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Metabolic Investigation in Hepatitis B Patients with or Without *Diabetes mellitus* to Determine the Impact of Anti-Oxidant Activity

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Abstract: To determine the impact of antioxidant activity by metabolic investigations in hepatitis B patients with or without diabetes mellitus. Fifty patients each were included in this study with chronic hepatitis B and 50 HBV patients with diabetic mellitus. Eligibility criteria included: HBsAg, HBeAg and HBV DNA positivity in blood for six months or more; elevation of serum aspartate aminotransferase or alanine aminotransferase levels for six months or more and exclusion of co-infection with hepatitis delta or acquired immunodeficiency virus, metabolic or autoimmune disorders. All subjects underwent blood testing after a 10-h overnight fast to measure serum levels of fasting plasma glucose, total cholesterol, triglyceride and alanine aminotransferase. The subjects were also assessed for urea, creatinin, copper, zinc and magnesium and total anti-oxidative activity was measured by reducing Fe+++ to Fe++ using vitamin c as reducing agent at pH 4 to 5. The urea, uric acid and creatinine levels are significantly increased in HBV, cholesterol (total, HDL and LDL) all decreased in HBV patient where as triglyceride levels significantly increased in HBV patients with DM. The AST increases significantly in both groups of HBV patients. Magnesium is significantly decreased in diabetic HBV patients and unchanged in HBV patients. Iron is increased and zinc is decreased in both groups of HBV patients. Additional factors in elevated level of iron resulting into low antioxidative activity. Urea and creatinine levels are significantly increased that shows not only heart and pancreas but kidney can be affected in HBV patients.

Key words: Hepatitis B virus · Antioxidant activity · Metal content · Enzyme activity
Abbreviations: ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; CPK creatinine phosphokinase; LDH, Lactate dehydrogenase; Mg, Magnesium; Zn, zinc; Cu, Copper; Fe, Iron

INTRODUCTION

Approximately 10% of people infected with the hepatitis B virus (HBV) develop a chronic, life-long infection. Of these, 15% to 65% of patients with chronic HBV develop cirrhosis, with a significant risk for the complications of portal hypertension, liver failure and hepatocellular carcinoma [1,2]. Age at onset of infection is an important factor affecting the outcome of HBV infection [3]. HBV infection is usually characterized by the presence of hepatitis B surface antigen. Other markers are used to determine if the virus is active and replicating, when it can cause serious liver damage. During the course of HBV, clearance of hepatitis B early antigen (HBeAg) represents a key event, because it implies that the host is no longer immuno tolerant and enters a low replication

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phase [4, 5]. Seroconversion coincides with a decrease in serum viral load and biochemical and histological remission in the majority of cases and may be followed by HBV surface antigen (HBsAg) seroclearance [6-8].

This bloodborne infection causes approximately 300,000 acute infections annually in the American population only and there are an estimated approximately 1 million HBV carriers [9]. Fortunately, the infection resolves in 90 to 95 percent of those infected with HBV and the rest develop chronic disease, persistent infection can lead to chronic progressive hepatitis, cirrhosis or hepatocellular carcinoma [10] and causes 5,000 to 6,000 deaths per year owing to liver failure [11].

HBV infection, a major world health problem, is hyper endemic in South-East Asia and sub-Saharan Africa. Being a major cause of morbidity and mortality, prophylaxis using the highly efficacious hepatitis B vaccine is recommended for those at risk. Despite the abundance of reports concerning the increased frequency of diabetes and impaired glucose tolerance in chronic liver diseases, the mechanisms underlying this phenomenon have not been resolved.

There are not many data available to compare patients with HBV with or without diabetes mellitus (DM) which can highlight biochemical mechanism involved helping in a design of chemotherapy suitable for these patients. In our study, the role of enzymes, kidney markers, cordial marker like cholesterol and metals such as iron, magnesium, calcium, zinc and copper along with the anti-oxidant activity are investigated in HBV patients with or without diabetic mellitus and comparison is made with healthy subjects.

