2. Phase-1 reactions are generally oxidative, reductive or hydrolytic processes and provide necessary chemical structure for phase-2 reactions, which are generally conjugation reactions. Phase-1 reactions may result in activation as well as detoxification of a compound, whereas phase-2 reactions, depending upon the conjugated cellular constituents, may lead either to detoxification or to the formation of biochemical lesions (Fig. 1.4).

Aflatoxin B₁ is oxidized by cytochrome P450 subfamilies and specific isoforms of enzymes to several products. Only one of these, the 2,3-oxide, appears to be mutagenic and others are detoxification products. A major detoxification pathway that the body utilizes is the conjugation of 2,3-oxide with glutathione. This process is catalysed by glutathione-S-transferase. The putative 2,3-oxide or AFB₁ epoxide (Swenson et al., 1974) is generally accepted as the active electrophilic form of AFB₁ which may attack nucleophilic nitrogen, oxygen and sulphur heteroatoms in cellular constituents (Fig. 1.4). Cytochrome P450 3A4 which can both activate and detoxicate AFB₁ is found in the liver and small intestine. In the small intestine, the first contact after oral exposure, epoxidation would not lead to liver cancer. The (non-enzymatic) half-life of the epoxide has been determined to be approximately 1 second at 23°C and neutral pH (Guengerich et al., 1996). P450 3A4 has been shown to play a major role in the activation of AFB₁ due to its intrinsic activity towards this substrate and the high level of this enzyme present in human liver (Raney et al., 1992; Ueng et al., 1995). P450 1A2 and some other human P450s also contribute, but they play a lesser role, even at relatively low AFB₁ concentration (Crespi et al., 1990; Yun et al., 1991). P450 3A4 forms mostly the
Fig. 1.4: Metabolic transformations of aflatoxin B1. Many of the pathways lead to detoxification while others lead to activation, particularly the 2,3-oxide which forms an N7 guanine adduct with hepatic DNA.
genotoxic AFB1-2,3-exo-oxide, whereas P450 1A2 forms both the exo- and nongenotoxic endo isomers (Ueng et al., 1995) (Fig. 1.5).

P450s can also detoxicate AFB1. P450 3A4 forms AFQ1, the 3 α-hydroxylation products which does not appear to be a good substrate for epoxidation (Raney et al., 1992). It is also reported that the phenobarbitol-induced cytochrome P450 is more susceptible to AFB1 than benzo (a) pyrene-induced cytochrome P450 in chicken (hepatic microsomal mixed function oxidase) (Govindwar and Adav, 1999).

AFB1 is converted in the adult liver by the cytochrome P450 enzyme, P450 3A4 and in the fetal liver with P450 3A6, to AFQ1, the major metabolite of AFB1. Other major metabolites in the human include AFM1, aflatoxicol (AFL), AFL H1, AFL M1; AFP1, AFB2α and AFB1-2, 3-dihydrodiol. The production of AFB1-dihydrodiol is an attempt made by the body at detoxification. This pathway is used to a lesser extent. The epoxide is converted to a dihydrodiol due to the actions of epoxide hydrolase. Aflatoxin B1-dihydrodiol is further metabolized to form AFM1, AFQ1 and AFP1, which form glucuronides and sulphate conjugates that are excreted in the urine and faeces. About 80% of a total dose of AFB1 is excreted in 1 week. AFM1 is mostly excreted within 48 h of ingestion. It is possible that its measurements give a reasonable estimate of recent aflatoxin ingestion (Hendrickse, 1991). AFM1 probably comprises 1 to 4% of consumed AFB1. Since it is conjugated and is easily detectable in urine, it may be useful in assessing aflatoxin conjugation. AFM1 is relatively stable metabolite, though it is definitely mutagenic and carcinogenic (Green et al., 1982). It is widely present in the excreta of animals exposed to AFB1 (Stoloff, 1980). Aflatoxin P1 is produced by demethylation of AFB1 by hepatic microsomes (Roebuck and Wogan, 1977). AFP1 is
Considerations:
AFB₁ intake
Relative levels of enzymes
  Regulation, genetics and environment (variation over time?)
  Influence of inhibitors and stimulators
Locations
DNA repair
Correlation of adducts with cancer
HBV status, inflammation, and other nongenotoxic influences

Fig 1.5 Complications involved in the metabolism of AFB₁ and relevance to hepatocellular cancer.
almost non-toxic and its occurrence in the excreta of animals orally given AFB\textsubscript{1} is also at very low levels (Dalezios et al., 1973). Aflatoxicol is considered to be reservoir of AFB\textsubscript{1}, since it can be readily converted back to AFB\textsubscript{1}, or into other metabolites by postmitochondrial fraction (Patterson and Roberts, 1972) and enhance toxicity for target cells (Fig 1.4).

Phase-2 reactions that leads to the detoxification involves conjugation to glucuronic acid, sulphate and glutathione. Conjugation to nucleic acid and protein on the other hand may produce biochemical lesions (Fig. 1.4).

Glucuronide and sulphate conjugate of AFM\textsubscript{1} are major detoxification products of AFB\textsubscript{1}. Rat hepatic microsomes in the presence of glutathione produced a compound believed to be a glutathione conjugate. A compound was isolated in the bile of rats that was identified to be 2,3-dihydroxy-2-glutathionyl-3-hydroxyl AFB\textsubscript{1} (Degen and Neumann, 1978). Alterations in the enzyme responsible for glutathione conjugation may be involved in the reduced susceptibility to AFB\textsubscript{1} cytotoxicity that was observed in carcinogenesis.

**Adduct formation**

From the carcinogenic point of view, AFB\textsubscript{1}-2,3-oxide is a highly reactive substance which can combine with DNA bases such as guanine to produce alterations in DNA (Hendrickse, 1991). Presence of AFB\textsubscript{1}-DNA adduct was identified both in vivo and in vitro (Groopman et al., 1980). The binding of AFB\textsubscript{1} residues to DNA in vivo is essentially a linear function of dose at a given time after treatment. A modification level of 125-1,100 AFB\textsubscript{1} residues/10\textsuperscript{7} nucleotides was observed in rat liver 2 h after
Intraperitoneal dosing with 0.125 to 1.0 mg AFB₁/kg (Croy et al., 1978). Initial binding levels in DNA have been observed to drop rapidly within h after the AFB₁ treatment (Groopman et al., 1980; 1988). For example, maximum modification of rat liver DNA (1,250 residues/10⁷ nucleotides) was noted not later than 30 min after 1 mg AFB₁/kg dose, but declined to a level of 160 residues/10⁷ nucleotides 36 h after treatment, giving an apparent half-life of AFB₁ binding to DNA of approximately 12 h (Cullen and Newberne, 1994; Groopman et al., 1996).

