

Relationship of Oxidative Stress in Hepatitis B Infection Activity with HBV DNA and Fibrosis

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Background: The aim of this study was to evaluate oxidative stress in various clinical forms of hepatitis B infection and to investigate its role in the development of the chronic form of the disease.

Methods: Ninety-three patients with inactive hepatitis B surface antigen (HbsAg) carrier state (IHBCS), 65 patients with chronic hepatitis B infection (CHB), and 42 healthy adults were included in the study. The following values were measured and compared in patient groups: total antioxidant status (TAS), total oxidative stress (TOS), oxidative stress index (OSI), sulfhydryl (SH), lipid peroxidation (LOOH), catalase (CAT), and ceruloplasmin. In patients with chronic hepatitis B, these values were compared with HBV DNA and fibrosis levels.

Results: ALT, TOS, LOOH, and OSI levels were higher in the CHB group compared to the other groups ($P < 0.001$). Catalase levels increased in the CHB and IHBCS groups compared to the control group ($P < 0.001$). Total aminooxidant and ceruloplasmin levels were found to be lowest in the CHB group and highest in the control group ($P < 0.001$). Sulfhydryl was higher in the control group compared to the other groups ($P < 0.001$). In the CHB group, there was no correlation between the HBV DNA and OSI ($P > 0.05$).

Conclusions: These findings suggested that oxidative stress is associated with hepatitis B activity.

Key Words: Hepatitis B, HBV DNA, Fibrosis, Oxidative stress, Catalase, Sulfhydryl

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INTRODUCTION

The hepatitis B virus (HBV), which is a chronic viral hepatitis factor, is a significant pathogen that causes fibrosis, cirrhosis, hepatocellular cancer as a result of the damage it causes to liver cells. Chronic hepatitis B (CHB) infection is a health issue that affects more than 400 million people globally. Although there is an effective vaccine and advances have been made in diagnostic and treatment methods, almost 1,000,000 people die every year due to HBV infection-related complications [1]. Hepatitis B infection exhibits a varied clinical course, which can range from asymptomatic infection to fulminant liver disease [2].

Cells continuously form free radicals and reactive oxygen species as part of metabolic processes. These free radicals and reactive oxygen species are neutralized by a complex antioxidant system. Oxidative stress is the imbalance that occurs between the reactive oxygen species or free radicals and the antioxidant system, and this imbalance may cause irreversible damage in important cellular compartments.

In chronic viral hepatitis, the role of oxidative stress in cell destruction and DNA and RNA damage has been established through experimental studies [3, 4]. In the present study, oxidative stress was measured in various clinical forms of the chronic hepatitis B and its role was investigated in the development of

chronic hepatitis.

METHODS

1. Patient selection and sample preparation

Patients infected with hepatitis B virus, who presented to Tokat State Hospital (in Tokat, Turkey) between June 2010 and May 2011, and healthy adults participated in the study. A total of 200 individuals participated in the study, comprising 65 chronic active hepatitis B patients in the 15-66 age range (32.5%), 93 patients with inactive hepatitis B infection (46.5%) and 42 healthy adults. All subjects who participated in the study gave informed consent and the study was approved by the Ethics Committee of the Harran University, Faculty of Medicine.

1) Diagnosis criteria

The disease was diagnosed according to the following criteria [5]. Chronic hepatitis B infection (CHB):

- (1) HBsAg-positive for more than 6 months,
- (2) ALT value greater than 1.5 times the normal value (normally, ALT value is less than 40 IU/mL),
- (3) HBV DNA value $\geq 100,000$ copies/mL (20,000 IU/mL) in those who were positive for the hepatitis Be antigen (HBeAg-positive),
- (4) Whereas it was $\geq 10,000$ copies/mL (2,000 IU/mL) in those who were HBeAg-negative, and
- (5) Fibrosis ≥ 2 in the histopathological evaluation of the liver

Inactive HbsAg Carrier State (IHBCS):

- (1) HBsAg-positive,
- (2) Normal ALT values,
- (3) HBeAg-negative, and
- (4) HBV DNA $\leq 10,000$ copies/mL (2,000 IU/mL)

The control group was formed from HBsAg-negative healthy adults whose anti-HBc total value was negative.

