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RESEARCH ARTICLE

AFLATOXIN EXPOSURE IN VIRAL HEPATITIS B ADULTS IN GHANA

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ABSTRACT

The consumption of foodstuffs contaminated with aflatoxins may lead to hepatotoxic effect particularly in coexistence with chronic viral hepatitis B infection. In Sub-Saharan Africa, hepatocellular carcinoma is one of the most common malignancies. The aim of this study was to measure the level of exposure of aflatoxin and liver damage markers in adults with viral hepatitis B infection in Ghana. The study was conducted in the Ejura-Sekyedumase district, Ghana. Five hundred adults were screened for viral hepatitis B surface antigen. Spot urine and blood samples were collected. Analysis of AFM_1 in the urine were measured using immunoaffinity column purification (AflaTest) followed by HPLC-fluorescence detection. Viral hepatitis B surface antigen and liver damage markers were detected using Clinogen rapid kit and automated calorimetric method respectively in the blood samples. Out of total number of 500 samples screened, only 43(8.6%) tested positive for HBsAg whiles 457 (91.4%) tested negative. Forty three controls (HBsAg negative) were selected. There was significant difference in the AFM_1 levels between the HBsAg positive adults (5255.0±2757.9) and their controls (374.8± 227.7) (P=0.001). There was no significant difference in the ALT, AST, and GGT concentrations between the Hepatitis B surface antigen positive and their control group. Four adults (9.3%) tested positive for HBeAg with mean concentration of AFM_1, ALT and AST of 6107.1 pg/dL 56.7 U/L and 78.8U/L respectively. Results from our study have demonstrated significant levels of aflatoxin in HBsAg positive subjects compared to HBsAg negative subjects. The observations emphasize the need for aflatoxin exposure intervention strategies in high-risk countries; possibly targeted at postharvest.

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INTRODUCTION

Aflatoxins are potent carcinogens that are produced as secondary metabolites by strains of the fungi *Aspergillus parasiticus* and *Aspergillus flavus* that grow and contaminate food crops such as groundnuts, maize and other oilseeds (Gourama and Bullerman, 1995). Aflatoxins exist in four major forms such as, B1, B2, G1 and G2 which can occur together in different proportions in various foods (Gourama and Bullerman, 1995). Aflatoxin B1 (AFB_1) is usually the predominant and most toxic form, and is classified by the World Health Organisation as a class 1 carcinogen (Tseng, 1994). A major metabolic product of AFB_1 is aflatoxin M1 (AFM_1) which can be excreted in milk and urine of dairy cattle and other animals (Gourama and Bullerman, 1995).

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Aflatoxins and hepatitis B viral (HBV) infections are major risk factors for the incidence of hepatocarcinoma in many parts developing countries. There are about 300–400 million chronic HBV carriers worldwide who continue to be at risk of developing hepatocellular carcinoma. Many of these HBV carriers reside in parts of the world with high exposure to aflatoxin-contaminated foods (Wild *et al.,* 1992). Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths worldwide, with over 500,000 people affected. The incidence is high in Africa and Asia, where high prevalence of hepatitis B strongly predisposes people to chronic liver disease and subsequent development of HCC (Cicalese and Luca, 2015). Approximately 250,000 deaths are caused by HCC in China and Sub-Saharan Africa annually and these have been attributed to factors such as aflatoxin intake and the incidence of viral hepatitis Binfection (Wild *et al.,* 1992). Hepatitis B virus primarily interferes with the functions of the liver by replicating in hepatocytes using a functional sodium-taurocholate cotransporting polypeptide (NTCP) (Li *et*

al., 2010). During HBV infection, the host immune response causes both hepatocellular damage and viral clearance. Cytotoxic T lymphocytes (CTLs), contributes to most of the liver injury associated with HBV infection by killing infected cells and producing antiviral cytokines, which are then used to purge HBV from viable hepatocytes (Coffin *et al.,* 2011). Aflatoxins, after entering the body are metabolized by the liver to a reactive epoxide which may intercalate into DNA and alkylate the bases through epoxide moiety. Chronic exposure increases the risk of developing liver cancer in the presence hepatitis B virus (Aguilar *et al.,* 1993). This causes mutations in the *p53* gene, an important gene in preventing cell cycle progression when there are DNA mutations, or signaling apoptosis (Aguilar *et al.,* 1993).

