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Aflatoxin Cause DNA Damage

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ABSTRACT Aflatoxins are food-borne secondary toxic fungal metabolites produced during the growth of *Aspergillus flavus* and *A. parasiticus*. Aflatoxins are well known hepatotoxic, hepatocarcinogenic and mutagenic agents. These effects are mainly due to adduct formation with DNA, RNA and protein. In addition, it also causes lipid peroxidation as well as oxidative damage to DNA. AFB1 possess genotoxic potential in a variety of test systems. Other aflatoxin has not been so extensively investigated, but in a variety of studies B2, G1, G2 and M1 have all shown evidence of genotoxicity.

Aflatoxins were initially isolated and identified as the causative toxins in 'Turkey-X-disease' in 1960 when 100,000 turkeys died in England (Asao et al. 1965). Now it is well known that aflatoxins (B1, B2, G1, G2) are food-borne secondary toxic metabolites produced during the growth of *Aspergillus flavus* and *A. parasiticus* group of fungi. These are highly substituted coumarin derivatives containing a fused dihydrofurofuran moiety. Aflatoxin B1 and AFB2 are named because of their strong blue fluorescence under UV light, whereas AFG1 and AFG2 fluoresced greenish yellow. The B-toxins are characterized by the fusion of a cyclopentenone ring to the lactone ring of the coumarin structure, while G-toxins contained an additional fused lactone ring. Aflatoxin B1 and to a lesser extent AFG1 are responsible for the biological potency of aflatoxin-contaminated feed. These two toxins possessed an unsaturated bond at the 8,9 position on the terminal furan ring. Aflatoxin B2 and AFG2 are essentially biologically inactive unless these toxins are first metabolically oxidized to AFB1 and AFG1 *in vivo*. AFM1 and M2 are hydroxylated derivatives of AFB1 and B2 that may be found in milk, milk products or meat (hence the designation M1). They are formed by the metabolism of B1 and B2 in the body of the animals following absorption of contaminated feeds.

Aflatoxins can contaminate corn, cereals, sorghum, peanuts and other oil seed crops. Although natural occurrence of aflatoxins in agricultural products has been reported from many countries and on vast array of crops, the levels of aflatoxins detected vary greatly from area to area. In general, higher quantities of aflatoxins were recorded in commodities from tropical and subtropical countries where environmental conditions are more congenial for mouldy growth and toxin production. In a survey of peanut products in North America, 19% of 1416 samples examined were contaminated with an average level of 1 µg/kg (Stoloff 1977), whereas, in Thailand 49% of 216 samples contained AFB1 at an average level of 424 µg/kg (Shank et al. 1972). As much as 260 µg of aflatoxin/kg was found in the sample of oat in Sweden shown contaminated with *A. flavus* (Smith and Moss 1985). Very severe 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 ìg/kg (Krishnamachari et al. 1975).

Aflatoxins are present in food chain. Consumption of aflatoxin in many parts of the world varies from 0 to 30 000 ng/kg/day (Denning 1987). Aflatoxins have also been found in human cord blood and apparently can enter the developing fetus in humans and animals (Denning et al. 1990; Applegren and Arora 1983). In addition, aflatoxins have been found in human breast milk, cow's milk and dairy products (Srivastava et al. 2001; Thirumala-Devi et al. 2001) and infant formula (Aksit et al. 1997).

Marvan and his colleagues (1983) have experimentally studied distribution of AFB1 in goslings and chickens and according to AFB1 concentrations the organs and tissues were categorized as follows: gonads; parenchymatous

organs-liver and kidney; lymphopoietic organs-spleen, bursa cloacalis and thymus; followed by the endocrine glands and muscles; lungs have low concentration 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 AFB1 has been detected in blood, liver, kidney and testis from minutes up to 8 to 10 hour after injection.

Once inside the body, 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. Aflatoxin B1 is converted in the adult liver by the cytochrome P450 enzyme, P450 III AY and in the fetal liver with P450 III A6 to AFQ1 the major metabolite of AFB1. Other major metabolites in the human include AFM1, aflatoxicol (AFL), AFLH1, AFP1, AFB2á and AFB1-2, 2-dihydrodiol. About 80% of a total dose of AFB1 is excreted in 1 week. The plasma half-life is 36.5 min, volume of distribution 14% of body weight and body clearance is 1.25 L/kg/h. Aflatoxin M1 is mostly excreted within 48 h of ingestion. It is possible that its measurement gives a reasonable estimate of recent aflatoxin ingestion (Hendrickse 1991).

Cytochrome P450 IIIA4, which can both, activate and detoxicate AFB1, is found. Only one of these, the 8, 9-exoepoxide appears to be mutagenic and others are detoxification products. The putative AFB1 epoxide is generally accepted as the active electrophilic form of AFB1 that may attack nucleophilic nitrogen, oxygen and sulphur heteroatoms in cellular constituents (Guengerich et al. 1996). This highly reactive substance may combine with DNA bases such as guanine to produce alterations in DNA (Hendrickse 1991). This may be the most important product from the carcinogenic point of view.

