World Journal of Medical Sciences 11 (4): 570-577, 2014
ISSN 1817-3055

DOI: 10.5829/idosi.wjms.2014.11.4.9181

Detection of Aflatoxins B_1 and M_1 in Serum of Different Types of Cancer Patients in Sana'a, Yemen and Their Effects on Liver and Kidney Function

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Abstract: This study was conducted to determine AFB_1 and AFM_1 in the serum of cancer patients to provide a better assessment on the extent of human exposure to aflatoxin in Yemen and to evaluate their effects on liver and kidney function in different types of cancer patients in Sana'a, Yemen. Forty two cancer patients and 40 matched controls volunteers were subjected to the current study. AFB_1 and AFM_1 were determined by HPLC using immunoaffenity column cleanup and liver and kidney functions were determined in serum of patients and controls. The results revealed the mean age of cancer patients was relatively higher than the control, the prevalence of cancer was higher in males than females and in rural than the urban residence. However no significant effect difference was found between the cancer patients and the control regarding the other social habits studied. The concentration of AFB_1 was found to be higher in all cancer patients except esophagus cancer patients compared to the control. However, AFM_1 was detected in a higher concentration in all types of cancer patients compared to the controls. No significant effect difference was observed in ALT and AST between cancer patients and the control although the other liver tests were found to be disturbance. The kidney function tests for the cancer patients were in the normal range of the control. It could be concluded that the occurrence of AFB_1 and AFM_1 in the serum of cancer patients and the controls volunteers is a risk factor which may be increase the prevalence of cancer in Yemeni population.

Key words: Aflatoxin · Cancer · Liver · Kidney · Yemen

INTRODUCTION

According to the National Oncology Center, Sana'a, Yemen in 2007, cancer is thought to be a major public health problem in Yemen, the size magnitude of the problem and underlying risk factors are not yet well studied. Yemen is geographically located in South West Asia at the southern tip of the Arabian Peninsula between Oman and Saudi Arabia. Due to its location, temperatures are generally very high in Yemen, particularly in the coastal regions with rainy summer. These conditions enhance the growth of mold in the food commodities where aflatoxins are produced favorably. Peanuts, cereals, spices and their products are the commodities most susceptible to aflatoxin contamination and the occurrence of aflatoxin in these foodstuffs is frequently reported in Yemen [1, 2].

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Aflatoxins are produced by *Aspergillus flavus*, A*spergillus paraciticus* and *Aspergillus nomius* [3] and are ubiquitously found in many food and agricultural commodities such as nuts [4], cereals [5, 6], milk and dairy products [7], spices and herbs [3]. The molds responsible for the production of aflatoxins are particularly adapted to the warm and humid climates in tropical and sub-tropical of many developing countries, where the conditions promote the growth of fungi and subsequently aflatoxin production [8]. Naturally, four metabolites of aflatoxin have been identified, namely aflatoxin B_1 (AFB), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) [9, 10]. The classification of B (blue) and G (green) series of aflatoxin is based on the fluorescence color produced under the UV light [11]. AFB_1 is the most dangerous metabolite as it is a known causal agent of human hepatocellular carcinoma (HCC) [12]. Moreover, several epidemiological studies have implicated AFB_1 in the increased incidence of human gastrointestinal (GI) and hepatic neoplasms and other organ cancer in Africa, the Philippines and China [13, 14]. AFB_1 itself is not a potent toxin and bioactivities by liver CYP450 enzymes, particularly CYP3A4 and CYP1A2 [9, 15-] to AFB_1-8, 9-epoxide to form adducts with DNA and protein albumin, where its toxicity and carcinogenic effects are evident [19]. This simultaneous reaction to DNA and protein albumin occurred due to the short half-life of the epoxide [19], but the intermediate also can undergo further degradation into metabolites such as aflatoxin Q_1 (AFQ_1), aflatoxin M_1 (AFM_1) and aflatoxin P_1 [16], where they are excreted in feces and urine. Sabran *et al*. [20] and Mohd Redzwan *et al.* [21] found the presence of urinary AFM_1 in almost all participated subjects in Penang, Malaysia and this finding was in line with Leong *et al*. [22] as 97% of 170 subjects had detectable level of serum AFB_1-lysine adduct. With the presence of aflatoxins in foods and other aspects of the human environment, an association between disease and exposure would be expected, *i.e*. the link between aflatoxin and liver cancer [23] or other organ cancer [14, 24, 25]. Direct evidences for human ingestion of aflatoxins or by other route of exposure have been found in a number of countries by the identification of aflatoxin or its metabolites in human biological samples [9, 26, 27]. This is a worrying issue as not just adults but infants and young children are also directly exposed to this food contaminant. The aim of the current study was to determine the level AFB_1 and AFM_1 in serum of patients with different types of cancer and their effects on different liver and kidney function.

