CHAPTER IV
DISCUSSION
Oral administration of aflatoxin (750 and 1500 μg/kg body weight/day) for 45 days in mice caused dullness and lethargy with signs of staggering. This could be due to reduction in aerobic metabolism and basal metabolic rate. Similar observations were also reported by Raval (1991) and Kolhe (1994) in rabbits. Ferrando and Henry (1965) recorded staggering, ataxia of nervous system, muscular spasms and characteristic stretching of the head and neck prior to death of animals under toxaemia.

Aflatoxin treatment for 45 days caused significant reduction in body weight gain of mice (Table 3.1). The decrease in body weight gain might be due to reduced feed intake, anorexia and altered biochemical changes in the skeletal muscles, which accounts for about 40% of total body weight. Reduced weight gain was also reported in rabbits (Clark et al., 1980; Verma and Raval, 1992; 1997; Salem et al., 2001; Soliman et al., 2001), turkeys (Klein et al., 2002), hens (Kim, J.G. et al., 2003), shrimps (Bintvihok et al., 2003), broilers (Oguz and Kurtoglu, 2000; Quezada et al., 2000; Raju and Devegowda, 2000; Aravind et al., 2003), pigs (Marin et al., 2002), mice (Kocabas et al., 2003) and rats (Pozzi et al., 2001). Dimitri et al. (1998) have reported significant weight loss equivalent to 13% mortality in aflatoxin-fed rabbits. Aflatoxin B1 and G2 were detected in muscle tissue at alarming concentration far exceeding the permissible levels in food for human consumption in the aflatoxin-fed groups (Dimitri et al., 1998). Verma and Chaudhari (1999) reported significant reduction in total acidic and basic proteins and
glycogen concentration in skeletal muscle of rabbits fed with aflatoxin-contaminated (7.5 mg/kg) feed.

**LIVER AND KIDNEY**

Aflatoxin treatment for 45 days caused an increase in absolute and relative weights of liver and kidney of mice (Tables 3.2 and 3.6). It might be due to accumulation of lipid in the liver, which produces characteristic enlarged, fragile fatty liver. Many investigators have reported increase in absolute (Quezada *et al.*, 2000) and relative (Miazzo *et al.*, 2000; Raju and Devegowda, 2000; Ortatatli and Oguz, 2001; Soliman *et al.*, 2001; Aravind *et al.*, 2003; Kim, J.G. *et al.*, 2003; Kocabas *et al.*, 2003) liver weights during induced aflatoxicosis. Increase in relative kidney weight was also reported (Raju and Devegowda, 2000; Ortatatli and Oguz, 2001).

Curcumin alone treatment (Group 3) did not have any significant effect on morphological changes, change in body weight gain as well as absolute and relative weights of liver and kidney as compared to other controls (Groups 1 and 2). However, administration of curcumin along with aflatoxin (low and high dose) (Groups 6 and 7) caused amelioration in morphological alterations, body weight gain as well as absolute and relative weights of liver and kidney as compared to aflatoxin alone treated animals (Groups 4 and 5). This might be due to the antioxidative property of curcumin, which causes reversal of aflatoxin-induced changes in liver and kidney of mice (Soni *et al.*, 1992).

Histopathological studies clearly indicate occurrence of pyknotic nuclei, increase in sinusoids and hepatocellular necrosis in the liver of aflatoxin-fed mice (Plate A,
7 - 11). It could be due to aflatoxin-induced cytotoxicity. It may be inferred that during aflatoxicosis higher concentration of aflatoxin reaches to the liver by hepatic portal system and affect the hepatic cells. *In vitro* studies of Raval and Verma (1997) have clearly indicated that when saline suspensions of hepatocytes were exposed to aflatoxin (0.35 to 3.5 µg/ml), swelling and lysis of cells occurred. Aflatoxin-induced cytotoxicity has also been reported by other investigators (Kaden *et al.*, 1987; Karenlampi, 1987; Verma and Raval, 1997). Aflatoxin-induced increases in lipid peroxidation have been observed both in *in vivo* and *in vitro* conditions in the liver of mice (Tables 3.5 and 3.39). Peroxidation of membrane lipids initiated loss of membrane integrity, membrane bound enzyme activity and cell lysis (Toskulkao and Glinsukon, 1988). Our results conform to the findings of Raval and Verma (1997), Towner *et al* (2000), Meki *et al.* (2001), Sahoo *et al.* (2001) and Bintvihok *et al.* (2003).

In addition, histopathological studies also revealed fatty infiltration of liver in aflatoxin-treated mice (Plate A; Figs. 7 - 11). Fatty infiltration of hepatocytes might be due to fat solubility of toxin. Once brought into the liver (through hepatic portal system), the fat present in the cells might dissolve toxin and retain it. This accumulation of fat along with toxin results in appearance of vacuolated cells. Aflatoxin-induced accumulation of lipid in liver has been reported in chicks, ducklings (Huff *et al.*, 1986), horses (Asquith, 1983), rats (Lin *et al.*, 2000) and laboratory animals (Newberne and Butler, 1969).