MATERIALS AND METHODS

Third-generation micro-ELISA assay was used for hepatitis B surface antigen (HBsAg), antibody to hepatitis B core (anti-HBc) and surface antibody (anti-HBs), secretory form of hepatitis B envelop antigen (HBeAg), antibody to secretory form of hepatitis B envelop antigen (anti-HBe) and ELISA for antibody to hepatitis C virus (anti-HCV). Clinical and laboratory features are helpful, but liver biopsy is essential for definitive diagnosis. Mild cases may have only minor hepatocellular necrosis and inflammatory cell infiltration, usually in portal regions, with normal acinar architecture and little or no fibrosis. In such cases, only uncommonly clinically important liver disease or cirrhosis develops. 50 patients each were included in this study with chronic hepatitis B and 50 HBV patients with diabetic mellitus. Eligibility criteria included: HbsAg, HbeAg and HBV DNA positivity in blood for six months or more; elevation of serum aspartate aminotransferase (AST) or alanine aminotransferase (ALT) levels for six months or more and exclusion of coinfection with hepatitis delta or acquired immunodeficiency virus and of other potential causes of liver damage such as metabolic or autoimmune disorders. HBsAg and antibody to HBs, HBeAg and anti-HBe and anti-delta antibody were measured using commercial assays (Abbott Laboratories, Chicago, Illinois, USA). HBV DNA was investigated by dot blot hybridisation using a quantitative method in the second trial [12] and a semiquantitative method in the first [13].

Informed consent was obtained from all patients and controls. Patients were assigned into the following three groups: chronic hepatitis B group, chronic hepatitis B group with diabetes and healthy controls. The ethical committee of Liaquat University Hospital, Hyderabad, Pakistan approved the study project before it began. All subjects underwent blood testing after a 10-h overnight fast to measure serum levels of fasting plasma glucose (FPG), total cholesterol (TC), triglyceride (TG) and alanine aminotransferase (ALT). Hepatitis B surface antigen (HBsAg) and HCV antibody (anti-HCV)were examined. Subjects with two FPG levels >126 mg/dL, a previously established diagnosis of DM or currently taking any form of hypoglycemic drugs or insulin injections were categorized as DM. HBsAg and anti-HCV were detected using a third generation, commercially available enzyme-linked immunosorbent assay kit (AxSYM 3.0, Abbott Laboratories, Chicago, IL). Detection of serum HCV RNA was performed using a standardized automated qualitative reverse transcription polymerase chain reaction assay (RT-PCR, COBAS AMPLICOR Hepatitis C Virus Test, version 2.0; Roche, Branchburg, NJ). Venous blood samples were collected from patients as well as from healthy subjects after a night fast in 10 ml tube. These blood samples were centrifuged for 20 minutes after clotting and the serum was separated. Serum levels of triglycerides, total cholesterol, all enzymes, HDL cholesterol and glucose were measured using enzymatic methods. The clinical data on HBV patients with or without diabetic mellitus are presented in Table 1. These enzymes were determined from the venous blood samples by centrifuging at 1500rpm. The serum was separated and stored at -40°C for the analysis using Microlab 300 (Merck, Darmstatd, Germany). Serum samples were treated with 1M sulphosylicylic acid (SSA) and centrifuged at 5000 r.p.m for 10 min. and the clear solution was subjected for the estimation of metals iron, copper, zinc and

Table 1: Clinical data on hepatitis patients with and without DM and Healthy controls.

	Control	Hepatitis B	Diabetics with Hbv
Age	40.46±1.55	40.46±1.85	48.92±1.58
Sex (M/F)	35/15	33/17	32/18
Albumin	3.68±0.83	3.20±0.079***	3.70±0.06
Protein	8.40±1.23	6.84±0.80**	7.25±0. 68*
Ca++	8.74±0.09	8.24±0.110**	8.62±0.08
Glucose (F)	81.56±1.47	77.94±1.27*	192.2±20.3***

* P < 0.05, P < 0.01, *** P < 0.001

Table 2: Serum levels of Urea, creatinine and uric acid in HBV patients with and without DM. The values are expressed as mean values (mg/dl) ± SEM.