Exclusion criteria: Patients with liver cirrhosis, diabetes mellitus, hypertension, coronary artery disease, acute infection, chronic obstructive pulmonary disease, corticosteroid usage, malignancy, morbid obesity, pregnancy, liver and kidney failure, and smokers were not included in the study.

Liver biopsy was performed on all patients with CHB prior to treatment. In order to rate the liver damage, histological activity (necroinflammatory lesion) and fibrosis (according to KNODELL fibrosis scoring system) [6] assessment was performed.

In addition to the patient's age, gender, HBsAg, HBeAg, anti-HBe, ALT, and AST values, HBV DNA levels and liver biopsy, fibrosis levels were also recorded in chronic hepatitis B patients.

2) Collection and storage of samples

Blood samples were collected in the form of venous blood following 12 hr of fasting. Physical examination was performed on the patients to exclude other diseases. Ten milliliter blood samples were collected into biochemistry tubes containing heparin. The plasma, which was obtained after centrifuging blood samples at 3,500 rpm for 10 min, was stored at -80°C until the study date.

2. Measurement of oxidative status

Total oxidative status (TOS), total antioxidant status (TAS), and oxidative stress index (OSI) were measured using the method developed by Erel [7]. In this method, long-lasting, durable, radical monocation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is formed. The color of this radical, which is characteristically blue-green, is reduced by the antioxidants and disappears. The lightening and/or discoloration of the color of the antioxidants in the samples is considered to be their total antioxidant capacity. Trolox, which is a water-soluble analog of vitamin E, was used as the standard, and the results were expressed in terms of μmol trolox equiv./L [7].

The serum catalase (CAT) activity was determined using Goth's colorimetric method [8], in which serum is incubated with H_2O_2 , and the enzyme reactions are stopped by the addition of ammonium molybdate. Serum CAT activity was expressed as kU/L.

Free sulfhydryl groups of serum samples were assayed according to the method of Ellman [9], as modified by Hu et al. [10]. Briefly, 1 mL of buffer containing 0.1 M Tris, 10 mM EDTA, pH 8.2, and 50 μL serum was added to cuvettes, followed by 50 μL 10 mM 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) in methanol. Blanks without DNTB in the methanol were run for each sample as a test. Following incubation for 15 min at room temperature, sample absorbance was measured at 412 nm using a spectrophotometer (CE1011; Cecil Instruments Ltd., Milton Technical Centre, Cambridge, England). The value of reagent blanks were subtracted from those of samples for calibration. The concentration of sulfhydryl groups was calculated using reduced glutathione as the free sulfhydryl group standard and the result was expressed as mmol/L.

Plasma lipid peroxidation (LOOH) was evaluated by the fluorimetric method based on the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA) [11]. Briefly, 50 μL of plasma was added to 1 mL of 10 mmol/L diethylthiobarbituric acid (DETBA)

reagent in phosphate buffer (0.1 mol/L, pH 3). The mixture was mixed for 5 sec and incubated for 60 min at 95°C. Samples were placed on ice for 5 min and then 5 mL of butanol was added. The mixture was shaken for 1 min to extract the DETBA-MDA adduct, and then centrifuged at 1,500×g for 10 min at 4°C. Fluorescence of the butanol extract was measured at an excitation wavelength of 539 nm and an emission wavelength of 553 nm. 1,1,3,3-tetraethoxypropane (Sigma Chemical Company, St. Louis, MO, USA) was used as a standard solution and the values were presented as mol/L.

Ceruloplasmin levels were assessed by measuring its oxidative activity using *p*-phenylenediamine as the substrate, according to the method of Sunderman and Nomoto [12].

3. Statistical analysis

Pearson's chi-square test was used to compare the categorical variables between groups. Categorical variables were presented as counts and percentages. The Shapiro-Wilk test was used to evaluate whether the distribution of variables was normal.

The t-test for two independent samples, or the Kruskal-Wallis

test, was used to compare continuous variables between two groups. Continuous variables were presented as mean (SD) or as median (interquartile range [IQR]). A *P* value of less than 0.05 was considered to be statistically significant. SPSS software 15.0 for Windows (Chicago, IL, USA) was used for all statistical analyses.

RESULTS

The chronic hepatitis B, inactive hepatitis B, and control groups were similar in terms of mean age of the subjects ($P > 0.05$). There was no significant difference in gender characteristics between the CHB group and the other groups (IHBCS, Control) ($P = 0.103$ and $P = 0.656$, respectively). The gender distribution of the IHBCS and control groups were also similar ($P = 0.35$).