Epidemiological studies (Ross *et al.,* 1992; Wang *et al.,* 1996) suggest that, risk of HCC is enhanced with chronic HBV infection and frequent exposure to dietary aflatoxins. Studies have also demonstrated correlation between aflatoxin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in mice induced with 750-1500ug/kg of AFB_1 (Wild *et al.,* 1992; Anamika *et al.,* 2013). Urinary markers (i.e. AFB-N7-guanine, AFB_1-formamidopyrimidine, AFB_1-albumin adduct, AF-mercapturic acid and AFM_1) have been developed and applied in multiple epidemiological studies and clinical intervention trials (Jolly *et al.,* 2006; Turner *et al.,* 2007).

The AFB_1-albumin marker indicates exposure over a number of weeks, whereas the AFM_1 marker is reflective of acute aflatoxin consumption allowing detection of AFM_1 24–48 hr after exposure. Two cohort studies in South-East Asia, have demonstrated an interaction between hepatitis B viral infection and aflatoxins in determining hepatocarcinoma risk. Both studies used biomarkers of aflatoxin M1 (AFM_1) to classify individuals for exposure status and showed significant association with chronic hepatitis B infection in relation to hepartocarcinoma (Qian *et al.,* 1994; Wang *et al.,* 1996). In Ghana where HCC mortality accounts for approximately 21% of all cancer related deaths in adults, high rates of aflatoxin exposure have been demonstrated (Jolly *et al.,* 2006; Kumi *et al.,* 2014; Wang *et al.,* 2008; Wiredu and Armah, 2006). Prevalence of hepatitis B and hepatitis C viral infection has been reported in Ghana as 10.53% and 5.63% respectively (Nkrumah *et al.,* 2011). However, data on human exposure to aflatoxin and the incidence of viral hepatitis is not readily available. Thus, the aim of the study was to measure biomarkers of aflatoxin exposure and liver damage in adults with viral hepatitis B infection.

Ethical approval

Ethical approval was given by the Ethical and Protocol Review Committee of the College of Health Sciences, University of Ghana, Legon with protocol identification number: MS-Et/M.7-P 3.6/2014-2015.

Sampling Methods

The study employed the modified World Health Organization (WHO) cluster sampling method to select eligible subjects. Inclusion criteria included: adults of 18yrs of age and above with Informed consent signed. Exclusion criteria include children below 18yrs. The Ejura-Sekyedumase district community in the Ashanti Region of Ghana was segmented into four clusters (communities) by natural/geographical boundaries. A cluster was chosen by a simple random sampling technique and all eligible subjects within households who consented were included. Homes in the community have standard house numbering systems, and a household within a house was numbered serially.

If more than one household existed within a home, then the first household interviewed was designated as (house number/001), the second household interviewed was designated as (house number/002), and serially if there are more eligible households within the same house. All households within a selected cluster were eligible for inclusion. One adult member of each household within a house was interviewed, and this continued until the number of subjects to be screened (500) was obtained. One hundred and twenty five subjects were selected from each community.

Study Population

A total of five hundred (500) subjects including three hundred and twenty (320) males and one hundred and eighty (180) females who consented were screened for viral hepatitis B surface antigen based on a prevalence rate of more than 10% of hepatitis B viral infection in the Ejura-Sekyedumase district (Ejura, 2014). Forty three (43) subjects (33males and 10 females) who tested positive for viral hepatitis B surface antigen were selected with age ranging from 18-47years. Based on the initial findings of 43 subjects who tested positive for hepatitis B surface antigen, forty three (43) subjects (30 males and 13 females) who tested negative for hepatitis B surface antigen were selected as controls with age ranging from 18-47 years similar to the positive group.