Once formed, the dihydrodiol may rearrange to a putative dialdehyde phenolate intermediate, which can condense with the primary amino groups of proteins and other cellular constituents to form a Schiff base. The Schiff base formation with a critical molecular receptor constitutes another way whereby a biochemical lesion may be produced from exposure to AFB₁. Thus the dihydrodiol can be considered as another form of a reactive metabolite. In fact, it has been demonstrated that the dihydrodiol binds to DNA in vitro and is a direct acting mutagen (Cole et al., 1980). Glutathione may form a Schiff base with the dihydrodiol of AFB₁ to result in detoxification. Similarly the dihydrodiol may be detoxified by forming a Schiff base with non-target proteins followed by their excretion and tissue turnover. Sotomayor et al. (2003) suggested that binding of AFB₁ to hepatic DNA in rat liver is a linear function of the dose, regardless of the way this is administered. The dose-response relationship for RNA adducts depends on the length of the no-dosing cycles and the turnover rate of RNA. As biomarkers of exposure, AFB₁-RNA adducts were three to nine times more sensitive than AFB₁-DNA adducts but showed greater variability.
Sabbioni et al. (1987) have elucidated the structure of the major aflatoxin-albumin adduct found in vivo. The protein adduct by binding of the 2,3-epoxy aflatoxin initially form dihydrodiol with sequential oxidation to dialdehyde and condensation with the \( \Sigma \) amino group of lysine. This adduct is a Schiff base that undergoes Amadori arrangement to an \( \alpha \)-aminoketone. This protein adduct is a completely modified aflatoxin structure retaining only the coumarin and cyclopentenone rings of the parent compound. These adducts represent the cumulative dose of aflatoxin intake over previous weeks. The average half-life of albumin in people is about 20 days. Therefore an accumulated dose of aflatoxin will be present in albumin long after the dietary exposure has ceased. This is a property not found for DNA adducts because the half-life of DNA adduct is about 12 h and then rapidly excreted in urine.

Excretion/Secretion

Aflatoxin concentration recorded in the serum of human beings varies with the amount and duration of aflatoxin-ingested and the physiological state of the body. Both unmetabolized \( (B_1, B_2, G_1, G_2) \) as well as metabolized forms \( (aflatoxicol, M_1 \text{ and } M_2) \) of aflatoxins get excreted in the urine (Jonsyn-Ellis, 2001; Nayak et al., 2001; Wang et al., 2001a), stool and milk (Martins and Martins, 2000; Dragacci et al., 2001; Hoogenboom et al., 2001; Panariti, 2001; Roussi et al., 2002; Battacone et al., 2003).

Verma and Chaudhari (1997) reported that out of a total 40 urine samples of human beings collected and analysed for presence of aflatoxin, only 24 samples were found positive. Maximum number of samples were positive for \( B_2 \) (47.5%) followed by \( G_2 \) (27.5%). Aflatoxicol was detected in 20% samples with as high as 67.63 ng/ml mean...
concentration. Aflatoxin B$_1$ was detected only in 7 samples (17.5%) with a mean concentration of 33.76 ng/ml; G$_1$ and M$_1$ were detected in 6 and 1 sample respectively. Wang et al. (2001a) also reported that urinary AFB$_1$ metabolites were detectable in 88.9% (24 of 27) samples (range, 0.9-3,569.7 ng/24 h urine). Nayak et al. (2001) reported that aflatoxin B$_1$-N$_7$ guanine (AFB$_1$-N$_7$ guanine) adduct is a useful dosimeter in molecular epidemiological studies. 63% of the total adduct was accounted in urine of female rats, whereas male rats excreted 47% of the total adduct in their urine.

Presence of much higher concentration of less toxic aflatoxicol in urine indicates higher rate of metabolic detoxification of AFB$_1$. Verma and Chaudhari (1997) have clearly indicated that although higher aflatoxin concentration in urine might be due to consumption of more aflatoxin – contaminated food-stuffs, the risk of aflatoxin poisoning is comparatively reduced because of the faster rate of metabolism, as the concentration of AFL is very high in urine.

Verma and Chaudhari (1998) also reported presence of seven different types of aflatoxins in saliva of human beings. Out of 44 samples analysed, 15 samples were positive for AFB$_1$ with mean concentration of 23 ng/ml. Highest concentration (36 ng/ml) was of G$_2$ recorded in 34% samples. M$_1$ and M$_2$ were detected in 5 and 12 samples. This explains a sort of recycling of aflatoxin in the body. Aflatoxin excreted/secreted through saliva might be getting absorbed in gastrointestinal tract and thus passing again to the bloodstream.

Thirumala-Devi et al. (2002) reported that out of 280 milk samples tested, 146 were found to contain <0.5 ng/ml of AFM$_1$; in 80 samples it varied from 0.6 to 15 ng/ml, in 42 samples from 16 to 30 ng/ml and in 12 samples from 31 to 48 ng/ml in the rural and
periurban areas in Andhra Pradesh. Garrido et al. (2003) analysed commercial milk samples of Brazil. Aflatoxin M$_1$ (AFM$_1$) was detected in 29 (20.9%) samples in the range of 50-240 ng/l.

AFLATOXICOSIS

Aflatoxicosis is a toxic disease caused by the consumption of aflatoxin-contaminated food/feed-stuffs such as maize, peanuts, barley, groundnut etc. Aflatoxin toxicities are strongly influenced by sex, age and strain of animals, administration routes (i.e. oral feeding or intubation, intravenous or interperitoneal injection), solvents of toxins, presence of other mycotoxins (Pozzi et al., 2001; Theumer et al., 2003) and composition of diet etc. These differences might be due to differences in cytochrome P450 activity, but in general, most animals including human beings are affected in the same manner (Smith and Moss, 1985).

Acute aflatoxin poisoning caused hepatocellular necrosis and derangement of hepatic functions (Cullen and Newberne, 1994; Ghebranious and Sell, 1998, Wasti et al., 1998). Subacute or chronic aflatoxicosis caused fatty changes in the liver, enlargement of the gall bladder and periportal fibrosis with proliferative changes in bile duct epithelium.

Aflatoxins cause wide range of pathological abnormalities in various organs of animals including human beings. Butler (1964) recorded damage in liver as well as bilateral adrenal haemorrhages and petechial haemorrhages in many organs particularly in congested lungs. Occassionally patchy necrosis have been observed in the myocardium, kidney and spleen during the first few days of aflatoxin B$_1$ feeding in male (7.2 mg/kg body weight) and female (17.9 mg/kg body weight) rats. Haemorrhages were
more extensive in the lungs, kidneys and adrenal glands after exposure to higher doses of aflatoxin.

Reduced feed conversion efficiency resulting in reduced weight gain and general lack of thrift in the animals have been associated with aflatoxin. Reduced milk yield in dairy cows and reduced egg production, immunosuppression and increased cracked eggs in poultry has been noted in mature animals exposed to aflatoxin. Immunosuppression is due to the reactivity of aflatoxins with T-cells, decrease in vitamin K activities, and a decrease in phagocytic activity in macrophages. Aflatoxin can cause an increased susceptibility to bruishings in chicken and a decreased market acceptability. Furthermore, this problem is only seen after the birds have been killed and prepared for market. Levels of aflatoxin required in feed to cause this effect in broiler chickens can be as low as 0.6 \( \mu g/gm \) feed which is less than half that is required to reduce growth (Smith and Moss, 1985). Toxic effects of aflatoxins in poultry is same as in mammals. A dose of 0.25 ppm in turkey poults and ducklings impairs growth, and a dose of 1.5 ppm in broilers and 4 ppm in Japanese quail also has a negative effect on growth. Aflatoxins impair the availability of bile salts. This causes a decrease in the absorption of fat soluble vitamins. Aflatoxins also decrease the production of vitamin A in the liver. It has secondary effects such as decreased blood calcium levels, decreased bone strength and a decreased tissue and serum tocopherol level. This decrease in tocopherol level can lead to vitamin A and E deficiencies (Smith and Moss, 1985).

