Aflatoxin M_1 in the urine of non-carriers and chronic carriers of hepatitis B virus in Maringa, Brazil

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Exposure to aflatoxins (AFs) in the diet may favour the development of hepatocellular carcinoma (HCC) and the acute exacerbation of hepatitis in chronic hepatitis B virus (HBV) carriers. Measurement of biomarkers such as aflatoxin M_1 (AFM_1), a metabolite of aflatoxin B_1 (AFB_1), in urine allows for the assessment of populations exposed to aflatoxins. The aim of this study was to investigate the occurrence of aflatoxin M_1 in the urine of HBV carrier and non-carrier patients. One group included 43 randomly selected HBV carriers treated at two hospitals in the city of Maringa, Brazil, from March to June 2008. Control group consisted of 29 healthy adult volunteers with anti-HBs positive and HBsAg negative test results. Detection of AFM_1 was performed by fluorescence using high performance liquid chromatography (HPLC) and post-column derivation with the Kobra Cell®. Of the 72 samples analysed, 05/29 (17.2%) AFM_1 positive samples were from HBV non-carriers, and 16/43 (37.2%) of samples were from chronic HBV carriers. This study showed AFM_1 in the urine of the two surveyed population. However, there is evidence that the chronic HBV carriers have a higher risk of developing HCC due to additive interaction between AFs and HBV.

Uniterms: Aflatoxins. Biomarkers. Hepatitis B. Hepatocellular carcinoma. Urina/análise toxicológica. Epidemiology.

A exposição às aflatoxinas (AFs) na dieta é um fator de risco para o desenvolvimento do carcinoma hepatocelular (CHC) e a exacerbação da hepatite aguda em indivíduos portadores do vírus da hepatite B (VHB). O uso de biomarcadores, como a aflatoxina M_1 (AFM_1) na urina, produto de biotransformação da aflatoxina B_1 (AFB_1), permite avaliar se a população está exposta às AFs. O objetivo do presente estudo foi investigar ocorrência de AFM_1 na urina de portadores e não portadores crônicos do VHB. Foi selecionado um grupo, de forma aleatória, representado por 43 portadores do VHB atendidos em dois hospitais da cidade de Maringá, Brasil, no período de Março a Junho/2008. O grupo controle foi composto por 29 voluntários adultos saudáveis anti-HBs positivo e HBsAg negativo. A determinação de AFM_1 foi realizada por meio de detecção por fluorescência em sistema de cromatografia a líquido de alta eficiência com derivação pós-coluna utilizando Kobra Cell®. Das 72 amostras analisadas, 05/29 (17,2%) foram positivas para AFM_1 em indivíduos não portadores do VHB, e 16/43 (37,2%) de pacientes portadores do VHB. Este estudo demonstrou a ocorrência de AFM_1 na urina dos dois grupos estudados. Entretanto, há evidências de que os portadores do VHB possuam alto risco no desenvolvimento do CHC devido ao efeito aditivo pela interação entre aflatoxinas e VHB.

Unitermos: Aflatoxinas. Biomarcadores. Hepatite B. Carcinoma hepatocelular. Análise toxicológica. Epidemiologia.

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INTRODUCTION

Aflatoxins (AFs) are a group of mycotoxins of major importance in foods such as peanuts and corn. The most important toxigenic fungi producing aflatoxins are *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. The four aflatoxins naturally found in foods are B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2). Aflatoxin M_1 (AFM_1) is a hydroxylated product of AFB_1 that can be secreted through body fluids (Creppy, 2002). The action of these toxins can cause alterations in the growth of children and adolescents, neurological and immunological disorders and hepatocellular carcinoma (Eaton, Gropman, 1994; Zhang *et al.*, 2012).

