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Correlation Study Between Aflatoxin M_1 and Hepatitis C Virus in Egyptian Patients with Chronic Liver Disease

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Abstract: Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. Chronic HCV infection causes progressive hepatic fibrosis and cirrhosis in up to 20% of patients and <10-20% of cirrhotic patients develop hepatocellular carcinoma (HCC) within 5 years. Chronic hepatitis C with cirrhosis is a major risk factor for HCC in Egypt. This community-based study evaluated the role of aflatoxin M_1 (AFM_1) in advanced liver disease with hepatitis C virus (HCV) in Egyptian patients. This study was performed on 70 Cases (60 suffering from hepatic disease and 10 healthy persons) aged 30-58 years. AFM_1 in serum was quantified by HPLC method and HCV was quantified by RT-PCR. The results indicated that AFM_1 was significantly higher in male and female patients with high HCV titer compared to the normal healthy controls. However, both male and female patients with moderate HCV titer showed concentrations of AFM_1 lower than those of the normal healthy controls. It is worthy to mention that within the same group AFM_1 was significantly higher in females than males in the high and moderate HCV titer; however, in the healthy control AFM_1 was higher in males than in females. It could be concluded that increased aflatoxin M_1 level is associated with high HCV titer patients with chronic liver disease.

Key words: HCV · Liver Diseases · Aflatoxin · HCC · Cancer · Egypt

INTRODUCTION

HCV infection is a major health problem with an estimated 175 million infected people worldwide [1]. At least 6 major HCV genotypes have been identified and each one differs from the others by 30-35% of its nucleotide site sequence [1, 2]. It was observed that HCV genotype 4 (HCV-4) is common in the Middle East and Africa, where it is responsible for more than 80% of HCV infections and the highest number of infections is reported in Egypt [3]. The use of paranteral antischistosomal therapy in Egypt is thought to have contributed to a prevalence of antibodies against HCV in various regions ranging from 6 to 28%, where hepatitis C is highly endemic, HCV-4 is the predominant subtype [4]. During an acute HCV infection only 20-30% of the infected persons develop symptoms. On the other hand, only about 20% of the infected persons can see a spontaneous clearance. The remaining 80% of the infected population develop a chronic infection, 10-20% of these 80% develop cirrhosis and 1-5% with a chronic infection develop liver cancer within 20-30 years [5].

Poynard *et al.* [6] reported that the risk and natural history of fibrosis associated with HCV have been greatly clarified as a result of several large clinical studies incorporating standardized assessments of fibrosis that combine detailed historical and clinical information. The disease can run a remarkably variable course, from decades of viremia with little fibrosis to a rapid onset of cirrhosis within 10-15 years. It appears to be host factors rather than viral factors that correlate with fibrosis progression in HCV. Recent reports showed that the combination of pegylated interferon α (PEG-IFN-α) with ribavirin markedly improves therapeutic outcomes, resulting in sustained virologic response (SVR) rates ranging between 44 and 69% [7, 8].

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On the other hand, the role of dietary exposure to aflatoxin B_1 (AFB_1), a category I known human carcinogen and a potent genotoxic agent, in the development of HCC has long been documented in many model systems [9-11]. A synergistic effect of AFB_1 and HBV on HCC risk has been reported in many studies [11, 12].

The occurrence of aflatoxins in food is a serious global health problem, particularly in developing countries. Aflatoxins are well documented as cancer potency factors as 4.6-28.2% of annual hepatocarcinoma cases worldwide are caused by theses toxins [13]. Aflatoxins are potent liver carcinogens [14] and chronic aflatoxin exposure at high levels has been associated with growth faltering in young children [15-18] and immune suppression [19, 20]. The four naturally occurring aflatoxins are aflatoxins B_1 (AFB_1), AFB_2, AFG_1 and AFG_2, though AFB_1 is the most commonly occurring and carcinogenic [15]. AFB_1 is the most hazardous mycotoxins, it is extremely toxic, mutagenic and carcinogenic [15, 21]. It poses a severe threat to both livestock productivity and human health and thus, brings huge worldwide economic losses each year [22]. It is also one of the most common mycotoxins found in foods processed for human consumption, such as peanuts corn, cotton seeds, Brazil nuts, pistachios, spices and dry fruits [23]. Once AFB_1 is absorbed into the cow's body, the clearance of AFM_1 in milk may take 5 to 7 days depending on the amount and duration of the AFB_1 consumption [24]. AFB_1 and AFM_1 (metabolite) are found in feeds and milk, respectively. Dairy cattle will produce milk contaminated with AFM_1 after consuming feeds contaminated with AFB_1. The AFB_1 is rapidly absorbed in the digestive tract and primarily metabolized by liver enzymes, converting it to AFM_1, which is then excreted in milk and urine. AFM_1 is less toxic than AFB_1; however, it has been demonstrated to be a carcinogen in rainbow trout and causes morphological changes in rat liver. The carcinogenic and highly toxic effects of aflatoxin and its metabolites have resulted in aflatoxin being highly regulated by most countries in the world [25]. The aim of the current study was to evaluate the relationship between HCV infection and aflatoxin M_1 in patients with chronic liver disease.

