Biomarkers of Aflatoxin Exposure and Its Relationship with the Hepatocellular Carcinoma

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1. Introduction

Mycotoxins are secondary metabolites produced by fungi that grow naturally in foodstuffs. They are able to generate a wide variety of toxic effects in vertebrates, including men (Coulombe, 1991). Toxigenic fungi may contaminate foodstuffs in the most different phases of production and processing, from cultivation to transport and storage. Mycotoxins show high chemical stability and may persist in the foodstuff even after fungi were removed by common manufacturing and packaging processes (Chu, 1991).

Diseases caused by mycotoxins are called mycotoxicoses. They are diffuse syndromes that cause lesions mainly in organs such as liver, kidneys, epithelial tissue (skin and mucous membranes) and central nervous system, depending on the type of the toxin. Two or more toxins may also occur simultaneously, leading to intensified toxic effects on the susceptible organism (Orsi et al., 2007).

Aflatoxins are mycotoxins produced by fungi in the genus Aspergillus, species A. flavus, A. parasiticus and A. nomius (Moss, 1998). These fungi are distributed worldwide, and their optimal growth conditions are relative humidity of 80-85% and temperature around 30°C (Coulombe, 1991).

Nowadays, 18 similar compounds are called aflatoxins. However, the most important in medical terms are types B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> e G<sub>2</sub> (Coulombe, 1991). Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), besides being the most frequently found in plant substrates, has the greatest toxigenic power. Aflatoxins B<sub>2</sub> (AFB<sub>2</sub>), G<sub>1</sub> (AFG<sub>1</sub>) and G<sub>2</sub> (AFG<sub>2</sub>) have about 50, 20 and 10% of AFB<sub>1</sub> toxigenic power, respectively (Leeson et al., 1995).

AFB<sub>1</sub> is a genotoxic compound, and is considered to be one of the most potent natural mutagens. Liver carcinogenesis is the most important effect of chronic aflatoxin exposure. This toxicity has been widely demonstrated – mainly in relation to AFB<sub>1</sub> - in many animal species, including fish, birds, rodents, carnivores and primates (Busby & Wogan, 1984). Based on available studies, the International Agency for Research on Cancer (IARC) concluded, in 1987, that there was enough evidence to classify AFB<sub>1</sub> in Group 1 - human carcinogen (Rothschild, 1992).

One of the most important aspects in risk analysis of chemical substances is to determine the degree of human exposure (World Health Organization [WHO], 2002), a particularly difficult task for contaminants present in foodstuffs. However, it is possible to indirectly estimate the degree of exposure based on data on consumption of contaminated foodstuffs, and on the average occurrence of the toxin. In this estimation, the degree of exposure is
measured in terms of probable daily intake (PDI) per unit of body weight, and is generally expressed in ng/kg of body weight (BW) / day. In risk analysis, PDI is compared with tolerable daily intake (TDI) determined in toxicological studies. In spite of the genotoxic characteristic of this toxin, there is no consensus on tolerable daily intake of AFB$_1$.

Taking into account aflatoxin toxicity and the lack of an established TDI, several countries determined regulations on maximum aflatoxin levels allowed in foodstuffs. Table 1 summarizes some data of a report by the Food and Agriculture Organization of the United Nations (FAO, 2004). It may be noted that the European Community and the Mercosur standardized their regulations, although some countries kept some food items with additional country regulations. Foodstuffs characteristic of each country, frequency of consumption of these items and climate characteristics apparently influence maximum limits adopted in each region, although there is a consensus that these limits should comply with the ALARA (as lowest as reasonable accepted) criterion recommended by the FAO (2004).

2. AFB$_1$ biotransformation

Biotransformation is a process by which the body transforms foreign substances (xenobiotics) in new chemical compounds (metabolites), that is, a process in which the initial compound is modified to be eliminated by the biological system (Guenguerich, 1999). After oral ingestion, AFB$_1$ is efficiently absorbed and biotransformed before urinary and fecal excretion (Figure 1).

Absorbed AFB$_1$ and its metabolites are excreted in urine and feces. Breastfeeding mothers who consume contaminated foodstuffs may also shed aflatoxins metabolites in their milk. Studies in animals demonstrated that in normal conditions, 50% of AFB$_1$ oral dose is quickly absorbed in the duodenum and reach the liver by the portal system (Wilson et al., 1985). AFB$_1$ is concentrated in the liver and, in lesser amounts, in the kidneys. It may also be found in mesenteric venous blood as free AFB$_1$ or as water-soluble metabolites (Wogan et al., 1967).

Enzymes of the cytochrome P450 (CYP) family, CYP1A2, CYP3A4 and CYP2A6, are responsible for the biotransformation of absorbed aflatoxins (Essigmann et al., 1982). These enzymes convert AFB$_1$ into its carcinogenic form, AFB-8,9-epoxide, which bonds covalently to DNA and serum albumin, producing AFB$_1$-N$^7$-guanine and lysine adducts, respectively (Essigmann et al. 1977; Sabbioni et al. 1987). The bond between AFB$_1$ and DNA modifies the structure and biological activity of DNA, leading to the basic mutagenic and carcinogenic mechanisms of the toxin. Studies with rat livers showed that AFB$_1$-N$^7$-guanine adducts may be removed after they are formed, leaving apurinic sites in the DNA molecule (Hsieh et al., 1991). Vacant sites tend to be filled with adenine, causing a guanine to thymine transversion and generating a highly significant point of mutation (Aguillar et al., 1993).