Human exposure to aflatoxins is a global concern, as studies have reported an association between exposure to aflatoxins in the diet and infection with hepatitis B virus (HBV) that may favour the development of hepatocellular carcinoma (HCC) and the acute exacerbation of hepatitis in chronic HBV carriers (Peers *et al.*, 1987; Kensler *et al.*, 2003). In areas with high incidence of HBV and aflatoxin contaminated foods, such as sub-Saharan Africa, Southeast Asia and China, the risk of HCC is high. Of the 550,000-600,000 new HCC cases worldwide each year, about 25,200-155,000 may be attributable to aflatoxin exposure (Liu, Wu, 2010).

One method for estimating exposure to aflatoxins in the diet is to determine the concentration and rate of consumption in contaminated foods, but several factors complicate this assessment. Thus, the detection of mycotoxins or their metabolites in body fluids such as milk, blood and urine allows a quantitative assessment of exposure (Groopman, Wogan, 1994; Bando *et al.*, 2007). AFB_1, an indicator of recent exposure and AFB-albumin adducts, which is indicator of long-term exposure, can be detected in blood samples (Wang *et al.*, 2001; Bando *et al.*, 2007). Aflatoxin M_1 (AFM_1), aflatoxin P_1 (AFP_1), aflatoxin Q_1 (AFQ_1) and AFB_1-N^7-guanine adducts are biotransformation products that can be detected in urine (Wang *et al.*, 2001). The determination of AFM_1 in urine is correlated with the amount of AFB_1 ingested, and as such is a good biomarker of exposure (Groopman *et al.*, 1992). The aim of this study was to evaluate the occurrence of AFM_1 in the urine of non-carriers and chronic carriers of HBV in the population of Maringa, southern Brazil.

MATERIAL AND METHODS

Study population

The study population consisted of 72 randomly se-

lected individuals divided into two groups: non-carriers and chronic HBV carriers. The group of non-carriers included 29 healthy adults with anti-HBs positive and HBsAg (surface antigen) negative results. All had been vaccinated against HBV. Age, race and schooling level similar to the group exposed. The group of chronic HBV carriers was composed of 43 patients adults diagnosed at two hospitals in the city of Maringa (Brazil) from March to June 2008, with HBsAg positive by more than six months. The voluntary participants each completed individual questionnaires. All participants signed a free and informed consent form. This study was approved by the Research Ethics Committee Involving Human Subjects (COPEP), State University of Maringa (CAAE N. 0007.0.093.000-08).

Data collection

The self-administered questionnaire for chronic HBV carriers covered personal data, alcohol and medication use, duration of HBV infection and laboratory data. Personal data including age and gender was collected from HBV non-carriers. Participants were provided with instructions regarding completion of the questionnaire and the collection of biological materials.

Blood samples were collected from HBV non-carriers for detection of anti-HBs and HBsAg serologic markers. For both study groups, samples from first morning urine were collected in a 300 mL polyethylene bottle, identified and stored at -20 °C until analysis for AFM_1.

Serological markers of HBV

Analysis of serologic markers for HBV antibody against surface antigen "S" (anti-HBs) and surface antigen S (HBsAg) was performed using the AxSym® and Chemiluminescence System methods, respectively, according to the manufacturer's instructions.

Urine AFM_1 analyses

AFM_1 and β-glucuronidase were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). The solvents and chemical reagents used were analytical grade acetonitrile (Mallinckrodt Baker, Xalostoc, Mexico), HPLC grade methanol (Mallinckrodt Baker, Phillipsburg, NJ, USA), potassium bromide (F. Maia Indústria e Comércio Ltda, Cotia, Brazil), acetone and anhydrous sodium sulfate (Vetec Ltda, Rio de Janeiro, Brazil), trimethylsilyl chloride (Merck, Hohenbrunn, Germany), acetic and nitric acid (Labsynth Ltda, Diadema, Brazil), toluene

(CAQ, Diadema, Brazil) and hydrochloric acid (Merck, Darmstadt, Germany). Nitrogen was purchased from AGA (Sundyberg, Switzerland) and Aflaprep®immunoaffinity columns were obtained from R-Biopharm Rhône Ltd. (Glasgow, Scotland).