MATERIALS AND METHODS

Patients: After having the approval of the Ethical Committee of NCI of Cairo University, the patients started recruiting and they signed the informed consents forms. Sixty patients (30 male and 30 female with a mean age >30 - <58 years old) were subjected to study the role of aflatoxin exposure on advanced liver disease (cirrhosis) in HCV patients. Ten matched controls volunteers (5 males and 5 females) were recruited from First lab Clinical Laboratory, Cairo, Egypt. Thirty of the selected patients were positive for HCV Abs and thirty were with moderate titer of HCV. The control volunteers were negative for anti-HCV. Advanced liver disease was diagnosed with liver cirrhosis and HCV titer was detected by RT-PCR technique.

Laboratory Assays: Liver function tests included alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and Gamma-Gglutamyl Transferase (Y-GT), albumin and total protein were determined on a Beckman LX20 Chemistry Analyzer (Beckman-Coulter, Inc., Brea, CA, USA). The quantitative measurement of serum AFP concentration was performed using ELISA kits purchased from Calbio Tech Co. (California, USA).

Assessment of HCV RNA load by real-time one step reverse transcriptase polymerase chain reaction for detection (RT-PCR) of HCV RNA was performed using a light cycler system (Roche Diagnostics GmbH, Mannheim, Germany). Amplification primers for HCV were 5' primer K78F (CAAGCACCCTATCAGGCAGT); and 3' primer K80R (AGCGTCTAGCCATGGCGT).

Hybridization probes; FL 5'(GCAGCCTCCAGGACCCCCC)3' and LC 5'(CCCGGGAGAGCCATAGTGGTCTG) 3' were used to detect the product. Reaction mixtures were included 7.5µl of Light cycler RNA Master HybProbe, 3.25mM Mn(OAc)2, 0.5µM concentration of each primer, 0.4µM of hybridization probe mix and 1µl of the RNA template in a total volume of 20µl. HCV RNA was first reverse-transcribed at 61°C for 20 minutes. Following denaturation for 30 seconds at 95°C, the Light Cycler amplification was performed for 45 cycles, each cycle consisting of 5 seconds at 95°C, annealing at 62°C for 15 seconds and extension at 72°C for 10 seconds. Fluorescence was monitored at 530/640nm.

Determination of AFM_1 by HPLC: Extraction of AFM_1 from serum samples was modified from the methods of Mokhles *et al.* [26] and AOAC [27]. HPLC grade methanol, acetonitrile and dichloromethane were purchased from Sigma Chemical Co. (St Louis MO, USA).

Statistical Analysis: All data were statistically analyzed using the General Linear Models Procedure of the Statistical Analysis System. The significance of the

differences among treatment groups was determined by Waller-Duncan k-ratio [28]. All statements of significance were based on probability of $P < 0.05$.

RESULTS

The present study was conducted on 60 patients and 10 matched control. Twenty patients were suffering from hepatocellular carcinoma (HCC) and 40 patients were suffering from liver cirrhosis. Demographic data comprising distribution of age, sex, smoking and residence between HCC and cirrhotic groups were represented in Table (1). The results indicated that the mean age of HCC patients (48.5±7.8) was significantly higher than that of cirrhotic ones (42.76±4.3). The distribution of sex among the HCC and cirrhotic patients showed the same percentage of males and females. However, the number of non-smoking cirrhotic patients (18) was higher than those of HCC patients (13). NO differences were found in residence between the HCC patient or the cirrhotic patients.