Besides being epoxided, AFB$_1$ can be also oxidized into several other derivatives. The main hydroxylated metabolites are aflatoxin M$_1$ (AFM$_1$), aflatoxin Q$_1$ (AFQ$_1$), a demethylated metabolite, aflatoxin P$_1$ (AFP$_1$), and a reduced metabolite, aflatoxicol (Figure 1). AFM$_1$ may be activated to form AFM$_1$-8,9-epoxide, which bonds to DNA and is excreted in urine as AFM$_1$-N$^7$-guanine (Egner et al, 2003). AFQ$_1$ and AFP$_1$ are not significantly oxidized by human microsomes, and are not considered to be genotoxic (Raney et. al, 1992). Metabolites AFM$_1$, AFQ$_1$ and AFP$_1$ are not good substrates for epoxidation, are less genotoxic than AFB$_1$, and consequently, are considered detoxification products. However, because of the high toxicity reported for AFM$_1$, researchers should be cautious when labeling this compound a “detoxification product” (Neal et al., 1998).
| Country                                    | Food Product                                                                 | Mycotoxin                        | Limit (µg/kg) |
|-------------------------------------------|-------------------------------------------------------------------------------|----------------------------------|---------------|
| United States                             | All foods except milk                                                         | AFB₁, AFB₂, AFG₁, AFG₂           | 20            |
|                                            | Milk                                                                          | AFB₁, AFB₂, AFG₁, AFG₂           | 0.5           |
| Canada                                    | Nuts and nut products                                                         | AFB₁, AFB₂, AFG₁, AFG₂           | 15            |
| Mercosur (Brazil, Argentina, Paraguay and Uruguay) | Peanuts, maize, and maize products                                          | AFB₁, AFB₂, AFG₁, AFG₂           | 20            |
|                                            | Fluid milk                                                                    | AFB₁, AFB₂, AFG₁, AFG₂           | 0.5           |
|                                            | Powdered milk                                                                 | AFM₁                             | 5             |
| Bosnia & Herzegovina                      | Wheat, maize, rice and cereals                                               | AFB₁, AFG₁                       | 1             |
|                                            | Beans                                                                         | AFB₁                             | 5             |
| China                                     | Maize and maize products, peanut and peanut products, peanut oil, irradiated peanut | AFB₁                             | 20            |
|                                            | Rice, irradiated rice, edible vegetable oil                                   |                                   | 10            |
|                                            | Soy bean sauce, grain paste, vinegar, other grains, beans, fermented foods, fermented bean products, starch products, fermented wine, red rice, butter cake, pastry biscuit and bread, food additive alpha-amylose, food additive glucoamylase preparation, salad oil |                                   | 5             |
| India                                     | All food products                                                             | AFB₁, AFB₂, AFG₁, AFG₂           | 30            |
|                                            | and AFM₁                                                                      |                                  |               |
| European Union                            | Groundnuts, nuts and dried fruits, processed products intended for direct human consumption or as ingredients in foodstuffs | AFB₁, AFB₂, AFG₁, AFG₂           | 2             |
|                                            |                                                                               |                                  | 4             |
| Australia and New Zealand                 | Peanuts and tree nuts                                                         | AFB₁, AFB₂, AFG₁, AFG₂           | 15            |
| Chile                                     | All foods                                                                     | AFB₁, AFB₂, AFG₁, AFG₂           | 5             |
|                                            | Milk                                                                          | AFM₁                             | 0.05          |
| Japan                                     | All foods                                                                     | AFB₁                             | 10            |
| Israel                                    | Nuts, peanuts, maize flour, figs and their products, and other foods          | AFB₁, AFB₂, AFG₁, AFG₂           | 5             |
|                                            |                                                                               |                                  | 15            |
| Italy                                     | Infusion plants                                                               | AFM₁                             | 0.05          |
|                                            |                                                                               | AFB₁                             | 5             |
|                                            |                                                                               | AFB₁, AFB₂, AFG₁, AFG₂           | 10            |
| Mexico                                    | Cereals and cereal products                                                   | AFB₁, AFB₂, AFG₁, AFG₂           | 20            |
|                                            | Corn flour for tortillas                                                      | AFM₁                             | 0.01          |

EU member states: Austria, Belgium, Denmark, Finland, France, Germany, Greece, Ireland, Italy, Luxembourg, the Netherlands, Portugal, Spain, Sweden, the United Kingdom

Table 1. Limits for mycotoxin contamination in food products destined for human consumption in different countries.
Source: Adapted from Mykkanen et al., 2005.

Fig. 1. Pathways of aflatoxin B<sub>1</sub> biotransformation and excretion in humans.
(a) Experimental and human evidence of excretion of this metabolite; (b) Scarce or no evidence available; (c) only experimental evidence available (no data for humans).
3. Role of aflatoxins in the etiology of hepatocellular carcinoma

Hepatocellular carcinoma (HCC) represents more than 80% of primary malignant tumors of the liver, and it is the 7th to 9th most common type of cancer worldwide affecting men and women, respectively. About 315,000 new cases of HCC are reported annually, a total of 4.1% of all malignant tumors in the world population. Although it is a relatively uncommon tumor, HCCs are aggressive, and mortality rates reach significant values, with about 312,000 deaths a year, and maximal survival rates of 5% in 5 years. Occurrence of HCC is associated with some degree of chronic liver disease in 90% of the cases, and it is an important cause of death in cirrhosis patients. HCC incidence has been growing, and may be directly related to the frequency of hepatitis C virus infection and longer survival of cirrhosis patients (Yang & Roberts, 2010).

