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Aflatoxin B_1 residues in human livers and their relationship with markers of hepatic carcinogenesis in São Paulo, Brazil

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ABSTRACT

In this study, hepatic biopsies from autopsy cases in São Paulo, Brazil, showing hepatocellular carcinoma (HCC, $n = 8$), cirrhosis associated with viral hepatitis (VC, $n = 20$), cirrhosis associated with alcoholism (AC, $n = 20$), and normal livers (NL or controls, $n = 10$) were subjected to determination of aflatoxin B_1 (AFB_1) and its main metabolites, and of markers of hepatic carcinogenesis Only non-metabolized AFB_1 was detected in 13 samples (27.1%, $N = 48$) of liver disorders (HCC, VC and AC), at levels between 10.0 and 418.0 pg/g (mean: 76.6 ± 107.7 pg/g). Immuno-labeling of p53, cyclin D1, p21, β-catenin, and Prohibitin (PB) increased mainly in HCC patients, in relation to the controls. AFB_1+ samples of HCC presented higher expressions of p53, cyclin D1, p21, and β-catenin compared with AFB_1-livers. In contrast, p27, p16, and Rb immuno-labeling decreased in HCC, VC, and AC samples, compared with NL, with lowest values in AFB_1+ samples for all liver disorders. Compared with NL, gene expression of cyclin D1 and PB in AFB_1+ samples of HCC and AC were also higher, along with higher gene expression of p21 in VC and AC AFB_1+ livers. Results indicated that patients with liver disorders were exposed to dietary aflatoxins, and that residual AFB_1 in liver negatively affected the p53 and protein Rb pathways in HCC. Moreover, the presence of AFB_1 in cirrhotic livers warrants concern about the potential contribution of dietary aflatoxin to disease progression during VC and AC.

1. Introduction

Aflatoxins are carcinogenic secondary metabolites produced by fungi in the genus *Aspergillus*, especially the species *A. flavus, A. parasiticus,* and *A. nomius*, which naturally grow in food products (Murphy et al.). There are 20 known similar compounds called aflatoxins, although the main types of interest in terms of health are B_1, B_2, G_1, and G_2 [1]. Aflatoxin B_1 (AFB_1), besides being the most frequently one found in plant substrates, is the one with the highest toxigenic power [2]. The toxin is ingested with contaminated food, mainly foodstuffs that undergo storage, such as peanuts, corn, beans, and rice. AFB_1 is genotoxic, and is considered one of the most potent natural mutagenic agents. Involvement in liver carcinogenesis is the most important effect of aflatoxin chronic toxicity [3]. Brazilian regulations determine maximum levels of aflatoxins (sum of AFB_1, AFB_2, AFG_1, and AFG_2) ranging from 1 to 20 μg/kg [4] in several foodstuffs.

Aflatoxins are absorbed in the gastrointestinal tract and primarily biotransformed in the liver by means of microsomal enzymes from the cytochrome P450 mixed function oxidase system [5]. AFB_1 requires metabolic activation to show its toxic effects., and its carcinogenic form is the highly active electrophilic metabolite AFB_1 8,9-oxide or AFB_1-epoxide, which originates from the epoxidation of the vinyl-ether double bond found in the di-furan structure of AFB_1 molecule [6,7]. AFB_1 activated form quickly reacts with covalent bonds of macromolecules, such as cellular deoxyribonucleic acid (DNA), ribonucleic acid (RNA), as well as proteins [6]. The bond with DNA leads to the production of adducts with guanine in the N_7 position at codon 249 of p53 tumor suppressor gene. Besides epoxidation, AFB_1 biotransformation includes hydroxylation originating aflatoxins M_1 (AFM_1), Q_1 (AFQ_1), and B_{2a} ($AFB_{2\alpha}$), as well as *O*-demethylation, originating aflatoxin P_1 (AFP_1), which may undergo conjugation with glucuronic acid or sulfates, and is excreted in urine, bile, and feces [8]. Aflatoxicol (AFL) can also be formed by the reduction of AFB_1 by means of an NADPH-dependent cytoplasmic enzyme found in the soluble fraction of

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liver homogenates [9]. All these biotransformation products of AFB_1 may remain in the form of residues in the liver, as observed in several species [10]. These residues are considered markers of toxin exposure in the diet.