The results of the correlations between HCV, AFM_1 and AFP were presented in Table (2). These results indicated that HCV/PCR qt in the high infected male patients was higher than the female patients; however the concentration of the virus in the moderate infected female was higher than the males. Serum AFM_1 was higher in the high and moderate-virus infected females than in males and in the high-infected than the moderate infected patients. However, the concentration of AFM_1 in the control males was higher than the control females. The same data revealed that AFP was higher in male patients with high HCV titer; however, it was higher in the female patients with moderate HCV titer. Moreover, the concentration of AFP in control patients was higher in females than in male.

The results presented in Table (3) showed that male patients with moderate HCV had a higher AST activity than those with high HCV. However, ALT activity was higher in female patients with moderate HCV titer. ALP showed a significant increase in male patients with high HCV than those with moderate HCV which recorded a lower value even under the control level; however, this enzyme activity was higher in the female patients with moderate HCV than those with high HCV titer. No significant differences were found in TP between male and female patients in the high HCV group. On the other hand, these parameters were comparable to the control levels in male and female patients in the moderate HCV group. TB was significantly increased in male patients with high and moderate HCV and only in female patients in the high HCV group compared to the control. This increase was more pronounced in the high HCV group. However, DB was significantly increased in the high HCV male and female patients and was comparable to the controls in the moderate HCV group. GGT was significantly higher in male and female patients with both high and moderate HCV titer compared to the control group. This increase was more pronounced in females with high HCV group compared to the other groups.

DISCUSSION

In the current study, patients were divided into high and moderate titer HCV groups and the control healthy volunteers who were negative for HCV based on RT-PCR technique. Alfa fetoprotein (AFP) and liver function tests were carried out to confirm the relation between HCV infection and advanced liver disease. Moreover, serum AFM_1 of all tested subjects was determined by HPLC. The results indicated that AFP was significantly high in both HCV groups; however, this increase was more pronounced in the group with high HCV titer. On the other hand, the healthy volunteers showed normal levels of AFP. AFP is considered as specific biomarker for liver cirrhosis and it is synthesized mainly in the fetal stage; practically no production of this marker occurs in the normal adult. However, when some adult cells are transformed to cancer cells, the synthesis of AFP commences again. Therefore, the current study affirmed a significant positive correlation between (AFP) and hepatitis C virus and confirmed the results recently reported by Abdel-Wahhab *et al*. [20].

The comparison of AFP between male and female in the high HCV titer group indicated that AFP level was higher in the male patients compared to the females. However, in moderate level of HCV, females showed a higher level of AFP than the males. Although the healthy volunteers have a normal level of AFP, the females showed a higher level of AFP compared to the males. Furthermore, the strong association between AFP and hepatitis C virus was noticed in patients with high titer of HCV infection. Similar to these observations Chen and Sung [29] pointed out that AFP and a 70-KDa glycoprotein tumor marker were increased in the majority of patients with HCC, liver cirrhosis, liver fibrosis and other gastrointestinal tumors. These authors suggested that AFP may be useful in the diagnosis and follow-up of cases with HCC, although increased levels are associated with malignancies other than primary HCC.

Table 1: Distribution of age, sex, residence and smoking between HCC group and cirrhotic patients

		HCC patients (n=20)	Cirrhotic patients (n=40)
Age		48.5±7.8	42.76±4.3
	<50	12 (60%)	30 (75%)
	>50	8 (40%)	10 (25%)
Gender	M	10 (50%)	20 (50%)
	F	10 (50%)	20 (50%)
Smoking	Yes	7 (35%)	22 (55%)
	No	13 (65%)	18 (45%)
Residence	Rural	10 (50%)	20 (50%)
	Urban	10 (50%)	20 (50%)

Table 2: HCV, AFM_1 and AFP in serum of male and females patients with high and moderate virus titer and their matched controls

Parameter	Gender	High	Moderate	Control
HCV (PCR qt)	M	$1.28 X10^6$	$239.3 X 10^3$	0
	F	$1.02 10^6$	$298.4 X 10^3$	0
AFM_1 (pg/ml)	M	$246±10.1^c$	$21±7.9^b$	$220±17.7^a$
	F	$467±34.1^d$	$27±6.8^b$	$114±7.2^a$
AFP (ng/ml)	M	$54.3±6.0^c$	$20.0±2.1^b$	$1.5±0.3^a$
	F	$44.3±4.5^c$	$27.9±3.2^b$	$4.9±0.2^a$

HCV: hepatitis C virus; AFM1: aflatoxin M1; AFP: Alpha-Fetal Protein; M: male; F: female
Within each raw, means superscripts with different letters are significantly different ($P \leq 0.05$)