HCC incidence in Africa and southeastern China is far greater than in the rest of the world. Besides known risk factors of western countries such as viral hepatitis and alcohol consumption, these populations are exposed to aflatoxin. The toxin is ingested in contaminated and stored foodstuffs, such as peanuts, maize, soybeans and rice. The association between AFB1 and HCC is based on the ability of the toxin to induce a specific mutation of gene p53 (Bressac et al., 1991).

In Brazil, HCC is not included among the ten most common types of cancers, probably because of underreporting. Estimates show that there are 2 to 3,000 diagnoses of the disease every year, with a national incidence of 1:100,000 inhabitants/year. Incidence of this neoplasm is greater in the north, northeast and southeast than in the south of the country. The greatest frequency occurs in the states of Amazonas, Bahia and Espírito Santo (Pimenta & Massabki, 2010). In São Paulo, incidence is a little greater than the mean of the country, affecting about 2:100,000 inhabitants/year. In terms of mortality, HCC is the 7th death cause and is responsible for 4% of the deaths by cancer in Brazil, annually. HCC incidence rate in Brazil is associated with advanced cirrhosis in 71.2% of the cases, as observed in the rest of the world. However, serology for viral hepatitis is negative in 42% of the cases HCC, even with regional discrepancies (Gonçalves et al., 1997). This difference may be related to the exposure to AFB1. This relationship, however, was not analyzed in the whole country.

Apparently, HCC progress is not an occasional event. Hepatocarcinogenesis seems to be a multifactorial process in which extrinsic stimuli induce gene changes in mature hepatocytes, leading to successive proliferation and cell death cycles that culminate in the production of monoclonal populations. Several lines of evidence suggest that hepatocarcinogenesis may begin in preneoplastic lesions, such as regenerative macronodules and low or high grade dysplastic nodules. Accumulation of genetic changes and new mutations in preneoplastic lesions would probably cause HCC (Theise et al., 2002).

In molecular terms, many derangements observed in HCC may be preferentially attributed to cirrhosis and inflammatory activity, and others are inherent to dysplastic nodules and to HCC itself. In early stages of chronic hepatitis, there are significant changes in the expression of growth factors, proteases and metalloproteinases, besides somatic changes, reduced apoptosis and increased expression of oncogenes and transcriptional factors. In general, these changes become more prominent and complex as the lesion progresses to fibrosis, cirrhosis, dysplastic nodules and, finally, HCC (Coleman, 2003). No tumor-suppressor gene exclusively associated with HCC has been identified. However, all molecular changes accumulated by chronic hepatitis and cirrhosis in repeated aggression / regeneration cycles, directly contribute to hepatocarcinogenesis (Fausto & Weber, 1993). Aneuploidy is also a common event in hepatocarcinogenesis. HCC is characterized by a considerable loss of heterozygosity, and includes several chromosomes, such as 1p, 4q, 6q, 8p, 8q, 9p, 13q, 16p, 16q, and 17p. Mutations
in several critical genes, such as p73, p53, Rb, APC, DLC-1 (deleted in liver cancer), p16, GSTP1, PTEN, IGF-2, BRCA2, SOCS-1, Smad2 and Smad4, \( \beta \)-catenine, c-myc, and cyclin D1 were also identified (Fujimori et al., 1991; Tsuda et al., 1992).

Impaired control of cell cycle is an important event in carcinogenesis. The first observations involving carcinogenesis and cyclins were related to detection of the incorporation of Hepatitis B virus DNA to cyclin A gene in HCC (Wang et al. 1990), and to amplification of cyclin D1 gene in some cell lineages of colon carcinoma (Leach et al., 1993). The p16/cyclin D1/RB pathway (retinoblastoma) may be considered the greatest cell cycle regulator. RB and p16 act as tumor suppressor genes, and cyclin D1 as an oncogene (Weinberg, 1995; Ito et al., 1999). Aberrant expression of both cyclin-dependent kinases (CDK) and CDK-inhibitors has an important role in HCC development. High expression of cyclin D1 in HCC is variable, ranging from 6 to 76% in different studies. Among positive regulators of cell cycle, changes in cyclin D1, A and B1 expression compared with normal tissues have been associated with increased cell growth and development of neoplasms (Ito et al., 1999).

Analysis of aberrant expression of cyclin D1, its biological role and its relationship with mutations in p53 in cases of HCC demonstrated that cyclin D1 of was normally expressed in healthy livers, but it was highly reduced in 40% of the livers affected by HCC (Peng et al., 1998). Lower expression of cyclin D1 RNAm was associated with larger and less differentiated tumors. Increased expression of cyclin D1 was observed in only 5.6% of the cases. On the other hand, cyclin E shows increased expression in 56% of the HCC cases. Overexpression of cyclin E was associated with little differentiation and with invasiveness, but not with tumor volume. Thus, decreased expression of cyclin D1 and increased expression of cyclin E are intimately associated with mutation in p53. Besides, overexpression of cyclin E and concomitant loss of p53 function seem to contribute to HCC progression (Peng et al., 1998; Jung et al., 2001).