Of the chronic hepatitis B patients, 32 (49.2%) were HBeAg-positive. The age and gender characteristics and ALT values of the patients are shown in Table 1. The ALT level was higher in the CHB group compared to the other two groups ($P < 0.001$). A positive correlation was found between ALT and HBV DNA ($r = 0.308$; $P = 0.012$). There was no correlation between ALT and oxidative stress ($P > 0.05$). The oxidative and antioxidative parameters in each group are shown in Table 2. The values of OSI, TAS, and ceruloplasmin of the patient and control groups are shown in Figs. 1-3, respectively. In the CHB group, no correlation was found between the fibrosis levels of the patients and oxidative stress ($P > 0.05$). In patients in the CHB group, HBV DNA was not correlated with OSI ($P > 0.05$). A strong negative correlation was observed between OSI and TAS ($r = -0.404$; $P < 0.001$) and between OSI and SH ($r = -0.333$; $P = 0.015$). A strong positive correlation was observed between OSI and LOOH ($r = 0.731$; $P < 0.001$) and TOS ($r = 0.731$; $P < 0.001$).

Table 1. The clinical and demographic data of the study groups

Item	Chronic hepatitis B (N=65)	Inactive HBsAg carrier state (N=93)	Control (N=42)	<i>P</i>
Age (yr), mean (SD)	31.82 ± 12.59	33.7 ± 12.81	33.29 ± 12.8	> 0.05
Male, n (%)	45 (48.4%)	40 (61.5%)	24 (57.1%)	> 0.05
Alanine Aminotransferase (IU/mL), median (IQR)	81 (53.5-133)	21 (16-31)	20.5 (11.75-29.25)	< 0.001*

*There was a statistically significant difference between the CHB group and the other groups.

Table 2. Oxidative and antioxidative parameters in each group

	Chronic hepatitis B (N=65)	Inactive HbsAg carrier state (N=93)	Control (N=42)	<i>P</i>
TAS (μmol Trolox equivalents/L), mean (SD)	0.74 ± 0.103	0.79 ± 0.107	0.91 ± 0.13	< 0.001*
CAT (kU/L), median (IQR)	17.33 (14.77-18.50)	17.95 (16.85-8.82)	8.5 (7.7-9.2)	< 0.001 [†]
SH, mean (SD)	0.23 ± 0.027	0.24 ± 0.026	0.28 ± 0.027	< 0.001 [‡]
Looh (mol/L), median (IQR)	6.82 (5.03-10.31)	5.19 (4.89-5.96)	5.94 (4.72-6.92)	< 0.001 [§]
TOS (μmol Trolox equivalents/L), median (IQR)	12.87 (8.62-16.93)	8.4 (7.38-10.77)	9.47 (7.61-10.43)	< 0.001 [§]
Serulop, median (IQR)	510.62 (486.25-544.37)	540 (504.21-588.12)	663.28 (624.60-746.87)	< 0.001*
OSI (μmol Trolox equivalents/L), median (IQR)	1.75 (1.15-2.13)	1.09 (0.93-1.4)	1.01 (0.88-1.16)	< 0.001 [§]

*There was a statistically significant difference between all groups; [†]There was a statistically significant difference between the control group and the other groups ($P < 0.001$) and no statistically significant difference between the chronic hepatitis B group and the inactive HBsAg carrier state group ($P < 0.005$); [‡]There was a statistically significant difference between the control group and the other groups but no statistically significant difference between the chronic hepatitis B group and the inactive HBsAg carrier state group; [§]There was a statistically significant difference between the chronic hepatitis B group and the other groups but no statistically significant difference between the inactive HBsAg carrier state group and the control group.

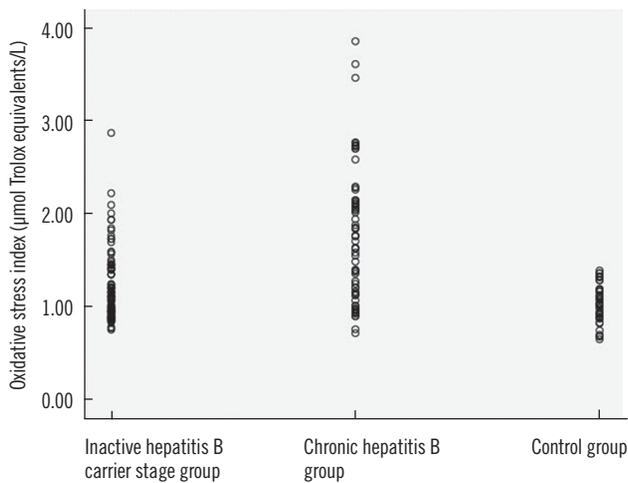


Fig. 1. Oxidative stress index values of patient groups and control group.