Hepatocellular carcinoma (HCC) is the most frequent malignant neoplasm found in the liver, and the third most important cause of cancer deaths in the world. Greater occurrence of HCC is associated with the presence of chronic liver disease in up to 90% of the cases [11]. Africa and China have the greatest incidence of HCC compared with the rest of the world due to aflatoxin exposure, besides the known risk factors of western countries, such as viral hepatitis and alcoholism. In Brazil, HCC is now the 7th cause of cancer death. However, different from the pattern in the rest of the world, 42% of the HCC cases in Brazil do not show positive serology to viral hepatitis, and there are regional differences [12]. These differences may correspond to AFB_1 exposure, taking into account the association between AFB_1, HCC the induction of a specific mutation in p53 gene [13]. However, this relationship has not been investigated in Brazil, and there are no previous studies on the occurrence of AFB_1 residues in human livers in this country.

Hepatocarcinogenesis seems to be a multifactorial process in which extrinsic stimuli induce gene changes in mature hepatocytes, leading to successive proliferation and death, and culminating in the production of monoclonal populations. Evidence suggests that hepatocarcinogenesis may begin with pre-neoplastic lesions, such as regenerative macronodules and hepatic nodules with low or high-grade dysplasia. Therefore, the accumulation of genetic changes and new mutations in pre-neoplastic lesions may probably cause HCC [14]. Mutations were identified in several critical genes involved in hepatocarcinogenesis, such as p73, p53, Rb, APC, DLC-1 (deleted in liver cancer), p16, GSTP1, PTEN, IGF-2, BRCA2, SOCS2, Smad2 and Smad4, ß-catenin, c-my + c, and cyclin D1 [15,16]. The p53 may also be related with other tumor suppressing gene, Prohibitin (PB). PB may be found in the cytoplasm or nucleus, according to the pathological characteristics of the cell [17]. In the nucleus, PB seems to be essential in the regulation of cell processes such as apoptosis, proliferation, and gene transcription [18].

Besides p53, other pathogenic mechanisms that may cause HCC after aflatoxin exposure still need to be studied. Cyclins are a family of proteins that are directly involved in the regulation of the cell cycle [19]. Changes in cyclin activities in transformed cells contribute to the acceleration of the cell cycle, which may be due to the lack of control of pathways that regulate cyclin synthesis, or to mutations that lead to the loss of function in inhibiting proteins [20]. The pathway p16/cyclin D1/Rb (retinoblastoma) may be considered the major cell cycle regulator. There are also three important inhibitors of cell cycle progression belonging to the Cip/Kip family: $p27^{KIP1}$, $p21^{WAF1}$, and $p57^{KIP2}$. However, little is known about the interference of major chronic liver diseases, such as cirrhosis associated with viral hepatitis or alcoholism, in AFB_1 biotransformation in the liver. The objective of the present study was to investigate the occurrence of residual AFB_1 or its metabolites in hepatic biopsies from autopsy cases, and the association of these residues with mechanisms related to hepatocarcinogenesis.

2. Material and methods

2.1. Sampling procedures

A total of 58 human liver samples were collected from patients (46 males and 12 females) with HCC ($n = 8$), cirrhosis associated with viral hepatitis (VC, $n = 20$), cirrhosis associated with alcoholism (AC, $n = 20$), and normal livers (NL or controls, $n = 10$). These livers came from autopsies performed at the Division of Pathology of a University Hospital in Ribeirão Preto, Brazil, from 2011 to 2014. Post-mortem time for sample collection was around 6 h. Clinical data were obtained from patients' records. Patients with evidence of human immunodeficiency virus infection, auto-immune or drug-induced hepatitis, endocrine disorders, or any other cause of liver disease were excluded from the study. The ethical guidelines from the Helsinki Declaration (1975) were followed in the study, and it also was approved by the Ethics Committee of the School of Medicine of the University of São Paulo at Ribeirão Preto (protocol no. 1611/2011). Each liver sample was subdivided in two parts: one was fixed in 10% buffered formalin for 48 h and then embedded in paraffin; and the other was placed in RNA stabilizing solution (RNAlater RNA Stabilization Reagent, Qiagen, Turnberry Lane, CA, EUA), frozen in liquid nitrogen, and stored at −80 °C. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) for confirmation of the original diagnosis.