There are three important inhibitors of cell cycle progression in the Cip/Kip family: p27Kip1, p21Waf1, and p57Kip2. The most comprehensively studied of these inhibitors, in terms of clinical significance in the evolution of human cancer, is p27Kip1. Expression of p27Kip1 is marked in non-proliferating cells, and it has important roles in the regulation of both quiescence and progression in G1 phase, by means of inhibition of cyclin / CDK complexes. Loss of p27Kip1 acts together with mutations of several oncogenes and suppressor genes, stimulating tumor growth. Reduced production of the protein synthesized by p27Kip1 is significantly involved in the stage and volume of primary tumors. Thus, p27Kip1 has been described as a crucial negative regulator of HCC progression. Its increased expression is considered an independent variable in favorable prognosis of HCC (Ito et al., 2001; Fiorentino et al., 2000). Reduced p21Waf1 expression is mainly related to mutation in gene p53 in HCC, and also contributes to hepatocarcinogenesis. However, p21Waf1 loss was not identified as an independent factor in HCC bad prognosis (Ito et al., 2001). Compared with healthy livers, expression of p57Kip2 is significantly decreased in HCC lesions. This decreased expression of p57Kip2 was associated with highly aggressive tumors, characterized by more advanced stages, little differentiation, larger size, portal invasion, intense cellular growth and low disease-free survival rates (Ito et al., 2001).

In terms of frequency, the most common molecular changes observed in HCC cases are p53 (20-70%), cyclin D (11%), p16Ink4 (0-50%), Rb (15%) and \( \beta \)-catenine (16-26%), from which only p53 mutation was reported to be associated with hepatitis B virus gene interaction and exposure to aflatoxin (Ozturk, 1999). Although mutations in p53 pathway have an important role in HCC pathogenesis in cases of cirrhosis, changes in cell cycle regulator genes p21waf1/cip1 and p27Kip1 are more involved in...
HCC cases unrelated to cirrhosis (Tretiakova et al., 2010). These cases could be, in part, attributed to aflatoxin. Therefore, other molecular changes in genes p21waf1/cip1 and p27Kip1 should also be assessed in individuals exposed to aflatoxin.

4. Biomarkers of aflatoxin exposure

Biomarkers measure cellular, biological or molecular changes in biological tissues, cells or fluids, providing information on disease or exposure to a given substance. As biomarkers are used to measure or indicate biological processes, detection of specific biomarkers may aid identification, diagnosis and treatment of individuals who are affected and at risk, but still asymptomatic. Development of biomarkers for environmental agents should be based on specific knowledge on metabolism, formation of by-products and general mechanism of action (Groopman & Kensler, 1999).

Biomarkers may be classified in four categories: internal dose, biologically effective dose, early biological response, and altered structure/function. (Figure 2). Internal dose is the amount of substance that is metabolized. Individual characteristics determine susceptibility to exposure, such as the ability to activate / detoxify carcinogens, ability to repair DNA changes, nutritional status and immunity, age, sex and socioeconomic status (WHO, 1993).

Biomarkers of exposure and effect for aflatoxins have been validated in comprehensive studies in animals and humans. Dose-response relationship between AFM1 and AFB1-N7-guanine levels and incidence of liver tumors was first established in animals (Groopman et al., 1992b). Biomarkers were then evaluated in humans to determine sensitivity, specificity, accuracy and reliability parameters. Later validation in epidemiological studies evaluated intra and inter-subject variability, the relationship biomarker–external dose and the feasibility of using them in large population studies (Groopman et al., 1992a; Groopman et al., 1992c).

In a study carried out in Shanghai, People’s Republic of China, 18,244 volunteers were followed up for three years. The analysis included individual interviews on eating habits, possible previous exposure to aflatoxins, and collection of urine samples (Ross et al., 1992; Qian et al., 1994). Cases and controls were compared to detect associations between aflatoxin markers, infection by Hepatitis B virus (HBV), and hepatocellular carcinoma. Data showed a 340% increase in the relative risk for HCC when aflatoxin biomarkers were detected in urine. Relative risk in individuals showing positive results for HBV was 730% greater. Subjects who showed aflatoxin markers in urine and were positive for HBV infection had a relative risk of developing HCC 5,900% greater. These data support the relationship between the two major causes of HCC: HBV infection and exposure to AFB1. Besides, when individual metabolites were stratified for HCC incidence, the presence of AFB1-N7-guanine adduct led to 200 to 300% increase in the relative risk of developing HCC.
After the Shanghai study, a trial developed in Taiwan with about 15,000 volunteers also analyzed the relationship between HBV, exposure to AFB₁, and incidence of HCC; the results of this trial confirmed the findings of the previous study (Yu et al., 1997). Risk of developing HCC along with AFB₁ exposure was more pronounced among those individuals infected by HBV and with detectable levels of AFB₁-N⁷-guanine in their urine.

