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Determination of Serum Aflatoxin B1 (Biomarker) Adducts in Chronic Liver Disease Patients Attending in 500 Bedded Specialty Hospital, Yangon

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Hepatocellular carcinoma and cirrhosis of liver diseases are common cases in the Department of Hepatology, 500 bedded Specialty Hospital, Yangon. Obesity and diabetes are closely associated with liver abnormality called Non Alcoholic Fatty Liver Disease (NAFLD) that may increase the risk of liver cancer. Environmental exposure to aflatoxin is one of the risk factors for development of liver cancer in underling chronic liver disease. A cross-sectional, hospital- and laboratory-based study was carried out on total 91 chronic liver disease patients (75 males and 16 females with mean age of 50±11 years) including 35 hepatocellular carcinoma (HCC) cases and 56 cirrhosis of liver (COL) cases attending the Department of Hepatology, 500 Bedded Specialty Hospital, Yangon. This study was aimed to determine AFB1-albumin adduct level in serum of all subjects by using enzyme-linked immunosorbent assay (Bio Scientific ELISA kit). The minimum detection level of AFB1 was 0.25 ng/ml in this ELISA kit. AFB1 was not detected in serum of 10 normal healthy subjects in this study. AFB1 was detected in 4 cases (11.4%) of HCC and 4 cases (7.1%) of COL. Mean AFB1 concentration was higher in HCC cases (0.38±0.09 ng/ml) than in COL cases (0.28±0.03 ng/ml). The number of hepatitis B virus surface antigen (HBsAg) positive cases were significantly higher (21, 60%) in HCC cases than in COL cases (16, 29%), ($p<0.05$). There was no significant difference in anti-HCV positive cases between two types of chronic liver diseases such as 11(31%) in HCC and 17(30%) in COL, respectively. All HCC cases with AFB1 positivity had markers of hepatitis B and C but three cases of COL with AFB1 positivity had no markers for hepatitis B and C. In this study, hepatitis B and C viral infections are main causes of hepatocellular carcinoma and aflatoxin B1 may be one of the risk factor for development of cirrhosis liver without hepatitis viral infections.

Key words: Hepatocellular carcinoma, Cirrhosis of liver diseases, AFB1-albumin adduct level, ELISA

INTRODUCTION

Chronic liver disease causes can be any condition that results in the gradual degradation and renewal of the tissue cells with a body's liver. This process usually results in fibrosis or cirrhosis and can be potentially fatal in cases of chronic liver failure. The classification of the sources of chronic liver diseases fall into five groupings: viral causes (hepatitis B and C or cytomegalo-virus), metabolic causes (haemochromatosis or Wilson's disease), autoimmune response causes (primary biliary cirrhosis or primary sclerosing cholangitis), toxin-related causes (alcoholic liver disease or nitrofurantoin), and other miscellaneous causes (right heart failure). However, the main cause of chronic liver disease is overuse of alcohol, leading to cirrhosis and hepatitis.[1]

Aflatoxin is secondary metabolites produced by *Aspergillus flavus, A. parasiticus* and

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A. nomius. There are four naturally occurring aflatoxins: aflatoxin B_1 (AFB1), B_2, G_1 and G_2, and all of them are toxic, mutagenic and carcinogenic compounds, especially AFB1. The World Health Organization has recently classified AFB1 as a class 1 carcinogen. In human beings, aflatoxins have been implicated in the etiology of hepatocelluar carcinoma (HCC). Ecological studies performed in Africa and Southeast Asia had revealed a significant correlation between the aflatoxin exposure and incidence of human HCC.[2]

Aflatoxins colonize a variety of food commodities, including maize, oilseeds, spices, groundnuts and tree nut in tropical and subtropical regions of the world. Aflatoxin exposure in food is a significant risk factor for HCC.[3] Exposure to aflatoxin can lead to several health-related conditions including acute and chionic aflatoxicosis, aflatoxin-related immune suppression, liver cancer, liver cirrhosis, as well as nutrition-related problems in children such as stunted growth.[4] European Union legislation has established maximum permissible levels of 2-8 µg/kg for AFB1 and 4-15 µg/kg for total aflatoxins, depending on the different foods used for direct human consumption or as ingredients in other food products.[5]

