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Aflatoxin exposure in viral hepatitis patients in Turkey

Türkiye'de viral hepatitli hastalarda aflatoksin maruziyeti

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Background/aims: *Hepatocellular carcinoma is the fifth most common cancer and a major public health problem worldwide. Differences in distribution of hepatocellular carcinoma incidence are probably due to different levels of exposure to hepatocellular carcinoma risk factors: chronic infections with hepatitis B virus (HBV) and aflatoxin exposure in developing countries, and smoking and alcohol abuse in developed countries. Aflatoxin is one of the most important of the environmental toxins that contribute to the pathogenesis of hepatocellular carcinoma, especially in the regions where dietary foodstuffs (peanuts, corn, Brazil nuts, pistachios, spices and figs) are highly contaminated. High aflatoxin levels have been shown in the foodstuffs that are produced in our country. The specific aim of this study was to assess the rate of aflatoxin exposure and to determine some clues about aflatoxin metabolism by measuring and comparing the levels of carcinogenic forms in healthy subjects, in different stages of viral disease, and in different viral hepatitis types.* ***Methods:*** *This was a cross-sectional observational, single-center study. A total of 203 (male/female: 119/84) viral hepatitis patients who were consecutively admitted to Ankara University, School of Medicine, Gastroenterology Clinic, between January 2006 and June 2007 were enrolled into the study. Sixty-two healthy subjects (male/female: 33/29) with normal blood chemistry and negative viral serology served as controls. Chemical forms AFB1, AFB2, AFG1, and AFG2 were assessed in plasma of study participants by high-performance liquid chromatography.* ***Results:*** *AFB1, AFB2, AFG1, and AFG2 were detected in 24.6%, 17.2%, 22.7%, 18.2% of the 203 patients, respectively, and were significantly higher than in the control group for all chemical forms. Percentage of AFB1-positive patients was significantly higher than in the control group irrespective of disease stage. There was no significant difference between chronic infected patients, cirrhotic patients and patients with Hepatocellular carcinoma with respect to percentage of aflatoxin-positive individuals.* ***Conclusions:*** *With this study, we have documented that in viral hepatitis patients, aflatoxin exposure is significantly higher than in healthy subjects in Turkey and it may play an important role in the development of hepatocellular carcinoma. Thus, large studies exploring the relation between aflatoxin exposure, viral hepatitis status, and risk of hepatocellular carcinoma development are needed.*

Key words: Aflatoxin, viral hepatitis, chronic hepatitis, cirrhosis, hepatocellular carcinoma

Amaç: *Hepatosellüler karsinom dünyada beşinci sırada en sık görülen, ciddi bir sağlık sorunudur. HCC insidansının dağılımındaki farklılık, muhtemelen risk faktörlerine farklı oranlarda maruz kalmaya bağlıdır. Gelişmekte olan ülkelerde kronik hepatit B virüs enfeksiyonu ve aflatoksin, gelişmiş ülkelerde ise sigara ve alkol kullanımı önemli risk faktörleridir. Aflatoksin, Hepatosellüler karsinom patogenezinde rol oynayan en önemli çevresel toksinlerden biridir. Yüksek aflatoksin düzeyleri, ülkemizde üretilen besin maddelerinde de gösterilmiştir. Bu çalışmanın amacı sağlıklı kontrol grubu, farklı viral hepatitler ve hastalığın farklı derecelerinde aflatoksin düzeylerinin karşılaştırılarak, aflatoksin maruziyet oranları ve aflatoksin metabolizması hakkında bazı ipuçlarının elde edilmesidir.* ***Yöntem:*** *Çalışmaya Ankara Üniversitesi Tıp Fakültesi Gastroenteroloji Kliniğine Ocak 2006 – Haziran 2007 tarihleri arasında ardışık olarak başvuran toplam 203 (erkek/kadın: 119/84) viral hepatitli hasta dahil edildi. Normal biyokimyasal değerleri ve negatif serolojisi olan 62 sağlıklı birey (erkek/kadın:33/29) kontrol grubu olarak çalışmaya dahil edildi. Plazma AFB1, AFB2, AFG1 ve AFG2 düzeyleri High-performance liquid chromatography yöntemiyle ölçüldü.* ***Bulgular:*** *AFB1, AFB2, AFG1 ve AFG2 sırayla hastaların %24.6, %17.2, %22.7, %18.2' sinde saptandı. Hastalığın derecesinden bağımsız olarak AFB1 pozitif hastaların yüzdesi, kontrol grubundan anlamlı olarak fazla idi. Ancak aflatoksin pozitif hasta yüzdesi açısından kronik hepatit, siroz ve hepatosellüler kanserli hasta grupları arasında analamalı farklılık saptanmadı.* ***Sonuç:*** *Bu çalışma ile Türkiye'de viral hepatitli hastalarda aflatoksin maruziyetinin, sağlıklı kontrol grubuna göre anlamlı olarak fazla olduğu gösterildi. Hepatosellüler karsinom gelişiminde bu maruziyetin önemli rolü olabilir ve bu konuda yapılacak daha geniş, randomize çalışmalar gerekmektedir.*