5. Occurrence of aflatoxin biomarkers in biological fluids

In past decades, several studies reported the presence of aflatoxins, metabolites and biomarkers in urine (Table 2). Zhu et al. (1987) analyzed 252 urine samples from inhabitants of Guangxi province, People’s Republic of China, and reported a correlation between total daily ingestion of AFB₁ and excretion of AFM₁. Between 1.2 and 2.2 of AFB₁ ingested daily was found in urine as AFM₁, in levels ranging from 0 to 3.2 ng/mL. In a later study, the same urine samples were analyzed again and levels of AFB₁-N⁷-guanine adduct were also correlated with AFB₁ ingestion (Groopman et al., 1992c). Total amounts of AFB₁-N⁷-guanine excreted in urine in a three-day period ranged from < 50 and 3250 ng and about 0.2% of AFB₁ ingested was excreted in urine as AFB₁-N⁷-guanine. In the same study, levels of the metabolite AFP₁ did not show a significant statistical correlation between dietary exposure and excretion in urine, and the metabolite AFQ₁ was observed in few samples. Percentage of AFB₁ excreted in urine as any of these metabolites was 4.4% in women and 7.6% in men. In another study, also carried out in Guangxi province, AFB₁-lysine adduct was determined in serum samples of 42 inhabitants and compared with AFB₁ ingestion and AFM₁ excretion in urine (Gan et al., 1988). Significant correlation coefficients were found between AFB₁-lysine levels in serum and AFM₁ in urine, and between AFB₁-lysine in serum and dietary exposure to AFB₁. It is estimated that 1.4 and 2.3% of AFB₁ ingested is covalently bound to albumin.

Qian et al. (1994) detected 55 cases of hepatocellular carcinoma. From these cases, urine samples of 50 individuals and 267 control samples were analyzed for levels of AFB₁-N⁷-guanine, AFM₁, AFP₁ and AFB₁. The metabolite detected in the greatest concentration was AFP₁, (0.59-16.0 ng/mL), whereas AFM₁ ranged from 0.17-5.2 ng/mL, and 0.3 to 1.81 ng/mL for AFB₁-N⁷-guanine adduct.

Wild et al. (1992) carried out a study with 20 individuals in Gambia, West Africa, and also confirmed the validity of AFB₁-lysine as a biomarker. Parallel evaluation of the same individuals by Groopman et al. (1992a) for AFB₁-N⁷-guanine in urine, confirmed not only the correlation between this metabolite and AFB₁ ingestion, but also demonstrated the correlation between levels of AFB₁-lysine in serum and AFB₁-N⁷-guanine in urine. AFG₁ was the most frequent metabolite observed in urine, as a consequence of the high concentration of aflatoxin found in the foodstuffs consumed by the individuals analyzed, compared with other studies in which the diet analyzed did not have AFG₁. Besides, metabolites AFQ₁ and AFP₁ were also determined, and AFM₁ was observed in some samples. Levels of AFB₁-N⁷-guanine adducts in urine (Groopman et al., 1992a; Groopman et al., 1992c) and AFB₁-lysine in blood (Gan et al., 1988) show the biological effective dose of aflatoxin to which the individual has been exposed. Concentration of AFB₁-N⁷-guanine in urine shows exposure to AFB₁ in a 1 to 2-day period, whereas concentration of AFB₁-lysine in serum indicates 2 to 3-month exposure (Wild et al., 1992).

Urinary and fecal excretion of metabolites AFQ₁ e AFM₁ and urinary excretion of AFB₁-N⁷-guanine were evaluated in 83 university students in China (Mykkanen et al., 2005). Mean fecal AFQ₁ concentration (137 ng/g, moist weight) was about 60 times greater than mean concentrations in urine of the same individuals.
AFM$_1$ concentration (2.3 ng/g, moist weight). In urine, mean AFQ$_1$ concentration was 10.4 ng/mL, and 0.04 ng/mL and 0.38 ng/mL for AFM$_1$ and AFB$_1$-N$^7$-guanine, respectively. The authors emphasized that, compared with other studies, differences in concentrations and frequencies of AFQ$_1$ and AFM$_1$ in their study may be attributed to differences in age and diet of the subjects. Participants of this study were young adults, 18-24 years of age, whereas in previous trials, individuals were 25 to 65 years old. Expression of CYP3A enzymes, which produce AFQ$_1$, decreases about 25-40% with age in animals and humans, and consumption of foodstuffs rich in flavonoids, such as green tea, may increase AFQ$_1$ formation by activation of these enzymes.

In Brazil, Scussel et al. (2006) evaluated the presence of AFB$_1$-lysine adduct in blood samples of 50 subjects in the city of Sao Paulo, in 1999. The adduct was detected in 62% of the samples, in a concentrations ranging from 0 – 57.3 pg AFB$_1$-lysine/ mg blood albumin. Mean concentration in positive samples was 14.9 pg/mg. Sixty-five urine samples from inhabitants of the city of Piracicaba, state of Sao Paulo, were analyzed for AFM$_1$ and 65% of them showed concentrations greater or equal to 1.8 pg/mL, with mean concentration of 5.96 pg/mL (Romero et al., 2010). Correlation between probable aflatoxin intake - estimated by means of questionnaires on the frequency of consumption – and AFM$_1$ levels in urine were not significantly correlated.