Aflatoxicosis in swine is mainly due to the fact that corn is a large part of their diet. Piglets are more susceptible than adults and it has been shown that feeding sows AFM1 during lactation, can cause stunted growth in her litter.
The effects of aflatoxin in ruminants are similar to those of non-ruminants. Young animals are comparatively more susceptible to aflatoxin than the adults (Smith and Moss, 1985). Lynch et al. (1970) recorded slight hyperplasia in hepatic cells from the end of the first month of aflatoxin feeding in cattle; this phenomenon increased during the second and third months until centrilobular degeneration of the hepatic cells became apparent. However, during the fourth month central necrosis of the hepatic cells was marked by proliferation of the bile ducts and occlusion of the centrilobular veins. Calves develop a disease that features blindness, circling and falling down, twitching of ears and grinding of teeth. Spasm of the rectum is seen in most cases. Death usually follows within two days of onset of severe clinical signs. Postmortem findings revealed pale, firm and fibrosed liver. Histologically the main changes in liver were centrilobular necrosis, bile duct proliferation and veno-occlusive disease. The kidneys are yellow and surrounded by wet fat. Ascites and edema of the mesentery (enteritis) and rectal eversion are common findings (Hintz, 1990).

Other pathological features in cattle are blood coagulation defects, which may involve impairment of prothrombin, factors VII and X and possibly factor IX. A single dose of aflatoxin causes increase in plasma enzymes (aspartate aminotransferase, lactate dehydrogenase, glutamate dehydrogenase, gamma-glutamyltransferase and alkaline phosphatase) and in bilirubin, probably reflecting liver damage. Other abnormal clinical findings are proteinuria, ketonuria, glycosuria and haematuria (Aflatoxins, 2002). Similar changes in blood coagulation parameters have been reported in dogs (Aflatoxins, 2002).

Horses are more susceptible to the adverse effects of aflatoxins than are cattle. The disease in horses is characterized by decreased feed intake, loss of body weight, liver
damage, centrilobular hepatic disease, brain, kidney and heart damage. Behavioural changes before death include belligerence, somnolence, excessive yawning, head pressing, circling, aimless walking and even blindness (Hintz, 1990).

It is not easy to establish a direct correlation of aflatoxin ingestion with a defined aflatoxicosis in human beings but circumstantial evidence suggests the involvement of this toxin in human disease and deaths. Direct ingestion of aflatoxins occur through consumption of contaminated food such as cereals, pulses, fruits, nuts and spices, whereas, indirect consumption takes place through contaminated milk, milk products, meat and eggs.

The possible acute and chronic effects of aflatoxin in human beings can only be estimated by observing suspected cases of aflatoxicosis and by analyzing the diets. Although incidence of acute poisoning in human beings is rare, prolonged exposure to subacute levels is particularly serious in developing countries due to poor food storage conditions and congenial environmental conditions for moldy growth and toxin production.

Suspected cases of aflatoxicosis have been reported in India. Over 200 villages in western India (Panchmahal district of Gujarat and Banswada district of Rajasthan) experienced an outbreak of disease affecting human beings and dogs. The disease was characterized by jaundice, rapidly developing ascites, portal hypertension and high mortality rate. Death usually occurred from massive gastrointestinal bleeding. The disease was confined to the very poor who ate badly molded corn containing aflatoxin at a concentration of 6.25 to 15.6 ppm. The average daily intake was 1-6 mg of aflatoxin. More than 990 individuals (2:1 male : female age 5 to 14 and over 30 years) were
affected and about 100 died. This disease was caused by the consumption of maize heavily contaminated with aflatoxin (Krishnamachari et al., 1975; 1977). Reports from another group of scientists (All India Institute of Medical Sciences, New Delhi) supported these findings (Tandon et al., 1978). This was perhaps the first report directly implicating aflatoxin in food as a human health hazard (Keeler and Tu, 1983; Aflatoxins, 2002).

An outbreak of food poisoning resulting in 13 deaths in children occurred in Malaysia during the Chinese festival of the Nine Emperor Gods in 1988. The food poisoning was attributable to aflatoxins and boric acid. Clinical features included an initial Reye-like syndrome with vomiting, fever, diarrhoea, abdominal pain, anorexia, seizures and coma. Patients died in acute hepatic and renal failure. High levels of aflatoxins B₁, B₂, G₁, M₁, M₂ and aflatoxicol were found in various organs (Chao et al., 1991). An incidence of 106 fatal cases of hepatic disease among 397 individuals who became ill after eating maize contaminated with aflatoxin also suggests that acute aflatoxicosis can occur in human beings (Haddad, 1990).

Aflatoxins have been reported to be associated with a Reye-like syndrome in Thailand, New Zealand, Czechoslovakia, United States (Aflatoxins, 2002), Malaysia (Chao et al., 1991), Venezuela (Burggra, 1986) and Europe (Dvorackova et al., 1977). The Reye-like syndrome reported in various places around the world was characterized by multiple symptoms and clinical findings that included disturbed consciousness, fever, convulsions, vomiting, disturbed respiratory rhythm, altered muscle tone and altered reflexes. Serum glutamate-pyruvate transaminase and glutamate oxaloacetate transaminase (mitochondrial) activities were elevated. Hypoglycaemia and low
cerebrospinal fluid glucose were observed. The onset of the illness included coughing, rhinorrhea, sore throat, earache, slightly enlarged, firm yellow liver, and a pale, slightly widened renal cortex. A high rate of mortality (81% of the diagnosed cases) occurred. Since Reye’s Syndrome is characterized by abnormal mitochondrial structure and function (Stein, 1990), it is of interest to note that aflatoxin B₁ causes abnormal mitochondrial structure and function (Sajan et al., 1996).

Indian childhood cirrhosis (ICC) mainly confined to the Indian sub-continent has been attributed by Amla et al. (1974) to aflatoxin consumption. Sreenivasamurthy (1975) has also reported correlation between aflatoxin and hepatomegaly in children of South Kanara district of Karnataka, India. Yadgiri and his associates (1970) analysed the presence of aflatoxin-like compounds in urine and liver extracts of children suffering with Indian childhood cirrhosis. The consumption of large amounts of corn, rice, peanuts and milk (food potentially high in aflatoxins) were significantly related to mental retardation of children with poor performance having higher levels of aflatoxins in the food supply in southern Georgia (Caster et al., 1986). Gong et al. (2003) measured aflatoxin exposure by estimating aflatoxin-albumin adduct in children (9 months to 5 years) from Benin and Tongo, West Africa. They correlated higher aflatoxin-albumin adduct in blood with reduced growth.

Many investigators have reported that protein malnutrition enhanced characteristic aflatoxin-induced liver injury in weanling rats and rhesus monkeys (Tulpule et al., 1964; Madhavan and Gopalan, 1965; Madhavan et al., 1965 a,b). Hendrickse (1985) has reported that a high protein intake protects, while a deficient protein intake increases susceptibility to aflatoxins. Enwonwu (1984) while speculating
on the role of dietary aflatoxin in the genesis of hepatocellular cancer suggests that the accumulation of AFB₁ in liver of Kwashiorkor victims is the result of impaired activity of liver microsomal mixed function oxidase system which is characteristic of protein malnutrition.

**IMMUNOTOXICITY**

Aflatoxins are immunosuppressive in a variety of animals making them more susceptible to infection by various micro-organisms (Sharma, 1993; Dimitri and Gabal, 1996; Celik et al., 2000; Aflatoxins, 2002).