MATERIALS AND METHODS

Participants: A total of 42 new diagnosed patients (26 male and 16 female with mean age >30 and <60 years old) with different types of cancer (Gastric cancer, intestinal cancer, colonic cancer, tongue cancer and esophagus cancer) were subjected to the current study at National Oncology Center, Sana'a, Yemen during a period of 5 months (December to April, 2012). All the selected cancer patients were not subjected to any chemical therapy. Forty matched control volunteers (33 male and 7 female) were recruited from Sana'a University. Blood samples were collected from the patients and control for the determination of AFB_1 and AFM_1 as well as different biochemical analysis.

Laboratory Assay: Serum was separated by centrifugation and the samples were used for the determination of biochemical parameters using a Diminsion Xp and Plus integrated clinical chemistry autoanalyzer (Seimens Healthcare Diagnostics, Dear field, IL, USA). The following tests were carried out: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), direct bilirubin, total bilirubin, albumin, creatinine, urea, cholesterol, low density lipoprotein (LDL), triglycerides, total protein and glucose. Determination of aflatoxins by HPLC: AFB_1 and AFM_1 from serum samples were extracted using the modified method of Mokhles *et al.* [28]. Briefly, 2 ml serum samples were warmed to 37°C and were shaken to distribute fat. The samples were centrifuged (3000 g, 15 min, 5°C) to remove fat and filtered via glass wool. To facilitate the passage through a C18 cartridge (Strata C18-E, 50um, 70A, Phenomenex, USA), the samples were diluted 1:1 with milli-Q water. The cartridge was pre-activated with 10 ml acetonitrile and then 10 ml of water, prior to passage of diluted serum at a flow rate of 3.5 ml/min. The loaded cartridge was then washed with 10 ml water, 10 ml basic acetonitrile/water (1% ammonia, 10% acetonitrile) and 10 ml acidic acetonitrile/water (1% acetic acid, 10% acetonitrile). AFM_1 was eluted with 5 ml acidic acetonitrile (1% acetic acid, 40% acetonitrile). AFM_1 was extracted twice from the eluent with 2 ml dichloromethane. Following centrifugation (3000g, 15 min) to separate the layers, the two dichloromethane fractions were pooled and dried under nitrogen gas. The residue was dissolved in 0.7 ml of methanol. Two hundred µl quantities of hexane were added to the clean up dry film of standard and samples followed by 50 µl trifluoraacetic acid (TFA) and they were mixed well for 30s and the mixture was let to

stand for 5 min. To the mixture, 450 ml H_2O: CH_3CN (9:1 v/v) were added and mixed well by vortex for 30s and the mixture was left to stand for 10 min [29].

Statistical Analysis: All data were statistically analyzed using the General Linear Models Procedure of the Statistical Analysis System [30]. The significance of the differences among study groups was determined by Waller-Duncan k-ratio [31]. All statements of significance were based on probability of $P < 0.05$.

RESULTS

The demographic data comprising distribution of age, sex, smoking and residence between control and cancer groups were represented in Table (1). These results indicated that the mean age of cancer patients (48.54 ± 5.6) was relatively higher than that of the control (42.5 ± 8.6). The distribution of sex among the cancer group showed that the percentage of male cancer patients (61.9 %) was significantly higher than female cancer patients (38.1 %). However, the percentage of cancer patients who are cigarettes smoking, medaah or shisha smoking and shamma consumption were significantly higher than the control however, chewing qat, has no significant effect since the percentage of cancer patients who did not chewing qat was higher than those chewing qat (Table 1). The results also revealed that the residence area has a significant effect on cancer disease since the percentage of patients live in rural area was significantly higher than those live in the urban area.