AFM$_1$ is also excreted in milk during lactation, and several studies demonstrated the presence of this metabolite in human milk. In the Arab Emirates, AFM$_1$ was detected in milk in concentrations ranging from 5 to 3400 pg/mL (Abdulrazzaq et al., 2003). In Australia, AFM$_1$ levels ranged from 28 to 1031 pg/mL, and in Thailand, from 39 to 1736 pg/mL (El-Nezami et al., 1995). In a study carried out in Gambia (Zarba et al., 1992), 0.09 to 0.43 % AFB$_1$ ingested in the diet was excreted in milk as AFM$_1$. In Brazil, this metabolite was studied in samples collected from human milk banks. From 50 samples analyzed, only one was contaminated by AFM$_1$ at a concentration of 0.024 ng/mL (Navas et al., 2005). In a recent study carried out with 160 lactating mothers in Iran, AFM$_1$ was detected in 157 samples, with concentrations ranging from 0.3 to 26.7 ng/kg (Sadeghi et al., 2009).

Aflatoxins were also detected in samples of umbilical cord blood, demonstrating they can cross the placenta, starting exposure to this carcinogen in the uterus (Wild et al., 1991; Turner et al., 2007).

Quantitative determination of several metabolites in complex matrices, such as serum and urine, requires specific and sensitive methods for a large number of samples. Particularly for AFB$_1$-lysine adduct in serum, methods may include radioimmunoassay (RIA; Gan et al., 1988), enzyme linked imunosorbent assay (ELISA; Wild et al., 1992), or purification with immunoaffinity columns followed by separation by high performance liquid chromatography (HPLC) and detection by fluorescence (Wild et al., 1992; Wang et al., 1996). As all these methods require antibodies for detection and/or purification, results will necessarily reflect the capacity, specificity and/or sensitivity of the antibody (Wang et al., 2001). Results obtained using ELISA, RIA and fluorescence were significantly different (Sheabar et al., 1993; Wild et al., 1990). ELISA is highly sensitive, but it is less specific and shows higher concentration of AFB$_1$-lysine due to the concomitant detection of adducts from reactions with other amino acids and ingestion of aflatoxins of similar structure, such as AFG$_1$. HPLC-fluorescence is specific for AFB$_1$-lysine, but it is not sensitive enough for epidemiological studies.

A recently developed method combines solid phase extraction and liquid chromatography-mass spectrometry (HPLC-MS/MS), showing high specificity and sensitivity (McCoy et al., 2005). The method uses a stable isotope internal standard to correct recovery and equipment variability. This method showed to be adequate for routine quantification of adducts in human serum (Scholl et al., 2006b).
| Sample/Country                   | Aflatoxin and metabolite | No. samples | % positive samples | Level *          | Ref.                      |
|---------------------------------|--------------------------|-------------|--------------------|------------------|--------------------------|
| Urine/Brazil                    | AFM₁                     | 69          | 65                 | 5.96 pg/mL       | 1.8-39.9 pg/mL           | Romero et al. (2010)     |
| Urine/Bosnia and Herzegovina    | AFB₁                     | 30          | 100                | N.S.             | 0.05-0.26 µg/kg          | Aljicevic & Hamzic (2010) |
| Urine/Bosnia and Herzegovina    | HCC patients             | 30          | 100                | N.S.             | 0.05-0.15 µg/kg          | Aljicevic & Hamzic (2010) |
| Urine/China                     | AFM₁                     | 145         | 54                 | NS               | 0.003-0.243 ng/mL        | Sun et al. (1999)        |
| Urine/China                     | AFM₁                     | 42          | NS                 | NS               | 0.01-3.2 ng/mL           | Zhu et al. (1987)        |
| Urine/China                     | AFM₁                     | 29          | 89                 | 192 ng/day       | 0.9-3569 ng/day          | Wang et al. (2001)       |
| Urine/China                     | AFB₁-N⁷-G                | 41          | 407                | 64.9-1789 ng/day |                         |                          |
| Urine/China                     | AFB₁-Merc                | 89          | 103                | 6.6-494          |                         |                          |
| Urine/China                     | AFQ₁                     | 26          | 92.2               | 77.3-137         |                         |                          |
| Urine/China                     | AFP₁                     | 30          | 664                | 80.4-3569        |                         |                          |
| Urine/China (placebo)           | AFB₁-N⁷-G                | 39          |                    |                  |                         | El-Nezami et al (2006)   |
| Urine/China (intervention with probiotics) | AFB₁-N⁷-G | 44          |                    |                  |                         |                          |
| Urine/China (intervention with GTP) | AFM₁                  | 352         | 100                |                  |                         | Tang et al. (2008)       |