AFM$_1$ standard was diluted in acetonitrile to create a stock solution of 7.61 µg/mL. Concentration of the prepared solution was performed according to the methodology 971.22 described by the *Association of Official Analytical Chemists* (AOAC, 2005). Working solutions for the calibration curve, prepared by diluting in acetonitrile-water (30:70 v/v), were 0.01, 0.03, 0.1, 0.2 and 0.3 ng/mL.

Urine samples were thawed at room temperature, homogenised by inversion and filtered through standard filter paper. Ten mL of 0.1 M sodium acetate buffer pH 5.0 containing 0.4 mg/mL (500 U/mL) β-glucuronidase was added to 10 mL of filtered urine and incubated in a EV 015T water bath (Evlab, Londrina, Brazil) at 37 °C for 17 hours (Kussak, Andersson, 1995).

After hydrolysis, AFM$_1$ was extracted from urine samples on immunoaffinity columns. The column was conditioned with 10 mL of Milli-Q Plus ultrapure water (Millipore, Bedford, MA, USA). Twenty mL of the sample was added, the column was washed with 10 mL water, dried by air flow, and AFM$_1$ was eluted with 2 mL of acetonitrile in previously silanised tubes under a flow of less than 2 mL/min. The eluate was concentrated under nitrogen flow in a concentrator Tech Vap TE 0194 (Tecnal, Piracicaba, Brazil). The residue was frozen at -20 °C until chromatographic determination.

The eluate was resuspended in 400 µL acetonitrile-water (30:70 v/v) and agitated in a KMC-1300V vortex (Vision, Korea). It was then filtered through a Sun-Sri (PTFE Titan 2 HPLC filter Yellow 17 mm, 0.45 µm) membrane. The standards and the filtered samples were analysed in an Alliance® HPLC system (Waters, Dublin, Ireland) attached to a quaternary pump and an automatic sample injection system at a volume of 100 µL. The fluorescence detector used was a 2475 model (Waters, Dublin, Ireland) with an excitation wavelength of 365 nm and emission at 440 nm. A phenyl C-18 column 250 x 4.6 mm with 5 µm particles was used (XTerra, Dublin, Ireland). The isocratic system included an acetonitrile-water (30:70 v/v) mobile phase containing 1 mM potassium bromide and 1 mM nitric acid at a flow rate of 1.5 mL/min. Post-column derivation was performed with Kobra Cell® (R-Biopharm Rhône Ltd., Glasgow, Scotland) at 20A connected to a 500 x 0.55 mm PTFE tube.

Quantification of AFM$_1$ was performed using the calibration curve obtained from working solutions (0.01 to 0.3 ng/mL or 0.8 to 24 pg/mL, depending on the dilution factor of the sample). Each solution was injected three times and the average of the replicates was used to construct the calibration curve. Linearity of the calibration curve was 0.9949. The detection limit was 0.13 pg/mL and the limit of quantification for the method was 0.40 pg/mL. The AFM$_1$ retention time was 5.0 minutes. Precision and accuracy (recovery) were 5.9% and 91.7% in a concentration of 10 pg/mL (n=6).

Statistical analysis

For the statistical evaluation between the means of AFM$_1$ detected in the urine of individuals in each group, the "t test" with a 95% confidence interval was performed using BioEstat 5.0 and Sigma Statistic 2.3.

RESULTS

The HBV non-carriers included 29 subjects with a mean age of 33 years (20 to 57 years) who presented anti-HBs positive and HBsAg antigen negative results. There were 5 males and 24 females in this group. There were 43 chronic HBV carriers with a mean age of 46 years (22 to 60 years). Of these, 26 were males and 17 were females. Questionnaires that were answered appropriately provided the following data: 86.5% of patients were Caucasian, 2.7% were Asian and 10.8% were African descent. The average time from diagnosis of HBV until the present study was six years. Laboratory tests previously carried out included: HBsAg (94.7%), anti-HBs (57.9%), anti-HBe (52.6%), anti-HBc (47.4%), HBeAg (36.8%) and anti-HBc IgM (31.6%). Only 9.5% of patients reported having undergone viral load testing for hepatitis B.