2.2. Determination of aflatoxin B_1 and metabolites

Liver samples stored at −80 °C were thawed at room temperature. Aflatoxin was extracted according to Chiavaro et al. [21] and purified through immunoaffinity columns (Aflatest WB®, Vicam, Watertown, MA, USA). Briefly, 1.0 g of liver sample was ground in a mortar and the resulting slurry was transferred to a 5-mL cryotube containing 4.0 mL of methanol/water (80:20, v/v) and 10 µL of internal standard (IS) working solution (described in the end of this section). The mixture was sonicated for 10 min, vortexed for 30 s and allowed to stand overnight at 7–10 °C. After that period, vortexing and sonication were repeated, and samples centrifuged (Sorvall® RC 3B Plus) at 3600 × G for 10 min. An aliquot (4 mL) of the supernatant was diluted with 25 mL of PBS and passed through the immunoaffinity cleanup column. The column was washed with 20 mL of PBS and then 2 mL of deionized water. After the washing steps, aflatoxins were eluted with 2 mL of methanol, dried, and reconstituted in 500 µL of acetonitrile/water (1:1, v/v). This solution was filtered (PTFE, 0.22 mm, Millex, Millipore Corp) and transferred to an amber glass vial.

Residual levels of AFB_1, AFM_1, AFP_1, AFQ_1, $AFB_{2\alpha}$, and AFL were determined by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS using [$^{13}C_{17}$]-AFB_1 (Sigma, St. Louis, MO, USA) and [$^{13}C_{17}$]-AFM_1 (Biopure, Romer Labs, Tulln, Austria) as internal standards. To prepare the calibration lines for the quantification assay, a standard working solution with a mixture of all standard aflatoxins (AFB_1, AFM_1, AFP_1, AFQ_1, $AFB_{2\alpha}$, and AFL at 10 µg L^{-1} each) were dissolved in acetonitrile/water (1:1, v/v) and diluted to the final concentrations of 1.0, 0.750, 0.5, 0.1, 0.05, and 0.01 ng/mL. An additional calibration line was constructed for 10, 8, 4, and 2 ng/mL AFB_1 by means of dilution of an individual AFB_1 standard solution (10 ng/mL). The IS working solution (25 ng/mL [$^{13}C_{17}$]-AFB_1 and 100 ng/mL [$^{13}C_{17}$]-AFM_1) was prepared in acetonitrile/water (1:1, v/v) and added to all composite standards at a final concentration of 0.5 ng/mL and 2.0 ng/mL, respectively. The curves were determined by plotting the ratio of the standard peak area to the IS peak area against the concentration of the calibration standards. For aflatoxin quantification, the peak area ratios of the analytes to the IS were calculated, and concentration was determined by the corresponding calibration line. LOD and LOQ values were calculated based on signal-to-noise ratios of 3:1 and 10:1, respectively, of peaks corresponding to the confirmatory Multi Reaction Monitoring (MRM) transitions.

Final extracts were injected into a Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA) coupled to a Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA). The column (BEH C_{18}, 2.1 × 50 mm, 1.7 µm) was kept at 40 °C during the analyses, and samples were maintained at 15 °C. The injection volume of extracted samples and standards was 10 µL. The mobile phase was composed by water (eluent A) and acetonitrile (eluent B), both containing 0.1% of formic acid. After an initial period of 0.5 min at 95% eluent A, the percentage of eluent B was linearly raised to 25% over 4.5 min (5.0 min). Then, eluent B was increased to 90% over 0.5 min, followed by a hold time of 0.25 min (5.5 min). After that, the percentage of eluent B was reduced to 5% over 0.5 min (6.0 min), and the column re-equilibrated to the initial conditions for 0.5 min. Total chromatographic run time was 6.5 min, and the flow rate was maintained at 0.5 mL/min.