Histopathological studies revealed degenerative changes, increased vacuolization, necrosis, disorganization of glomerulus and increased space between the glomerulus and the capsule wall in the kidney of aflatoxin-treated mice (Plate B; Figs. 7 - 11). These
degenerative changes could be due to increased lipid peroxidation and reduced antioxidative capacity of the kidney in mice (Table 3.9). The kidney helps to control the composition and volume of blood through its excretory and reabsorptive capacity. Also urine is the principal route through which most toxicants are excreted. The kidney has a high rate of blood flow, concentrates toxicants in the filtrate, transports toxicants across the tubular cells and bioactivates certain toxicants (Lu, 1996) It is, therefore, a major target organ for toxic effects. Similar results were also reported by Nair (2000) and Sahoo et al. (2001).

Oral administration of aflatoxin and curcumin caused amelioration in all histopathological changes observed in liver (Plate A; Figs. 12 - 15) and kidney (Plate A; Figs. 12 - 15). It might be due to antioxidative effect of curcumin as an active polyphenols. Results shown in Table 3.5 and 3.9 indicates significant reduction in lipid peroxidation in liver and kidney of mice treated with aflatoxin along with curcumin. This effect is mainly due to increased antioxidative capacity of the cells.

Aflatoxin treatment caused significant, dose-dependent reduction in concentration of DNA, RNA and protein in the liver and kidney of mice (Tables 3.3 and 3.7). Aflatoxin is known to impair protein biosynthesis by forming adducts with DNA, RNA and proteins (Busby and Wogan, 1984), inhibits RNA synthesis, DNA-dependent RNA polymerase activity and causes degranulation of endoplasmic reticulum (Groopman et al., 1988; 1996; Cullen and Newberne, 1994; Verma and Nair, 2001a; 2003). Reduction in protein content could also be due to increased necrosis in liver (Plate A; Figs. 7 - 11) Thus reduction in protein biosynthesis as well as increased necrosis could be responsible for decrease in protein content. Many other investigators have also reported decrease in

Our results showed that curcumin treatment along with aflatoxin significantly ameliorates aflatoxin-induced changes in DNA, RNA and protein contents in the liver and kidney of mice. The amelioration in these contents might be due to increased DNA synthesis and reduction in harmful adduct formation. Cheng et al. (2003) investigated the inhibitory effects of curcumin, garlic squeeze, grape seed extract, tea polyphenols, vitamin C and vitamin E on nicotine-DNA adduction in vivo. They suggested that these dietary constituents are beneficial to prevent the harmful adduct formation and thus to block the potential carcinogenesis induced by nicotine.

Oral administration of aflatoxin for 45 days caused significant increase in lipid peroxidation in liver and kidney of aflatoxin-treated mice, as compared to controls. Lipid peroxidation is regarded as one of the primary key events in cellular damage (Mead, 1976; Plaa and Witschi, 1976) and the relationship between GSH levels, lipid peroxidation and cell lysis has been reported (Anundi et al., 1978). Carcinogens like aflatoxin B1, which generate epoxides, have been found to conjugate readily with GSH (Degen and Neumann, 1978). Liver cells, which are lethally injured by several toxins, exhibit marked alterations in intracellular Ca^{2+} homeostasis after excessive accumulation of Ca^{2+} (Nicotera et al., 1992). During hepatocellular necrosis, excessive intracellular Ca^{2+} is known to thrust the metabolism in an unmanageable disorder, which leads to mitochondrial dysfunction, inhibition of enzymes and denaturation of structural proteins (Fagian et al., 1990). Verma et al. (1998) showed that aflatoxin treatment caused
significant increase in intracellular calcium in the liver and kidney of rabbits. Toskulkao and Glinsukon (1988) reported that excessive accumulation of hepatic intracellular Ca\(^{2+}\) might be responsible for potentiation of hepatotoxicity in rats treated with both ethanol and AFB\(_1\). It is a secondary effect of the dysfunction of mitochondria caused by lipid peroxidation and reduction of hepatic adenosine triphosphatase activity, which participate in Ca\(^{2+}\) extrusion and uptake mechanisms (Toskulkao and Glinsukon, 1986). The increased lipid peroxidation in aflatoxin-treated animals is in agreement with findings reported previously for rat liver (Toskulkao and Glinsukon, 1988; Shen et al., 1994; Rastogi et al., 2000, 2001b; Meki et al., 2001; Abdel-Wahhab and Aly, 2003; El-Gibaly et al., 2003) as well as liver, kidney (Verma and Nair, 1999) and testis (Verma and Nair, 2001b) in mice.