Anahtar kelimeler: Aflatoksin, viral hepatit, kronik hepatit, siroz, hepatosellüler karsinom

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and a major public health problem worldwide (1). In 1990, the global number of new cases was estimated as 316,300 for males and 121,100 for females, accounting for 7.4% (males) and 3.2% (females) of all malignancies, excluding skin cancer (2). Its incidence varies largely according to the geographic region, and evidence has been accumulating in different countries that the incidence is increasing. These differences in distribution of HCC incidence are probably due to different levels of exposure to HCC risk factors: chronic infections with hepatitis B virus (HBV) and aflatoxin (AF) exposure in developing countries, and smoking and alcohol abuse in developed countries (3). Additionally, this exposure with the trophic effect of androgens makes males more likely to develop HCC than females (4). The majority of HCCs occur in older patients with long-standing chronic liver disease. However, in regions with high frequency of HBV carriers and AF exposure, like sub-Saharan Africa, the mean age of presentation of HCC is decreased to as low as 33 years (5).

HCC risk is increased with coexistence of risk factors, e.g., HCV infection and alcohol use or HBV infection and exposure to AF (6, 7).

Aflatoxin is one of the most important of the environmental toxins that contribute to the pathogenesis of HCC, especially in the regions where dietary foodstuffs (peanuts, corn, Brazil nuts, pistachios, spices and figs) are highly contaminated. It is produced by *Aspergillus parasiticus,* A. flavus, and A. nomius and occurs in several chemical forms: aflatoxin B1, B2, G1, and G2, all of them toxic, especially AFB1. The World Health Organization has classified AFB1 as a class 1 carcinogen (8).

AFB1 is metabolized by the liver through the cytochrome p450 enzyme system to the major carcinogenic metabolite AFB1-8,9-epoxide, which binds to cellular macromolecules including proteins and DNA to form adducts. Reaction with DNA at the N7 position of guanine preferentially causes a G:C > T:A mutation in codon 249 of the p53 tumor suppressor gene (9). Mutations of the p53 tumor suppressor gene have been demonstrated in patients with HCC who have chronically been exposed to AF, and this mutation is also induced in cultured Hep G2 human hepatocytes exposed to AFB1 (10-12).

It is known that dietary ingestion of AF has an important effect, particularly in the context of coexisting chronic HBV infection, which leads to a more than 50-fold increase in the risk of developing HCC (7, 13). A number of mechanisms have been suggested for the interaction between the two risk factors (14). The first is that HBV infection sensitizes hepatocytes to the carcinogenic effects of AF, which must be correlated with hepatocyte damage, thus the stage of disease. The second mechanism is that the two risk factors together can activate phase II detoxification enzymes, which can play a role in the genesis of HCC (14).

High AF levels have been shown in the foodstuffs produced in our country (15-20). To date, there has been no study about serum AF levels of healthy and chronic viral hepatitis patients in our country, which is thought to be a direct evidence of exposure (11). The specific aim of this study was to assess the rate of AF exposure and to determine some clues about AF metabolism by measuring and comparing the levels of carcinogenic forms in healthy subjects, in different stages of disease (chronic hepatitis, cirrhosis, and HCC) and in different viral hepatitis types. We also aimed to determine if the AF exposure rates are different in different socioeconomic conditions, since it is known that chronic viral hepatitis is more common in low socioeconomic populations.

MATERIALS AND METHODS

This was a cross-sectional observational, single-center study. A total of 203 (male/female: 119/84) viral hepatitis patients, who were consecutively admitted to Ankara University School of Medicine, Gastroenterology Clinic, between January 2006 and June 2007, were enrolled into the study. Sixty-two healthy subjects (male/female: 33/29) with normal blood chemistry and negative viral serology served as controls. All individuals in this study signed an informed consent before any procedure.

One hundred and five of the patients were infected with HBV, 16 with HBV and HDV, and 82 with HCV (Table 1).