Formation of these adducts disrupts the normal working process of the cell and in the case of DNA adducts, can ultimately lead to a loss of control over cellular growth and division. Human metabolised AFB1 to the major aflatoxin B1-N7 guanine adducts at levels comparable to those in species, which are susceptible to aflatoxin-induced hepatocarcinogenicity such as the rat.

However, both humans and animals possess enzymes system, which are capable of reducing the damage to DNA and other cellular constituents caused by the 8,9-epoxide. For example glutathione-S-transferase mediates the reaction (termed conjugation) of the 8,9-epoxide to the endogenous compound glutathione. This essentially neutralizes its toxic potential. Animal species such as the mouse that are resistant to aflatoxin carcinogenesis have 3-5 times more glutathione-S-transferase activity than susceptible species such as the rat. Humans have less glutathione-S-transferase activity or 8,9-epoxide conjugation than rats or mice suggesting that humans are less capable of detoxifying this important metabolite.

Presence of AFB1-DNA adduct was identified both *in vivo* and *in vitro* (Groopman et al. 1980). The binding of AFB1 residues to DNA in vivo is essentially a linear function of dose at a given time after treatment. A modification level of 125-1100 AFB1 residues/10^7 nucleotides was observed in rat liver 2 hour after i.p. 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 hour after the AFB1 treatment (Groopman et al. 1980, 1988). For example maximum modification of rat liver DNA (1250 residues/10^7 nucleotides) was noted not later than 30 min after 1 mg AFB1/kg dose but declined to a level of 160 residues/10^7 nucleotides 36 hour after treatment, giving an apparent half-life of AFB1 binding to DNA of approximately 12 hour (Pohland and Wood 1987; Cullen and Newberne 1994; Groopman et al. 1996).

In circulation, aflatoxin binds with plasma proteins especially albumin to form aflatoxin-albumin adduct (Autrup et al. 1991). Sabbioni et al. (1987) have elucidated the structure of the major aflatoxin-albumin adduct found *in vivo*. The protein adduct by binding of the 8,9 epoxy aflatoxin initially forming dihydrodiol with sequential oxidation to dialdehyde and condensation with the S-amino group of lysine. This adduct is an Schiff base that undergoes Amadori arrangement to an µ-aminoketone. This protein adduct is a completely modified aflatoxin structure retaining only the coumarin and cyclopentenone rings of the parent compound. These adduct 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 adduct because the half-life of DNA

adduct is about 12 hour and then rapidly excreted in urine.

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 (B1, B2, G1, G2) as well as metabolized forms (aflatoxicol, M1 and M2) of aflatoxins get excreted in the urine, stool and milk (Coulter et al. 1986; De Vries et al. 1987; Verma and Chaudhari 1997). Verma and Chaudhari (1998) also reported presence of seven different types of aflatoxins (B1, B2, G1, G2, M1, M2, aflatoxicol) in the saliva of human beings. Aflatoxin excreted/secreted through saliva might be getting absorbed in gastrointestinal tract and passing again to the blood stream. This explains a sort of recycling of aflatoxin in the body.

Aflatoxin (0.35- 3.5 µg/ml) exposure to hepatocytes *in vitro* caused pronounced swelling, polymorphic condition, bleb formation and lysis (Raval and Verma 1997). Aflatoxin B1 is reported to induce cytotoxicity and transformation in culture cells (Schwartz and Perantoni 1975). Kaden et al. (1987) noted mutations besides toxicity as a result of AFB1 exposure to TK6 and HrM1 cells in culture. Cytotoxicity of aflatoxin on mouse hepatoma cell line HePa-1 was reported by Karenlampi (1987). 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). The earliest effect of aflatoxin is to reduce protein biosynthesis by forming adducts with DNA, RNA and protein, to inhibit RNA synthesis and DNA dependent RNA polymerase activity and to cause degranulation of the endoplasmic reticulum (Pohland and Wood 1987; Cullen and Newberne 1994; Groopman et al. 1996).

Intracellular calcium accumulation had been reported in the liver, kidney, testis, adipose tissue, heart and skeletal muscle of rabbits (Verma et al. 1998). Increased accumulation of calcium causes mitochondrial swelling (Rainbow et al. 1994) and reduced mitochondrial activity and ATP content (Toskulkao and Glinsukon 1988) thus impairing the operation of the sodium pump. Aflatoxin B1 preferentially attacks mitochondrial DNA during hepatocarcinogenesis vs. nuclear DNA (Niranjan et al. 1982). Mitochondrial DNA is protected in aflatoxicosis resistant rodents from DNA adducts that affect mitochondrial transcription and translation (Niranjan et al. 1986). The mycotoxin alters energy-linked functions of ADP phosphorylation and FAD and NAD-linked oxidizing substrates (Sajan et al. 1996) and a-ketoglutarate-succinate cytochrome reductases (Obasi 2001). It causes ultrastructural changes in mitochondria (Shanks et al. 1986; Rainbow et al. 1994) and also induces mitochondrial directed apoptosis (Pasupathy et al. 1999).