MATERIALS

Vicam AflaTest kits for the detection and quantification of aflatoxin were used according to the Association of Official Analytical Chemists' method (AOAC 993.31, V1 series 4).Sodium chloride (NaCl) and methanol (HPLC grade) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). AflaTest columns were purchased from VICAM (Watertown, MA, USA). All other chemicals and reagents used were of the highest purity that was commercially available.

Specimen Collection and Processing

Urine Collection

Spot urine samples were successfully collected from 86 adults into a sterile specimen cup at the Ejura-Sekyedumase district hospital laboratory. The urine samples were frozen at -20^{o}C and transported to Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon, in a leak proof cool box. The urine samples were kept frozen at -20^{o}C prior to the analysis of AFM_1 levels at NMIMR, University of Ghana, Legon.

Blood Collection

Whole blood (5mls) was collected from each study participant by venipucture into a labeled serum clotter tube at the Ejura-Sekyedumase district hospital laboratory. The blood was centrifuged at 2,000rpm for 10 minutes to collect the serum. Aliquots of serum samples were stored in freezing vials, labeled with each participant's identification number. The serum samples were frozen and transported to NMIMR in a leak proof cool box. The serum samples were kept at -20ºC until prior to analysis.

Sample Analysis

Urinary Aflatoxin M1 (AFM_1)

Aflatoxin M_1 levels were analyzed with immunoaffinity column purification (AflaTest) followed by with HPLC-flourescence detection. Each urine sample (5ml) was acidified with 0.5ml of 1.0M ammonium formate (pH 4.5) and diluted to a volume of 10ml with deionised water. Samples were then allowed to flow through immunoaffinity columns by gravity at a flow rate of 1-2 drops per seconds. Columns were washed with phosphate buffered saline (PBS) and deionised water before elution of AFM_1 with 80% methanol. The eluants were then dried under nitrogen gas and resuspended in methanol: ammoniumformate (ratio 1:1) solution for analysis using a Shimadzu HPLC system with fluorescence detection (Shimadzu Corporation, Japan). A 250 x 4.6 mm LiCrospher RP-18EC end capped column with a pore size of 100 and a particle size of 5u (Alltech) was used to resolve aflatoxin metabolites. The mobile phase was composed of 22% ethanol in deionised water buffered with 20 mM ammonium formate (pH 3.0). Samples (100 ul) were injected at an elution rate of 1ml/min. The limit of detection was 0.5 pg AFM_1/ml urine. Analysis of AFM_1 was done at the Clinical Laboratory Department of the Noguchi Memorial Institute for Medical Research.

Liver Damage Markers

The levels of liver damage markers in the serum; alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB) and gamma-glutamyl transferase (GGT) were measured using an automated calorimetric method by (Flexor E, endpoint automation, Vital Scientific, Netherlands).

Viral Hepatitis B surface antigen screening

Screening of viral hepatitis B surface antigen was done using the Clinogen rapid kit from Warehouse/Robin Willis Way, Windsor SL4 2PX, United Kingdom, with a specificity of 98.5%, sensitivity 99.0% and an accuracy of 98.5%. A 50 ul aliquot of each serum sample was used for hepatitis B surface antigen screening.

Hepatitis B viral Profile

Hepatitis B core antibody (HBcAb), Hepatitis B envelope Antigen (HBeAg), Hepatitis B envelope Antibody (HBeAb) and Hepatitis B surface Antibody (HBsAb) were determined for samples which tested positive for hepatitis B surface antigen using a rapid assay from Clinogen Laboratories.

Statistical Analysis

The data of AFM_1, ALT, AST and GGT were analysed for range, mean (n) and standard deviation (SD) using and an IBM Statistical Package (version 20). Of our data Statistical significance was determined using the t-test analysis. $P < 0.05$ was considered to be significant.