Oxidative stress was originally defined as the disequilibrium between prooxidants and antioxidants in biological systems (Sies, 1991). Therefore, increase in lipid peroxidation could be due to significant reduction in the activities of enzymatic antioxidants such as catalase, superoxide dismutase and glutathione peroxidase as well as non-enzymatic antioxidants such as glutathione and total ascorbic acid contents in the liver and kidney of aflatoxin-treated mice, as compared to the controls. Superoxide dismutase protects cells from oxidative damage by breaking down a potentially hazardous free radical superoxide (O\(_2^.-\)) to H\(_2\)O\(_2\) and O\(_2\). The H\(_2\)O\(_2\) produced can then be decomposed enzymatically by catalase and glutathione peroxidase (GSH-Px). Glutathione peroxidase not only decomposes H\(_2\)O\(_2\) but can also interact with lipid peroxidation (Venkateshwaran et al., 1987). Thus significant reduction in these enzyme activities (Tables 3.5 and 3.9) could be responsible for increased lipid peroxidation.
observed during aflatoxicosis. Significant reductions in glutathione peroxidase (Meki et al., 2001; Rastogi et al., 2001a; Sheen et al., 2001; El-Gibaly et al., 2003), superoxide dismutase (Rastogi et al., 2001a; Abdel-Wahhab and Aly, 2003) and catalase (Rastogi et al., 2001a) have been reported in aflatoxin-fed rat liver.

The decline in enzymatic antioxidants could be due to the reduction in protein biosynthesis. Aflatoxin impairs protein biosynthesis by forming adducts with DNA, RNA and proteins, inhibits RNA synthesis and DNA-dependent RNA polymerase activity as well as causing degranulation of endoplasmic reticulum (Cullen and Newberne, 1994, Groopman et al., 1996) as mentioned earlier. In addition, oxidative stress may result in damage to critical cellular macromolecules including DNA, lipids and proteins (Shen et al., 1994). Cellular fatty acids are readily oxidized by ROS to produce lipid peroxyl radicals which can subsequently propagate into MDA, may result in interaction with cellular DNA-MDA adducts (Shen et al., 1994; 1995). Proteins are also easily attacked by ROS directly or indirectly through lipid peroxidation modifying their enzyme activity (Clayson et al., 1994).

Glutathione content decreased significantly in liver and kidney of aflatoxin-treated mice (Table 3.5 and 3.9), suggesting its rapid oxidation. Glutathione has a beneficial effect by virtue of possessing -SH groups. It helps to protect biological membranes, which are readily susceptible to injury by peroxidation (Venkateshwaran et al., 1987). The aflatoxin 2,3-epoxide may be inactivated by hydrolysis to the dihydrodiol, either spontaneously or via epoxide hydrolase or by conjugation with glutathione by glutathione-S-transferase. Thus, significantly lower GSH level would further aggravate the toxic effects of aflatoxin. Many investigators (Meki et al., 2001; Abdel-Wahhab and
Aly, 2003; El-Gibaly et al., 2003; Huber et al., 2003; Shin and Kwon, 2003) have reported significant reduction in glutathione content in aflatoxin-fed rat liver.

Present study shows significant reduction in ascorbic acid content in the liver and kidney of aflatoxin-treated mice (Tables 3.5 and 3.9). During free radical scavenging action, ascorbic acid is transformed into L-dehydroascorbate (Breimer, 1990). Reduced glutathione is required for the conversion of L-dehydroascorbate back to ascorbate (Breimer, 1990). The fall in the level of reduced glutathione decreases the conversion of L-dehydroascorbate to ascorbate and this probably explains the lowered level of ascorbic acid in aflatoxin-treated animals.

Oral administration of curcumin along with aflatoxin for 45 days caused significant amelioration in aflatoxin-induced lipid peroxidation by increasing the antioxidative activity of the cells. Activities of enzymatic antioxidants (catalase, superoxide dismutase and glutathione peroxidase) as well as contents of non-enzymatic antioxidants (glutathione and ascorbic acid) were significantly increased in liver and kidney of aflatoxin plus curcumin-treated mice than that of aflatoxin alone treated mice (Table 3.5 and 3.9).

Curcumin shows many health promoting activities including chemopreventive action during carcinogenesis due to antioxidative polyphenolic constituents (Surh et al., 2001). Smoke shield, which is a formulation containing extracts of turmeric together with extracts of green tea and other spices, was found to elevate antioxidant enzymes such as catalase and superoxide dismutase in blood as well as in liver and kidney of mice. Glutathione levels were also significantly elevated in blood. Administration of smoke shield decreased the lipid peroxidation in serum, liver and kidney. Sreekanth et al. (2003)
thus explained that smoke shield had potent antioxidant activity, could inhibit phase I enzymes and increase detoxifying enzymes which makes it an effective chemopreventive herbal formulation.

Eid et al. (2003) studied the effects of dietary polyphenols (PP) on growth and oxidative stress in the corticosterone (CTC)-treated broiler chickens. They suggested that muscle and liver thiobarbituric acid reactive substances (TBARS) were elevated by CTC and these effects were reduced by PP. Polyphenols can thus minimize growth inhibition, hyperlipidaemia and oxidative stress-induced by CTC treatment in broiler chickens.