The results also indicated that serum AFB_1 and AFM_1 concentration were higher in cancer patients compared to the control (Fig. 1) except AFB_1 in the esophagus cancer patients who showed a concentration of AFB_1 lower than the control (Table 2). No significant difference was observed in ALT and AST between the cancer group and the control however; the other liver function parameters including total protein, albumin, total bilirubin, triglycerides and LDL were significantly higher in the cancer patients compared to the controls. The activity of alkaline phosphatase and the level of direct bilirubin and cholesterol showed a significant decrease in cancer patients compared to the control (Table 3). On the other hand, no significant difference was observed between the cancer group and the control in createnine and urea as kidney function tests (Table 4). The same table also showed that random blood glucose was insignificantly higher in the cancer patients compared to the control.

Table 1: Distribution of age, sex, Qat consumption, smoking and residence and between cancer group and control under study

		Control (n=40)	Cancer patients (n=42)
Age		42.5 ± 8.6	48.54 ± 5.6
	<50	14 (35 %)	15 (35.7 %)
	>50	26 (65 %)	27 (64.3 %)
Gender	M	33 (82.5 %%)	26 (61.9 %)
	F	7 (17.5 %%)	16 (38.1 %)
Qat-chewing	Yes	29 (72.5 %)	20 (47.6 %)
	No	11 (27.5 %)	22 (52.4 %)
Smoking cigarettes	Yes	7 (17.5%)	9 (21.4%)
	No	33 (82.5%)	33 (78.6%)
smoking medaah or shisha	Yes	2 (5.0%)	7 (16.7%)
	No	38 (95.0%)	35 (83.3%)
consume shamma	Yes	0	7 (16.7%)
	No	40 (100%)	35 (83.3%)
Residence	Rural	6 (15.0%)	35 (83.3%)
	Urban	34 (85.0%)	7 (16.7%)

Table 2: Serum AFB_1 and AFM_1 concentration in patients with different type of cancer

Groups	AFB_1 (pg/ml)	AFM_1 (pg/ml)
Control	0.09 ± 0.03^a	1.08 ± 0.63^a
Gastric cancer	0.10 ± 0.03^a	2.27 ± 0.45^b
Intestinal cancer	0.22 ± 0.13^b	5.5 ± 2.38^c
Colonic cancer	0.17 ± 0.097^b	6.75 ± 3.67^d
Tongue cancer	0.13 ± 0.09^c	2.05 ± 1.22^b
Esophagus cancer	0.04 ± 0.02^d	5.22 ± 1.43^c

Within each column, means superscript with different letters are significantly difference (P= 0.05)

Table 3: Liver function test in control and cancer patients

Parameters	Control	Cancer group
ALT (IU/L)	27.93 ± 1.06[a]	29.52 ± 1.02[a]
AST (IU/L)	29.05 ± 0.86[a]	31.25 ± 1.06[a]
ALP (IU/L)	110.79 ± 4.11[a]	99.55 ± 3.29[b]
TP (mg/dl)	3.86 ± 0.04[a]	4.58 ± 0.20[b]
Alb (mg/dl)	0.57 ± 0.09[a]	1.73 ± 0.24[b]
TB (mg/dl)	0.14 ± 0.01[a]	0.34 ± 0.07[b]
DB (mg/dl)	151.7 ± 10.43[a]	69.58 ± 9.41[b]
Cholesterol (mg/dl)	165.1 ± 19.52[a]	115.57 ± 4.42[b]
TriG (mg/dl)	58.1 ± 4.26[a]	89.19 ± 5.71[b]
LDL (mg/dl)	5.68 ± 0.07[a]	32.18 ± 5.22[b]

Within each row, means superscript with different letters are significantly difference (P= 0.05)

Table 4: Kidney function in control and cancer patients

Parameters	Control	Cancer group
Createnine (mg/dl)	0.68 ± 0.03[a]	0.68 ± 0.03[a]
Urea (mg/dl)	27.78 ± 0.98[a]	27.60 ± 1.54[a]
RBS (mg/dl)	105.03 ± 2.98[a]	110.52 ± 3.64[a]