Aflatoxin albumin adducts are found in peripheral blood after exposure to AFB1 and the measurement of these adducts is potentially a useful tool in the epidemiological study of the role of AFB1 in the etiology of liver cancer.[6]

Chronic hepatitis B virus infection and food aflatoxin B1 contamination have been identified as the major and possibly synergistic risk factors for HCC in endemic areas. Chronic hepatitis C virus is also an important risk factor. Cirrhosis of any etiology, particularly viral and alcoholic, is an important step toward HCC. Many other factors, such as alcohol drinking, cigarette smoking, homone and even vitamins may also contribute to the development of HCC.[7] Aflatoxin exposure is an associated risk factor for advanced liver diseases including liver cirrhosis (COL) or hepatocellular carcinoma (HCC) in patients with chronic hepatitis C.[8]

MATERIALS AND METHODS

After getting approval from the Ethics Review Committee of Department of Medical Research (DMR), a cross-sectional, hospital- and laboratory-based study was carried out in 91 patients including 35 HCC cases and 56 COL cases attending the Department of Hepatology, 500 Bedded Special Hospital (YSH) and 10 normal healthy persons from November 2014 to May 2015.

After getting informed consent, clinical history, drug history, history of alcohol drinking and ultrasound report of these patients were recorded according to the proforma.

Sample preparation and extraction

Venous blood 3 ml from ante-cubital vein of all subjects was collected into vaccutainer tube and transported to the laboratory with crushed ice. After collection, blood was centrifuged and serum was separated into aliquots. These serum samples were stored at -70º C for AFB1, HBsAg and HCV tests. These serum samples were tested for AFB1 by MaxSignal Total Aflatoxin ELISA Test Kit (Bioo Scientific) and for HBsAg and Anti-HCV by using SD Rapid Test.

All serum samples were extracted with equal volume of hexane for ELISA. After centrifugation for 5 minutes at 4,000 rpm, top hexane layer was completely removed, including milky interphase. Two hundred microliter of the lower aqueous layer was transferred to a new tube. Phosphate buffered saline (450 µl) and 100% methanol (350 µl) were added to this tube. This mixture was mixed with vortex for 30 seconds and centrifuged for 5 minutes at 4,000 rpm. Fifty microlitre of the extracted diluted sample were used per well in the test. Dilution Factor was 5.

Enzyme-linked immunosorbent assay (ELISA)

MaxSignal Total Aflatoxin ELISA Test Kit included the following components: aflatoxin coated 96-well plate; Aflatoxin B1 standards: 0, 0.05, 0.1, 0.2, 0.4, 0.8, 100 ng/ml; aflatoxin antibody #1; HRP-Conjugated Antibody #2; TMB substrate; Stop Buffer; PBS; wash solution. Fifty microlitre of each Aflatoxin B1 Standards and each extracted sample were added in duplicate into different 96 wells. Antibody #1 100 µl was added and mixed well gently for 1 minute and the plate was incubated for 30 minutes. After washing, this plate was incubated with HRP-Conjugated Antibody #2 150 µl for 30 minutes and washed.

Then, the plate was incubated with TMB substrate 100 µl for 15 minutes and Stop Buffer 100 µl was added. The plate was read on a plate reader with 450 nm wavelength. Sensitivity (Detection Limit) of this ELISA Test Kit was 0.25 ng/ml in serum. A standard curve was constructed by plotting the mean relative absorbance (%) obtained from each reference standard against its concentration in ng/ml on a logarithmic curve (Fig. 1).

$$\text{Relative absorbance (\%)} = \frac{\text{Absorbance standard (or sample)}}{\text{Absorbance zero standard}} \times 100$$