Level * includes Mean and Range.
| Sample/Country | Aflatoxin and metabolite | Mean | Range |
|----------------|--------------------------|------|-------|
|                | 500 mg GTP               | crea | crea  |
|                | 1000 mg GTP              | crea | crea  |
| 1st month      | Placebo                  | 61.67| 0.52-881.39 |
|                | 500 mg GTP               | 15.03| 0.38-64.27 |
|                | 1000 mg GTP              | 20.06| 0.77-51.50 |
| 3rd month      | Placebo                  | 78.66| 0.24-1276.25 |
|                | 500 mg GTP               | 16.12| 0.18-222.35 |
|                | 1000 mg GTP              | 25.95| 0.12-338.85 |
| AFB\(_1\) - Merc | Beginning               |      |       |
|                | Placebo                  | 8.67 pg/mg | 0.43-41.15 pg/mg |
|                | 500 mg GTP               | 10.31| 0.38-50.77 |
|                | 1000 mg GTP              | 9.32 | 0.60-67.71 |
| 1st month      | Placebo                  | 9.95 | 0.09-57.92 |
|                | 500 mg GTP               | 79.53| 1.57-362.47 |
|                | 1000 mg GTP              | 79.48| 0.30-465.62 |
| 3rd month      | Placebo                  | 6.11 | 0.43-50.58 |
|                | 500 mg GTP               | 97.76| 11.32-501.48 |
|                | 1000 mg GTP              | 96.60| 18.20-560.30 |
| Urine/China    | Total aflatoxin (AFB\(_1\)-N\(_7\)-G, AFP\(_1\), AFB\(_1\), AFQ\(_1\)) | 42   | NS    |
|                | NS                       | NS   | 1.5-2.3 ng/mL |
|                | NS                       | NS   | 3300-6600 ng/day |
| Urine/China    | AFM\(_1\)               | 317  | 67    |
|                | NS                       | NS   | 0.17-5.2 ng/mL |
|                | AFB\(_1\)-N\(_7\)-G      | 49   | NS   |
|                | NS                       | NS   | 0.3-1.81 |
|                | AFP\(_1\)                | 53   | NS   |
|                | NS                       | NS   | 0.59-16 |
|                | AFB\(_1\)                | 71   | NS   |
|                | NE                       | NE   | NE   |

Ref. Groopman et al (1992c)
| Sample/Country       | Aflatoxin and metabolite | No. of samples | % positive samples | Level * |
|----------------------|--------------------------|----------------|--------------------|---------|
|                      |                          |                |                    | Mean    | Range            | Ref.                      |
| Urine/China          | AFM<sub>1</sub>          | 83             | 83                 | 0.04 ng/mL | 0.01-0.33 ng/mL | Mykkanen et al. (2005)    |
|                      | AFQ<sub>1</sub>          |                |                    | 10.4     | 3.4-23.3        |                          |
|                      | AFB<sub>1</sub>-N<sub>7</sub>-G |                |                    | 0.38     | 0.0-2.15        |                          |
| Urine/Egypt          | AFB<sub>1</sub>          | 20             | 30                 | NS       | <1.5 ng/mL      | Al-Saadany (1993)         |
|                      | AFM<sub>1</sub>          |                |                    | NS       | <2.5 ng/mL      |                          |
|                      | AFG<sub>1</sub>          |                | 1.1<sup>b</sup>   |          |                |                          |
| Urine/Egypt          | AFB<sub>1</sub>          | 60             | 61                 | NS       | 0.01-0.15 ng/mL | Hatem et al. (2005)       |
| Urine/Egypt (Children 1-2.5 years old) | AFB<sub>1</sub> | 50             | 38                 | 189<sup>b</sup> pg/mL | - pg/mL | 0.8-2.2 | Polychonaki et al. (2008) |
|                      | AFB<sub>2</sub>          |                |                    | 1.4      | 72.1-81.1       |                          |
|                      | AFG<sub>1</sub>          |                |                    | 76.6     |                |                          |
|                      | AFG<sub>2</sub>          |                |                    | 2.2      | 9.9-8.0         |                          |
|                      | AFM<sub>1</sub>          |                |                    | 5.5      | 5.0-6.2         |                          |
| Guinea (Children 2-4 years old) | AFB<sub>1</sub> | 50             | 86                 | 2682 pg/mL | 179-18000 pg/mL | Groopman et al (1992b)    |
|                      | AFB<sub>2</sub>          |                |                    | 5.7      | 0.6-43          |                          |
|                      | AFG<sub>1</sub>          |                |                    | 709<sup>b</sup> | -       | 1.4-199 |                          |
|                      | AFG<sub>2</sub>          |                |                    | 19.0     | 8.0-801         |                          |
|                      | AFM<sub>1</sub>          |                |                    | 97.0     |                |                          |
| Urine/Gambia         | AFB<sub>1</sub>-N<sub>7</sub>-G (AFG<sub>1</sub>, AFM<sub>1</sub>, AFB<sub>1</sub>, AFQ<sub>2</sub>; also detected) | 20             | NS                 | NS       | 48.2-7099 ng/day     | Groopman et al (1992b)    |
| Urine/United States  | AFB<sub>1</sub>-lys      | 184            | 20.6               | 3.84 pg/mg alb | 1.01-16.57 pg/mg alb | Johnson et al. (2010)     |
|                      | AFM<sub>1</sub>          |                | 11.7               | 223.85 pg/mg crea | 1.89-935.49 pg/mg alb |                          |
| Serum/Benin          | AFB<sub>1</sub>-alb      | 480            | 99                 | NS       | 5-1064 pg/mg alb | Gong et al. (2002)        |
| Serum/Brazil         | AFB<sub>1</sub>-alb      | 50             | 62                 | 14.9 pg/mg alb  | 0-57.3 pg/mg alb   | Scussel et al. (2006)     |
| Serum/Egypt          | AFB<sub>1</sub>          | 60             | 61                 | NS       | 0.04-0.69 ng/mL  | Hatem et al. (2005)       |
| Serum/Egypt          | AFB<sub>1</sub>          | 20             | 55                 | NS       | <4.5 ng/mL      | Al-Saadany (1993)         |
|                      | AFM<sub>1</sub>          |                |                    | NS       | <0.5            |                          |
|                      | AFM<sub>2</sub>          |                |                    | 0.2<sup>c</sup> ng/mL | -       |                |                          |
Table 2. Aflatoxins and metabolites in human urine and serum. (a) The unit is expressed only in the first row; (b) Only one positive sample; (c) *Lactobacillus rhamnosus* LC705 and *Propionibacterium freudenreichii* subsp. *shermanii* (1:1, m:m), 2 – 5 x 10^10 colony forming units/d. NS, not specified; GTP, green tea polyphenols; alb, albumin; crea, creatinine.