Aflatoxin B₁ altered the immune response of mice (Reddy et al., 1987) and showed a decreased activity of B- and T-cells in AFB₁ exposed animals. Inhibition of protein synthesis caused by aflatoxins alters serum protein composition, resulting in the suppression of the production of non-specific humoral substances important to native defense (Thaxton et al., 1974). At higher doses, AFB₁ lowers the level of IgG and IgA in chick (Tung et al., 1975) resulting in decreased acquired immunity.

Antibodies to aflatoxin B₁ have been reported in humans. At present, these antibodies are considered indicative of exposure and may or may not be related to disease (Wang et al., 2001b). Workers handling animal feed exposed to mycotoxins, including aflatoxins, were examined for alterations in plasma alpha tumor necrosing factor (TNF-α). Air-borne concentration of aflatoxin was 0.99 ng/m³. Exposed workers had increased levels of TNF-α and concomitant changes in serum lactate dehydrogenase isoenzyme activity (Nuntharatanpong et al., 2001).
Aflatoxin is an immunomodulating agent that acts primarily on cell-mediated immunity and phagocytic cell functions (Bondy and Pestka, 2000).

Moon and Pyo (2000) indicated that the reduced nitric oxide (NO) production in murine peritoneal macrophages by AFB₁ pretreatment is related to the suppressed expression of CD_{4} on macrophage membrane and to the increased secretion of it to culture medium after lipopolysaccharide-stimulation. Liu, B.H. et al. (2002) reported AFB₁ toxicity on primary cultures of swine alveolar macrophages.

Sahoo and Mukherjee (2001) reported that a single intraperitoneal injection of AFB₁ at 1.25 mg/kg body weight caused a significant reduction in non-specific immunity as measured through neutrophil phagocytic indices, serum bacterial activity and specific immunity as measured through bacterial agglutination titre against Edwardsiella tarda, as well as reduced protection against Aeromonas hydrophila challenge in comparison to control.

Theumer et al. (2003) reported that mitogenic response of spleen mononuclear cells (SMC) in vivo was higher in animals fed with AFB₁. In in vitro studies lower proliferations of SMC pre-exposed to AFB₁ and to the mixture of toxins were detected.

Hinton et al. (2003) reported that AFB₁ effects on the immune system can be either stimulatory or suppressive depending on a critical exposure window of dose and time. Immune cells in spleen such as T-lymphocytes and macrophages, both important mediators of inflammatory responses to tissues damage, were affected differently in the continuous and intermittent exposure.

23
CARCINOGENICITY

Aflatoxin B₁ primarily causes hepatocellular carcinoma and cholangiocarcinoma in the liver (Busby and Wogan, 1984). Amongst various types of aflatoxins known, B₁ is the most potent hepatocarcinogen but G₁ and B₂ have also been shown to cause cancers but with reduced potency (Butler, 1974). It causes liver tumors in mice, rats, fish, marmosets, tree shrews and monkeys following administration by various routes. Types of cancers described in research animals include hepatocellular carcinoma, cholangiocellular cancer and adenocarcinoma of the gall bladder (Butler, 1964; Butler et al, 1969, IARC, 1972)

Besides the liver, tumors have also been reported due to aflatoxin feeding in lacrimal glands (Goodal and Butler, 1969), squamous cells of the tongue (Ward et al., 1975), oesophagus (Butler et al., 1969), trachea (Dickens et al., 1966), kidney, lung adenomas, osteogenic sarcomas and carcinoma of the pancreas (IARC, 1972). Carcinoma of the colon has been reported by many investigators (Newberne and Butler, 1969; Ward et al., 1975). Aflatoxin exposure contributes to the risk for development of hepatocellular carcinoma (HCC) in ducklings (Sell et al., 1998).

AFB₁ can cause hepatocarcinogenesis and mutation in rat liver (Lee et al., 1998). In vitro studies have shown that AFB₁, major causative factor for hepatocellular carcinoma, cytochrome P450 1A2 and c-myc, can transform rat hepatocytes which could grow beyond two months limit in primary culture. Ghebranious and Sell (1998) proposed that some mutant protein may act as a promoting agent for AFB₁ hepatocarcinogenesis in mice. Hepatitis B virus (HBV), male gender, aflatoxin and p53 expression interact to
produce malignant liver tumors in transgenic mice (Ghebranious and Sell, 1998).

Epidemiological studies from Africa and Asia suggest direct correlation between the occurrence of high level of aflatoxin in staple food and the incidence of primary liver cancer (Alpert et al., 1971; Shank et al., 1972 a,b).

In China, a strong correlation between the intake of peanut, peanut oil and corn and increased mortality rates for liver cancer were reported in five groups of inhabitants from four villages. The median intake of aflatoxin B1 for each group was 6.05, 6.36, 2.69, 1.83 and 0 µg/day. The median daily urine concentrations of M1 metabolite were 16.46, 8, 29, 4.78 and 1.21 ng/person. A significant correlation was found between the mortality rates of primary liver cancer and intake of aflatoxin B1 (Aflatoxins, 2002).

Overall summary evaluation of carcinogenic risk to humans is Group 1 (Dominguez-Malagon and Gaytan-Graham, 2001). Many investigators have also reported carcinogenic effect of aflatoxin in human beings (Chen et al., 2001; Li et al., 2001; Okuda, 2002). Primary hepatocellular carcinoma is one of the most common malignancies and has the fourth highest mortality rate worldwide. The major risk factors, including chronic infections with the hepatitis B or C virus, are exposure to dietary aflatoxin B1 (AFB1) (Omer et al., 2001; Wild and Turner, 2001; Wang et al., 2001a).

One of the characteristic of hepatocellular carcinoma in China is the selective mutation resulting in a serine substitution at codon 249 of the p53 gene and it has been identified as a 'hotspot' mutation in hepatocellular carcinomas occurring in population exposed to aflatoxin and with high prevalence of hepatitis B virus carriers. 249 (Ser) p53 mutation, as it gives more information in early diagnosis of HCC (Stern et al., 2001; Ming et al., 2002; Huang et al., 2003) should be developed to a new early diagnostic
marker of HCC (Huang et al., 2003). It was reported that in hepatocellular carcinoma cases exposed to aflatoxin B$_1$ mutation of p53 gene is fixed at codon 249 third base and takes the form of G to T transversion (Liu et al., 2002; Smela et al., 2002). It appears from the reported observations that it is a definite marker of mutation, which is induced by aflatoxin B$_1$ mutagen and is applicable for molecular epidemiology survey of sufferers of aflatoxin B$_1$ exposure among hepatocellular carcinoma cases (Deng and Ma, 1998; Ndububa et al., 2001; Edamoto et al., 2003).

Aflatoxin-albumin adduct, a biomarker for aflatoxin exposure, was higher in the serum of individuals at risk for hepatocellular carcinoma (Turner et al., 2000; Ahsan et al., 2001, Sun, G. et al., 2001; Sun, C.A. et al., 2002). Martins et al. (1999) reported that microsatellite/ genomic instability may play a role in the pathogenesis of a subset of hepatocellular carcinomas in black Africans.