Oral administration of aflatoxin for 45 days caused significant reduction in the activities of succinate dehydrogenase and ATPase in liver and kidney of mice. The effect was comparatively more pronounced in high dose aflatoxin-treated group than that of low dose (Tables 3.4 and 3.8). Succinate dehydrogenase (SDH) is a key enzyme in the mitochondrial Kreb's cycle, which is mainly concerned with the aerobic oxidation of acetyl CoA and the generation of ATP. Putlina and Eschanko (1969) explained that among the Kreb's cycle dehydrogenases, SDH is very active than any other enzyme. Therefore reduction in SDH activity clearly indicates reduction in aerobic metabolism, which might be the result of reduced oxygen transport to tissues.

Aflatoxin caused ultrastructural changes in mitochondria (Shanks et al., 1986; Rainbow et al., 1994). Roy (1968) have reported mitochondrial swelling during aflatoxicosis. This could be due to accumulation of calcium which is known to cause mitochondrial dysfunction and reduced ATP generation (Toskulkao and Glinsukon, 1988; Verma et al., 1998). Aflatoxin alters energy-linked functions of ADP phosphorylation, FAD$^+$ and NAD$^+$ linked oxidizing substrates (Sajan et al., 1996) and $\alpha$-ketoglutarate-
succinate cytochrome reductases (Obasi, 2001). Reduction in hepatic succinate dehydrogenase activity was also reported in broilers (Quezada et al., 2000), rats (Rastogi et al., 2000, 2001a), rabbits (Verma and Raval, 1992) and mice (Verma and Nair, 2001a). Inhibition of liver mitochondria and electron transport flow by aflatoxin has been reported by Doherty and Campbell (1972; 1973) in rat.

The enzymatic hydrolysis of ATP by ATPase in an ubiquitous property of cells which is important for intracellular transfer of energy. Reduction in ATPase activity in liver and kidney suggests reduced utilization of ATP produced in the cell. The reduced aerobic oxidation and ATP generation could be responsible for the reduction in ATPase activity. Verma and Nair (2001a) also reported reduction in ATPase activity in the testis of aflatoxin-treated mice.

Treatment with curcumin along with aflatoxin significantly ameliorates aflatoxin-induced changes in SDH and ATPase activities in liver and kidney of mice. The ameliorative effect of curcumin might be due to its polyphenolic nature having antioxidative property. Antioxidants have the property to protect all membrane lipids and unsaturated fatty acids against oxidative degeneration (Dean and Cheeseman, 1987). Histopathological studies in the present experiments also revealed amelioration in histoarchitecture of liver (Plate A; Figs. 12 - 15) and kidney (Plate B; Figs. 12 - 15). The cells were almost normal.

REPRODUCTIVE ORGANS

Oral administration of aflatoxin (750 and 1500 μg/kg body weight/day) for 45 days caused significant reduction in absolute and relative weights of reproductive organs
such as testis, caput and cauda epididymides, seminal vesicle and ventral prostate gland. The reduction in weights of testis could be due to degenerative changes, reduced spermatogenesis and tubular lumen devoid of sperm bundles. Also the reduction in weights of caput and cauda epididymides could be due to degenerative changes in the epithelial lining and tubular lumen devoid of sperm bundles. The protein content was significantly reduced in the testis, caput and cauda epididymides and ventral prostate gland of aflatoxin-treated mice. It corroborates with earlier findings reported in skeletal muscle (Raval and Verma, 1992; Verma and Chaudhari, 1999), heart (Verma and Kolhe, 1997), liver (Verma and Raval, 1996) and kidney (Verma and Raval, 1997) of aflatoxin-fed rabbits. Aflatoxin impairs protein biosynthesis by forming adducts with DNA, RNA and protein, inhibits RNA synthesis and DNA dependent RNA polymerase activity and causes degranulation of endoplasmic reticulum as mentioned earlier (Cullen and Newberne, 1994; Groopman et al., 1996).

Aflatoxin treatment caused significant reduction in DNA and RNA contents in the testis. It could be due to formation of aflatoxin adduct with DNA and RNA (Busby and Wogan, 1984; Groopman et al., 1988; 1996) and thereby reducing its biosynthesis.

Cholesterol is the principal sterol found in all tissues and body fluids of animals and human beings. In addition to dietary sources, cholesterol can be biosynthesized actively and gets distributed all over the body through blood. Aflatoxin treatment for 45 days caused significant rise in testicular cholesterol concentration in mice (Table 3.11). Increase in testicular cholesterol could also be due to its reduced utilization in the steroidogenesis. Verma et al. (1998) have also reported significant rise in testicular cholesterol and hypercholesterolaemia in aflatoxin-fed rabbits.
The present study clearly indicates significant reduction in 3β- and 17β-hydroxysteroid dehydrogenases in the testis of aflatoxin-treated mice (Table 3.11). Bashandy et al. (1994) also reported similar changes in the gonadal activity of male rats. Significantly reduced testosterone concentration during induced aflatoxicosis has been reported in mice (Verma and Nair, 2002). Aflatoxin administration in adult rats caused a significant decrease in androgen receptors in the testicular cytosol and androgen profile (Srivastava and Singh, 1985).