Several studies (Nicotera et al. 1992) have also 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 H2O2. Turrens et al. (1991) demonstrated that accumulation of calcium in mitochondria mobilized iron which in turn could stimulate the production of OH• from H2O2.

The results of Hoehler et al. (1996) suggest that ochratoxin A increases the permeability of the cell to calcium. They also indicated that both the enhanced cellular concentration of calcium and the presence of prooxidant OA uncoupled oxidative phosphorylation resulting increased leakage of electrons from the respiratory chain producing O_2• and hence H2O2. Lack of an adequate supply of NADPH and GSH to permit H2O2 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.

Possibly an increase in AFB1-8, 9-epoxide (an active metabolite of AFB1) cause significant increases in hepatic lipid peroxide level (Toskulkao et al. 1982). Peroxidation of membrane lipids initiated loss of membrane integrity; membrane bound enzyme activity and cell lysis (Younes and Siegers 1984; Toskulkao et al. 1982; Toskulkao and Glinsukon 1988). 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). Lipid peroxidation was significantly increased in the liver, kidney (Verma and Nair

1999) and testis (Verma and Nair 2000) of aflatoxin-treated mice as compared to controls. The oxidative damage in a cell or tissue occurs when the concentration of reactive oxygen species (O_2 •, H2O2, and OH •) generated exceeds the antioxidant capability of the cell (Sies 1991). Therefore, it could be due to significant decreases in the levels of non-enzymatic antioxidant (e.g. vitamin C, vitamin E, glutathione) and enzymatic antioxidants (superoxide dismutase, glutathione peroxidase and catalase), which are the main determinants of the antioxidant defence mechanism of the cell.

Superoxide dismutase protects cells from oxidative damage by breaking down a potentially hazardous free radical superoxide (O_2 •) to H2O2 and O_2 • The H2O2 produced can then be decomposed enzymatically by catalase and glutathione peroxidase (GSH-Px). GSH-Px not only decomposes H2O2 but can also interact with lipid peroxidation (Venkateswaran et al. 1967). The decline in these enzyme activities could be due to a reduction in protein biosynthesis. Glutathione levels declined significantly in the liver, kidney and testis after 45 days of aflatoxin treatment, which suggests its rapid oxidation. GSH can inhibit peroxidation, scavenge free radicals and protect cell membranes (Patel 1987). Thus significantly lower GSH levels would further aggravate the toxic effects of aflatoxin.

During the free radical scavenging action, ascorbic acid is transformed into L-dehydro-ascorbate (Eastwood 1997). Reduced glutathione is required for the conversion of L-dehydro-ascorbate back to ascorbate (Eastwood 1997). The fall in the level of reduced glutathione decreases the conversion of L-dehydroascorbate to ascorbate and this probably explained the lowered level of ascorbic acid in the aflatoxin-treated animals.

Glutathione has a beneficial effect by virtue of possessing –SH groups that help to protect biological membranes, which are readily susceptible to injury by peroxidation. Breimer (1990) reported that free radicals produced in biological membranes rapidly react with alpha tocopheryl radicals. Cytosolic GSH and ascorbic acid help in the regeneration of alpha tocopherol.

In addition, oxidative stress may result in damage to critical cellular macromolecules including DNA, lipids and proteins (Breimer 1990). Cellular fatty acids are readily oxidized by ROS to produce lipid peroxyl radicals which can subsequently propagate into MDA may result in the interaction with cellular DNA-MDA adducts (Shen et al. 1994, 1995). A time- and dose-dependent increase in 8-hydroxydeoxy-guanosine (8-OHdG) was observed in rat hepatic DNA after a single intraperitoneal injection of AFB1. It indicates that AFB1 causes oxidative DNA damage in rat liver that may involve hydroxyl radicals as the initiation species (Shen et al. 1995; Gradelet et al 1998). Therefore, factors interfering with the generation or action of hydroxyl radical would affect the formation of 8-OHdG. Proteins is also easily attacked by ROS directly or indirectly through lipid peroxidation modify their enzyme activity (Clayson et al. 1994).

There is considerable *in vitro* and *in vivo* evidence to support the view that humans possess the biochemical processes necessary for aflatoxin-induced carcinogenesis. Thus, the presence of DNA and protein aflatoxin adducts, urinary excretion of aflatoxin B1-N7-guanine adducts and the ability of tissues to activate aflatoxin B1 have all been demonstrated for humans. In addition, studies have suggested that oncogenes are critical molecular targets for aflatoxin B1. A high frequency of mutations at a mutational 'hotspot' has been found in p53 tumour suppressor genes in hepatocellular carcinomas from the patients residing in areas considered to offer a high risk of exposure to aflatoxins, and where there is a high incidence of hepatocellular carcinoma (Deng and Ma 1998).

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