**MUTAGENICITY**

Aflatoxin B$_1$ is the most potent mutagen among aflatoxins and a strong parallel correlation exists between the ability of the aflatoxins to be mutagenic and carcinogenic. Microsomal activation is an absolute necessity for mutagenicity with aflatoxin. Aflatoxin B$_1$ cause chromosomal aberrations and DNA breakage in plant and animal cells (Ong, 1975) Bilgrami and Sinha (1988) recorded chromosomal abnormalities such as clumping, fragmentation, meiotic polyplody and euploidy in mice fed with aflatoxin-contaminated diets. These results underscore the mutagenic potential of the toxin and also recorded dominant lethal mutations in mice. Significant decrease in the fertility of females mated to treated males was observed. This was caused by gross genetic damage.
induced by aflatoxin in the germ cells of treated mates. Yakicier et al. (2001) revealed a relationship between chromosome 16q homozygous deletions and R249S p53 mutations in tumors where the patient had been exposed to aflatoxin B₁ (p = 0.002).

Loarca et al. (1998) reported that AFB₁ is a direct acting mutagen and that ellagic acid inhibits AFB₁ direct acting mutagenicity. It is also observed that comutagenicity of coumarin (1,2-benzopyrene) with AFB₁ and human liver S9 fraction increased due to enhanced AFB₁ bioactivation (Goeger et al., 1999).

TERATOGENICITY

Teratogenicity of aflatoxin B₁ has been established (Ong, 1975; Heathcote and Hibbert, 1978) Vesley et al. (1983) have reported teratogenic effects of AFB₁ in chick embryos including retarded growth, exencephaly, anophthalmia, microphthalmia, cleft palate and malformation of the maxilla. However, no teratogenic effect was observed in C₁H mice at the same level of exposure.

Prenatal and early postnatal exposures of aflatoxin were also found to have teratogenic effect (Doi et al., 2002). Kihara et al. (2000) indicated that prenatal exposure to AFB₁ produced a delay in early response development, impaired locomotor coordination and impaired learning ability in the offspring of rats. The early gestational exposure appears to produce more effects than latter exposure. Abdulrazzaq et al. (2002) measured aflatoxins levels in umbilical cord blood in women of Taiwan and reported significant negative correlation between birth weight and levels of aflatoxin in foetuses.
CYTOTOXICITY

Goeger et al. (1999) have reported cytotoxic effects of aflatoxin on several different cell types. Aflatoxin exposure to hepatocytes in vitro caused pronounced swelling, polymorphic condition, bleb formation and lysis (Raval and Verma, 1997). When RBC suspension was treated with aflatoxin in vitro a concentration-dependent swelling followed by lysis was observed indicating permeability alterations and membrane destabilization (Verma and Raval, 1991; Raval and Verma, 1997). Exact mechanism of aflatoxin-induced cytotoxicity is not clearly understood. It could be due to its effect on mitochondria, increased lipid peroxidation, increased adduct formation with DNA, RNA and protein or all the three.

The damage can be to mitochondrial DNA (adducts and mutation), mitochondrial membranes, as well as to disruption of energy production (production of ATP) (Wallace, 1997, Thrasher, 2000). The mycotoxin alters energy linked functions of ADP phosphorylation and FAD and NAD-linked oxidizing substrates (Sajan et al., 1996) and α-ketoglutarate-succinate cytochrome reductases (Obasi, 2001). It causes ultrastructural changes in mitochondria (Rainbow et al., 1994) and also induces mitochondria directed apoptosis (Pasupathy et al., 1999; Meki et al., 2001).

Free radicals are highly reactive species that have an unpaired electron e.g. the hydroxyl and superoxide radicals which have potential to cause tissue damage. Also some of the reactions which involve oxygen derived free radicals give rise to compounds which are not themselves free radicals, e.g. H$_2$O$_2$. They are nevertheless reactive and for this reason oxygen derived free radicals and related non-radical compounds are referred to as reactive oxygen species (ROS).
Oxygen radicals are produced as a consequence of the normal process of reduction of oxygen to water and represent by-products of oxidative cellular metabolism. Fig. 1.6 shows the series of reactions that take place in the conversion of oxygen to water and demonstrate the site of formation of the superoxide (O$_2^-$) and the hydroxyl (OH') free radical series (Dickerson and Williams, 1990).

Oxygen free radicals can react with DNA to cause breaks in the DNA chain and mutation which could initiate carcinogenesis. Free radicals can react with serum low density lipoprotein (LDL) which is damaging the arterial walls than LDL. They can also react with membrane lipids leading to peroxidation of polyunsaturated fatty acid (PUFA) residues.

\[
\begin{align*}
O_2 & \rightarrow O_2^- & \text{Superoxide anion} \\
O_2^- & \rightarrow O_2^{2-} & \text{Peroxy anion} \\
O_2^{2-} \rightarrow [O_2H]^- & \text{Hydrated Peroxy anion} \\
[O_2H]^- \rightarrow H_2O_2 & \text{Hydrogen peroxide} \\
H_2O_2 & \rightarrow OH^- + OH^- \\
20H^- & \rightarrow 2H_2O \\
OH^- & \rightarrow OH^-
\end{align*}
\]

*Fig. 1.6. Site of formation of free radical in the reduction of oxygen to water (Source: Dickerson and Williams, 1990).*
These free radicals are highly reactive species and may attack the double bonds of PUFA chains of membrane phospholipids. The lipoperoxyl free radicals thus formed can attack adjacent PUFA residues and thereby initiate a chain of free radical reactions with widespread harmful consequence to membrane structure. Peroxidation chain reactions are characterized by initiation, propagation and termination stages (Fig. 1.7) with lipid peroxides as the primary products. The generally accepted mechanism of peroxide formation involves the formation of a free radical (R') by a PUFA (RH) molecule followed by the addition of oxygen to form lipid peroxyl radical (ROO'). The lipid peroxyl radical can react with another PUFA molecule to produce another free radical, thus the reaction is propagated.

\[
RH \rightarrow R^\bullet + H^+ \\
R^\bullet + O_2 \rightarrow ROO^\bullet \\
ROO^\bullet + RH \rightarrow ROOH + R^\bullet
\]

Fig. 1.7. Initiation, propagation and termination of peroxidation chain reaction (RH: PUFA; ROO': lipid peroxyl radical; ROOH: lipid hydroperoxide)

Free radicals are normal by-products of the oxidative processes in cells and there are several physiological mechanisms that dispose off free radicals and limit their tissue-damaging effects. There are a number of metal containing enzymes like superoxide dismutase (Zn, Cu), glutathione peroxidase (Se), catalase (Fe) and glutathione reductase
(flavoprotein), the sole function of which appears to scavenge and dispose off free radicals and thus prevent or limit their tissue damaging effects. Fig. 1.8 shows some of the mechanisms for safe disposal of free radicals (Webb, 1995).

Superoxide dismutase (zinc-containing) converts superoxide radicals to hydrogen peroxide:

\[
2H^+ + O_2^- + O_2 \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2
\]

Glutathione peroxidase (selenium-containing) converts hydrogen peroxide to water:

\[
2H_2O_2 + \text{reduced glutathione} \xrightarrow{\text{peroxidase}} 2H_2O + O_2 + \text{oxidized glutathione}
\]

Glutathione reductase (riboflavin-containing) regenerates reduced glutathione:

\[
\text{Oxidized glutathione} \xrightarrow{\text{reductase}} \text{reduced glutathione}
\]

The enzyme catalase (iron-containing) converts hydrogen peroxide to water and oxygen:

\[
2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2
\]

Fig. 1.8 Mechanisms for safe disposal of free radicals
Possibly an increase in AFB$_1$ cause significant increase in hepatic lipid peroxide level. Shen et al. (1994) found that AFB$_1$-induced lipid peroxidation in rat liver and this lipid peroxidation was closely related to liver cell injury. A time- and dose-dependent increase in 8-hydroxy- deoxyguanosine (8-OHdG) was observed in DNA after a single intraperitoneal injection of AFB$_1$. It indicates that AFB$_1$ causes oxidative DNA damage in rat liver, which may involve, hydroxyl radicals as the initiating species (Shen et al., 1995; Gradelet et al., 1998). Therefore, factors interfering with the generation or action of OH' would affect the formation of 8-OHdG.