RESULTS

The study recruited five hundred participants (320 males and 180 females) who were screened for hepatitis B viral surface antigen. Out of the 500 subjects screened, 43 (8.6%) including 33 (6.6%) males and 10 (2%) females tested positive for hepatitis B surface antigen only (Table 1). Four hundred and fifty seven (457) subjects representing 91.4% of the total subjects screened, tested negative for hepatitis B surface antigen. Four subjects (0.8%) including 3 females and 1 male tested positive for hepatitis B envelope antigen (Table 1).

Table 1. Gender and age distribution of hepatitis B surface antigen positives

	Number of HBsAg Positives		Number of HBsAb Positives		Number of HBcAb Positives		Number of HBeAg Positives		Number of HBeAb Positives	
	M	F	M	F	M	F	M	F	M	F
Total	33	10	--	----	33	10	3	1	26	7

Total number of participants tested (N) =500.

Table 2. Results of urinary AFM_1 and liver damage markers in hepatitis B surface antigen (HBsAg) positive subjects and controls

Markers	HBsAg Positive Subjects		HBsAg Negative Subjects		t-Test
	Mean±SD	Range	Mean±SD	Range	P-value
AFM_1 (pg/dL)	5255.0±2757.9	768-12427	374.8±227.7	61.3-717.6	0.001*
ALT (U/L)	25.6±16.1	5.4-70	19.0±11.4	2-49	0.066
AST (U/L)	41.8±27.6	13.5-153	37.8±16.4	6.9-67	0.367
GGT (U/L)	46.1±26.4	5.9-215	22.4±12.1	5.2-49.3	0.021*

Data is presented as mean±SD. There was a significant difference in the mean AFM_1 and GGT of group A and group B (p < 0.05). Normal ranges: AFM_1=0.5 ug/kg (FAO, 2003). AST=13-60 U/L, ALT=6-54 U/L, GGT=6-71 U/L and ALB=32.7-49.8 g/L. (Dosoo et al., 2012).*

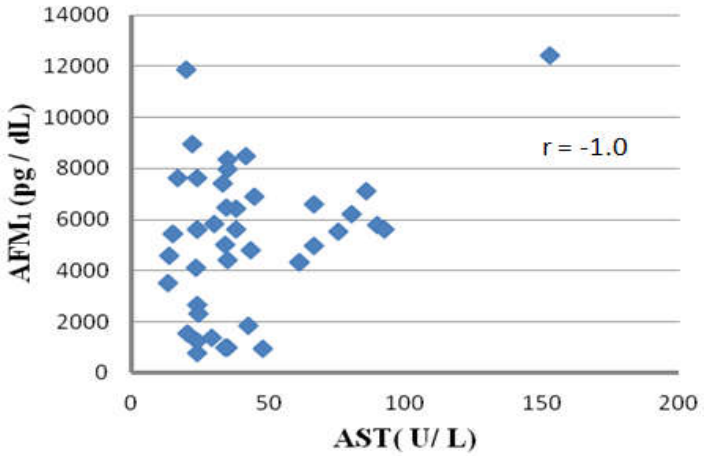

Figure 1a. Association between urinary AFM_1 values and serum AST values in HBsAg positive subjects (N=43). There was no association between urinary AFM_1 and serum AST of HBsAg positive subjects (r = -1.0). ***r = 0.9: strong positive association, r = 0.2: weak association and r = - 0.9: strong negative association***