Within each row, means superscript with different letters are significantly difference (P= 0.05)

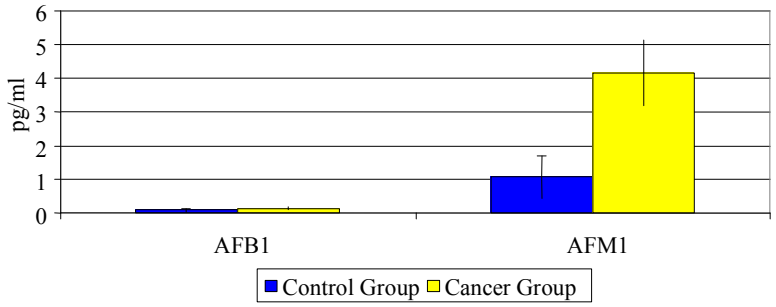

Fig. 1: AFB_1 and AFM_1 concentration in serum of cancer patients and their matched control

DISCUSSION

The current study represents the first comprehensive attempt to outline whether aflatoxin is a risk factor in different types of cancer patients in Yemen. The current study revealed that cancer was more prevalence in patients more than 50 years old, in male than females and in those resident in rural than in urban. No significant correlation was found between cancer incidence and qat-chewing, however; smoking cigarettes, medaah or shisha and shamma consumption showed a significant effect on cancer incidence. These results were in agreement with the previous studies of Bawazir *et al*. [32] and AL-Thobhani *et al*. [33]. The current results also detecte AFB_1 and AFM_1 in all samples of the control and cancer patients. However, the concentration of AFB_1 in all types of cancer patients was higher than the control group except the esophagus cancer patients. Patients with intestinal, colonic and esophagus cancer showed higher concentrations of serum AFM_1 compared to the controls. These results provide an interesting insight of human exposure to aflatoxin in Yemen via foodstuffs. Although no available data on human dietary exposure to aflatoxin in Yemen, Alghalibi and Shater [1] determined AFB_1 level in date samples in the range of 110-180 ìg/ kg which exceed the permissible level adopted by the FDA. These data provides an interesting insight on the extent of human exposure to aflatoxin in Yemen. Nonetheless, such exposure could be due to the consumption of aflatoxin-contaminated foods that may be a result of low knowledge and awareness on fungal and aflatoxin contaminations in the diets. In this respect, Jolly *et al*. [27] stated that people do not identify fungal contaminations until there are obvious sign of spoilage such as discoloration, insect infestation or rotting.

On comparing the levels of AFB_1 and AFM_1 detected in our study with other available data in the literature, it can be assumed that subjects in this study were moderately exposed to aflatoxin. For instance, the highest level of AFB_1 and AFM_1 detected was 0.128 and 4.1537 pg/ml respectively and it is considered low when compared to countries such as in Africa and East Asia

(China) where aflatoxin contaminations in the food and agricultural commodities are prevalent [27, 34]. Albeit the detectable level of AFB_1 and AFM_1 in the current study can be considered low than those high-risk populations, the level is higher than reported in America and some European countries. Johnson *et al*. [35] found AFB_1-lysine adduct in 20.6% of serum samples from San Antonio with an average of 3.84 pg/mg albumin, which is about two times higher than our average value. Thus, it is could be hypothesized that subjects in the Yemeni population had moderate exposure to aflatoxin. Recent exposure to aflatoxin is reflected in the urine or serum as directly excreted AFM_1 and other detoxification products. Measurements of aflatoxin and its byproducts in serum have been found to be highly variable from day to day, which reflects the wide variability in the contamination of food samples so the measurement of AFM_1 on a single day may not be a reliable indicator of a person's chronic exposure [36].

As stated earlier, most of the information indicated that there is an evidence that aflatoxin affects human immunity and nutrition. AFM_1 is a detoxification product of AFB_1 that is rapidly excreted by the liver [9, 37-39]. Moreover, AFM_1 toxicities may be modified by the dietary intakes of antioxidant vitamins, such as vitamins A, C and E [28, 40]. A previous study of human liver biopsies indicated that cases classified as mild chronic active hepatitis exhibit modestly increased aflatoxin activation, but no increase was observed in severe chronic hepatitis or cirrhosis [41].