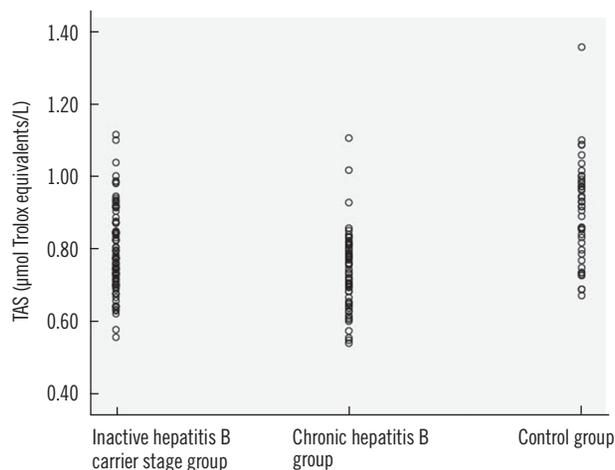


Fig. 2. Totally antioxidant status values of patient groups and control group.
Abbreviation: TAS, totally antioxidant status.

DISCUSSION

In this study, we showed that in hepatitis B infection, oxidative stress indices such as TOS, LOOH, OSI, and CAT are increased, and TAS, sulfhydryl, and ceruloplasmin levels, which are indicative of antioxidant status, are decreased.

The HBV is one of the important factors of acute/chronic hepatitis, cirrhosis and hepatocellular carcinoma. More than 400 million people are known to be chronically infected with HBV [13]. Five percent of the world population are HBV carriers and CHB infection is cited by WHO to be the ninth most common cause of death [1].

Hepatitis B infection exhibits a varied clinical course, which

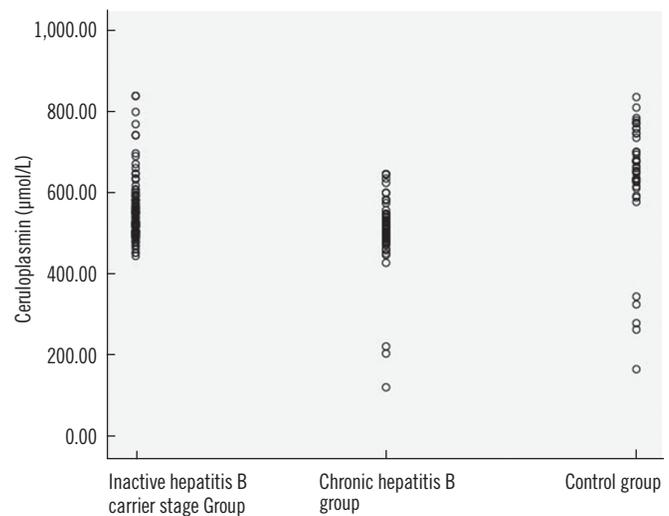


Fig. 3. Ceruloplasmin values of patient groups and control group.

can range from asymptomatic infection to fulminant liver disease [2]. The disease is diagnosed through clinical, microbiological (ELISA, PCR), biochemical (ALT, AST), and pathological assessment [14]. In the present study, oxidative stress parameters in two different forms of the disease (CHB and IHBCS) were compared to those in healthy adults.

The liver fibrosis levels of the patients were determined through the histological assessment of the liver biopsy material. The diagnosis of the patients included in the study was made based on these four methods, i.e. clinical, microbiological, biochemical, and pathological method. HBV DNA analysis and histological assessments of liver biopsy samples were performed for all CHB patients.

Free radicals are defined as short-lived, unstable, and highly active molecules with one or more unpaired electrons, and with low molecular weight [15, 16]. The substances that can prevent or delay the effects of molecules that may cause oxidation of essential substances in an organism are called antioxidants [17]. Oxidative stress is defined simply as the imbalance between the body's antioxidant defense and the production of the free radicals, which can cause peroxidation of the lipid layer of the cells [18, 19]. The main intracellular antioxidants in humans are the superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) enzymes. In contrast to the intracellular environment, vitamins E and C, transferrin, haptoglobin, ceruloplasmin, albumin, bilirubin, β -carotene, and alpha-1-antitrypsin are responsible for antioxidant defense in the extracellular environment [20].