	Control	Hepatitis B	Diabetics with Hbv
Urea	28.84±0.89	24.28±0.779***	29.18±0.95
Creatinine	0.73±0.021	0.59±0.015***	0.74±0.02
Uric Acid	3.68±0.069	5.13±0.22***	3.86±0.09

*** P < 0.001

magnesium using atomic absorption spectrophotometer (Model A 20 Hitachi, Japan). Total anti-oxidative activity was measured by reducing Fe+++ to Fe++ using vitamin c as reducing agent at pH 4 to 5. Iron II by spectrophotometry using 2, 4,6-(2-pyridyl)-s-triazine (TPTZ). The colored complex so produces is directly proportional to the concentration of Fe II produced in presence of antioxidant was determined at 595 nm. The results were quantified giving RSD less than 3% [14]. Student's t test, Mann-Whitney U, ANOVA were used for comparisons between groups. A p-value <0.05 was considered statistically significant.

RESULTS

Table 2 showed the levels of urea, creatinine and uric acid in healthy controls and HBV patients with or without diabetes mellitus. The urea, uric acid and creatinine levels are significantly increased in HBV but no change was found in HBV patients with DM. Table 3 shows the level of Cholesterol (total, HDL and LDL fractions) and triglycerides in HBV patients with and without DM and healthy controls. Cholesterol (total, HDL and LDL) all decreased in HBV patient and remained unchanged in HBV patients with DM where as triglyceride levels remained unchanged in HBV patient and significantly increased in HBV patients with DM.

The Table 3 showed serum levels of total Cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides in HBV patients with and without DM.

The Table 4 showed the enzyme levels of ALK, ALT, CPK, LDH and AST in HBV patients with or with DM and healthy controls. The level of ALK significantly increased

Table 3: Serum levels of total Cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides in HBV patients with and without DM. The values are expressed as mean values (mg/dl) ± SEM.

	Control	Hepatitis B	Diabetics with Hbv
Cholesterol	172.44±3.03	127.56±1.70***	172.8±2.9
HDL	30.42±0.57	27.96±0.36**	30.3±0.51
LDL	112.34±2.98	97.88±1.87***	116.12±2.54
Triglycerides	96.00±4.33	95.30±3.76	116.72±5.83**

**P < 0.01, *** P < 0.001

Table 4: Serum levels of enzyme activity of ALK, ALT, CPK, LDH and AST in HBV patients with and without DM. The values are expressed as mean values (U/l) ± SEM.

	Control	Hepatitis B	Diabetics with Hbv
ALK	115.64±3.98	145.82±8.47**	274.3±15.1***
ALT	22.34±1.67	59.24±3.28***	24.28±1.61*
CPK	106.56±6.52	86.10±3.34*	103.2±4.7
LDH	331.06±9.13	353.72±9.37*	338.0±7.40
AST	18.86±0.71	40.88±1.44***	21.22±0.94*
ALT:AST ratio	1.13 ±0.071	1.56±0.083***	1.17±.11

* P < 0.05, P < 0.01, *** P < 0.001

Table 5: Serum levels of total Iron, Magnesium, Zinc and Copper in HBV patients with and without DM. The values are expressed as mean values (ppm) ± SEM.

	Control	Hepatitis B	Diabetics with Hbv
Magnesium	19.76±0.59	20.49±0.701	16.66±0.60**
Iron	2.93±0.137	3.14±0.052**	3.39±0.10***
Zinc	4.16±0.120	3.26±0.107***	4.00±0.10*
Copper	2.03±0.052	2.08±0.021	1.99±0.024
Anti-oxidant activity	1.52±0.05	0.37±0.05***	1.05±0.04**

*P < 0.05, P < 0.01, *** P < 0.001

in both HBV patients. ALT shows the similar increasing tendency in both HBV patients whereas CPK is significantly decreased in HBV patients but remained unchanged in HBV with DM. LDH is significantly increased in HBV patients but remained unchanged in HBV patients with DM. AST increases significantly in both groups of HBV patients.

The ratio between ALT and AST increased in HBV patients but remained unchanged in diabetic HBV patients.

Table 5 showed the serum levels of magnesium, iron, zinc and copper in HBV patients with and without DM and healthy controls. Magnesium is significantly decreased in diabetic HBV patients where as it remained unchanged in HBV patients. Iron is significantly increased and zinc is significantly decreased in both groups of HBV patients. Copper remained unchanged in both groups of HBV patients. Total antioxidant activity is decreased in both groups of HBV patients.