Table 1. Patient numbers according to disease stage and viral serology

n	Chronic hepatitis	Cirrhosis	HCC	Total
HBV	37	38	30	105
HDV	2	4	10	16
HCV	54	22	6	82

HCC: Hepatocellular carcinoma

Table 2. Approximate retention time of samples in HPLC and minimum detected concentration according to AF chemical form

Aflatoxin	Approximate retention time in HPLC (minute)	Minimum detected concentration (pg/ml)
B1	15.5	1.6
B2	12.8	1.9
G1	10.9	1.6
G2	9.1	1.9

HPLC: High-performance liquid chromatography

A blood sample was drawn from each participant and separated plasma samples were frozen at -80ºC until subsequent analysis.

Aflatoxins are present in serum as AFB1, AFB2, AFG1, and AFG2-albumin adducts reflecting AF exposure in the previous 2-3 months and the promutagenic lesion AFB1-N7-guanine DNA adduct in the liver (21). Proteins of serum were digested with pronase and bounded AFB1, AFB2, AFG1, and AFG2 were extracted with methanol and analyzed by HPLC (high-performance liquid chromatography), according to the modified method of the Association of Official Analytical Chemists (AOAC, method no 999.07) procedure.

The HPLC detector was programmed to have excitation/emission wavelengths at 360 nm and 430 nm, respectively. The column was octadecyl silica gel C18, 25 cm x 3.8 mm (Waters Spherisorb S5ODS 2.5 µm). For chromatographic determination, a water/acetonitrile/methanol (62:16:22) mobile phase system having a flow rate of 1 ml/min was used. The injection volume was 500 µL.

The detection limits of AFB1, AFB2, AFG1, and AFG2 were 1.6, 1.9, 1.6, and 1.9, respectively (Table 2).

Statistical Analyses

All patients and control subjects were included in the data analysis. Mann-Whitney U test was used to compare mean AFB1 levels of patients and controls. Chi-square test was used to compare the patient group and controls for the rate of AF (AFB1, AFB2, AFG1, and AFG2) exposure and odds ratio was given with upper and lower values with 95% confidence interval. Patients according to disease stage (chronic hepatitis, cirrhosis, HCC) and viral serology (HBV, HDV, HCV) were also compared with each other and the control group with Pearson chi-square tests in terms of AFB1 exposure rate. Kruskal Wallis test was used to compare mean AFB1 levels of the patients, in different stage of disease and with different viral serology, with each other and with the control group. For all tests, a two-tailed p value of less than 0.05 was considered statistically significant. Analyses were performed with SPSS for Windows 11.5.

RESULTS

AFB1, AFB2, AFG1, and AFG2 were detected in 24.6%, 17.2%, 22.7%, 18.2% of the 203 patients, respectively, and were significantly higher than in the control group for all chemical forms (Table 3). The odds ratio (95% confidence interval) of being viral hepatitis-positive was 3.7 (1.4-9.8) for the detectable serum level of AFB1. Mean AFB2, AFG1, and AFG2 levels were significantly higher than in the control group, whereas mean AFB1 level was significantly lower than in the controls (Table 4).

Percentage of AFB1-positive patients was significantly higher than in the control group irrespective of disease stage. There was no significant difference between chronic infected patients, cirrhotic

Table 3. Percentage and number of aflatoxin-positive samples

	AFB1	AFB2	AFG1	AFG2
Patients (n=203)	50 (24.6%)	35 (17.2%)	46 (22.7%)	37 (18.2%)
Controls (n=62)	5 (8.1%)	3 (4.8%)	6 (9.7%)	2 (3.2%)
p	0.005	0.015	0.024	0.004
Odds ratio (95% Confidence interval)	3.7 (1.4-9.8)	4.0 (1.2-13.8)	2.7 (1.1-6.7)	6.6 (1.5-28.5)

Table 4. Mean aflatoxin levels detected in patients and controls

	AFB1 (pg/ml)	AFB2 (pg/ml)	AFG1 (pg/ml)	AFG2 (pg/ml)
Patients (mean±SD)	36.1±25.3 (n=50)	28.4±77.5 (n=35)	92.0±193.0 (n=46)	18.2±14.4 (n=37)
Controls (mean±SD)	101.2±28.1 (n=5)	18.8±16.0 (n=3)	32.5±17.5 (n=6)	10.4±10.0 (n=2)
p	<0.05	<0.05	<0.05	<0.05

patients and patients with HCC with respect to percentage of AF-positive individuals (Table 5).

AFB1 was positive in 26.7% of HBV-positive patients (p=0.004), 25% of HDV-positive patients (p=0.08) and 22.0% of HCV-positive patients (p=0.024), whereas it was positive in only 8.1% of the control group (Table 6). There was no significant difference between the different viral serologies.