Succinate dehydrogenase (SDH) is a key enzyme of mitochondrial Kreb's cycle which is mainly concerned with aerobic oxidation of acetyl CoA and generation of ATP. Decrease in SDH activity observed in testis, caput and cauda epididymides (Tables 3.12, 3.14 and 3.15) indicate a reduction in aerobic oxidation, which could be in response to reduced oxygen transport to tissues.

Another possible reason could be due to accumulation of calcium, which is known to cause mitochondrial dysfunction, and reduced ATP generation (Toskulkao and Glinsukon, 1988). Roy (1968) has reported mitochondrial swelling during aflatoxicosis. In addition, the altered calcium/magnesium ratio, reduced oxidative phosphorylation and ATP generation in heart and skeletal muscle might be a key factor causing development of lethargy.

The enzymatic hydrolysis of ATP by ATPase is a ubiquitous property of cells which is important for intracellular transfer of energy. The ATPase activity showed decline in testis, caput and cauda epididymides suggesting a reduced utilization of ATP produced in the cell. Toskulkao and Glinsukon (1988) have reported increased
accumulation of Ca\textsuperscript{2+} inside mitochondria causing mitochondrial dysfunction and reduction in hepatic ATP content.

Intracellularly acid phosphatase is restricted to the membrane bound vesicle, lysosomes. Acid phosphatase (AcPase) is very important for the tissue reorganization and repair. AcPase is also concerned with uptake of glucose and its metabolism in cytoplasm. Activity of AcPase was significantly increased in ventral prostate gland (Table 3.17) during aflatoxicosis, which could be due to increased leakage of lysosomal enzymes as well as lysis of the cells causing enhanced release of enzymes. Our data matches with the findings of Raval (1991) and Raval and Verma (1997). Other researchers (Clark et al., 1980; Kolhe, 1994) have also reported similar changes. The recovery in the group administered with curcumin plus aflatoxin may be due to the prevention of damage of the tissue (oxidative damage and hydrolysis).

Sialic acid is a sialomucoprotein, essential for the maintenance of the structural integrity of the sperm membrane and sperm maturation (Chinoy and Sequeira, 1989, Chinoy et al., 1994). The altered sialic acid content in the testis, caput and cauda epididymides (Tables 3.12, 3.14 and 3.15) indicate that the structural integrity of acrosomal membranes of the sperm might be affected. Reduction in sialic acid content during induced aflatoxicosis in mice has been reported by Verma and Nair (2001).

Results shown in table 3.13 clearly indicate significantly increased lipid peroxidation in the testis of aflatoxin-treated mice as compared to controls. Castilho et al. (1995) proposed that calcium plus prooxidant significantly reduced mitochondrial glutathione and NADPH, substrates of the antioxidant enzymes glutathione peroxidase and glutathione reductase respectively, favouring accumulation of H\textsubscript{2}O\textsubscript{2}. 

134
Lack of an adequate supply of NADPH and GSH to permit H$_2$O$_2$ consumption by the GSH-dependent glutathione peroxidase and NADPH-dependent glutathione reductase together with an increased concentration of free iron within the cell stimulates the production of OH$^-$ via a Fenton reaction due to mobilization of ferrous by calcium. Castilho et al. (1995) have shown that in the absence of molecular oxygen and Fe$^{2+}$, Ca$^{2+}$ is not toxic to mitochondria either in the absence or presence of t-butyl hydroperoxide. These results and those showing protection by catalase strongly support the notion that ROS generated by the respiratory chain and the Fenton reaction are involved in this process. The decline in these enzyme activities could be due to a decline in protein biosynthesis or oxidative damage (Shen et al., 1994; 1995, Clayson et al., 1994).

Aflatoxin treatment caused degenerative changes in the testis of mice with evidence of distortion, intra-epithelial vacuolization in the germinal epithelium, depletion of germ cells and cellular debris in the testicular lumen (Plate C, Figs. 7 - 10). Similar degenerative changes have been reported by Piskac et al. (1982). The degenerative changes observed in the testis, caput and cauda epididymides, seminal vesicle and ventral prostate gland could be attributed to oxidative stress, which is generally correlated with cellular damage (Shen et al., 1994; Romero et al., 1998). Also, as testis, caput and cauda epididymides, seminal vesicle and ventral prostate gland are androgen-dependent organs, histological and biochemical changes observed in these organs could be due to reduced testosterone level in aflatoxin-treated mice. Significantly decreased 3β- and 17β-hydroxysteroid dehydrogenase activities and serum testosterone levels were significantly reduced in aflatoxin-treated mice as compared to controls (Verma and Nair, 2002).
Administration of curcumin along with aflatoxin caused recovery in all histopathological, biochemical and enzymatic changes in all reproductive organs. The recovery was more pronounced in animals receiving low dose aflatoxin as compared to those receiving the high dose. Amelioration is due to antioxidative property of curcumin.