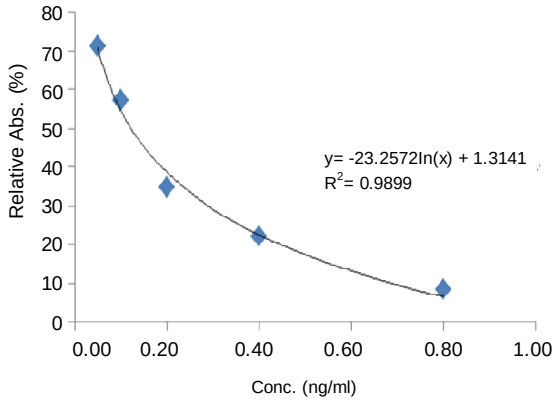

Fig. 1. Aflatoxin B1 standard curve

RESULTS

Table 1 shows ninety-one chronic liver disease patients were 75 males (82%) and 16 females (18%) with mean age of 50±11 years. There were 35 cases of HCC (38%) and 56 cases of COL (62%).

Table 1. General characteristics of patients with chronic liver disease

Characteristics	n=91	(%)
Age (year)		
Mean± SD	50±11	
Mode	40	
Range	30 to 84	
Sex		
Male	75	82
Female	16	18
Cases		
HCC	35	38
COL	56	62

HCC=Hepatocellular carcinoma
COL=Cirrhosis of liver

Table 2. Specific characteristics of patients with chronic liver disease

Variable	HCC case		COL case	
	No.	%	No.	%
HBsAg carrier status				
Negative	14	40	40	71
Positive	21	60	16	29
Anti-HCV status				
Negative	24	69	39	70
Positive	11	31	17	30
Habitual cigarette smoking				
Non-smokers	19	54	35	63
Smokers	16	45	21	37
Habitual alcohol drinking				
Non-drinkers	21	60	18	32
Drinkers	14	40	38	68
Level of aflatoxin B1 in serum				
Nondetectable	31	89	52	93
Detectable	4	11	4	7

HCC=Hepatocellular carcinoma
COL=Cirrhosis of liver

Table 2 shows that HBsAg carrier status was 60% in HCC and 29% in COL. Anti-HCV status was found in 31% of HCC and 30% of COL. AFB1 was not detected in serum of 10 normal healthy subjects in this study but detected in 4 cases (11.4%) of HCC and 4 cases (7.1%) of COL. Among 4 cases of HCC with aflatoxin B1 positive, one patient had both markers of HBsAg and anti-HCV. Other two cases of AFB1 positive HCC had presence of markers for

hepatitis B (HBsAg) and one case was anti-HCV positive. One case of aflatoxin B1 detectable COL was positive in anti-HCV and other three cases were negative for both markers of hepatitis B and C infection.

According to MaxSignal Total Aflatoxin ELISA Test Kit, the upper detection limit of AFB1 in serum is 0.25 ng/ml. Detectable aflatoxin B1 concentrations of HCC were 0.27 ng/ml, 0.41 ng/ml, 0.33 ng/ml and 0.48 ng/ml, and those of AFB1 COL were 0.25 ng/ml, 0.31 ng/ml, 0.31 ng/ml and 0.27 ng/ml, respectively. Among these HCC cases, history of alcohol drinking was found in one case and smoking status in two cases. History of alcohol drinking status was found in one case of aflatoxin B1 positive COL cases (Table 3).

Table 3. Status of aflatoxin B1 detectable hepatocellur carcinoma (HCC) and cirrhosis of liver (COL) cases

Cases	Age	Sex	AFB1 (ng/ml)	HBsAg	Anti-HCV	Smok-ing	Alcohol drinking
HCC							
1	34	M	0.27	+	+	+	+
2	73	F	0.41	-	+	-	-
3	50	M	0.33	+	-	-	-
4	58	M	0.48	+	-	+	-
COL							
1	53	M	0.25	-	-	-	+
2	54	M	0.31	-	-	-	-
3	39	M	0.31	-	-	-	-
4	50	F	0.26	-	+	-	-

M= Male, F= female

Mean concentrations of aflatoxin B1 were 0.38±0.09 ng/ml in HCC cases and 0.28±0.03 ng/ml in COL cases. Increased concentration of AFB1 was found in HCC cases in this study (Table 4).