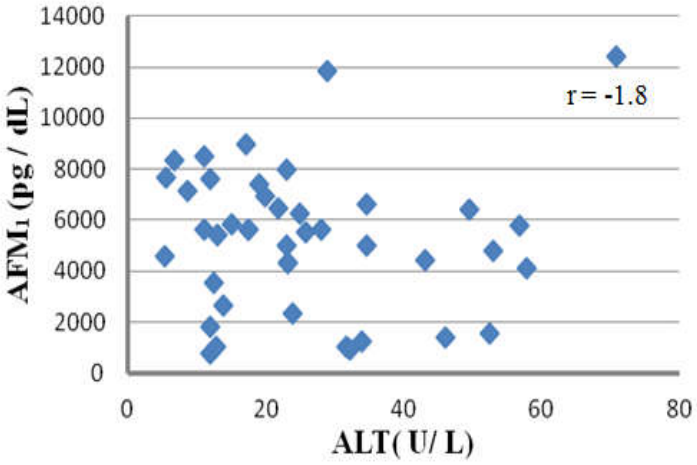

Figure 1b. Association between urinary AFM_1 values and serum ALT values in HBsAg positive subjects (N=43). There was no association between urinary AFM_1 and serum ALT of HBsAg positive subjects (r = - 1.8). ***r = 0.9: strong positive association, r = 0.2: weak association and r = - 0.9: strong negative association***

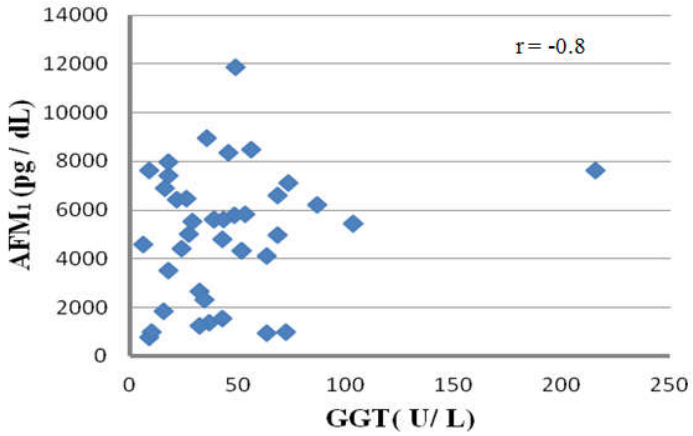

Figure 1c. Association between urinary AFM_1 values and serum GGT values in HBsAg positive subjects (N=43). There was no association between urinary AFM_1 and serum GGT of HBsAg positive subjects (r = - 0.8). ***r = 0.9: strong positive association, r = 0.2: weak association and r = - 0.9: strong negative association***

Comparison of urinary AFM_1 level and biomarkers liver damage

Table 2 shows the comparison of urinary AFM_1 levels and the levels of biomarkers of liver damage between hepatitis B surface antigen positive and hepatitis B surface antigen negative subjects. The average values of urinary AFM_1, serum AST, ALT and GGT for HBsAg positive subjects were 5,255.0 pg/dL, 41.8 U/L, 25.6 U/L and 46.1 U/L respectively. The average values of urinary AFM_1, serum AST, ALT, and GGT for HBsAg control group were 374.8 pg/dL, 37.8U/L, 19.0 U/L and 22.4 U/L respectively (Table 2). There was a 14 fold increase of urinary AFM_1 in hepatitis B surface antigen positive participants compared to hepatitis B surface antigen negative participants (Table 2). Four samples showed positive for hepatitis B envelope antigen, had mean values of AFM_1= 6536.7 pg/dl, AST = 78.8 U/L and ALT= 58.8 U/L. Figure 1a shows a correlation graph of urinary AFM_1 vrs serum AST of hepatitis B surface antigen (HBsAg) positive subjects. There was no association between AFM_1 and AST of hepatitis B surface antigen positive subjects (r = - 1.0).

Figure 1b shows a correlation graph of urinary AFM_1 vrs serum ALT of hepatitis B surface antigen (HBsAg) positive subjects. There was no association between urinary AFM_1 and serum AST of hepatitis B surface antigen (HBsAg) positive subjects (r = - 1.8). Figure 1c shows a correlation graph of urinary AFM_1 vrs serum gamma-glutamyl transferase (GGT) of hepatitis B surface antigen (HBsAg) positive subjects. There was no association between urinary AFM_1 and serum GGT of hepatitis B surface antigen (HBsAg) positive subjects (r = - 0.8).