DISCUSSION

HBV is strongly associated with diabetes mellitus (DM) among Asian Americans, but not in Pacific Islanders, whereas HBV infection was associated with DM in both ethnic groups [16]. Insulin resistance (IR) plays an important role in this development. The liver helps to maintain normal blood glucose concentration in the fasting and postprandial states. Loss of insulin effect on the liver leads to glycogenolysis and an increase in hepatic glucose production. Abnormalities of triglyceride storage and lipolysis in insulin-sensitive tissues such as the liver are an early manifestation of conditions characterized by insulin resistance and are detectable earlier than fasting hyperglycemia. The precise genetic, environmental and metabolic factors and sequence of events is still unknown. One study [17] has shown that IR precedes the onset of diabetes by 10-20 yearand impaired glucose tolerance and overt DM frequently occurs in patients with chronic liver disease, the main contributing factors being hyperinsulinaemia and peripheral insulin resistance to the development of DM in these patients. This study also showed that in cirrhotic patients a high prevalence of DM, which is more frequently associated with HCV infection than HBV. Clinically, chronic hepatitis B is a mild disease in both infants and children and cirrhosis is rare; however, viral replication and liver damage may persist for several years. The strict control of blood glucose and the control of infection could be important in prolonging the survival in compensated cirrhotic patients with DM. In endemic areas, infection with HBV is a major cause of liver disease in childhood.

Albumin synthesis is an important function of the liver. With progressive liver disease serum albumin levels fall, reflecting decreased synthesis as we have observed in HBV patients (Table 1). Albumin levels are dependant on a number of other factors such as the nutritional status, catabolism, hormonal factors and urinary and gastrointestinal losses. Significant reduced serum urea, creatinine is found in HBV patient where as these levels remained unchanged in diabetic HBV patients. Uric acid is elevated in HBV patients; however, it remained unchanged in diabetic HBV patients (Table 2). Total Cholesterol as well in both HDL and LDL fractions is decreased in HBV patients. Triglyricide is increased in diabetic HBV (Table 3). Elevated levels of plasma triglycerides (TG) and reduced concentrations of HDL-Cholesterol are very common in patients with DM, particularly in NIDDM [18, 19]. One study on symptomatic chronic HBV infection patients showed lower serum levels of total- and HDL-Cholesterol. Elevation of ALT was also an indicator of lower levels of HDL-C in patients with chronic HBV and lower serum HDL-Cholesterol and higher TG levels in patients without HBV infection. These findings also indicated the need to monitor the risk of atherosclerotic diseases in patients with asymptomatic chronic HBV infection, especially those with lower HDL-Cholesterol levels [20].

The occurrence of liver disease and raised liver enzymes is common in Type 2 diabetes and may be multifactorial in origin. Recent study in diabetics with hepatitis showed ALT, AST and Gamma Glutamyl transferase (GGT) levels exceeding the upper limit of normal level and the prevalence being higher in males, increasing with obesity class and poor metabolic control and decreasing with age. Elevated enzymes were systematically associated with most parameters of the metabolic syndrome [21]. It is well understood that glucose intolerance is associated with chronic liver disease, particularly cirrhosis and overt DM is two to four times more common than in the general population. The relationship between the cause of cirrhosis and the development of glucose intolerance or whether cirrhosis is a prerequisite is unknown, however, it is shown that there appears to be an association between DM and chronic HCV that is not present in patients with chronic HBV [22]. Among the enzymes selected, ALT is specific for liver disease of a hepatocellular injury type and this enzyme is 4 times elevated in HBV patients, however, no change is found in diabetic HBV patients (Table 4). Hence this rise in ALT can be interpreted as indicative of liver disease and not due to DM. AST shows the similar increasing tendency in all HBV patient groups (Table 4). This enzyme is less sensitive and specific for liver disease but still should be employed as a screening test because the ALT to AST ratio can often be used to suggest the cause and/or extent of liver disease [23]. We have also shown ALK elevation in both HBV patients which originates predominantly from two sources, liver and bone [24]. There is decreased activity of CPK in HBV patients, however no change in activity was found in diabetic HBV patients. LDH is significantly increased in only HBV patients but remained unchanged in diabetic HBV patients. It is pointed in an earlier study that HCV and HBV are strongly associated with early atherosclerosis independent of classical risk factrs, insulin resistance and metabolic syndrome components [25].