Verma et al. (1998) showed that aflatoxin treatment caused significant rises in intracellular calcium in liver, kidney, testis, adipose tissues, heart and skeletal muscle of rabbits. Several studies (Nicotera et al., 1992) have suggested that calcium activated catabolic processes are involved in cytotoxicity. Fagian et al. (1990) demonstrated that reversible permeabilization induced by calcium plus pro-oxidant is associated with oxidation of membrane protein thiols, forming cross-linked aggregates. Castilho et al. (1995) proposed that calcium plus pro-oxidant significantly reduced mitochondrial GSH and NADPH, substrates of the antioxidant enzyme glutathione peroxidase and glutathione reductase respectively favouring accumulation of H$_2$O$_2$. Turrens et al. (1991) demonstrated that accumulation of calcium in mitochondria mobilized iron which in turn could stimulate the production of OH' from H$_2$O$_2$.

**ANTIOXIDANTS**

Antioxidants are compounds that help to inhibit many oxidation reactions caused by free radicals thereby preventing or delaying damage to the cells and tissues. There are
many evidence in highlighting the role of antioxidants which may protect our body against certain conditions such as heart disease, stroke and cancers. It has been proposed that the mechanisms leading to these diseases may be promoted by free radicals and that antioxidants may oppose the action of these molecules. Now the concept has come of "eco-friendly antioxidants" which are obtained from nature and its use do not show any side effects and also they are friendly to environment and economically affordable.

The human diet is an unavoidable source of exposure not only to toxicants (Ames, 1983) but also to a variety of compounds which can inhibit toxicities in experimental animals and humans (Fiala et al., 1985). Many plant products like neem (Allameh et al., 2002; Tepsuwan et al., 2002), chlorophyllin (Egner et al., 2001; 2003; Kensler et al., 2002; Sugiyama et al., 2002; Diaz et al., 2003; Sudakin, 2003), soyabean paste (Jun et al., 2002; Kim J.G. et al., 2003; Park et al., 2003), live yeast (Parlat et al., 2001), grape fruit juice (Miyata et al., 2004), garlic (Sheen et al., 2001; Guyonnet et al., 2002; Abdel-Wahhab and Aly, 2003), coffee (Cavin et al., 2001; 2002), common bean (Cardador-Martinez et al., 2002); flavonoids (Ngombo et al., 2000; Ueng et al., 2001; Horn and Vargas, 2003); green tea (Qin et al., 2000; Muto et al., 2001) and dietary compounds (Lee et al., 2001; Sahoo and Mukherjee, 2002) may have significant inhibitory and protective effects against aflatoxin-induced toxicities. In addition, the antimutagenic chemicals present in food products belong to several chemical groups such as vitamins (Salem et al., 2001; Sahoo and Mukherjee, 2003; Yousef et al., 2003) and plant products (Wood et al., 1982; Huang et al., 1983; Steele et al., 1985).
TURMERIC-CURCUMIN

India has a rich history of using plants for medicinal purposes. Turmeric \((\text{Curcuma longa L.})\) is a medicinal plant extensively used in Ayurveda, Unani and Siddha medicine as home remedy for various diseases (Ammon and Wahl, 1991). Turmeric is used as a food additive (spice), preservative and colouring agent in Asian countries, including China and South East Asia.

Turmeric was described as *Curcuma longa* by Linnaeus and its taxonomic position is as follows:

- **Class** – Liliopsida
- **Sub-class** – Commelinids
- **Order** – Zingiberales
- **Family** – Zingiberaceae
- **Genus** – *Curcuma*
- **Species** – *Curcuma longa*

The wild turmeric is called *Curcuma aromatica* and the domestic species is called *Curuma longa*.

**Chemical composition of turmeric (Kapoor, 1990)**

- Protein – 6.3%
- Fat – 5.1%
- Minerals – 3.5%
- Carbohydrates – 69.4%
- Moisture – 13.1%
α-phellandrene – 1%
Sabinene – 0.6%
Limeol – 1%
Borneol – 0.5%
Zingiberene – 25%
Sesquiterpines – 53%
Curcumin (diferuloylmethane) – 3-4 %

Molecular weight of biphenolic curcumin is 368.4. Curcumin (diferuloylmethane) is responsible for the yellow colour of turmeric. It comprises of:

Curcumin I – 94%
Curcumin II – 6%
Curcumin III – 0.3% (Ruby et al., 1995)

Curcumin was first isolated by Vogel and Pelletier (1815) and its chemical structure was determined by Roughley and Whiting (1973). It has a melting point at 176-177°C, forms a reddish-brown salt with alkali and is soluble in ethanol, alkali, ketone, acetic acid and chloroform. Curcumin is found to be the biologically active compound in turmeric. The structures of some of these compounds are shown in Fig.1.9 (Araujo and Leon, 2001).

**Biological activity of Curcumin**

Curcumin and its derivatives have high anti-inflammatory activity against carrageenin-induced rat paw oedema (Srihari et al., 1982) and also on formalin-induced
CURCUMIN I

CURCUMIN II

CURCUMIN III

AR-TUMERONE

METHYLCURCUMIN
Fig. 1.9: STRUCTURES OF NATURAL CURCUMINOIDS
Curcumin reduces carbon tetrachloride and D-galactosamine-induced glutamate oxaloacetate transaminase and glutamate pyruvate transaminase levels (Kiso et al., 1983; Hikino, 1985). Curcumin has capacity of lowering cholesterol, fatty acids and triglycerides in alcohol-induced toxicity (Rukkumani et al., 2003). It is also reported to have anti-HIV activities (Mazumdar et al., 1995), anti-tumour (Bhaumik et al., 2000; Surh et al., 2001) and anti-carcinogenic activities (Goel et al., 2001; Shao et al., 2002; Choudhari et al., 2002). Curcumin has anti-spasmodic effect in isolated guinea pig ileum (Srihari et al., 1982). Curcumin also increases the activity of pancreatic lipase, amylase, trypsin and chymotrypsin (Platel and Srinivasan, 2000). Curcumin decreases the severity of pathological changes and thus protects from damage caused by myocardial infarction (Nirmala and Pavanakrishnan, 1996). Curcumin offers protective action against vascular dementia by exerting antioxidant activity (Vajragupta et al., 2003; Thiyagarajan and Sharma, 2004). The increase in fatty acid content after ethanol-induced liver damage is significantly decreased by curcumin treatment and arachidonic acid level is increased (Akrishnan and Menon, 2001). Alcohol and water extracts of Curcuma longa shows anti-inflammatory effects (Yegnarayanan et al., 1976). The anti-rheumatic activity of curcumin has also been established in patients who showed significant improvement of symptoms after treatment (Deodhar et al., 1980).