**SERUM**

Significant decrease in serum protein was noted in aflatoxin-fed mice (Table 3.18). Almost all the serum proteins are invariably secreted by liver. Decreased biosynthesis and secretion of protein might be due to formation of aflatoxin adducts with DNA, RNA and protein. Extensive hepatocellular necrosis, fatty infiltration and bile duct proliferation have also been observed in aflatoxin-fed mice (Plate A; Figs. 7 - 11). Aflatoxins have previously been shown to lower the total protein concentration in serum of rabbits (Verma and Raval, 1997; Yousef et al., 2003) and broilers (Quezada et al., 2000; Raju and Devegowda, 2000). Biosynthesis and secretion of serum protein becomes normalized as no necrotic changes were observed in histopathological study of liver of aflatoxin plus curcumin-treated mice (Plate A. Figs. 12 - 15).

Oral administration of aflatoxin for 45 days caused, as compared to the controls, significantly higher level of creatinine in the serum of mice (Table 3.18). Creatine is synthesized in the liver, passes into circulation and is taken up almost entirely by skeletal muscle for conversion to creatine phosphate, which acts as an energy reservoir. Creatine and its phosphate are converted spontaneously into creatinine (Mc Lauchlan, 1988). The two substances are handled differently by the kidney. Both are filtered at glomerulus although there may be some additional secretion of creatinine by renal tubules, creatine
is reabsorbed by the tubules at low plasma concentration. This ensures that there is little, or no creatine in urine (Mc Lauchlan, 1988). The heightened appearance (p<0.05) of creatinine in the serum of aflatoxin-fed mice indicates the increased transformation of phosphocreatine to creatinine in muscle which might be due to lesser utilization of phosphocreatine in muscular contraction. The kidney rapidly excretes creatinine. Histopathological studies revealed glomerular damage and tubular degeneration in the kidney of aflatoxin-fed mice (Plate B; Figs. 7 - 11). Thus significant increase in creatinine concentration in serum could be due to increased release from muscles and/or decrease excretion from the kidney. Verma and Raval (1997) reported the occurrence of nephrotoxicity and the elevation of creatinine in serum and urine of rabbits receiving aflatoxin-contaminated feed (15 mg/kg) for 60 days. Verma and Kolhe (1998) showed time-dependent rises in creatine and creatinine concentrations in the serum and urine of aflatoxin-fed rabbits. This suggests that aflatoxin causes adverse changes in skeletal muscle and kidney at a very early stage. They also suggested the occurrence of cumulative toxicity during aflatoxicosis.

Curcumin alone treatment did not have any effect on serum creatinine levels. However, curcumin when given along with aflatoxin, it ameliorates aflatoxin-induced effects in the serum parameters as compared to the aflatoxin alone treated mice. These changes could be due to amelioration in aflatoxin-induced histopathological changes in kidney (Plate B; Figs. 12 - 15).

Enzymes glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) are present in the cytosol of the hepatocytes. The GPT is also localized in the mitochondria. Whenever liver hepatocytes are damaged, these enzymes
released into the blood. Significant increase in GOT and GPT activities indicates damage
to the cytosol and also to mitochondria. The results obtained in the present study indicate
significant increase in SGPT and SGOT activities in the aflatoxin alone treated mice
(Table 3.18). On the other hand, aflatoxin treatment with curcumin showed marked
recovery. Similar results have been reported by Koul and Kapil (1994) with CCl₄ and
Trivedi (1999) with BHC.

BLOOD

Treatment with aflatoxin for 45 days caused decrease in RBC count and
haemoglobin (Hb) concentration (Table 3.19). Exact mechanism of decreased RBC count
and Hb concentration is not clearly understood. It could be due to inhibition of
haematopoiesis, defective haematopoiesis, increased rate of destruction of RBC or a
combination of all three. It could be due to direct action of aflatoxin on the plasma
membrane causing lipid peroxidation, membrane permeability alterations and cell lysis
(Verma and Nair, 1999). Yousef et al. (2003) showed significant decrease in
haemoglobin (Hb), total erythrocyte count (TEC) and packed cell volume (PCV), in a
dose-dependent manner in aflatoxin-induced rabbits. Similar results were also reported
by Verma et al. (2001c).

The results revealed that treatment with curcumin alone did not show any altered
effect on Hb and RBC (Table 3.19). However, treatment of aflatoxin plus curcumin
shows the almost marked recovery in Hb content and RBC along with their normal
membrane structure. The protective effect of curcumin on tissue lipid peroxidation as an
antioxidant is shown in nicotine-treated Wistar rats (Kalpana and Menon, 2004).
Sambaiah et al. (1982) have also reported that curcumin ameliorates erythrocytes and leucocytes count, blood constituents such as haemoglobin, total serum protein and alkaline phosphatase etc.

**SPERM PARAMETERS**

Cauda epididymal sperm count reduced significantly in aflatoxin-treated mice (Table 3.20). It could be due to reduced spermatogenesis. Histopathological studies revealed reduced spermatogenesis and reduction in sperm bundles in the lumen of seminiferous tubules, as well as, caput (Plate D; Figs. 7 - 10) and cauda epididymal lumen (Plate E; Figs. 7 - 10).