DISCUSSION

A significant association between aflatoxin exposure and human liver disease has been reported in endemic areas in Sub-Sahara Africa, East and Southeast Asia (Qian *et al.,* 1994; Colombo, 1992). Reported rates of hepatitis B viral (HBV) positivity in The Gambia, China and Guinea are 15% (Allen *et al.,* 1992) 14-20% (Wang *et al.,* 2001) and 10% (Diallo *et al.,* 1995) respectively. The effect of hepatitis B viral infection and the efficiency with which aflatoxin is detoxified is affected by biological exposure to aflatoxin (Kelly *et al.,* 1997). Two cohort studies in South-East Asia, have demonstrated an interaction between hepatitis B virus and aflatoxins in determining hepatocarcinoma risk. Both studies used biomarkers of aflatoxin M1 (AFM_1) to classify individuals for exposure status and showed significant association with chronic infection in relation to HCC (Wang *et al.,* 1996; Qian *et al.,* 1995). The present study measured the levels of aflatoxin exposure and liver damage biomarkers in adults with viral hepatitis B infection. The levels of urinary AFM_1 concentrations found in subjects in the present study (both control and hepatitis B surface antigen positive) ranged from 61.3 to 12,427pg/dL (Table 2).

Aflatoxin was detected in urine samples of 45 subjects in the range of 180 to 3,990 pg/ dL in a study involving a Brazilian population (Alessandra de Cássia Romero *et al.,* 2009). The results showed a lower incidence range of urinary AFM_1 concentration as compared to the current study. In a study involving aflatoxin exposure, malaria and hepatitis B infection in rural Gambia reported that, aflatoxin exposure was much lower in the rainy season compared to the dry season (Allen *et al.,* 1992). However, the current study was not conducted in the rainy season in Ghana. The difference in urinary AFM_1 concentration between the findings in the present study and the report by Alessandra de Cássia Romero and his colleagues (2009) may have occurred due to seasonal variation and dietary exposure to aflatoxin. Aflatoxin exposure in hepatitis B surface antigen positive individuals is higher when compared to hepatitis B surface antigen negative individuals (Henry *et al.,* 2002). Thus, reduction in the intake of aflatoxins in populations with a high prevalence of hepatitis B surface antigen positive (HBsAg positive) group will have greater impact on reducing liver disease (Henry *et al.,* 2002). Prospective studies in Shanghai, China by Qian *et al.,* (1994) showed that urinary excretion of aflatoxin metabolite is a useful indicator for the increased risk of hepatocarcinoma. The present study demonstrated significant differences in urinary aflatoxin M1 levels in subjects (43) who were positive for HBsAg when compared to the control group (Table 2). The findings from the present studies are comparable to the study by (Mizrak *et al.,* 2009) who reported that, in viral hepatitis B positive subjects, aflatoxin concentration is significantly higher than hepatitis B surface antigen negative subjects in Turkey.

Although aflatoxin B1 (AFB_1) might contribute to hepatocarcinogenesis by other mechanisms, its role in pathogenesis of hepatocarcinoma is primarily mediated by its effects on chronic hepatitis B viral infection. Various mechanisms of interaction between aflatoxin and HBV in hepatocarcinogenesis have been proposed. It may reflect changes in metabolism of aflatoxin B1 (AFB_1) with coexistence of HBsAg. Hepatitis B viral infection sensitizes hepatocytes to the carcinogenic effects of aflatoxin B_1 (Kew, 2003) by inducing cytochrome P450 enzyme that metabolize AFB_1 to the toxic metabolite AFB_1-8,9-epoxide (Chemin *et al.,* 1999). However, other studies suggest that positive interaction between HBV and aflatoxin B1 (AFB_1) seemed to depend on the absence of detoxification enzymes like glutathione-S-transferase, which converts the carcinogenic AFB_1-8, 9-epoxide to non-reactive metabolites (Chemin *et al.,* 1999; Chen *et al.,* 1996; Yu *et al.,* 1997; Sun *et al.,* 2001). A factor in this greater potency of aflatoxin in HBV-positive people is that, HBV positivity reduces the person's ability to detoxify aflatoxin (Allen *et al.,* 1992). In a report by Qian *et al.,* (1994) exposure of aflatoxin in HBV positive subjects increased HCC risk up the risk to up 7-fold. However, the present study demonstrated a 14 fold (Table 2) increase of urinary aflatoxin M1 (AFM_1) in HBsAg positive subjects compared to the control group and a 2 fold difference when compared to the study by (Qian *et al.,* 1994). Differences in exposure risk of aflatoxin in HBV positive subjects could be due to the concentrations of aflatoxin B1 ingested and the efficiency with which aflatoxin is detoxified by the liver (Kelly *et al.,* 1997). Primary prevention, such as vaccination for hepatitis B virus and control of aflatoxin contamination of food offers strategies for lowering HCC rates. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are commonly measured as a clinical biomarker for the health of the liver (Hou *et al.,* 2013).