In vitro curcumin can significantly inhibit the generation of reactive oxygen species (ROS) like superoxide anions, $H_2O_2$ and nitrite radical generation by activated macrophages, which play an important role in inflammation also. Curcumin lowers the production of ROS in vivo (Joe and Lokesh, 1994). Curcumin reduces oxidized proteins in amyloid pathology in Alzheimer transgenic mice (Lim et al., 2001). It also decreases
lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates (Pulla and Lokesh, 1994). Curcumin shows anti-coagulant activity by inhibiting collagen and adrenaline-induced platelet aggregation in vitro as well as in vivo in rat thoracic aorta (Srivastava et al., 1985). Both turmeric and curcumin decreases blood sugar level in alloxan-induced diabetes in rat (Arun and Nalini, 2002). Curcumin also decreases advanced glycation end products induced complications in diabetes mellitus (Sajitlal et al., 1998). In patients undergoing surgery, oral application of curcumin reduces post-operative inflammation (Satoskar et al., 1986).

Curcumin was given to Wistar rats, guinea pigs and monkeys of both sexes at a dose of 300 mg/kg body weight. No pathological, behavioural abnormalities or lethality was observed (Bhavani Shankar et al., 1980). No adverse effects were observed on both growth and the level of erythrocytes, leucocytes, blood constituents such as haemoglobin, total serum protein, alkaline phosphatase etc. (Sambaiah et al., 1982). Human clinical trials also indicate that curcumin has no toxicity when administered at doses of 1-8 g/day (Chainani-Wu, 2003) and 10 g/day (Aggarwal et al., 2003).

An ethanolic extract of turmeric (Curcuma longa) as well as an ointment of curcumin were found to produce remarkable symptomatic relief in patients with external cancerous lesions. Reduction in swelling were noted in 90% of the cases and reduction in itching in almost all cases. Dry lesions were observed in 70% of the cases, and a small number of patients (10%) had a reduction in lesion size and pain. In many patients the effect continued for several months (Kuttan et al., 1987). It has also been indicated that both turmeric and curcumin increased the survival of experimental animals with lymphoma (Kuttan et al., 1985). Turmeric extract was found to interfere in chromosome
condensation and chromosome banding in vitro (Goodpasture and Arrighi, 1982), although it was shown to be non-mutagenic (Jensen, 1982). These reports indicate the possible use of curcumin as an antimitotic and anti-inflammatory agent which could be beneficial in cancer therapy.

The survival rate of tumor bearing mice, induced by using B16F10 melanoma cells shows an average of 88.2 days with an increase in life span of 143.85% when treated with curcumin, while when mice treated with other polyphenols such as catechin and rutin had an increase of 80.81% and 63.59% respectively in their life span (Menon et al., 1995). Thus curcumin along with catechin were found to be excellent antimitagens and anticarcinogens and are in chemopreventive trail in the National Cancer Institute, India. These compounds are also reported to be non-toxic, non-mutagenic and non-teratogenic. Result indicate the use of these compounds in clinical trials as they could not only prevent cancer but may also be able to inhibit metastasis. Curcumin has also been shown to inhibit the lysosomal enzymes by stabilizing the membrane (Menon et al., 1995).

Turmeric (Curcuma longa) and its active ingredient curcumin have been shown to scavenge the free radicals and thereby act as good antioxidants (Sharma, 1976). It has been investigated that the food additives such as turmeric when added to the medium containing Aspergillus parasiticus inhibited the growth of mycelium. The concentration needed for 50% inhibition was approximately 2.5 mg/ml of the medium (Soni et al., 1992). Both turmeric and curcumin inhibited the aflatoxin-induced toxicity in experimental ducklings. The weight change induced by the toxin is partially reversed by the simultaneous administration of turmeric and curcumin. The most remarkable feature
is the histopathological findings in which the aflatoxin-induced injury is remarkably reduced in animals treated with curcumin and turmeric (Soni et al., 1992). Repeated applications of turmeric extract and curcumin in the promotion phase produced a reduction in the expression of papillomas in mouse skin induced by 7, 12-dimethylbenz[a]anthracene followed by croton oil promotion. Also the anticarcinogenic potential was substantiated by the reduction in tumour formation induced by subcutaneous injection of 20-methylcholanthrene (Soudamini and Kuttan, 1989). Curcumin reduces tumour induced in mice by injecting one million cells of Datton's lymphoma tumour cells in intraperitoneal cavity. This property of curcumin would be an ideal drug which could be of value as a chemopreventive agent against carcinogen activation (Soudamini and Kuttan, 1988).

Statistically non-significant incidence of structural aberrations and absence of numerical aberrations showed *Curcuma longa* to be neither clastogenic nor mitoclastic. Thus turmeric when incorporated into the diet in different amounts had no adverse cytogenetic and mutagenic effects in the rats test system as it is revealed by the long-term observation for a period of four generations (Krishnamoorthy and Abdul Rahiman, 1986).

Researchers at Yale University discovered that curcumin may correct a cellular malformation that causes cystic fibrosis. In experiments with mice, curcumin corrected the cystic fibrosis defect and significantly increased the survival of mice (Egan et al., 2004). The researchers conducted *in vivo* studies by implanting squamous cell tumors in mice. Curcumin was applied as a non-invasive topical paste to the tumors and inhibition of tumour growth was observed (Lo Tempio et al., 2005). Curcumin can stop the growth of human pancreatic cancer cells, according to a study in the Journal of Cancer.
Researchers found that curcumin inhibited the production of interleukin-8, a protein produced by white blood cells that contributes to tumour growth (Hidaka et al., 2002). A recent study shows that curcumin can inhibit the accumulation of prions in vitro. Prions are proteins that are responsible for bovine spongiform encephalopathy, the scientific term for "Mad Cow Disease" and "Creutfeld-Jakob disease" as it is called in humans. In this study, curcumin potently inhibited the accumulation of a type of prion called protease-resistant prion protein. Prions must convert from their original state to this protease-resistant state in order to cause disease (Caughey et al., 2003).

One study showed curcumin to be eight times more powerful than that of vitamin E in preventing lipid peroxidation. Taken in group arrangements such as C-complex, curcuminoids are three times as potent in neutralizing free-radical molecules. Several studies have demonstrated curcumin's ability to reduce oxidative stress (Dikshit et al., 1995; Brozet and Ohshima, 1995; Mortellini et al., 2000). It appears that curcumin's role as an antioxidant may be due in part to its ability to down regulate nitric oxide formation, which is a key element in inflammation and may contribute to carcinogenesis. Curcumin lowers cholesterol and triglyceride levels, decreases susceptibility of low density lipoprotein (LDL) to lipid peroxidation, and inhibiting platelet aggregation. These effects have been noted even with low doses of turmeric (Srivastava et al., 1986).