Regarding the consumption of alcoholic beverages, 73.7% of individuals reported that they do not drink and 26.3% reported that they consume alcohol 'frequently'. Of the 25% of patients that underwent treatment, lamivudine (14.3%) and interferon (3.6%) were the most common medications used. Other unspecified medications accounted for 7.1% of the HBV carrier population.

Of 72 urine samples obtained from non-carriers and chronic HBV carriers, 21 (29.2%) presented quantifiable levels of AFM$_1$ in urine, ranging from 0.67 to 7.87 pg/mL (Table I).

HBV non-carriers had detectable levels of AFM$_1$ in 5 (17.2%) of 29 analysed samples (Figure 1). All of the positive samples, 5/24 (20.8%), were from females. Two had concentrations between 0.6 and 3.0 pg/mL and 3 had concentrations ranging from 3.01 to 5.50 pg/mL. None of the samples had concentrations greater than or equal to 5.51 pg/mL. Sixteen (37.2%) of 43 analysed samples were

TABLE I - AFM$_1$ in 72 urine samples from non-carriers and chronic HBV carriers from Maringa, Brazil

Population	Gender	N	Positive Samples		AFM$_1$ in urine (pg/mL)	
			n	(%)	Range	Average ± Standard Deviation
Non-carriers HBV	Male	05	nd	0	--	--
	Female	24	05	20.8	1.76-4.15[a]	2.81 ± 1.04
HBV carriers	Male	26	07	26.9	0.74-6.77[a]	2.03 ± 2.13
	Female	17	09	52.9	0.67-7.87[a]	3.24 ± 2.26
Total		72	21	29.1	0.67-7.87	2.74 ± 1.98

[a] = no significant statistical difference between the groups (p > 0.05); nd = not detected
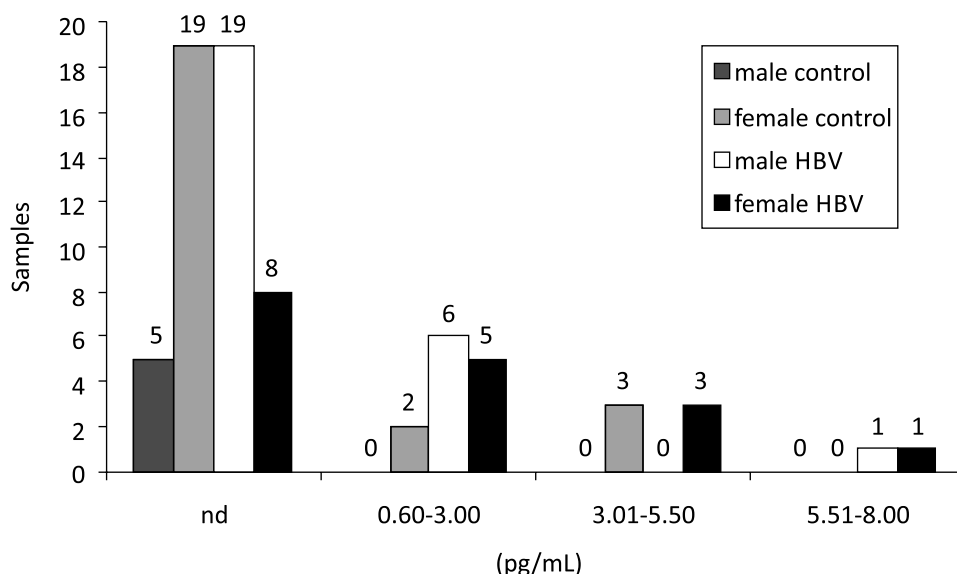
FIGURE 1 - Distribution of AFM$_1$ levels in the urine of 29 HBV non-carriers (control) and 43 chronic HBV carriers in Maringá, Brazil. (nd = not detected)

positive for AFM$_1$ in chronic HBV carriers. Nine (56.3%) were from female patients and 7 (43.8%) were from males, ranging between 0.6 to 8.0 pg/mL. The number of samples with concentrations ranging from 0.6 to 3.0, 3.01 to 5.50 and 5.51 to 8.00 pg/mL were 5, 3 and 1 for females, and 6, 0 and 1 for males, respectively.