Cauda epididymal sperm viability and motility reduced significantly in aflatoxin-treated mice (Table 3.20). This could be due to reduced mitochondrial function, decreased ATP and ATPase activity. Roy (1968) reported mitochondrial swelling. Reduction in SDH activity has been discussed earlier. Reduction in sperm motility could also be due to reduction in testicular and epididymal proteins. Androgen binding proteins are important for several functions in the testis. The proteins of epididymis are important for sperm maturation, motility and fertility rate. Acidic epididymal glycoprotein of epididymal luminal fluid of various species induces forward motility of caput epididymal spermatozoa (Chinoy, 1984). Therefore, reduction in protein synthesis during aflatoxicosis (Tables 3.11, 3.14, 3.15) might affect sperm function. Shahin (1993) showed depression of sexual behaviour in adult male rats during aflatoxicosis. The evaluation of treated spermatozoa stained with trypan blue showed a large number of dead spermatozoa, probably due to loss of membrane integrity. The effect of aflatoxin on
membrane permeability might be another major factor in the loss of sperm motility. Similar results have been reported with fluoride treatment in rat (Chinoy et al., 1995).

Sialic acid is a sialomuco-protein, essential for the maintenance of the structural integrity of the sperm membrane. The altered sialic acid in the testis and epididymis during aflatoxicosis (Tables 3.12, 3.14, 3.15) indicates that the structural integrity of acrosomal membranes of the sperm might be affected. This affects sperm morphology. Moreover, sialic acid is important for sperm maturation (Chinoy and Sequeira, 1989; Chinoy et al., 1994).

The results revealed that there was no adverse effect of curcumin treatment alone on sperm count, viability, motility, morphological structures as well as fertility rate (Table 3.20). National Cancer Institute reported that curcumin is non-toxic, non-mutagenic and non-teratogenic. Curcumin has ability to inhibit the lysosomal enzymes by stabilizing the membrane. Positive effect of curcumin was observed on sialic acid and on prostate cancer (Dorai and Katz, 2001). This indirectly supports that curcumin significantly ameliorates aflatoxin-induced toxicity in sperm parameters.

**IN VITRO STUDIES**

Addition of aflatoxin to RBC suspension caused a significant rise in haemolysis and swelling of the cells. The cell pellets in the bottom of the tubes reduced with reddish coloured supernatant indicating haemolysis due to bursting of the cells due to excess swelling (Table 3.21). It could be due to the direct action of aflatoxin on the plasma membrane causing lipid peroxidation, membrane permeability alterations and cell lysis (Verma and Nair, 1999).
The concurrent addition of an aqueous/ethanolic extracts of turmeric and curcumin to the RBC suspension significantly reduced aflatoxin-induced haemolysis (Table 3.22). An almost concentration-dependent effect was observed. Curcumin was found to be most effective rather than ethanolic and aqueous extracts. It could be due to antioxidative property of curcumin and other compounds present in ethanolic and aqueous extracts. The protective effect of curcumin on tissue lipid peroxidation as an antioxidant is shown in nicotine-treated Wistar rats (Kalpana and Menon, 2004).

Addition of aflatoxin to human semen suspension caused significant reduction in sperm viability, motility and increased sperm morphological alterations. The effects were concentration-dependent (Tables 3.23, 3.27, 3.31 and 3.35). The swelling of sperm head might be due to loss of membrane integrity. These alterations could be correlated with direct action of aflatoxin on the plasma membrane causing lipid peroxidation, membrane permeability alterations and cell lysis (Verma and Nair, 1999). Another possible reason could be due to the accumulation of intracellular calcium, which is known to cause mitochondrial dysfunction and reduce ATP generation during aflatoxicosis (Verma et al., 1998). Also reduced aerobic oxidation and ATP generation might be responsible for the reduction in ATPase activity. Reduction in ATPase activity during aflatoxicosis has been reported in testis of mice (Verma and Nair, 2001b). Male fertility has been deteriorated in many countries during the last few decades due to poor sperm quality (Irvine et al., 1996; Toppari et al., 1996). This may be due to oxidative stress, which causes cellular damage (Romero et al., 1998).

Concurrent addition of curcumin/turmeric extracts (ethanolic and aqueous) and aflatoxin to human semen suspension showed gradual recovery in sperm viability (Tables

141
3.24 - 3.26) and motility (Tables 3.28 - 3.30) along with recovery in altered sperm morphology (Tables 3.36 - 3.38) and recovered sperm abnormalities (Tables 3.32 - 3.34). These ameliorative changes could be due to antioxidative property of curcumin. This is indirectly related with the positive effect of curcumin on sialic acid (Dorai and Katz, 2001).

Present *in vitro* studies revealed that addition of aflatoxin to liver, kidney and testis homogenates caused significant, persistant and concentration-dependent increase in H$_2$O$_2$ induced TBARS (Table 3.39) suggesting that it causes lipid peroxidation. It is known that H$_2$O$_2$ is an oxidant, which can form free radicals with ferrous ion and with oxygen (Haber Weiss reaction).