Several animal studies have demonstrated that turmeric extract and curcumin inhibits the growth of a variety of bacteria, parasites and pathogenic fungi. Turmeric reduced the lesions caused by intestinal parasites, dermatophytes, pathogenic fungi, yeast, Plasmodium falciparum and leishmania organisms. Topical applications of curcumin extract was also effective (Allen et al., 1998; Rasmussen et al., 2000).
Turmeric and curcumin has been found to have hepatoprotective effects in protecting animal livers from a variety of hepatotoxic insults induced by chemicals and drugs. Turmeric and curcumin were also been found to reverse biliary hyperplasia, fatty liver and liver necrosis induced by aflatoxin (Donatus et al., 1990). Dietary administration of turmeric (0.05%) and curcumin (0.05% each) to rats significantly reduced the number of gamma-glutamyl transpeptidase positive foci induced by aflatoxin B1 which is considered as the precursor of hepatocellular neoplasm. These studies indicate the usefulness of antioxidant food additives in ameliorating aflatoxin-induced mutagenicity and carcinogenicity (Soni et al., 1997). One of the study suggests that curcuminoids could be considered as promising leader in the design of more efficacious (Multidrug resistance) MDR modulators, and bisdemethoxycurcumin is the curcuminoids present in turmeric for the modulation of MDR-1 gene expression in drug-resistant KB-VI cells. Curcumin, the main component in turmeric, increases sensitivity to vinblastine, which was consistent with a decreased P-glycoprotein (Pgp-170) level (Limtrakul et al., 2004). Curcumin and related analogues inhibited the production of aflatoxicol, another toxic metabolite from the breakdown of aflatoxin (Mark, 2002).

In South Korea, scientists did experiment on rats to see the comparative effects of aqueous extract of turmeric and aqueous ranitidine which is commonly prescribed for gastric and duodenal ulcers and gastroesophageal reflux. In both the turmeric extract-treated and ranitidine-treated rats, however, ulceration was markedly reduced; both of these agents strongly suppressed hyperacidity, apparently through their inhibitory effect on certain histamine receptors called H2R, which are involved in gastric acid secretion (Kim et al., 2005) Curcumin could be a potentially therapeutic anticancer agent, as it
significantly inhibits prostate cancer growth, and has the potential to prevent the progression of this cancer to its hormone refractory state (Dorai and Katz, 2001). According to University of Chicago scientists, curcumin inhibits a cancer-provoking bacteria (*Helicobacter pylori*) associated with gastric and colon cancer (Magad, 2002). Curcumin suppresses a number of key elements in cellular signal transduction pathways pertinent to growth, differentiation and malignant transformation. Among signalling events inhibited by curcumin are protein kinases and others (Liu *et al*., 1993).

Anti-cancer effect of curcumin seems to be potentialized in the presence of estrogen in breast cancer cells and it inhibits genes which are under the influence of the estrogen receptor (Shao *et al*., 2002). Curcumin also displays an inhibiting effect on human telomerase reverse transcriptase (hTERT) expression, reducing telomerase activity in MCF-7 cells (Ramachandran *et al*., 2002). Moreover, it allows sensitizing ovarian cancer cells to cisplatin, enhancing chemotherapeutic treatment (Chan *et al*., 2003). NF-Kappa B-dependent genes inhibition by curcumin is certainly an interesting strategy against diseases such as the pathogenesis of alcoholic liver disease, in which NF-KB is activated (Nanji *et al*., 2003). Curcumin also leads to apoptosis in scleroderma lung fibroblasts (SLF) without affecting normal lung fibroblasts (NLF) (Tourkina *et al*., 2004).

A study suggests a therapeutic role for dietary curcumin in patients with homocysteinemia, thereby reducing cardiovascular morbidity and mortality (Ramaswami *et al*., 2004). The ability of curcumin was also examined to protect against lead-induced damage to hippocampal cells of male Wistar rats, as well as lipid peroxidation induced by lead and cadmium in rat brain homogenate (Daniel *et al*., 2004). Some protective effect of curcumin against lead-induced neurotoxicity in rats was examined by Shukla *et al*.
Epidemiological data also suggest that curcumin may be responsible for the lower rate of colorectal cancer in some countries and is naturally occurring powerful anti-inflammatory medicine (Chauhan, 2002). Curcumin has been studied as an alternative non-toxic means of inducing the apoptosis potential in both androgen-dependent and hormone refractory prostate cancer cells (Dorai et al., 2000). Also curcumin proved to be a potent anti-proliferative agent for breast tumour cells and may have potential as an anti-cancer agent (Mehta et al., 1997). On particular interest is the ability of dietary curcumin to interfere with colon carcinogenesis in chemical and genetic rodent models (Mahmoud et al., 2000). Curcumin being the major constituent of the spice turmeric, which is abundantly used in the diet on the Indian subcontinent, an area showing low incidence of colorectal cancer (Greenlee et al., 2000; Ireson et al., 2001). Turmeric and curcumin provide protection against oxidative stress in a renal cell line injured by induced H2O2 (Cohly et al., 1998). Curcumin has moderate antimicrobial effect against Plasmodium falciparum and Leishmania major organisms (Rasmussen et al., 2000).

Curcumin caused marked inhibition in NF-KB and AP-1 activation, assessed by DNA binding and degradation of inhibitory IKB proteins and the induction of mRNAs for cytokines IL-6 (Interleukin-6) and TIVF-α, the chemokine KC, and inducible nitric oxide synthase in pancreas. Curcumin also blocked (Cholecystokinin) CCK-induced NF-KB and AP-1 activation in isolated pancreatic acini. Findings indicate that blocking key signals of the inflammatory response ameliorates pancreatitis in both ethanol and non-ethanolic models. This suggests that curcumin, which is currently in clinical trials for cancer prevention may be useful for treatment of pancreatitis (Gukovsky et al., 2003). As the gastrointestinal tract seems to be exposed more prominently to unmetabolized
curcumin than any other tissue, the results support the clinical evaluation of curcumin as a colorectal cancer chemopreventive agent (Ireson et al., 2001). Curcumin stimulates gall bladder contraction, and so could prevent risks in individual with gall bladder disease (Lal et al., 1999). One of the result indicate that curcumin effectively inhibits diethylnitrosamine (DEN)-induced hepatocarcinogenesis in the mice (Chuang et al., 2000). Significant cardioprotection and functional recovery has been demonstrated by curcumin (Mohanty et al., 2006).

The various studies conducted at the NIN (National Institute for Nutrition), Hyderabad, India, on turmeric and its active principle curcumin suggest that it can have impact on all the stages of carcinogenesis. It prevents activation of carcinogens and attack of electrophiles on DNA, acts as antioxidant and antipromoter, retards the conversion of preneoplasia in addition to repairing the damage to DNA.

It is apparent from experiments with animals and from clinical observations in man under toxaemia that short exposures to large doses of aflatoxin produces acute toxicity which may be lethal; while exposure to small doses over a protracted period of time is carcinogenic. Little is known about the effects of frequent exposure to moderate amount of dietary aflatoxins which appears to be of common occurrence in many countries.

Curcumin is proved to be more powerful than that of vitamin E in preventing lipid peroxidation. Taken in group arrangements such as C-complex, curcuminoids are three times as potent in neutralizing free radical molecules. Several studies have demonstrated curcumin’s ability to reduce oxidative stress (Dikshit et al., 1995; Brouet and Ohshima, 1995; Mortellini et al., 2000). As, all round the world researchers are making their best to
make “Curcumin – a boon drug” against cancer and other diseases in human as well as other animals, and its effectiveness has also been known against aflatoxin-induced cancer, which is world-wide problem.

The present study is an attempt to investigate aflatoxin-induced histopathological and biochemical changes in vital organs such as liver and kidney along with reproductive organs and also few parameters of blood and serum along with sperm study in Swiss strain male albino mice (*Mus musculus*). In addition, ameliorative effect of turmeric/curcumin, on aflatoxin-induced toxicity were also evaluated.