Increasing tendency of blood iron is alarming in HBV patients as is seen in our study. Iron is essential for its metabolic functions, makes iron potentially hazardous

because of its ability to participate in the generation of powerful oxidant species such as hydroxyl radical [26]. However, an iron store does not accurately reflect hepatic iron content, or predict clinically important endpoints such as response to interferon and disease progression. Because iron participates in the formation of reactive oxygen species, organisms take great care in the handling of iron. Indeed, iron sequestration in transport and storage proteins may contribute to antioxidant defenses. It is now well established that oxidants can cause the release of catalytic iron [27]; thus, a vicious cycle is initiated that leads to the formation of more reactive oxygen species. Haemochromatosis gene mutations were more frequent in chronic viral hepatitis patients with iron overload than in those without iron overload and in healthy controls suggesting they contribute to pathogenesis of hepatic iron accumulation. The correlation between hepatic fibrosis and portal iron supports the fibrogenetic role of iron in chronic viral hepatitis [28].

The role of zinc has long been considered very vital in hepatitis patients. The possibility that zinc might increase sustained virologic response has been proposed. A link between zinc and immune deficiency has been reported [29]. Zinc inhibits replication of diverse viruses in vitro, including HIV [29]. Zinc deficiency is common in cirrhosis as it is involved in altered nitrogen metabolism. It is concluded that zinc deficiency in cirrhotic patients appears to be due to low absorption and to high urinary excretion, partly due to excessive diuretic administration. Changes in the hormonal drive and/or the antioxidant activity of zinc might be involved in the general improvement in liver function by decreasing ammonia production.

Copper and magnesium are unaltered in their serum content in HBV patients, however, magnesium shows decreasing tendency in diabetic HBV patients. We have already shown magnesium deficiency in diabetic patients. Mg depletion has a negative impact on glucose homeostasis and insulin sensitivity in type 2 diabetic patients. Low plasma magnesium concentration is a highly specific indicator of poor magnesium status. In the USA and some European countries, blood magnesium concentrations have been found to be decreased in diabetics and similar is the case in our diabetic patients (Table 4). It is shown that oral Mg supplementation in the doses and duration studied is modestly effective in reducing systolic blood pressure in patients with NIDDM but has little impact on other important biochemical parameters related to diabetes-associated end-organ disease [30].

One of the major pathogenic mechanisms for progression of liver disease is oxidative stress. Recently, some studies [31-32] have demonstrated the role of oxidative stress in liver diseases; however, studies describing the mechanism behind antioxidant status in these patients are still unknown. Larger studies and newer markers of oxidative stress are required to clarify the association between oxidative stress and histological severity in viral hepatitis patients. Few studies tested the hypothesis that enzymes conventionally association with liver dysfunction (aspartate aminotransferase, alanine aminotransferase, gamma-glutamyltransferase [GGT] and alkaline phosphatase) for the prediction of diabetes. According to these studies although mild elevations in liver enzymes are considered associated with features of the metabolic syndrome, only raised GGT was reckoned as an independent predictor of deterioration of glucose tolerance to diabetes. And it was spectulated that GGT signals oxidative stress, the association with diabetes may reflect both hepatic steatosis and enhanced oxidative stress [31-32]. Hepatic iron may promote liver injury, whereas antioxidant vitamins and minerals may inhibit it, but few clinical studies have examined such relationships. In this large, national, population-based study, the risk for apparent liver injury was associated with increased iron and decreased antioxidants, particularly carotenoids [33].Our results also confirmed significant low antioxidant activity and higher iron serum level in both HBV patients with or without DM (Table 5).

CONCLUSION

The present study concluded that an additional factors in elevated level of iron resulting into low antioxidative activity and low urea, creatinine and high uric acid showed that not only heart and pancreas but also, kidney can be affected in HBV patients.

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