Concurrent addition of curcumin and aflatoxin to liver, kidney and testis homogenates significantly reduced aflatoxin-induced lipid peroxidation (Table 3.40). This could be due to antioxidative property of curcumin. The result revealed that addition of curcumin to homogenates shows decrease in lipid peroxidation. The effect was concentration-dependent. One of the study showed curcumin to be eight times more powerful antioxidative agent than that of vitamin E in preventing lipid peroxidation. Several studies have demonstrated curcumin's ability to reduce oxidative stress (Mortellini *et al.*, 2000; Dikshit *et al.*, 1995; Brouet and Ohshima., 1995).

**Mechanism of action of curcumin**

It has been suggested that the apparent chemopreventive action of curcumin is related to its ability to competitively inhibit cytochrome P450 isoenzymes responsible for the metabolic activation of carcinogens such as benzo[a] pyrene and aflatoxin B$_1$.  

142
Curcumin selectively inhibits the CYP 1A1/1A2 and 2B1/2B2 isozymes in the high-nanomolar to low-micromolar range (Oetari et al., 1996), levels that are certainly achievable, even given the poor absorption kinetics for this agent.

The antioxidant mechanism of curcumin is attributed to its unique conjugated structure, which includes two methoxylated phenols and an enol form of \(-diketone; the structure of curcumin shows typical radical trapping ability as a chain-breaking antioxidant (Rao, 1994; Masuda et al., 2001). Generally the nonenzymatic antioxidant process of the phenolic material is thought to be mediated through the following two stages:

\[
S - O + AH \rightarrow SOOH + A^o
\]

\[
A^o + X^o \rightarrow \text{Nonradical materials}
\]

Where S is the substance oxidized, AH is the phenolic antioxidant, A\(^o\) is the antioxidant radical and X\(^o\) is another radical species or the same species (Frankel, 1998) as A\(^o\). A\(^o\) and X\(^o\) dimerize to form the non-radical product. Masuda et al. (2001) further studied the antioxidant mechanism of curcumin using linoleate as an oxidizable polyunsaturated lipid and proposed that the mechanism involves oxidative coupling reaction at the 3\(^\prime\) position of the curcumin with the lipid and a subsequent intramolecular Diels-Alder reaction.

**Beneficial effects of curcumin**

To evaluate possible ameliorative effect of curcumin/turmeric extracts on aflatoxin-induced toxicity in mice, the animals were administered with curcumin (2.0 mg/0.2 ml olive oil/animal/day) along with aflatoxin.
The results revealed that administration of curcumin along with aflatoxin caused significant amelioration in aflatoxin-induced changes in mice. Curcumin reduces carbon tetrachloride and D-galactosamine-induced glutamate oxaloacetate transaminase and glutamate pyruvate transaminase levels (Kiso et al., 1983). Curcumin have capacity of lowering cholesterol, fatty acids and triglycerides in alcohol-induced toxicity (Rukkumani et al., 2003). Alcoholic and water extracts of Curcuma longa shows antiinflammatory effects (Yegnanarayan et al., 1976). Curcumin reduces oxidized proteins in amyloid pathology in Alzheimer transgenic mice (Lim et al., 2001). It also decreases lipid peroxidation in rat liver microsomes, erythrocytes membrane and brain homogenates (Pulla and Lokesh, 1994). No adverse effects of curcumin treatment 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). An ethanolic extract of turmeric as well as an ointment of curcumin were found to produce remarkable symptomatic relief in patients with external cancerous lesions. 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). Curcumin and related analogues inhibited the production of aflatoxicol, another toxic metabolite from the breakdown of aflatoxin (Mark, 2002). Turmeric and curcumin provide protection against oxidative stress in a renal cell line injury by induced H₂O₂ (Cohly et al., 1998). Curcumin is capable to treat prostate cancer (Dorai and Katz, 2001).
Some other functions of curcumin/turmeric extracts

- Curcumin has effect on gastrointestinal system (Lee et al., 2003).
- It has effect on cardiovascular system (Nirmala and Pavanakrishnan, 1996).
- It has effect on nervous system (Vajragupta et al., 2003).
- Curcumin has effects on lipid metabolism (Kamal-Eldin et al., 2000).
- It has antiinflammatory activity (Ghatak and Basu, 1972).
- It has anticarcinogenic and chemopreventive effect (Chen and Huang, 1998).
- Curcumin shows antimitagenic activity along with anticoagulant, antidiabetic, antibacterial, antifungal, antiprotozoan, antiviral, antifibrotic, antivenom, antiseptic activities etc.
- Leukaemia, breast, colon, hepatocellular and ovarian carcinoma cells undergo apoptosis in the presence of curcumin (Khar et al., 2001).

It can be said that if curcumin/turmeric are added to food/feed-stuffs, it increases its nutritive value, restricts fungal growth and aflatoxin production as well as reduces cytotoxicity in the body.