Table 5. Percentage of AFB1-positive individuals and mean AFB1 levels of these individuals according to disease stage

	AFB1 (%)	AFB1 (mean±SD)
Chronic hepatitis (n=93)	25 (26.9%)	35.9±27.5
Cirrhosis (n=64)	15 (23.4%)	28.9±26.4
HCC (n=46)	10 (21.7%)	48.1±11.1
Controls (n=62)	5 (8.1%)	101.2±28.1

HCC: Hepatocellular carcinoma

DISCUSSION

High worldwide incidence of HCC may be attributable to both environmental and genetic risk factors. HBV infection and dietary exposure to AF coexist in those countries with highest incidence, which raises the possibility of a synergistic carcinogenic interaction between the two factors. To our knowledge, there are no data about interactions between HCV infection and AF exposure.

In the present study, AF exposure rates in viral hepatitis patients were found significantly higher than in the control group. AF contamination of foods occurs predominantly in countries with hot, humid climates (22). Because we did record data regarding from which part of country our study group patients originated, we cannot comment regarding any difference between AF exposure rates in different regions of the country. The decrease in AF contamination in the food supply in Singapore and Shangai resulted in a decreasing burden of HCC, and there was a relatively short time interval between the increase in the country's living standards and a decrease in the HCC rate, suggesting that AF exerts a major effect on late-stage carcinogenesis (23). Primary prevention, such as vaccination for HBV and control of AF contamination of food, offers strategies for lowering HCC rates; however, positive outcomes will not be observed for many years. Nevertheless, it would be wise to try to protect HBsAg-positive individuals from AF exposure. Thus, it is important to know the exposure rates of these people. AF-contaminated foodstuffs produced in our country were shown in several studies. However, to our knowledge, there has been no previous report published from Turkey regarding the detection of AFs using serum biomarkers, which is a more accurate and reliable indicator of AFB1 exposure, in healthy subjects and viral hepatitis-positive patients.

It is also important that the present study investigates AF exposure in different stages of disease, in three different viral serologies, and compares groups to each other and against healthy controls. Two cohort studies in Southeast Asia and experimental studies in HBV-transgenic mice and woodchucks infected with woodchuck hepatitis virus showed a synergistic hepatocarcinogenic effect between viral infection and AF exposure (11, 24-26). We still do not know the mechanism of interaction between AFB1 and HBV in hepatocarcinogenesis. It may reflect changes in metabolism of AFB1 with coexistence of HBsAg or only reflect the high prevalence of two risk factors together in a low socioeconomic population. A number of mechanisms have been suggested, the most widely accepted of which is that HBV infection sensitizes hepatocytes to the carcinogenic effects of AFB1 (14). It can do this by inducing the specific cytochrome p450s that metabolize AFB1 to the toxic metabolite, AFB1-8,9-epoxide. Induction of this enzyme has been described in HBV transgenic mice, where this effect appeared to result from hepatocyte injury rather than the presence of the virus (27). In some of the studies, a positive interaction between HBV and AFB1 seemed to depend on the absence of detoxification enzymes like glutathione-S-transferase, which converts the carcinogenic AFB1-8,9-epoxide to non-reactive metabolites (28-30). Another possibility is that increased hepatocyte necrosis and proliferation caused by chronic HBV infection increases the likelihood of AFB1-induced mutations like 249ser mutation, but there is inconsistent data about this hypothesis (31, 32). Interestingly, in the present study, we showed no significant difference between mean AFB1 levels between different stages of disease, and moreover,

Table 6. Percentage of AFB1-positive individuals and mean AFB1 levels of these individuals according to viral serology

	AFB1 (%)	AFB1 (mean±SD)
HBV (n=105)	28 (26.7%)	35.2±24.5
HDV (n=16)	4 (25.0%)	49.7±2.6
HCV (n=82)	18 (22.0%)	34.6±28.9
Controls (n=62)	5 (8.1%)	101.2±28.1

mean AFB1 level was higher in healthy subjects. Thus, it seems the HBV-AFB1 interaction is independent of disease stage and enzyme induction. Of course, the results can be attributable to the small sample size; larger studies are needed.

There was no significant difference between mean AFB1 levels in the different viral serologies, so we were unable to determine any additional synergistic effect of HBV to HCV in the development of HCC.

Because we determined significantly high AFB1 exposure rates in patients compared to healthy subjects, it is important to investigate AFB1 exposure according to different regions of the country with more humid and hot climates and according to the sociocultural situation of subjects by questioning their foodstuff consumption.

With this study, we have documented that in viral hepatitis patients, AF exposure is significantly higher than in healthy subjects in Turkey, and it may play an important role in the development of HCC. Large studies exploring the relation between AF exposure, viral hepatitis status, and risk of HCC development are needed.

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