The free fatty acid (FFA) oxidation products (lipid peroxide, superoxide, and hydrogen peroxide radicals) can generate oxi-

oxidative stress and result in subsequent lipid peroxidation. Lipid hydroperoxide (LOOH), which is a non-radical free oxygen particle, is one of the indicators for oxidative stress [21]. In recent years, the role of the imbalance between oxidative stress and the antioxidant status in cell destruction has become better understood [3, 7, 22].

Free oxygen radicals are effective in the pathogenesis of many diseases such as carbon tetrachloride-associated liver damage, glomerulonephritis, arteriosclerosis, diabetes mellitus, vitamin C-E deficiency, malignancy, emphysema, hyperoxia, bronchopulmonary dysplasia, pancreatitis, and rheumatoid arthritis [22-24].

In infectious diseases, inflammatory cells have been shown to become activated and secrete reactive oxygen and nitrogen species. Studies on oxidative stress have been conducted in many infectious diseases and oxidative stress has been demonstrated to increase in leishmania, measles, *Neisseria gonorrhoeae* infections, sepsis, urinary system infections, and *Paracoccidioides brasiliensis* infections [25-30].

There have been various studies indicating that oxidative stress is increased in hepatitis B and hepatitis C infections and in liver disease [31-35]. Increased oxidative stress in HBV infection was demonstrated to have an effect on DNA damage and hepatocarcinogenesis [16]. It is known that of the 530,000 hepatocellular carcinoma cases seen each year, 316,000 are associated with HBV [2]. Therefore, we believe oxidative stress, which is known to play a significant role in hepatocarcinogenesis, is important in CHB patients.

Various indicators of oxidative stress have been studied in various clinical forms of hepatitis B and have been demonstrated to increase as the disease becomes chronic [4, 35].

Measurement of the overall antioxidant status may yield more valuable information than the measurement of individual antioxidants. In our study, we chose to use the total antioxidant capacity measurement method developed in recent years by Erel, which measures total SH, vitamin C, uric acid, vitamin E, bilirubin, and many other antioxidants precisely, and the total oxidant capacity measurement method, which measures the oxidative stress caused by free radicals in the plasma such as hydroxyl (OH), hydrogen peroxide (H₂O₂), singlet oxygen (O₂¹), lipid hydroperoxide (LOOH), and superoxide (O₂⁻), which can all be measured with ease due to the fact that these measurements can be performed by fully automated colorimetric methods [7, 19]. In the present study, OSI, TOS, and TAS measurements were performed using this method. Additionally, ceruloplasmin, LOOH, and SH, which are also oxidative stress indicators, and SH and CAT enzymes, which are antioxidant status indicators, were assessed

and compared in IHBCS, CHB and control groups.

The values of OSI, TOS, and LOOH, which are oxidative stress indicators, were higher in the CHB group compared to the other groups ($P < 0.001$). The values of the antioxidant status indicator TAS, the intracellular antioxidant CAT, and the extracellular antioxidant ceruloplasmin were different in all three groups, and thus they decreased with increasing severity of the disease ($P < 0.001$). This suggested that oxidative stress may play a role in the pathogenesis of CHB and in exacerbation of the disease. This study is the first study to compare oxidative stress with HBV DNA. Our data indicate no correlation between OSI and HBV DNA.

In chronic hepatitis B, the gold standard in assessing fibrosis is the histopathological examination of the liver biopsy material. The histological activity index (HAI) and fibrosis level in the liver indicates the progression of the disease toward cirrhosis. This study investigated the relationship of oxidative stress with fibrosis. We did not find a correlation between HAI and fibrosis and the indicators of oxidative stress. We believe this may be in part due to the homogeneity of the patients in whom the biopsies were performed, exclusion of patients with liver cirrhosis from the study, and the presence of moderate chronic active hepatitis in all patients.

In conclusion, oxidative stress increases and antioxidant values decrease as the disease becomes chronic in patients with hepatitis B infection. This study suggests that oxidative stress may be associated with hepatitis B activity.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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