Table 3: Serum biochemical parameters of liver function in male and female patients with high and moderate HCV infection and their matched controls

Parameter	Gender	High	Moderate	Control
AST(U/L)	M	$47.4±7.3^c$	$64±3.9^b$	$20±2.6^a$
	F	$68±6.1^c$	$44±3.5^b$	$26±5.3^a$
ALT(U/L)	M	$92±10.9^c$	$53±4.3^b$	$23±2.2^a$
	F	$92±7.6^c$	$59±6.3^b$	$28±4.0^a$
ALP(U/L)	M	$199±17^c$	$165±11^b$	$180±18.7^a$
	F	$172±14.7^a$	$180±9.6^b$	$168±17^a$
TP (g/dl)	M	$6.1±0.13^b$	$6.9±0.14^a$	$7.1±0.12^a$
	F	$6.2±0.09^b$	$6.6±0.07^a$	$7.0±0.2^a$
Alb (g/dl)	M	$3.2±0.08^b$	$3.9±0.13^a$	$4.1±0.04^a$
	F	$3.2±0.06^c$	$3.6±0.07^b$	$4.2±0.01^a$
TB (mg/dl)	M	$1.1±0.08^c$	$0.9±0.03^b$	$0.7±0.04^a$
	F	$1.0±0.07^b$	$0.9±0.06^a$	$0.9±0.09^a$
DB (mg/dl)	M	$0.3±0.04^b$	$0.2±0.01^a$	$0.2±0.02^a$
	F	$0.1±0.004^b$	$0.02±0.002^a$	$0.02±0.002^a$
GGT (U/L)	M	$24±3.3^b$	$24.7±1.7^b$	$16.8±2.7^a$
	F	$40.5±2.9^c$	$27.0±2.1^b$	$15.2±1.9^a$

AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; TP: total protein; Alb: albumin; TB total bilirubin; DB: direct bilirubin; GGT: Gamma-Gglutamyl Transferase; M: male; F: female
Within each raw, means superscripts with different letters are significantly different ($P \leq 0.05$)

Several studies suggested for patients thought to have HCC on clinical grounds, AFP levels should strongly confirm the presence of HCC and liver cirrhosis by a tissue diagnosis [30]. In the same concern, Lehmann and Wegener [31] determined AFP in HCC, cirrhosis and CLD cases. These authors concluded that it is generally accepted that a considerable number of patients with acute and chronic hepatitis and cirrhosis without malignancy might also have slight but significant elevation of serum AFP. Frequent rises in adult serum level of AFP are seen in patients with hepatitis and cirrhosis [20]. Moreover, Peng *et al*. [32] noted that AFP levels are more likely to be elevated with the stage of such cancers and are of prognostic value to check the efficacy of treatment of HCC. Consequently, levels of serum AFP are widely used for HCC screening in patients with chronic liver disease (CLD).

Reactive oxygen species (ROS) are potential carcinogens because of their roles in mutagenesis, tumor promotion and progression [38]. ROS and oxidative damage have been shown to contribute to the genotoxicity of AFB_1 [9, 15, 17]. AFB_1 is metabolized by

constitutive cellular enzymes, during which there is formation of free radicals [34]. The process results in both lipid peroxidation and covalent adducts with DNA and proteins [9].

In the present study, liver function tests ALT, AST, ALP and GGT were significantly elevated in HCV-infected patients and the highest levels were found in the high titer HCV group. However, there is mild increase in these parameters in the moderate HCV group. On the other hand, liver function tests were in the normal range in the healthy volunteers. Conversely, serum albumin and total protein levels were significantly decreased in the high titer HCV group than those with moderate titer of HCV; however, these parameters were in the normal range in the healthy control volunteers. Similar to the current observations, Liu *et al.* [35] reported that total protein and albumin levels are lower in patients with HCV exposed to AFB_1. Oxidative damage to proteins may be a critical pathological event because enzyme inactivation can have rapid effects, by nature of their catalytic functions [36]. It is hypothesized that oxygen uptake changes due to the viral infection process are responsible for the increase of protein oxidation and the death of virally infected cells [37]. This is supported by the elevation of liver function enzymes in HCV carriers [38], but seems not in accordance with the consistent associations observed between AFM_1 and ALT and AST levels in all HCV groups.