The current results indicated that no significant changes between control and cancer patient in serum ALT and AST. ALT can be normal even in those with liver damage and can be elevated in diseases other than chronic liver disease. However, AST can be very high in acute hepatitis and drop to normal or slightly elevated in chronic hepatitis [42]. Moreover, a number of studies have reported the importance of aflatoxin in the development of liver cancer especially in the presence of hepatitis virus infection [43-46]. On the other hand, the animal studies had shown the effect of aflatoxin exposure on kidney functions as it reduced sodium phosphorus co-transport in proximal renal epithelium in opossum kidney cells [47], affected renal transport of calcium and phosphorus in avians [48] and impaired kidney function during the elimination of aflatoxin metabolites [49] and an autopsy on kidney of children exposed to aflatoxin in Nigeria showed detectable amount of aflatoxin [50]. Therefore, the association between liver and kidney biomarkers with aflatoxin could provide additional information on toxicity of aflatoxin on liver and kidney. Although no liver or urinary tract cancer patients were subjected to the current study, the occurrence of AFB_1 or its metabolite AFM_1 in serum of control or cancer patients may be suggested to be a risk factor of different organ cancer. In the current study, the levels of total bilirubin were positively correlated with the control level. Bilirubin is used as an indicator of hepatic injury as a rise in the level of total bilirubin was reported in an animal exposed to aflatoxin [51]. In this concern, Tao *et al.* [52] reported a close association between indirect (Total) bilirubin and aflatoxin level in non-infected HIV adults. Jolly *et al.* [53] suggested that the higher total bilirubin indicated liver damage or bile duct damage within the liver, but subjects in our study had normal value of total bilirubin and it is unlikely that aflatoxin caused the elevation of bilirubin. The low level of AFB_1 reported in the current study suggested that the reactive intermediate of AFB_1 (AFB_1-8,9-epoxide) can form glutathione conjugate (AFB_1-GSH) [16], the substrate for enzyme GGT [54] since the exposure of aflatoxin in rats elevated the glutathione level in the liver [55]. The serum creatinine is used as one of the indicators for renal health. Creatinine is produced from the breakdown of creatine phosphate in the skeletal muscle. However, diet containing high protein also can raise the creatinine level [56]. In aflatoxin-fed mice, Mathura and Verma [57] explained that high level of serum creatinine could be due to the increased transformation of phosphocreatinine to creatinine in muscle and inability of kidney to excrete creatinine in the urine. Although this finding was not agree with many animal studies such as in chicken [58] and rat [14, 55] as long term exposure to aflatoxin increased the creatinine level. However, it agrees with Desalegn *et al*. [59] who suggested that higher amount of aflatoxin was detected among those with early stage of chronic kidney disease, supporting the neprotoxicitity of aflatoxin. Therefore, the association between serum creatinine and aflatoxin level showed a promising finding on the impact of aflatoxin to kidney functions.

CONCLUSION

It can be concluded that mean age of cancer patients was higher than the control, the prevalence of cancer was higher in males than females in rural than the urban residence. However no significant impact of other social habits studied was found between the cancer patients and the control. Although the levels of serum AFB_1 and AFM_1 detected in the current study were not high compared to those high risk-populations, the serum samples analyzed

contained these carcinogenic mycotoxins and it may be associated with health risk after long term exposure. Furthermore, the association found between total bilirubin and creatinine level with the levels of serum AFB_1 and AFM_1 may indicate a possible toxicity effect of aflatoxin on liver and kidney. Even though, all the subjects had normal liver and kidney functions, this finding provides valuable reference for future studies to investigate the link between liver and renal injuries with serum aflatoxin especially among those who are at risk in Yemeni population. Intervention should be planned to reduce to limit the presence of aflatoxin in the diets.

ACKNOWLEDGMENTS

This work was supported by 48-Model Hospital, The authors would like to express there gratitude to the management of 48-Model Hospital, Sana'a-Yemen for the permanent attention and support decent for this search.

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