DISCUSSION

Aflatoxin is a hepatotoxic, carcinogenic, immunosuppressive, antinutritional contaminant of many staple food commodities. Contamination may develop as a result of fungal action before and during harvest and also during storage. Studies show that aflatoxin is a factor with established or potential influence on 6 of the 10 most important health risks identified by the WHO for developing countries where short lifespan is prevalent.[9] Due to highly immunosuppressive and carcinogenic nature of AFB1, even low-level contamination is important. Monitoring of their concentration in body fluids requires the determination of even trace amounts because of their potent biological activity. Also, their measurement in fluids would give a direct measurement of exposure.[10] Therefore, AFB1 (Biomarker) is also increased risk of development of liver cancer by presence of underlying oncogenic viral infection especially hepatitis B and C in this study. Chronic low-level exposure to aflatoxin particularly AFB1 is associated with increased risk of developing liver cancer. In people with chronic low-level aflatoxin exposure, HBsAg enhances the risk of developing liver cancer.[11]

Our study measured AFB1 in 91 serum samples of HCC and cirrhosis cases by ELISA. AFB1 was detected 11.4% (4/35, mean 0.38 ng/ml) in HCC cases and 7.1% (4/56, mean 0.28 ng/ml) in COL cases. In this study, all HCC cases with detectable AFB1 had presence of viral marker for hepatitis B and C. Most of the COL with detectable AFB1 had absence of viral markers for chronic hepatitis infections except only one case who had positive of anti-HCV. Previous studies, including animal, epidemiological and molecular studies, suggest that aflatoxin exposure may amplify the hepatic carcinogenic potential of HBV infection.[12]

In human studies, mean aflatoxin-albumin adducts in HBsAg-positive children in Gambia, West Africa were higher than those in non-carrier children. An interaction between aflatoxin and HBV is also suggested by the early onset of HCC in cases with detectable aflatoxin-DNA in liver tissue.[13] Epidemio-logical evidence suggests that dietary exposure to AFB1 and chronic infection with hepatitis B virus (HBV) are major risk factors for HCC.[14] Aflatoxin and hepatitis B virus exposure appeared to

interact synergistically to substantially increase the risk of cirrhosis, although this was not statistically significant.[15]

Aflatoxin also appears to have a synergistic effect on hepatitis C virus (HCV)-induced liver cancer, although the quantitative relationship is not as well established as that for aflatoxin and HBV in inducing HCC. Other important causative factors in the development of HCC, in addition to HBV or HCV infection and aflatoxin exposure, are the genetic characteristics of the virus, alcohol consumption, and the age and sex of the infected person.[3] The risk factors associated with hepatocellular carcinoma (HCC) include infection with HBV and HCV viruses, exposure to AFB1, and liver cirrhosis, due primarily to alcohol consumption.[16, 17]

Epidemiologic studies have clearly linked dietary exposure with increased risk of hepatocellular carcinoma. These investigations have been markedly improved by mechanism-based biomarkers that provide individual exposure assessment. Among these biomarkers, aflatoxin serum albumin adducts have been shown to be highly correlated with dietary aflatoxin intake and resultant cancer risk.[18] Aflatoxin contamination of different commodities should be monitored and toxicological data should be complied in order to set up the maximum permissible limit of aflatoxin in Myanmar based on the aflatoxin levels of different commodities of neighboring countries.[19]

Therefore, AFB1 contents should be determined in all kind of food and foodstaff of storage for long period especially all kinds of bean, corn, peanut, animal food staff, milk and milk product. In this study, both aflatoxin exposure and hepatitis B, C viral infections are common causes of hepatocellular carcinoma and interacted synergistically. The risk of HCC is greatly increased in chronic viral carriers exposed to other recognized risk factors, including exposure to AFB1. In cirrhosis of liver, aflatoxin B1 may be one of the risk factors for development without underlying hepatitis viral infections. Liver cancer is one of the main clinical outcomes from COL.

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