Alterations in serum levels of ALT and AST have been considered as a tool for studying varying cell viability and changes in cell membrane permeability (El-Zayadi, 2008). Albumin is the main protein made by the liver, circulates in the bloodstream and is affected by liver disorders. In a study involving children in The Gambia, blood samples were analyzed for aflatoxin-albumin adducts with serum alanine aminotransferase (ALT) as a marker of liver damage. The subjects were followed for one year. The study reported no significant difference between aflatoxin-albumin adduct and ALT (Wild *et al*., 1993). In table 2 of the present study there was no significant difference of the ALT biomarker between urinary AFM_1 HBsAg positive group and urinary AFM_1 HBsAg negative group. These findings concur to the study conducted by (Wild *et al.,* 1993). An association was not found between Urinary AFM_1 and AST/ALT/GGT in the HBsAg positive group (Figures 1a, 1b and 1c). Gamma-glutamyl transferase (GGT) is a prime marker of bile duct epithelial proliferation that is typical of aflatoxicosis (Kramah, 1989). In a study conducted by Mohgah and his group (2014) in Egypt, flour mill workers who were exposed to aflatoxin between 7-38 years had a significant difference of GGT between the exposed workers and their controls.

There was a significant difference of GGT between urinary AFM_1 of HBsAg positive subjects and their control group in the current study. Gamma-glutamyl transferase is also used to evaluate bile duct epithelial proliferation that is typical of aflatoxicosis and screen for chronic alcohol abuse in people who are receiving treatment on alcoholic hepatitis (Kramer, 1989). In the present study, chronic alcohol abuse and bile duct obstructions were not evaluated as an objective. Therefore the significant difference in GGT (Table 2) between the hepatitis B surface antigen positive group and the control group may not be due to aflatoxin only, but other contributing factors such as alcohol and bile duct obstructions. Increase in liver damage markers in chronic hepatitis B infection may also depend on the chronic phases of hepatitis B viral infection (McMahon, 2005). Four (4) subjects with hepatitis B envelope antigen positive showed high mean concentrations of urinary AFM_1 and liver damage markers as compared to those without hepatitis B envelope antigen.

Four subjects with HBeAg positive in relation to liver damage markers and aflatoxin exposure were not large enough to confirm the relationship between aflatoxin and hepatitis B envelope antigen. Studies involving aflatoxin exposure and hepatitis B envelope antigen are not readily available. The findings of the present studies warrant further studies to confirm the relationship between hepatitis B envelope antigen and aflatoxin exposure using a larger sample size. Results from this study have demonstrated significant levels of aflatoxin in hepatitis B surface antigen positive subjects as compared to hepatitis B surface antigen negative subjects and have provided for the first time in Ghana, information on aflatoxin levels in hepatitis B positive subjects.

The present studies employed subjects who were exposed to hepatitis B virus with no liver diseases. Therefore aflatoxin exposure in people with liver disease warrants further studies especially in an aflatoxin endemic area. The observations of aflatoxin exposure emphasize the need for aflatoxin exposure intervention strategies in high-risk countries; possibly targeted at postharvest stage. Therefore there is a critical need to educate people on the dangers of aflatoxin exposure to humans and to develop an economically feasible strategy to eliminate aflatoxin.

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