DISCUSSION

The levels found of AFM$_1$ in urine, 0.67 to 7.87 pg/mL (29.2%), were lower than the value reported by Romero *et al.* (2010), who showed a prevalence of 65%, with levels ranging from 1.8 to 39.9 pg/mL in a Brazilian population. The difference between the data may have occurred, since in our study only one sample was analyzed for each subject, and the seasonal variation and dietary data were not investigated.

These results showed a lower incidence and concentration of AFM$_1$ in urine than other studies. Jolly *et al.*

(2006) conducted a cross-sectional study in Ghana and detected AFM$_1$ in the urine of 83 (91.2%) of the 91 participants studied, ranging between 10 and 11,562.36 pg/mg creatinine. In the Czech Republic, the occurrence of AFM$_1$ in urine was 57.6% of samples, ranging between 0.019 and 19.219 pg/mg creatinine (Malir *et al.*, 2006). Cheng *et al.* (1997) analysed 32 urine samples from patients in Taiwan and 138 from China, and AFM$_1$ was found in 66% and 64% of the samples, respectively.

In China, Mykkanen *et al.* (2005) demonstrated the presence of AFM$_1$ in the urine of 47.3% of young individuals (18-24 years) with an average level of 40 pg/mL. Concentrations ranged between 10 and 330 pg/mL. The presence of this biotransformation product in urine was not statistically different between subjects presenting HBsAg positive and HBsAg negative results. Our results were similar to this study in that there was no significant statistical difference between the average level of AFM$_1$ in the urine of individuals from the non-carrier group

and from the chronic HBV carrier group (p=0.925). There was no significant statistical difference when levels of AFM$_1$ in the urine of females and males were compared (p=0.257).

In our study, the group of chronic HBV carriers showed AFM$_1$ in 16 (37.2%) of 43 samples analysed. This result showed that this population was exposed to AFB$_1$, and is therefore at risk of developing HCC, as several studies have shown high morbidity from HCC due to exposure to aflatoxins and a high rate of chronic HBV infection (Qian *et al.,* 1994; Sun *et al.*, 1999; Wang *et al.*, 2001). Sun *et al.* (1999) demonstrated that detectable urinary AFM$_1$ levels above 3.6 ng/L were associated with increased 3.3-fold risk of HCC in male HBsAg carriers with chronic hepatitis. This fact is due to mutations in the third base of codon 249 of the p53 gene, since the studies showed a positive correlation between aflatoxin exposure and HCC with mutation 249[ser] (Sun *et al.*, 1999; Bando *et al.*, 2007). Anwar *et al.* (2008) concluded that have strategies to reduce HCC with the use of markers of HBV, HCV (hepatitis C virus) and AFB$_1$ exposure.

AFM$_1$ is the major metabolite of AFB$_1$ in quantitative terms and excretion represents exposure within a period of 24 hours of sampling (Eaton, Gropman. 1994). Thus, the levels of AFM$_1$ in urine were used as a biomarker for short-term exposure to AFB$_1$ (Gan *et al.*, 1988). This study showed AFM$_1$ in the urine of 29.2% of the surveyed population, although differences were not statistically significant between the non-carrier group and chronic HBV carriers regarding recent exposure to AFB$_1$. However, there is evidence that the chronic HBV carriers have a higher risk of developing HCC due to additive interaction between AFB$_1$ and HBV (Wu *et al.*, 2009). Further studies using long-term exposure biomarkers, such as AFB$_1$-N^7-guanine and AF-albumin adducts, are required to assess the actual levels of long-term exposure to aflatoxins in the Brazilian population.

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