These results are in agreement with the finding of Lopez *et al*. [39] who reported that with the exception of bilirubin, the liver function tests are abnormal more frequently in liver cirrhosis than in chronic hepatitis and fibrosis. Also, Fahim *et al*. [40] found that AST/ALT ratio is high in HCV infection with decompensate liver cirrhosis. Serum total protein and albumin levels showed the highest reduction concomitantly with the highest increase in gamma globulin level in HCV infection with decompensate liver cirrhosis than those without liver cirrhosis.

In general, chronic hepatitis C patients with elevated ALT levels and high HCV-RNA titers in the sera are considered to have active HCV replication in the liver and to be at risk for continued liver injury in a clinical basis. While, Puoti *et al*. [41] stated that clinical and virological features of HCV infected patients do not differ between subjects with ALT flares and those with persistently normal ALT. However, a number of studies showed ambivalent results in the relationships among the degree of histological damage, serum ALT level and HCV-RNA titers in chronic hepatitis C [42]. Hoofnagle *et al*. [43] reported that ALT, AST, ALP and GGT are enzymes produced in the liver and ALT and AST are used as rough indicators (or markers) of the degree of liver cell inflammation. In fact, there is probably only a weak association between the ALT levels and inflammatory changes. Although elevations of these liver enzymes are frequently seen in both acute and chronic hepatic C, they are not related to severity or outcome of the disease or to inflammatory changes or degree of fibrosis. ALT can be normal even in those with liver damage and can be elevated in diseases other than CLD. However, AST can be very high in acute hepatitis and drop to normal or slightly elevated in chronic hepatitis. Consequently, ALT is very useful for monitoring the effectiveness of antiviral drug treatment for CLD [44].

Several factors are known to affect the progression of HCV-related liver diseases [45]. However, it remains unclear whether aflatoxin exposure is an associated risk factor for developing advanced liver disease in patients with chronic hepatitis C [46]. The impact of aflatoxin on human health is substantial and has been documented in both acute and chronic exposure settings [47, 48]. Acute exposure to high levels of aflatoxin (aflatoxicosis) can result in acute toxicity, which often presents clinically as fulminant liver failure [49].

The current study revealed that aflatoxin M_1 (AFM_1) showed a significant increase in serum of patients infected with high HCV titer followed by samples collected from the healthy volunteers. Furthermore, the comparison of AFM_1 between male and female in the two HCV groups indicated that AFM_1 was high in the high level HCV patients. Moreover, it was found that AFM_1 level was higher in females than in males. However, in the control volunteers AFM_1 was higher in females than males. A synergistic interaction between AFM_1 exposure and HCV infection on liver cirrhosis (LC) risk has also been reported. However, this study showed mild difference in serum AFM_1 levels between male patients with high HCV titer and matched controls while male and female patients with moderate HCV titer displayed AFM_1 levels lower than their matched controls. Thus, AFM_1 apparently plays a relatively minor role in the prevalence of advanced liver disease in HCV infected patients.

Groopman [50] stated that each biochemical process results in AFB_1 derivatives that have a characteristic half-life within the body and thus the exposure over a period of days, weeks, or months can be assessed. Recent exposure to aflatoxin is reflected in the urine as directly excreted AFM_1 and other detoxification products, but only a small fraction of the dose is excreted in this way. Measurements of aflatoxin and its byproducts in

urine have been found to be highly variable from day to day, which reflects the wide variability in the contamination of food samples and for this reason, the measurement of AFM_1 on a single day may not be a reliable indicator of a person's chronic exposure [51].

As stated earlier, most of the information indicated that there is an evidence that aflatoxin affects early growth and at least some aspects of human immunity and nutrition. AFM_1 is a detoxification product that is rapidly excreted, but it may have significant immunologic and nutritional consequences in the nursing young [52]. AFM_1 toxicities may be modified by the dietary intakes of antioxidant vitamins, such as vitamins A, C and E and the amount of biological exposure is conditioned by infection with HBV and HCV [53]. A previous study of human liver biopsies indicated that cases classified as mild chronic active hepatitis exhibit modestly increased aflatoxin activation, but no increase is observed in severe chronic hepatitis or cirrhosis [47]. Moreover, this study showed that AFM_1 levels were associated with advanced liver disease (cirrhosis) in anti-HCV positive patients. However, AFM_1 level was not an important factor in liver in persons without anti HCV. Thus, advanced liver disease is apparently not a factor causing increased AFM_1 levels.

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