Methods used to determine AFB1-N7-guanine adduct include immunoassays (Groopman et al., 1992a), HPLC with UV detection (Groopman et al., 1992c), or fluorescence (Wang et al., 1999; Mykkanen et al., 2005). Egner et al. (2006) described a method using HPLC-MS/MS in the analysis of AFB1-N7-guanine in urine, also based on the use of stable isotope internal standards.

| Sample/Country | Aflatoxin and metabolite | no. samples | % positive samples | Level * | Ref. |
|----------------|--------------------------|-------------|-------------------|--------|-----|
| Serum/Gambia   | AFB1-alb                 | 20          | NS                | 44 pg/mg alb | NS    | Wild et al. (1992) |
| Serum/Gambia   | AFB1-alb                 | 357         | 100               | 83.2 pg/mg alb | NS    | Wild et al. (2000) |
| July/Aug       | AFB1-alb                 | 64          | 100               | 0.9972 pmol/mg alb | 0.3325-2.2703 pmol/mg alb | Jiang et al. (2005) |
| Serum/China    | AFB1-alb                 | 444         | 100               | NS                | 2.2-459 pg/mg alb | Turner et al. (2000) |
| Serum/Gambia   | AFB1-alb                 | 117         | 100               | 29.3 pg/mg alb | 2.2-254 pg/mg alb | Wild et al. (1993) |
| Serum/Guinea   | AFB1-alb                 | 600         | 95                | NS                | 9.4-22 pg/mg alb | Sylla et al. (1999) |
| Serum/Ghana    | AFB1-alb (pregnant women)| 755         | 100               | 10.9 pg/mg alb | 0.44-268.73 pg/mg alb | Shuaib et al. (2010) |
| Serum/Ghana    | AFB1-alb                 | 507         | 100               | 0.94 pmol/mg alb | 0.1-4.4 pmol/mg alb | Tang et al. (2009) |
| Serum/Gambia   | AFB1-alb                 | 119         | 100               | 38.9 pg/mg alb | 23.3-64.1 pg/mg alb | Turner et al. (2007) |
|                | Mothers                  |             |                   |                    |                   |                   |
| Serum/China    | AFB1-alb                 | 99          | 48.5              | 2.5               | 2.5-7.9 |               |
|                | Children (4 months)      | 118         | 11                | 2.5               | 2.5-2.5 |               |
| Serum/China    | AFB1-alb                 | 42          | NS                | NS                | 30-340 pg/mg alb | Gan et al. (1988) |
standard. Precision and accuracy were far superior than previous procedures. Together with the analysis of AFB1-lysin, determination of these two biomarkers in urine and serum samples is precise, accurate, specific and selective. Determination of residual aflatoxin and metabolites AFM₁, AFP₁ and AFQ₁ in urine has been carried out using HPLC-fluorescence (Tang et al., 2008; Polychronaki et al., 2008; Romero et al., 2010). However, HPLC-MS/MS has recently been used successfully to determine AFB1, AFB2, AFG1, AFG2, AFM1 and AFP1 in urine (Everley et al., 2007).

6. Concluding remarks

Current concepts derived from intensive research on biotransformation, mechanisms of toxicity and evidence of the role of aflatoxins in the etiology of human liver cancer were summarily presented in this chapter. AFB1 exerts its effects after conversion to the reactive compound AFB1-epoxide by means of cytochrome P450-dependent enzymes. This epoxide can form derivatives with cellular macromolecules, including proteins, RNA and DNA. Reaction with DNA occurs with guanines in codon 249 of tumor suppressor gene p53. Although mutations in p53 pathway have an important role in HCC pathogenesis other molecular changes in genes p21waf1/cip1 and p27kip1 should also be assessed in individuals exposed to aflatoxin. Primary biotransformation of AFB1 also produces hydroxylated and less toxic derivatives, such as aflatoxins Q₁ and P₁. Intra and interspecies differences in the pathways of activation/detoxification are directly related to the susceptibility of animals to aflatoxin effects. In humans, individual biomonitoring of AFB1 metabolites such as AFB1-N⁷-guanine have demonstrated that aflatoxins constitute an important risk factor for hepatocellular carcinoma in highly exposed populations. Some of these studies also show synergism between aflatoxins and hepatitis B virus in the development of human HCC. Based on these concepts, and taking into account the frequent detection of aflatoxins in foodstuffs worldwide, further investigations are needed to assess the level of dietary exposure to these toxins and its impact on human health.

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Aflatoxins – Biochemistry and Molecular Biology is a book that has been thought to present the most significant advances in these disciplines focused on the knowledge of such toxins. All authors, who supported the excellent work showed in every chapter of this book, are placed at the frontier of knowledge on this subject, thus, this book will be obligated reference to issue upon its publication. Finally, this book has been published in an attempt to present a written forum for researchers and teachers interested in the subject, having a current picture in this field of research about these interesting and intriguing toxins.

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